ISSN 0971-6777 (Print)
ISSN 2277-8934 (Online)

JOURNAL OF CAMEL PRACTICE AND RESEARCH

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Volume 28 April 2021 Number 1

In This Issue

JOURNAL OF CAMEL PRACTICE AND RESEARCH

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Editor, Journal of Camel Practice and Research
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Bikaner-334001, INDIA

Phone : 0091-151-2527029 (R)
Mobile : 0091-9414137029
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Publisher : The **Journal of Camel Practice and Research** (Triannual) is published by the **"Camel Publishing House"** 67, Gandhi Nagar West, Near Lalgarh Palace, Bikaner-334001, India. Phone : 0091-151-2527029, email: tkcamelvet@yahoo.com

Cover Design: T.K. Gahlot

Courtesy: Bernard Faye [Camels in Lanzarote (Canarias Islands)]

Printer: Sankhla Printers, Vinayak Shikhar, Near Polytechnic College, Bikaner-334003, India.

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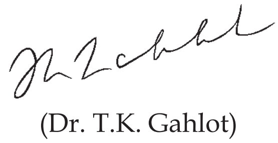
NO SPECIFIC IMPACT OF COVID-19 ON THE CAMEL SECTOR

The impact of the COVID-19 pandemic on camel sector was viewed through many ways, i.e. infection and disease of the owners or staff in camel farms, difficulties in the local and international distribution network of camel products due to the restriction of movements, changes in the consumers' behavior toward the unexpected health crisis, cancellation of touristic or sport event linked to camel breeding and national and international travel restriction of professionals, service personals, scientists etc. International travel restrictions seriously impacted ongoing and future international technical and scientific cooperation. These observations were critically evaluated and published by the Peter Nagy, Ulrich Wernery, Pamela Burger, Judit Juhasz, Bernard Faye in their recent publication cited below. They emphasised the role of extraordinary immunology of camelids in fighting infectious diseases. These nanobodies, due to their small size, have an enormous potential for diagnostic use and therapeutics. The peripheral blood mononuclear cells of camelids can be used to produce specific nanobodies that effectively neutralises beta coronaviruses. These nanobodies are excellent candidates for antiviral therapy. There is no specific impact of COVID-19 on the camel sector compared to other livestock sectors or agricultural sector.

(Peter Nagy, Ulrich Wernery, Pamela Burger, Judit Juhasz, Bernard Faye, The impact of COVID-19 on Old World Camelids and their potential role to combat a human pandemic, Animal Frontiers, Volume 11, Issue 1, January 2021, Pages 60–66, https://doi.org/10.1093/af/vfaa048)

Journal of Camel Practice and Research is proudly releasing the first issue of volume 28th. A big leap from first issue of June 1994 to April 2021. I am thankful to my team of editorial board and authors who are continuously contributing their manuscripts. The current issue is rich in the research contents of dromedary and Bactrian camels. These include a new milking technology: "Stimulactor", intramuscular myxoma, caseous lymphadenitis (Pseudotuberculosis), association of vitamin B12, cobalt and sulfur levels in serum and cerebrospinal fluid, comparative transcriptome analysis provides potential insights into the mechanism of camel milk in regulating alcoholic liver disease in mice, Crimean-congo haemorrhagic fever, ER-α expression in the hypothalamus-pituitary-gonad axis of the Bactrian camel, evaluation of transtracheal wash (TTW) and tracheal wash (TW) in camels with respiratory disorders, immunoreactivity of alpha smooth muscle actin in the epididymis of the dromedary camel, influence of 8 km training on cardiac biomarkers, immunomodulatory effect of *Escherichia coli* lipopolysaccharide on phenotype and function of blood monocytes in camels, molecular characterisation of growth hormone (GH) gene in Indian dromedary and Bactrian camel, molecular detection of *Trypanosoma evansi* in camel using internal transcribed spacer 1 of Ribosomal DNA, obstructive urolithiasis in dromedary camels: clinical, ultrasonographic and postmortem findings, prevalence of Rotavirus infection, prominent prescapular caseous lymphadenitis abscess, prozone reaction in an antibody ELISA of a brucellosis positive dromedary camel, scanning electron microscopic studies on the thyroid gland, immunophenotype of camel blood eosinophils and fatalities in dromedary camels across the Arabian peninsula caused by plastic waste.

I am sure that all camel researchers and scientists will keep strengthening their support to the biggest platform of camelid research literature- JCPR. Wishing all the editors and authors a corona free year to stay healthy.



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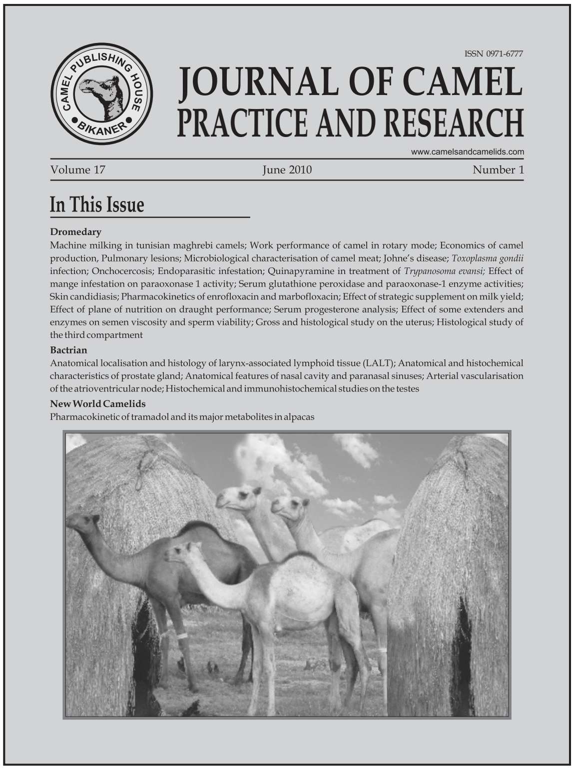
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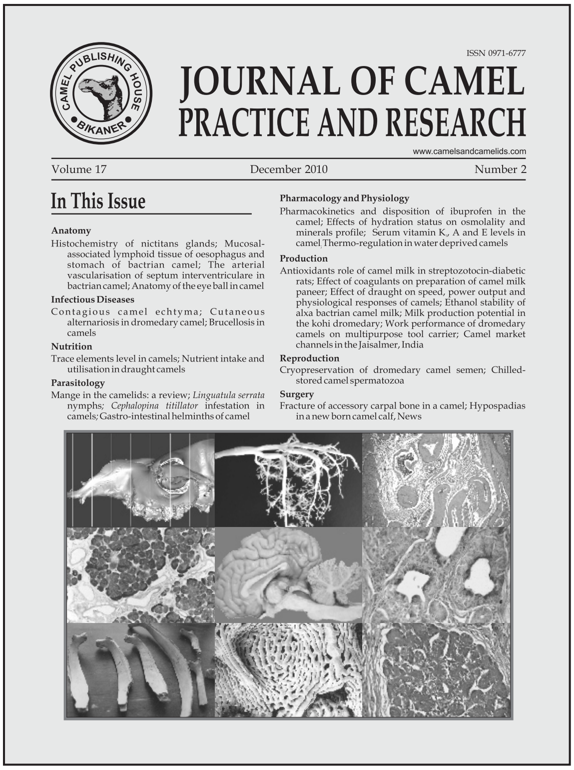
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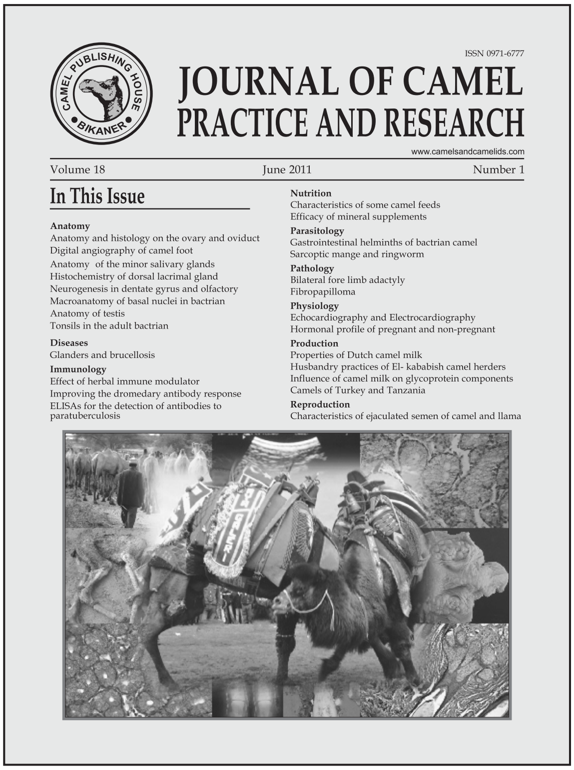
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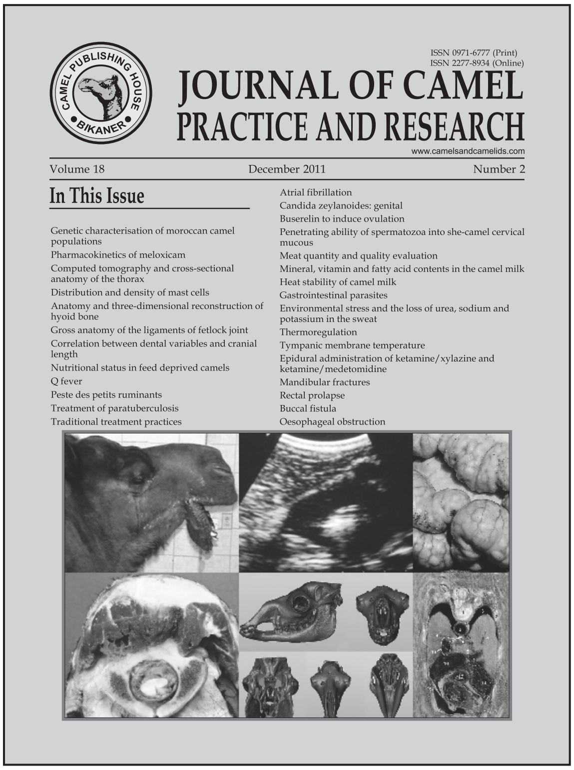
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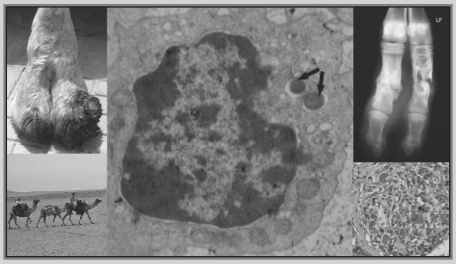

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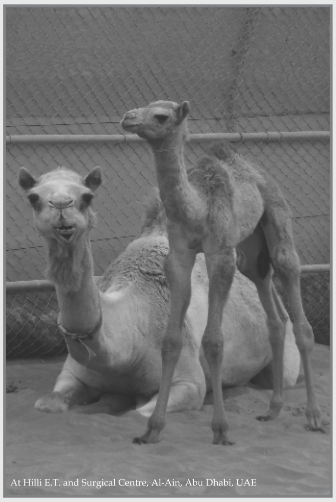

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DOI : 10.5958/2277-8934.2021.00001.1 *Vol 28 No 1, p 1-9*

A NEW MILKING TECHNOLOGY: "STIMULACTOR" FOR LACTATING CAMELS

S. Kaskous

Department of Research and Development, Siliconform, Schelmengriesstrasse 1, D-86842 Türkheim, Germany

ABSTRACT

The aim of this study was to develop a new milking machine for camels that guarantees high milk yield and quality whilst maintaining udder health. After a great effort, the new milking machine "StimuLactor for Camels" has been developed by Siliconform Germany. All the requirements of camels were tested on 5 dromedary camels over a period of one year. Our development was based on two main aspects: first, on the technical basis of the milking machine (type of machine, with or without a claw piece, kind of pulsation, vacuum level, type of teat cup liner and pulsation rate and ratio) which have to be adapted to the requirements of a camel's udder and teats. The second important aspect was the calf's suckling behaviour. The first results with the new milking technology have clearly shown that the system milks as the calf sucks. It can be used without the presence of the calf during the course of milk removal. Furthermore, the results proved that milk ejection reflex was induced and the milk was let down during the milking process. In conclusion, the new milking technology "StimuLactor for Camels" was adapted to the physiological, morphological and anatomical requirements of lactating camels.

Key words: Camel, dromedary, lactation, milking technology, stimulactor

Despite the importance of the milking machine for milk removal in camels, it is common only in a few farms in the world. There are several reasons hindering the use of milking machines for camels. First, differences in milk yield and lactation length. The daily milk yield varies between 0.5 and 35 kg and the length of lactation varies between 6 and 18 months or more (Khan and Iqbal, 2001; Wernery, 2006; Razig *et al,* 2008; Nagy *et al,* 2013; Zayed *et al,* 2014; Kaskous and Fadlelmoula, 2014; Dowelmadina *et al,* 2015; Jemmali *et al,* 2016; Hadef *et al,* 2018; Gebremichael *et al,* 2019; Boujenane, 2020). Second, there are very strong differences with regard to udder and teat shapes as well as udder measurements between the camels, including within the herd in the same farm (Kaskous and Fadlelmoula, 2014; Kaskous, 2018a). The third challenge is that most camels milking necessitate the presence of calves beside their mothers to stimulate the udder and for the induction of the milk ejection reflex and milk let-down (Kaskous, 2018b). But to increase the milk yield for each camel and to improve the quality as well as the safety of raw camel milk, machine milking must be used instead of hand milking. However, the daily milk yield was 38% higher in machine compared with hand milking of camels (Hammadi *et al,* 2010). Saleh *et al* (2013) reported that the use of milking machines can reduce the contamination of camel's milk as compared with hand milking. Currently, milking machines are limited to intensive dairy camel farms in a few countries (Nagy and Juhasz, 2016; Ayadi *et al,* 2018; Kaskous, 2018b). The amount of residual milk after machine milking was found high and up to 30% or even more of the stored milk (Ayadi *et al,* 2014; 2018). Milking machine used, therefore, needed to be improved to fit the camel's udder, hence improving milk ejection reflex (Nagy and Juhasz, 2016) and avoiding the problems with the use of the milking machine (Aljumaah *et al,* 2012).

Many studies have shown the impact of teat cup liners on milk performance and udder health in cows (Schmidt *et al,* 1963; Gleeson *et al,* 2004; Zwertvaegher *et al,* 2012). Marnet *et al* (2016) recommended that setting the optimal vacuum level is necessary before definition of the best liner shape and quality for camels.

The pulsation ratio of the milking machine affects milk flow rate and milking time (Thomas *et al,* 1991; Pfeilsticker *et al,* 1995; Hamann and Mein, 1996; Ambord and Bruckmaier, 2009). Bade *et al* (2009) found that increasing the vacuum and b-phase duration increased peak milk flow rate. Hamann and Mein (1996) observed that a d-phase duration of at least 150 ms was enough to relieve congestion and keep the teat healthy.

SEND REPRINT REQUEST TO S. KASKOUS email: skaskous@siliconform.com

Present study was, therefore, aimed to develop a milking machine for camels that is suitable for all teat shapes and measurements, needs a low vacuum to open the teat effectively, have the right kind of pulsation for a stable vacuum on the teat area during milking, have the right type of teat cup liner to quickly and completely extract the milk from the udder, can form the milk ejection reflex without the presence of the calf during milking and can provide the right pulsation rate and right pulsation ratio to achieve an ideal milking process for an increasing milk production with high quality standards.

Materials and Methods

This experimental work was carried out by the Department of Research and Development of Siliconform Company located in Türkheim, Germany on 5 dromedary she-camels with their calves, in different parity and stage of lactation. The animals were kept out on the pasture most of the time. At night and during the cold winter season the camels were kept in the barn in a loose housing system. Camels were fed primarily pasture grass and were also provided with grass hay and supplements of vitamins and minerals. Drinking water was given *ad lib*. The camels were milked once a day (11:00 a.m.) with a unit milking machine-StimuLactor for camels over a period of one year.

Study parameters

This project was done to achieve the right milking technology for camels and to answer the target questions: first, on the technical basis of the milking machine (type of machine, with or without a claw piece, vacuum level, kind of pulsation, type of teat cup liner and pulsation rate and ratio) adapted to the requirements of a camel's udder and teats. Second, on the basis of the suckling behaviour of the calf. The following phases were carried out for the development of the milking machine for camels:

1st phase: A milking machine for camels was made analogous to "MultiLactor" milking machine for cows, which was based on a claw-free quarter separation. The quarter-individual milking system is particularly suitable for different udder and teat shapes, which is of great importance in the case of camels. The claw piece centre was omitted. There was an even distribution of the forces acting on the teat. However, a quarter individual adaptation to each udder quarter was possible*. The developed milking machine was called "StimuLactor" for camels.

* (ST-C), Siliconfom company, Türkheim Germany (2018) (www.siliconform.com).

2nd phase: Determination of the vacuum in the milking machine:

A low vacuum (36 kPa) was tested on lactating camels and was found sufficient for the teat opening and for the milking process as well as without the teat cups dropping during milking.

3rd phase: Determination of the kind of pulsation:

In order to achieve a stable vacuum in the teat cup and a regular milk flow as well as to take into account observations of the calf while sucking, sequential pulsation was used in the new milking machine, which proved to be ideal here.

4th phase: Testing the best teat cup liner for the camel teat:

Seven teat cup liners (1 to 7) were tested with constant vacuum (36 kPa) and pulse rate (90 cycles/min) and pulsation ratio (60:40).

5th phase: Establishment of the pulsation rate and pulsation ratio. Two pulsation rates (60 and 90 cycles/minute) and two pulsation ratios (50:50 and 65:35) were tested.

Characteristics of the new milking technology

StimuLactor for camels (ST-C) was found an easily handled and animal- as well as person-friendly semiautomatic milking system. It was based on a quarter-individual milking system and milking cups worked completely independently from each other (without a claw). Furthermore, the system provided periodic air inlet into the teat cups and was equipped with silicone liners (Figs 1A, B).

The working vacuum level was set to 36 kPa and sequential pulsation (25% each quarter) was adopted. The pulsation rate was 90 cycles/min with a 65:35 pulsation ratio during the milking time. In addition, the system included a very special pre-stimulation program and an excellent cleaning and sanitary process.

The milking routine

First, the animals were gradually trained to the new milking machine technology with the presence of calves for a month, to avoid the physiological-psychological effects at the beginning of machine milking with new technology, since the lactating camels had never been milked either by hand or by machine. Subsequently, the system was successfully installed and the camels became fully acclimatised to it. After the training phase, milking was started without the presence of calves during the milking

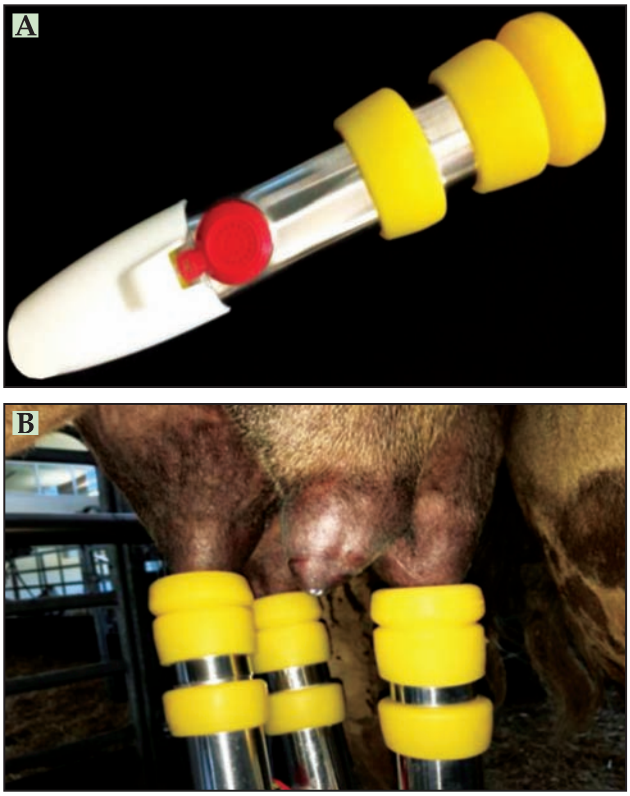

Fig 1. **A)** Teat cup with periodic air inlet for camels, **B)** The StimuLactor during attachment.

process. At the beginning, the milking routine started with pre-milking preparations, in which the teats were cleaned with a wet udder tissue and afterwards dried with another tissue. Then, each teat cup was individually or in pairs manually attached to the teats. Subsequent to this step, the system was started on the control display and the pre-stimulation began. The pre-stimulation is programmed to be intensively stimulated with a normal pulse rate (90 cycles/ min) and reduces the milking phase (b-phase) to 10 % over a period of 90 s. Simultaneously, intensive movement of the teat cups is regulated as an additional stimulation by an actuator. This is an arm on which four milk tubes are placed. With this methods, the liners apply a gentle vibratory massage to the teats, similar to the tongue of a calf. During the pre-stimulation and the milking phases this arm moves up and down. This movement is transferred to the teat cups and make the teats erect. After stimulation the main milk phase begins and the milk flow is observed on the display. When the milk flow has decreased to a certain level, the milking process is automatically stopped by detaching the milking unit. After all animals have been milked, the milking system is cleaned.

Statistical analyses

The received data were processed with Excel and evaluated using statistics program SAS (SAS, 1999). The data were checked for normal distribution. Then, the data were analysed by ANOVA. Significant differences ($P<0.05$) of the study parameters were localised by F-Test.

Results and Discussion

The goal of a camel milking machine is to harvest the total quantity of milk fast and completely whilst maintaining good udder health. However, the characteristics of the milking machine play a crucial role. But camel milking machine and routine needed to be adjusted according to the animals' physiological mechanism in order to achieve optimal milk removal and minimise stress factors during the course of milking (Bruckmaier and Blum, 1998; Marnet *et al,* 2016; Kaskous, 2018b). Although camels are known to be difficult to milk using the existing milking machine (Wernery, 2006; Nagy and Juhasz, 2016). Atigui *et al* (2015) emphasised that the cow's liner used in camels was not adapted to a large basis and short teats. The machine used in present study was improved to fit the camel's udder. Thus, milking machine design and function are critical for rapid and efficient removal of milk without damage to the teat and with minimal risk for transmitting pathogenic microorganisms that might cause mastitis.The new milking machine thus developed was "StimuLactor for Camel" and had many merits when used for camels.

An optimal seat of the milking equipment on the camel udder with an even distribution of the vertical forces acting on the four teats by the milking machine is an important factor for good milking technology. The new milking system used in present study had a cluster-free milking unit, i.e. the teat cups work completely independently of each other. This ensured an even weight distribution per quarter over the entire milking period. There were no disruptions in milk let down due to uncontrolled penetration of air into the teat cup. It offered advantage of no cross contamination with StimuLactor for camels since the milking cups were not connected to each other.

A low vacuum (36 kPa) was sufficient to successfully carry out the milking process in camels to milk gently and to avoid strain on the udder. Since it was a quarter individual milking machine, no loss of vacuum was shown on the teat area during milking. On the other hand, low vacuum was enough to open the teat during milking. Conversely, high vacuum levels were recommended to ensure efficient machine milking for Tunisian Maghrebi camels (Atigui *et al,* 2011). Similar results were shown by Ayadi *et al* (2014; 2018) and milk yield was increased significantly by using higher vacuum (50 kPa). However, these technical settings of the milking machine were not sufficient to empty the udder completely. The amount of residual milk remaining in the udder after milking by injection of oxytocin (20 UI/camel) was estimated to be 30%. It is known that the level of the operating vacuum in machine milking is one of the principal factors which influence the integrity of the tissues and the milk quality (Caria *et al,* 2013). Therefore, Marnet *et al* (2016) recommended that setting the optimal vacuum level is necessary before definition of the best liner shape and quality for camels. Due to the slower induction of milk ejection in camels and a short milking time, many authors use high vacuum levels of machine milking to increase their efficiency (Ayadi *et al,* 2014; 2015; Atigui *et al,* 2014). However, they emphasised that camels can readily be milked efficiently at 50 kPa and 60 pulsations/min without negatively affecting teat condition or udder health (Ayadi *et al,* 2018). The effect of using higher vacuum on udder health and teat condition need to be examined for a long period (not just 10 or 12 weeks). Gleeson *et al* (2003) reported that reducing the vacuum level minimised teat tissue reaction, but extended the cluster-on time and reduced the peak flow rate without affecting milk yield or milk composition. Furthermore, scientists have tried to reduce the vacuum level in the milking machine used on sheep, goats and buffaloes in order to avoid the problems with higher vacuum. The results of present study showed that a low vacuum level modifies the kinetics of milk removal. However, the milk yield was satisfactory at any level tested, showing that low vacuums can be adequate to completely empty the udder (Caria *et al,* 2013).

It is noteworthy that the vacuum level in the range of 37 to 52 kPa did not significantly affect the individual milk production per milking in Mediterranean Italian buffalo cows (Caria *et al,* 2012). Conversely, with increasing vacuum level and wider ratio, the average and peak milk flow rates increased, whereas milking duration decreased (Spencer *et al,* 2007). Atigui *et al* (2015) showed the same results and the best combination of settings for camel milking machines was high vacuum and low pulsation rate (48 kPa/60 cycles per min). A lower vacuum level extended the milking time by more than 100% and was not enough to extract the milk completely from the udder. These results do not agree with our results possibly due to differences in the machine technology. High vacuum levels and vacuum fluctuations that occurred in cows during the milking process had a negative impact on teat conditions and udder health (Hamann, 1990; Hamann *et al,* 1993; Neijenhuis *et al,* 2001; Gleeson *et al,* 2004; Besier *et al,* 2016). High vacuum levels can also lead to increased teat wall thickness (Hamann *et al,* 1993), tissue damage and the development of hyperkeratosis (Bade *et al,* 2007, 2009).

Penry *et al* (2018) reported that increasing teat-end vacuum and suction phase time in the milking machine increased the milk flow rate, but reduced cross sectional area of the teat canal (indicates an increased congestion at the teat-end).

Using a high milking vacuum for camels could lead to udder health problems, which is reflected in a high somatic cell count in the produced milk and a negative impact on the health status of the teats. A positive relationship between increasing working vacuum and somatic cell counts in the milk has been found in buffalo (Pazzona and Murgia, 1992) and other dairy species (Hamann, 1990; Sinapis and Vlachos, 1999; Rasmussen and Madsen, 2000; Mein *et al,* 2003). In addition, it must also be noted that our milking machine used in present study works with a quarter-individual milking system and always provide a constant vacuum on the teats during the suction phase, and there are no fluctuations in the vacuum, as in the case with claw piece milking machines. The investigations by Ströbel *et al* (2016) confirm this statement and the authors observed that a sequential pulsation regime leads to a lower range of vacuum reductions during the suction phase. As a result, these settings can help to improve the udder health of a dairy herd.

Among testing of seven different teat cup liners made from silicon, the best type of liner for camels was number 7 (Fig 2). The amount of harvested milk is essential parameter for camel breeders as it reflects the state of the milking process.

The examined teat cup liners are significantly different from each other, especially as regards structure, density, hardness, elasticity, and head structure and dimensions.

The liner is the only component of the milking machine that comes into direct contact with the camel's teat. However, the liner has the greatest impact on milking efficiency, hygiene and camel comfort in comparison with any other milking machine component. Hence, the use of unsuitable milking liners leads to the occurrence of oedema and enhances colonisation of *Staphylococcus aureus* during the period of machine milking in camels (Juhasz and Nagy, 2008). Model and Rudovsky (1999) observed that bad application of teat cup liner in the milking machine with claw, the germs can be transferred to the next 6-8 cows after milking a cow infected with streptococci. Thus, the kink point of the liner is generally situated in the middle of its barrel (Marnet *et al,* 2016). The shape of the liner barrel (conical or tubular), the diameter of the mouthpiece and softness of the lip, the quality of rubber used are some of the liner's parameters to adapt to avoid too much elongation of the teat or compression ring at the teat base, leading to retention of milk in cisterns (Marnet *et al,* 2015). Silicone teat cup liners used in present study harmonised due to their outstanding milk physiological properties. Many studies have shown the impact of teat cup liners on milk performance and udder health in cows (Schmidt *et al,* 1963; Gleeson *et al,* 2004; Zwertvaegher *et al,* 2012). However, studies on camels have been lacking. Badly slipping teat cup liners may increase new mastitis infection rate by 10-15%, therefore, teat cup liner slip appears to have a most significant impact on udder health (Tranel, 2018). Results from Spencer and Rogers (1991) indicated that machine liner design and construction as well as operating vacuum influence the occurrence of liner slips. Therefore, optimisation of vacuum setting and liner design improved machine milking in present study. An interaction between liner slippage and the mean machine yield per cow and milking was detected; the amount of slippage increased significantly as milk yield increased (O'Callaghan and Harrington, 2000). It is important that teats penetrate into the liner barrel to provide for relief of the teats

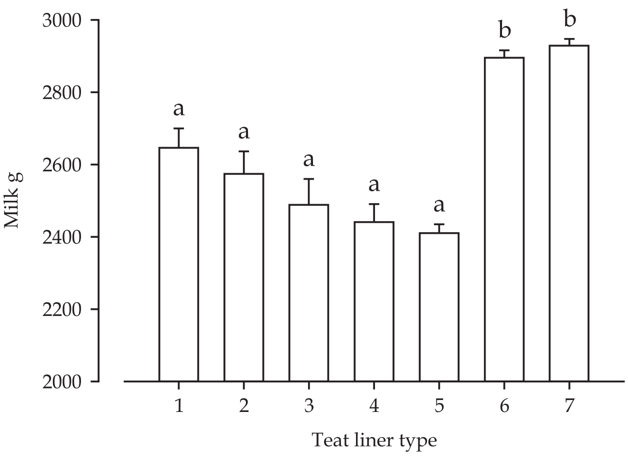

Fig 2. Average daily milk yield (LSM±SE) in examined camels after the application of various teat cup liners in the new milking technology.

during the rest phase. In some liners, teats less than two inches may not be massaged adequately (Tranel, 2018). Finally, the following point must be considered: depending on the teats shape of the majority of the herd, the appropriate size and shape of the liner should be selected. The opening of the teat cup liner head should only be large as big as necessary, but never too small.

Each pulsation cycle contains two phases, the suction phase and the rest phase. During the suction phase of the pulsation cycle the liner is open and milk flows through the teat. During the rest phase, the liner collapses, preventing milk flow. The timing of these two phases is determined by the pulsator's pulsation rate and pulsation ratio settings. However, the pulsation system of the milking machine impacts milk flow rate, milk harvesting time, udder health and milk let-down, which are important factors in animal farm productivity and profit (Spencer *et al,* 2007; Kaskous, 2018c). Hamann (1987) concluded that mastitis could be caused by improper milking techniques, such as inappropriate pulsation settings. In our study, 2 pulsation rates (60 and 90 cycles/min) and 2 pulsation ratios (50:50 and 65:35) were tested. Figs 4 and 5 show the effect of the pulsation rate and the ratio on the daily milk yield after using new milking technology for camels in present study. As shown in Fig (4), a pulsation rate of 90 cycles/min produced a higher daily milk yield compared to 60 cycles/min and the difference was significant ($P<0.05$). The investigations of Atigui *et al* (2015) showed different results. Higher pulsation rate did not improve stimulation of the camel's udder during milking, on the contrary, it induced more bimodality and lower milk flow rate, and the best combination of setting the milking machine for camels was high vacuum and low pulsation rate (48 kPa/60 cycles per min). In our study, the better milk yield after applying the pulsation rate of 90 cycles/min compared to 60 cycles/min is due to two factors, namely using a quarter-individual pulsation system and sequential pulsation (25% each quarter) (Fig 3).

Neijenhuis *et al* (2000) reported that quarter-individual pulsation systems might prevent over-milking and improve the tissue of the teatend. However, the use of a quarter-individual pulsation system led to a positive trend (Sterrett *et al,* 2013), but the author did not find a significant effect on the teat-end condition.

The pulsation ratio is the percentage of time in each cycle spent in the suction phase versus the rest phase. As shown in Fig 5, the pulsation ratio 65:35

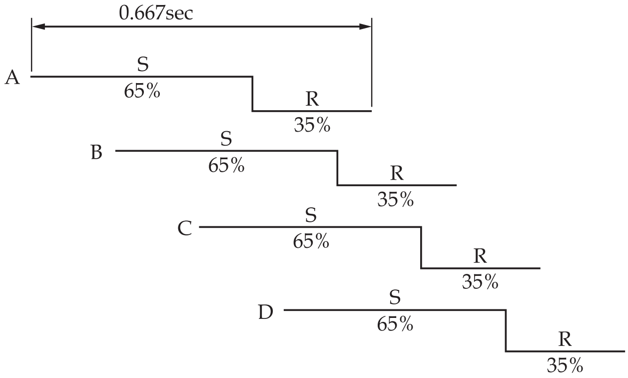

Fig 3. Sequential pulsation in the new milking technology for camels. (0.667 sec.: each cycle in the pulsation rate 90 cycles/min; S: suction phase 65%: R: rest phase 35% and A, B, C and D are four quarters with sequential pulsation 25%).

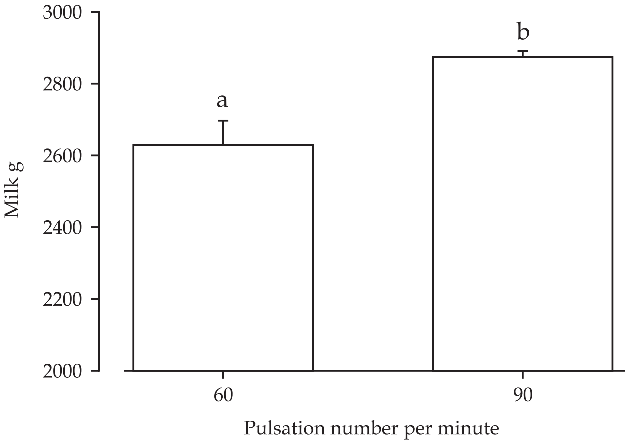

Fig 4. Daily milk yield (LSM±SE) of tested camels after using 2 pulsation rates (60 and 90 cycles/min) in the new milking technology.

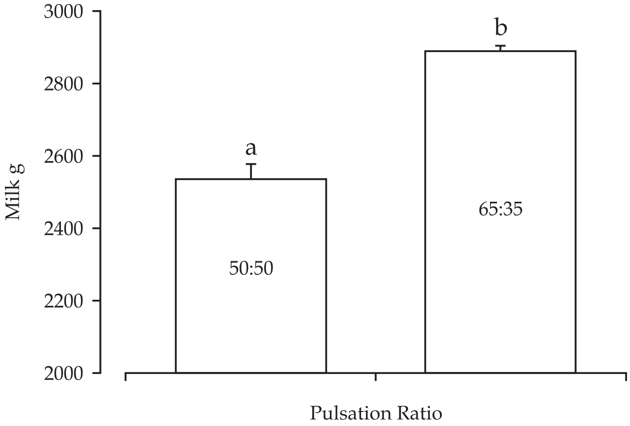

Fig 5. Daily milk yield (LSM±SE) in camels after the application of 2 pulsation ratios (50:50 and 65:35) in the new milking technology.

produced a higher daily milk yield compared to 50:50 and the difference was significant (P<0.05), with no changes on the teat tissue shown. Similar results have been observed in cows in many studies (Gleeson *et al,* 2004; Kaskous, 2018c). It is known that the pulsation ratio of the milking machine affects milk flow rate and milking time (Thomas *et al,* 1991; Pfeilsticker *et al,* 1995; Hamann and Mein, 1996; Ambord and Bruckmaier, 2009). Thus, Bade *et al* (2009) found that increasing the vacuum and b-phase duration increased peak milk flow rate. Hamann and Mein (1996) observed that a d-phase duration of at least 150 ms was enough to relieve congestion and ensure that teat stays healthy. In this study, the rest phase was 230 ms and the sucking phase was 430 ms. These data are calculated with respect to the pulsation rate (90 cycles/min) and pulsation ratio (65:35) used (Fig 3). That is why the teats stay healthy after milking. However, a rest phase which is too short may not allow enough time for blood to move away from the teat-end, resulting in increased teat damage. Kaskous (2018c) showed in dairy cows that milking efficiency could be increased by raising the pulsation ratio from 60:40 to 65:35 without negative effects on udder health in a conventional milking parlor with MultiLactor milking system. The explanation for higher milk yield after changing the pulsation ratio from 50:50 to 65:35 in this study is due to the rapidly harvested milk yield during the milking process. As we know, camel milking time is short and with increasing suction phase, more milk is harvested from the udder in a shorter time. Of course, the amount of stored milk in the udder before milking plays a significant role in the harvested milk, milk flow rate and milking on-time (Kaskous, 2018c). Furthermore, Spencer *et al,* (2007) observed that pulsation ratio and vacuum level are important operating parameters that affect the performance of milking machines. They tested three different pulsation ratios, 60:40, 65:35 and 70:30, and found that the interaction between vacuum level and pulsation ratio had a significant effect on peak flow rate, average flow rate and milking on-time.

Experience through present study with camels has shown that the function of the milking machine must be modelled on the natural sucking process of the calf and principle of the milking machine should be akin to imitate the suckling of calf. Observations on suckling calves clearly showed that a calf is able to extract the total milk yield of a she-camel, including that from the alveoli (Kaskous, 2018b). However, she-camels are sensitive, respond slowly and have difficulty particularly with machine milking. Consequently, camels must be accustomed to entering the milking parlour and being milked by machine, and the farmer must have a basic knowledge of camel behaviour and field experience in dealing with such animals (Wernery, 2006). Camels need more stimulation (up to 2 minutes) than cows in order to evoke the milk ejection reflex (Kaskous, 2018b). An incorrect application of the milking machine, inappropriate use of the milking technique, or a change in milking routines can inhibit milk let down, thus negatively affecting milk production.

Usually, the suckling is a cyclic process, divided into active and resting phases. During the active phase, the calf produces a vacuum at the teat-end within the oral cavity and creates pressure within the teat cistern. In the rest phase, the mouth of the calf relaxes, and consequently vacuum at the teat-end is relieved and tissue rebound is ensured. These effects are mechanically reproduced by the new milking technology StimuLactor for camels used in present study.

Conclusion

- After analysing the first results of the use of StimuLactor for camels, it was shown that a quarter individual milking technology was adapted to the physiological requirements of dairy camels.
- This new milking machine is easy to use for the milker and it requires less effort when attaching the individual quarters compared to the conventional milking machine.
- This milking machine exhibits optimally positioned milking cups, which are necessary to milk at a high level and to keep the animals healthy.
- The calves do not have to be present during the milking process, because this new milking technology reproduces the way the calf suckles.

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DOI : 10.5958/2277-8934.2021.00002.3 Vol 28 No 1, p 11-16

ASSOCIATION OF VITAMIN B12, COBALT AND SULFUR LEVELS IN SERUM AND CEREBROSPINAL FLUID OF DROMEDARY CAMELS WITH NEUROLOGICAL SIGNS

Turke Shawaf

Department of Clinical Sciences, College of Veterinary Medicine, King Faisal University, Al-Hasa, Saudi Arabia

ABSTRACT

The hypothesis of this study was that higher levels of sulfur following chronic digestive disorders in camels could decrease the concentration of cobalt and vitamin B12, which possibly might cause neurological disorders. Therefore, this study was aimed to determine levels of vitamin B12, cobalt and sulfur in serum and CSF of camels with neurological signs. Five apparently healthy camels and 7 dromedary camels with neurological signs like shivering, tremor, staggering, rotation of the head, slight vision impairment and progressive worsening general condition were included in the present study. The diseased animals showed previously chronic digestive problems like constipation, emaciation and weakness. Clinical examination and collection of blood and cerebrospinal fluid samples were done. The concentrations of vitamins B12, cobalt and sulfur in cerebrospinal fluid and serum samples were determined using HPLC assay. There was a decrease in levels of vitamin B12 and cobalt in serum and CSF for affected camels, while there was an increase in the level of sulfur in serum and CSF of affected camels as compared to healthy. The obtained results of serum and CSF in healthy and diseased animals could help in early diagnosis of neurological disorders in camel.

Key words: Camel, cobalt, CSF, neurological, serum, sulfur, vitamin

Neurological signs in camels can be categorised according to its origin into infectious or noninfectious causes (Baaissa *et al,* 2018; Shoeib *et al,* 2019). The neurological signs in camels can be characterised by behavioural and neurological changes, meningitis, encephalitis, meningoencephalitis, stillbirth and abortion (El Dobab *et al,* 2008). Vitamin B12 (cobalamin), which is synthesised in ruminants by ruminal flora (Mohamed, 2006), is closely associated with neurological functions (Nijst *et al,* 1990). Cobalt plays an essential role for ruminal synthesis of vitamin B12 as Co resides at the centre of the circle of vitamin B12 (McDowell, 2000). There are no clinical disorders reported in the literature due to deficiency of cobalt in the diet (Faye and Bengoumi, 2018). Determination of serum or plasma cobalt concentration in dromedary camel is very rare. However, researchers (Deen *et al,* 2004; Shen and X, 2010) studied the levels of cobalt on Bactrian camel. It found in ruminants also that the toxicity of sulfur is mostly related to an increased sulfide production due to the microbial reduction of sulfate in the rumen, which could cause diarrhoea, respiratory and nervous symptoms (Alves de Oliveira *et al,* 1996). However, Hooshmand *et al* (2016) reported a close connection between vitamin B12 and sulfur, which could cause the decrease concentration of vitamin B12 with increased concentration of sulfur neurological disorders (Stabler, 2013). Clinical observation of many camels with neurological symptoms in the eastern region of the Saudi Arabia revealed that they had suffered previously from digestive problems like chronic constipation or that they were in an environment in which the soil and water contained high concentrations of sulfur. However, El Dobab *et al* (2008) observed a high neurological injury in camels consuming water containing high levels of sulfur. Changes in the cerebrospinal fluid composition may reflect central nervous system (CNS) injuries and many pathological processes, because this fluid originates from CNS structures (Lardinois *et al,* 2015). Cerebrospinal fluid analysis forms the essential diagnostic evaluation of ruminants with clinical symptoms involving the central nervous system (Camara *et al,* 2020). Recently, Shawaf *et al* (2020) reported some values of CSF

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constituents from healthy and affected camels with neurological disorders in Saudi Arabia. Although the debate of valuableness of cerebrospinal fluid analysis in the diagnosis of neurological disorders, serum, cerebrospinal fluid and their comparisons may provide a wide range of valuable biochemical and cellular information that help in evaluation of nervous system health of animals (AI-Sagair *et al,* 2005; Camara *et al,* 2020; Welles *et al,* 1992). The hypotheses of the study were the higher levels of sulfur after digestive disorders in camels could cause later decrease concentration of cobalt and vitamin B12, which could cause neurological disorders. Therefore, this study was aimed to determine levels of vitamin B12, cobalt and sulfur in serum and CSF of camels with neurological manifestations and correlate these finding with diagnosis of these cases.

Materials and Methods

In this study, seven diseased dromedary camels (aging 5-14 years) presented to Veterinary Teaching Hospital, King Faisal University were included and these camels showed neurological signs with previous history of chronic digestive problems causing constipation, emaciation and weakness. The predominant neurological signs observed were shivering, tremor, staggering, rotation of the head, slight vision impairment and progressive worsening of general condition. Five apparently healthy camels were used for comparison. All animals were examined clinically and blood samples were obtained from the jugular vein. CSF samples were taken from the atlanto-occipital articulation under sedation (Shawaf *et al,* 2018).

Vitamin B12 analysis

The concentrations of Vitamins B12 in cerebrospinal fluid and serum samples were determined using HPLC assay. Vitamins B12 were purchased from ACROS ORGANICS (New Jersey, USA, 1-800-ACROS-01, Geel, Belgium). Cyanocobalamin (B12, 96% extra pure, HPLC assay, lot: A0304024, code: 405920010). Ethyl acetate, water, ammonium acetate and methanol HPLC grade were purchased from Sigma Aldrich (USA). Serum samples (0.250 mL), 25 µL NaOH (0.5 mM) and 25 µL IS (1µg mL^{-1}) were vortex-mixed for 0.5 min, then 2 mL of ethyl acetate was added and vortexed again for 2 min, followed by centrifugation at 5000 rpm for 6 min at 4°C. The organic layer was separated and transferred into another polypropylene tube, and evaporated to dryness at 37°C under gentle flow of nitrogen gas. The residue was reconstituted with 100 µL mobile phase, and 7 µL was injected for analysis. UPLC/ESI–MS/MS analysis was done according to (Geng *et al,* 2017). An ultra-performance liquid chromatography (UPLCTM) system Acquity (Waters, Mildford, MA, USA) was interfaced to a triple quadruple mass spectrometer (UPLC/MSMS) (TQDTM, Waters Micro mass, Manchester, UK) using an electrospray interface. Vitamins B12 of serum and CSF were separated, using an Acquity UPLC BEH C18 analytical column, 1.7 µm particle size, 2.1mm × 50 mm (Waters). The column was eluted with the mobile phase of methanol: ammonium acetate 5 mM (60:40, v/v) at a flow rate of 0.3 mL min^{-1} with column oven 30°C. Calibration curves were prepared for control and quantification purposes according to Geng *et al* (2017). Serum and CSF samples extracts (after reconstitution in mobile phase) were spiked with different aliquots of Vitamins B12 standard solution to give final concentrations of 6.25, 12.5, 25, 50, 100, 200 and 400 ng/ml.

Cobalt analysis

Concentrations of cobalt were estimated in serum and CSF samples by using AA-7000 Shimadzu (Koyoto, Japan) atomic absorption spectrophotometer coupled with a FAAS Flame Atomic Absorption, GFA-7000 graphite furnace atomizer, and ASC-7000 auto sampler from Shimadzu (Koyoto, Japan) was used. A high-density graphite tube was used for atomisation (Meligy, 2018). The digestion procedures were done by using the microwave method Usero *et al* (2005) by using the Microwave digestion system Model MARSXpress 907511 (CEM Cooperation, Mathews, North Carolina, USA) according to USEPA method 3051. 0.5 gm of each serum samples were placed in [polytetrafluoroethylene (PTFE)] digestion vessels with 6mL of nitric acid (65%) and 2mL of hydrogen peroxide (30%).The samples in the vessels were then digested using an optimised microwave method as described (Meligy, 2018). The contents of the tubes were cooled then diluted to 50 mL with Deionised doubly distilled water (DDDW).

Sulfur analysis

Concentrations of Sulfur were estimated in serum and CSF samples by using high-resolution continuum source atomic and molecular absorption spectrometer (Analytik Jena, Jena, Germany) (Andrade-Carpente *et al,* 2016). The digestion procedures were done by using the Microwave digestion system Model MARSXpress 907511 (CEM Cooperation, Mathews, North Carolina, USA) according to USEPA method 3051 (Usero *et al,* 2005). Each serum sample (0.5

gm) was placed in [polytetrafluoroethylene (PTFE)] digestion vessels with 6mL of nitric acid (65%) and 2mL of hydrogen peroxide (30%). The samples in the vessels were then digested using an optimised microwave method (Meligy, 2018).

Statistical analysis

Statistical analysis was performed after the data has been recorded in Excel spreadsheets and imported into Stata version 14 (Stata Corp., TX, USA) using the GraphPad Prism (v. 5) software. Results were expressed as means ± S.E. of the mean (SEM). Student's t test was used for difference analysis between means. Variation within each parameter was evaluated using coefficient of variation (CV). Effects were considered statistically significant at p value of less than 0.05.

Results and Discussion

Table 1 and figs 1, 2 and 3 showed the serum and CSF levels of vitamin B1, sulfur and cobalt in healthy and camels with neurological signs, respectively. Vitamin B12 levels for serum and CSF of affected camel were 27.3±0.94 ng/dL and 10.3±0.47 ng/dL, respectively which were lower as compared to healthy camels where these were 46.79±1.77 ng/dL; 16.21±1.09 ng/dL, respectively. On the other hand, there were higher concentration of vitamin B12 in serum for healthy and diseased camels as compared to their values in CSF. However, vitamin B12 deficiency was certainly not clinically reported in camel (Faye and Bengoumi, 2018). Lower results for B12 in serum of healthy camel was reported in camel (Mohamed, 2006) and in sheep (Clark *et al,* 1989) compared to its levels in the present study. However, Kather *et al* (2020) reported similar results for vitamin B12 in serum of healthy dogs. CSF vitamin B12 could deliver significant additional evidence to understand some neurological disorders in human and animals (Gianazza *et al,* 2003). In agreement to the present study Christine *et al* (2020) stated a closer relationship between the concentrations of vitamin B12 in serum and CSF. Lower levels for vitamin B12 in CSF of healthy camels in the present study was reported previously in CSF of healthy people (Nijst *et al,* 1990). Christine *et al* (2020) also reported similar results for levels of vitamin B12 in CSF of people affected with neurological disorders. The decreased levels of vitamin B12 in serum and CSF for diseased animals compared to healthy in the present study could be explained by considering that the diseased camels showed neurological symptoms with a previous history of chronic digestive disorders causing emaciation and weakness, which may cause a decreased vitamin B12 levels in the serum and CSF (Friesecke, 1980). The difference values for B12 in serum and CSF of healthy and affected camels in the present study may be due to several factors, including the pathogenesis of the neurological diseases in camels, which are still not well studied (El Dobab *et al,* 2008).

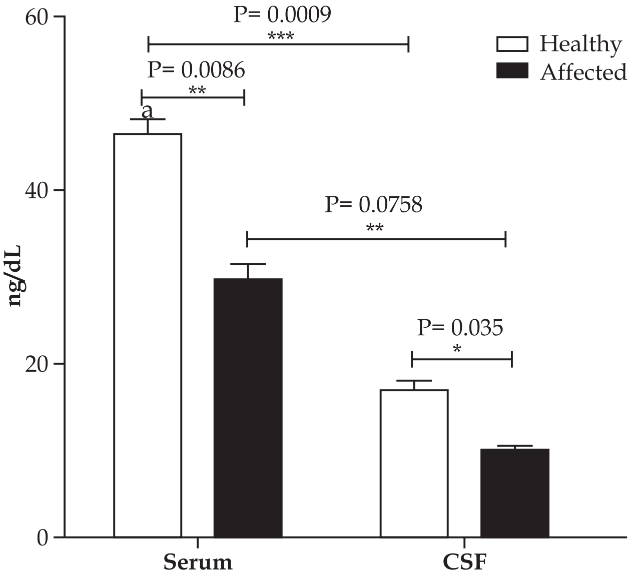

Fig 1. Vitamin B12 levels (ng/dL) in serum and cerebrospinal fluid (CSF) of healthy and camel with neurological signs. *<0.05, (01, ***<0.001.

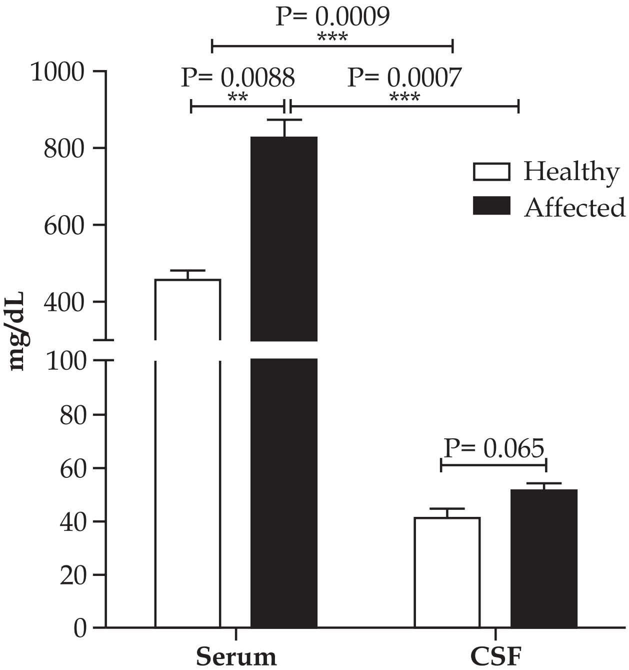

Fig 2. The concentration of sulfur (mg/dL) in serum and cerebrospinal fluid in healthy and camel with neurological signs. NS>0.05, **<0.01, ***<0.001.

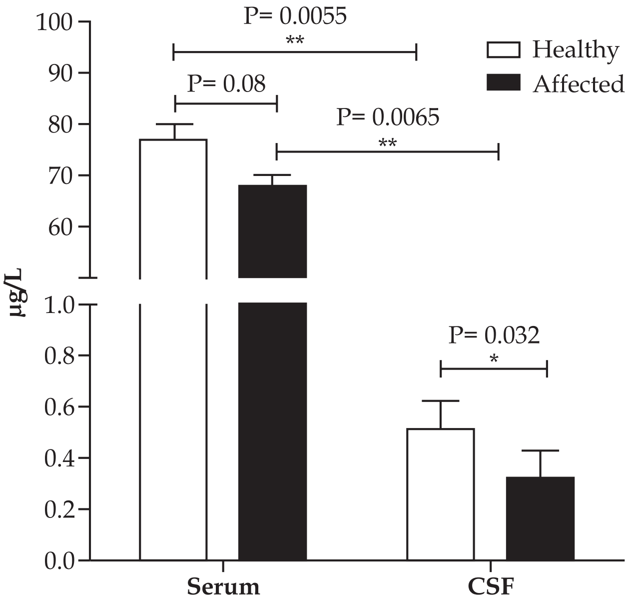

Fig 3. Cobalt levels (μg/L) in serum and cerebrospinal fluid in healthy and camel with neurological signs (NS>0.05, *<0.05, **<0.01).

Cobalt is an essential mineral to mammals in the form of methyl cobalamin (Roos *et al,* 2013). A closer relationship between vitamin B12 and cobalt is important in ruminants. Cobalt is a part of the molecule and essential to its synthesis in the rumen, its deficiency can result in vitamin B12 deficiency (Faye and Bengoumi, 2018). In the present study, there was no significant difference in serum of cobalt levels among healthy (77.18±2.68 μg/L) and diseased (67.56±2.54 μg/L) camels, while the cobalt levels in CSF for affected camel (0.32±0.1 μg/L) was lower than in healthy camels (0.51±0.11 μg/L). In agreement with the present study, Burenbayar (1989) reported similar levels for cobalt in serum of healthy camels, while Shen and Li (2010) and Zongping (2003) reported higher levels for cobalt in serum of healthy Bactrian camels. Similar results for decreased cobalt in diseased sheep was reported by MacPherson *et al* (1976), who stated that the animals with cobalt deficiency showed cerebrocortical necrosis. Sanyal *et al* (2016) reported decreased levels in serum cobalt in people with neurological disorders. The ratio of cobalt concentration in CSF/serum in healthy camels was about 1% in present study, while Stojsavljevic *et al* (2020) reported 10% ratio in human. Contrary to the results of present study, Sanyal *et al* (2016) found equal levels for cobalt in serum and CSF for healthy people. Similar results for decreased levels of cobalt in CSF in diseased camels were reported in people with neurological disorders (Sanyal *et al,* 2016).

In ruminants, it is evident that a diet or drinking water containing high levels of sulfur caused neurological disorders (Alswailem *et al,* 2009; Niles *et al,* 2000) resulting from brain malacia (Rousseaux *et al,* 1991). Sulfur levels in the present study for serum of affected camels (824.6±47.91 mg/dL) was higher as compared to its levels in serum of healthy camels (456.9±23.7 mg/dL). In central Saudi Arabia, Alswailem *et al* (2009) reported diseased camels with neurological disorders living in environment with high concentrations of sulfur in soil and water. The possible relationship between higher levels of sulfur in serum and CSF with neurological symptoms in diseased camels in this study could be explained by the fact that the animals with a previous digestive system disorders could cause the high sulfide production due to the microbial reduction of sulfate in the rumen (Alves de Oliveira *et al,* 1996). There were no significant changes for sulfur levels for CSF in healthy and affected animals (41.5±3.54 mg/dL; 51.87±2.84 mg/dL), respectively. Lower concentrations for sulfur in CSF was reported in healthy people (Gellein *et al,* 2008). In agreement with the results in this study for diseased camels, Gellein *et al* (2008) reported similar values for sulfur concentration in CSF in people affected with neurological disorders. However, Batista *et al* (2013) and Camara *et al* (2020) presented neurological

Table 1. The mean ± SEM with p values of B12, cobalt and sulfur levels in serum and CSF of healthy camels and those with neurological signs.

Parameter		Healthy (N=5)		Diseased (N=7)	
		Mean ± SEM	Range	Mean ± SEM	Range
Vitamin B12 (ng/dL)	Serum	46.79 ± 1.77	28-59.9	27.03 ± 0.94	18.6-35
	CSF	16.21 ± 1.09	9-27.3	10.03 ±0.47	5.9-13.2
Cobalt (μg/L)	Serum	77.18 ± 2.68	69.32-84.12	67.56 ± 2.54	60.14-75.48
	CSF	0.51 ± 0.11	0.12-0.9	0.32 ± 0.1	0.09-0.9
Sulfur (mg/dL)	Serum	456.9 ± 23.7	380-570	824.6 ± 47.91	712-1100
	CSF	41.5 ± 3.54	28.2-52.9	51.87 ± 2.84	38.8-60.3

disorders (Polioencephalomalacia) with significant changes in CSF of sheep and goats correlated with sulfur poisoning from water contaminated by petroleum. In the present study, higher levels for serum sulfur than that in CSF of healthy and affected camels were reported, which is in agreement with previous studies (Gellein *et al,* 2008).

In conclusion, higher levels of sulfur in serum and CSF could decrease the levels of vitamin B12 and cobalt in camels, which possibly precipitated neurological signs. Therefore, vitamin B12 and cobalt might prove logical in treatment of chronic digestive disorders in these animals. The obtained results provide reference values for serum and CSF vitamins B12, cobalt and sulfur levels for further studies and could assist in the diagnosis and treatments of camel neurological disorders.

Acknowledgements

The author acknowledges the Deanship of Scientific Research at King Faisal University for the financial support under Nasher Track (Grant No. 206120). The author appreciate Dr. Melegi, A for his kind cooperation in chemical analysis.

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DOI : 10.5958/2277-8934.2021.00003.5 Vol 28 No 1, p 17-24

COMPARATIVE TRANSCRIPTOME ANALYSIS PROVIDES POTENTIAL INSIGHTS INTO THE MECHANISM OF CAMEL MILK IN REGULATING ALCOHOLIC LIVER DISEASE IN MICE

Xiaoyun Wu[1], Naqin[1], Shiqi Hao[1], Rimutu Ji[1,2] and Liang Ming[1,2]

[1]Key Laboratory of Dairy Biotechnology and Bioengineering, Ministry of Education, College of Food Science and Engineering, Inner Mongolia Agricultural University, 010018, Hohhot, China

[2]Camel Research Institute of Inner Mongolia, 737300, Alashan, China

ABSTRACT

Alcoholic liver disease (ALD) is a general term used to refer to these alcohol-related liver damage. In this study, we investigated the hepatoprotective effect of camel milk (CM) in an ALD mouse model and its underlying mechanism at the transcriptome level. Male C57BL/6NCr were divided into 3 groups: normal diet (NC) ; normal diet, then ethanol (ET); and normal diet, then ethanol and camel milk (ET+CM). Comparative hepatic transcriptome analysis among the groups was performed by Illumina RNA sequencing. The result showed that a total of 526.76±19.87, 563.04±17.84, and 513.56±20.41 million clean reads were obtained for the NC, ET, and ET+CM groups, respectively. Compared with the Et group, 423 differentially expressed genes (DEGs) (including 160 upregulated and 263 downregulated genes) were identified in the NC group, and 186 differentially expressed genes (including 62 upregulated and 124 downregulated genes) were identified in the ET+CM group. The enrichment analyses revealed that the NOD-like receptor signaling pathway, the Toll-like receptor signaling pathway, the MAPK signaling pathway, and mTOR signaling pathway enriched the most differentially expressed genes. The findings of this study provide insights into the development of nutrition-related therapies for alcoholic liver disease (ALD) with camel milk.

Key words: Alcoholic liver disease, camel milk, mice transcriptome analyses

Alcoholic liver disease (ALD) is one of the main causes of chronic liver disease worldwide, including fatty liver, alcoholic hepatitis (AH) and cirrhosis and its complications (Singal *et al,* 2018). ALD can also be superimposed on other common liver diseases, including nonalcoholic liver disease (NAFLD) and hepatitis C virus (HCV) infection, accentuating their prevalence and severity (Dunn and Shah, 2016). Ethanol oxidative metabolism influences intracellular signaling pathways and deranges the transcriptional control of several genes, leading to fat accumulation, fibrogenesis and activation of innate and adaptive immunity (Ceni *et al,* 2014). The investigations have found that intracellular signal transduction pathways, transcription factors, innate immunity and chemokines participate in the pathogenesis of ALD (Gao and Bataller, 2011).

Camel milk has been reported to possess various human health benefits and used as a medicine to treat human diseases such as hepatitis, spleen problems etc. Camel milk is known to exhibit significant antioxidant effects as well as possess protective proteins which includes lysozyme, lactoperoxidase and lactoferrin (Krishnankutty *et al,* 2018). Studies have shown that the milk intake of camels may play an important role in reducing alcoholic liver damage (Ming *et al,* 2020 and Darwish *et al,* 2012). Therefore, CM can be used as a supplementary member in the treatment and management of ALD. Our previous study suggested that camel milk (CM) modulates liver inflammation and alleviates the intestinal microbial disorder caused by acute alcohol injury. In the present study, we investigated the hepatoprotective effects of CM and the underlying mechanism at the transcriptome levels in a mouse model of chronic alcoholic liver disease.

Materials and Methods

Ethics statement and animals

This study was approved by the Review Committee for the Use of Human or Animal Subjects of the Food Science and Engineering College of Inner

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Mongolia Agricultural University (Hohhot, China). All procedures were conducted according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (Publication No.85-23, revised 1985).

SPF male C57BL/6NCr mice (21±23 g) were obtained from Beijing Weitong Lihua Laboratory Animal Technology Co., Ltd. (China; license number SCXK 2016-0006). Mice were maintained in ventilated cages (3 per cage) under the following conditions: 22±2°C; 50 to 60% relative humidity; 12h light and dark cycle. Mice were given free access to food and water. All food, water, and experimental equipment were sterilised before use.

Camel milk administration

Bactrian camel milk was collected from a private camel farm in Bayan Nur City, Inner Mongolia, China, and transported to the laboratory in cool boxes. Milk samples were centrifuged at 3,500 r/min at 4°C for 40 min to remove the milk fat, heated in a water bath at 65°C for 30 min, and freeze-dried under vacuum. Skimmed CM powder was stored at -20°C.

Experimental groups and treatment protocol

In this experiment, the NIAAA mouse model was established by referring to the previous research (Darwish *et al,* 2012 and Ming *et al,* 2020). After one week acclimation, a total of 9 mice were randomly divided into 3 groups: (1) the NC group (n = 3), given a ordinary maintenance feed for 10 weeks; (2) the ET group (n = 3), given a Lieber–DeCarli liquid diet for the first weeks, then an ethanol-containing Lieber–DeCarli liquid diet (i.e., 5% ethanol v/v accounted for 36% the total caloric intake) for another 9 weeks; (3) the ET+CM group (n = 3), given a Lieber–DeCarli liquid diet for the first weeks, then an ethanol-containing Lieber–DeCarli liquid diet and skimmed CM powder (3g/kg of BW; MOH, 2003) dissolved in 0.3mL of double-distilled water and fed for another 9 weeks.

After modeling, the animals were fasted for 9h and anesthetised with isoflurane gas. Liver samples were collected and stored at -80°C.

mRNA sequencing of liver tissue

To clarify the mechanism responsible for the protective effect of CM on liver tissue, RNA sequencing was performed on 9 liver samples: NC0101, NC0112, and NC0113 from the NC group; ET0132, ET0202, and ET0212 from the ET group; and CE0221, CE0242, and CE0321 from the ET+CM group after intervention. High-throughput mRNA sequencing was performed at the Shanghai Ma-jorbio Bio-Pharm Technology Co.

Total RNA was extracted using TRIzol reagent (In-vitrogen, Carlsbad, CA), and the transcriptome library was constructed using the TruSeq RNA Sample Preparation Kit (Illumina), according to the manufacturers' instructions. The libraries were sequenced on a HiSeq 4000 ultra-high-throughput sequencing system (Illu-mina), and all sequences were submitted to the NCBI SRA under accession no. PRJNA680682.

After obtaining the raw data, the sequencing adapters, low-quality reads, and those containing ploy-N were removed using in-house perl scripts. The Q20, Q30, GC content, reads, and bases were then calculated from cleaned raw data. The clean reads were mapped to the reference genome of *Mus musculus* (GRCm38.p6) using TopHat2 software (v. 2.1.1; Dong *et al,* 2019). Read counts for all mapped genes were calculated using RSEM (v.1.3.3; http://deweylab.biostat.wisc.edu/rsem/). Differentially expressed genes (DEGs) were identified using the edgeR package (v. 3.24.3; R Foundation for Statistical Computing, Vienna, Austria) based on $P < 0.05$ and $|\log2\text{-fold change}| \geq 2$. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses of DEG were performed using the relevant databases. The Benjamini–Hochberg approach was used to adjust P-values for controlling the false discovery rate (Benjamini and Hochberg, 1995) and a false discovery rate <0.05 indicated significant enrichment.

Statistical analysis

Statistical significance in the DEG analyses was performed using the R statistical package. Values of $p < 0.05$ were considered statistically significant.

Results and Discussion

Overview of RNA sequencing analysis

After removing the low-quality reads and quality control, a total of 526.76±19.87, 563.04±17.84, and 513.56±20.41 million clean reads were obtained for the NC, ET, and ET+CM groups, respectively (Table 1). The clean GC content of each group ranged from 49.17 to 50.68%, the value of Q20 ranged from 98.92 to 99.05%, and the value of Q30 ranged from 96.29 to 96.71% (Table S1). To evaluate the quality of the RNA-Seq data, the total clean reads were mapped to the reference genome. A high proportion of the clean reads were mapped to the mouse reference genome using TopHat2 (http://ccb.jhu.edu/software/tophat/index.shtml) ; that is, 86.80% from NC, 85.12% from ET, and 85.21% from ET+CM (Table 1). Through TopHat2 analysis, more than 94% of the reads of each group were mapped to known genes, and more than 83% of the reads were mapped to exons. These results indicated the reliability of the RNA-Seq data.

Table S1. Statistics of RNA-seq.

Sample	Reads Number	Bases (bp)	Q20 (%)	Q30 (%)	GC (%)
NC0101	53773458	8037719775	98.97	96.51	50.68
NC0112	53871652	7984617085	99.04	96.69	49.17
NC0113	50382590	7515918115	98.9	96.32	49.69
ET0132	56078612	8370264136	98.92	96.29	49.73
ET0202	58189636	8704281225	98.99	96.49	49.72
ET0212	54642576	8126486791	99.01	96.59	49.5
CE0221	53187818	7909360831	99.05	96.71	49.6
CE0242	49155490	7259103921	99.02	96.65	49.81
CE0321	51725642	7693583159	98.97	96.46	49.49

Table 1. Summary of RNA-sequencing data.

Sample	NC	ET	CE
Total reads ($\times10^5$)	526.76± 19.87	563.04± 17.84	513.56± 20.41
Total mapped reads ($\times10^5$)	500.90± 22.60	532.48± 18.80	485.01± 19.59
Mapped to reference genome (%)	86.80%	85.12%	85.21%
Mapped to gene (%)	95.08%	94.57%	94.44%
Mapped to exon (%)	83.46%	83.87%	83.28%
Mapped to intergene (%)	0.46%	0.42%	0.43%

Gene annotation and functional analysis

The genes were aligned with public databases, such as the RefSeq non-redundant proteins (NR), the Gene Ontology (GO) database, the Cluster of Orthologous Groups of proteins (COG), the Swiss-Port, the Kyoto Encyclopedia of Genes and Genomes (KEGG), and the Protein families (Pfam). As shown in table 2, most of the genes were annotated using the NR database (93.46%), followed by GO (85.83%), COG (84.81%), Swiss-Port (83.12%), KEGG (64.98%) and Pfam (64.59%).

GO is an international standardised gene functional classification system. In total, there were 22287 genes mapped in the GO database (Fig S1). The biological process group possessed more terms than the cellular component and molecular function groups and 65 terms were enriched in biological process (n = 21), cellular component (n = 15), and molecular function (n = 9; Fig S1). The highly enriched GO terms were in the binding (GO: 0005488), cell part (GO: 0044464), cellular process (GO: 0009987), biological regulation (GO: 0065007), organelle (GO: 0043226), and metabolic process (GO: 0008152) groups.

Furthermore, the genes were annotated and classified using the KEGG database. As shown in Fig S2, genes assigned to human diseases (48) occupied the maximum proportion, followed by those assigned to environmental information processing (34), organismal systems (33), metabolism (22), cellular processes (19) and genetic information processing (4).

Table 2. Functional annotation of transcriptome data in three public databases.

Data base	Annotated	Per cent
NR	24269	93.46
GO	22287	85.83
COG	22022	84.81
Swiss-Port	21583	83.12
KEGG	16873	64.98
Pfam	16772	64.59

Analysis of differentially expressed genes (DEGs)

Gene expression levels of NC, ET, and ET+CM were quantified and compared (Fig 1). The genes with a reads per kilobases per million (RPKM) ratio greater than two fold were defined as DEGs. As shown in Fig 1A, a total of 11,817, 11,092, and 10,987 DEGs were identified in the NC, ET, and ET+CM groups, respectively. Among these DEGs, there were 853, 212, and 180 DEGs uniquely expressed in NC, ET, and ET+CM, respectively. Moreover, 10,489 DEGs were commonly expressed in all the groups.

Significant DEGs, including upregulated or downregulated genes, were identified by DEGseq (Fig 1B). Compared with the Et group, 423 DEGs (including 160 upregulated and 263 downregulated genes) were identified in the NC group; 186 DEGs (including 62 upregulated and 124 downregulated genes) were identified in the ET+CM group.

KEGG enrichment analyses of DEGs

To gain insight into the potential mechanisms responsible for the protective effects of camel milk against ALD, we performed KEGG enrichment analysis of DEG in the liver identified by comparisons of the ET and NC groups and the ET+CM and ET groups. Table S2 and Table 3 summarise the KEGG pathways that were significantly enriched in each comparison group.

Through analysis, we found compared with NC group, DEGs related to NOD-like receptor signaling pathway (map04621) and Toll-like receptor signaling pathway (map04620) were significantly down-regulated in ET group (Table S2). Based on this result, it is speculated that ethanol may destroy the NOD-like receptor pathway and activate the Toll-like receptor pathway, and aggravate alcoholic liver damage in mice.

As shown in table 3, the DEGs related to the MAPK signaling pathway (map04010) and the mTOR signaling pathway (map04150) in the ET+CM group were significantly down-regulated compared with the ET group. Therefore, targeting the MAPKs signaling pathway and mTOR signaling pathway may be an effective treatment strategy to inhibit the deterioration of liver injury.

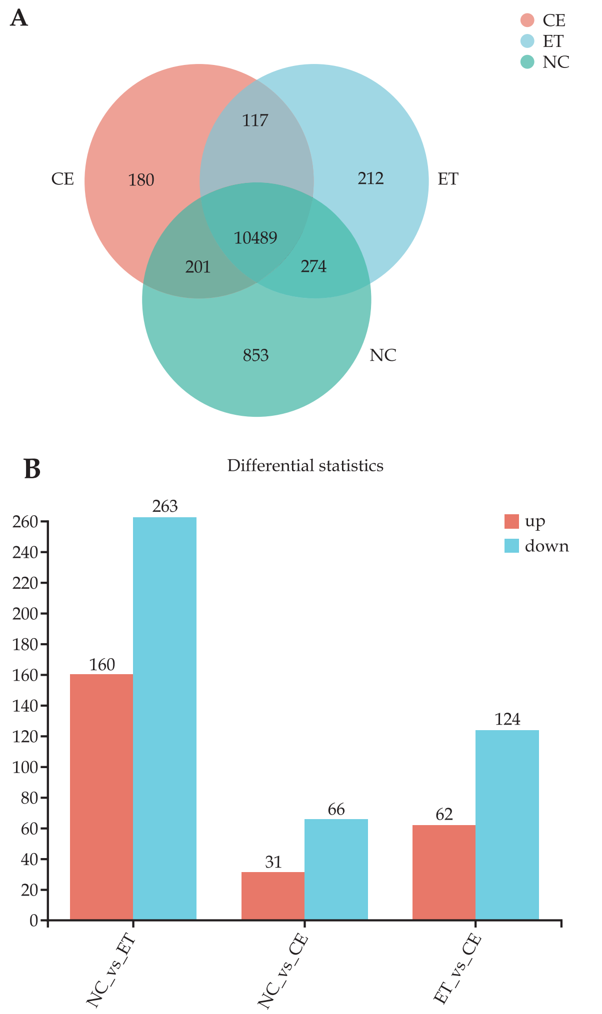

Fig 1. Statistical analysis of the gene expression detected by RNA-sequencing (RNA-Seq). **(A)** Venn diagram of gene counts expressed in the NC, ET and ET+CM groups. **(B)** Number of total differentially expressed genes (DEGs) and down or upregulated DEGs, respectively.

Table 3. Statistics on the KEGG pathway enrichment of DEGs between ET+CM and ET.

Pathway ID	Pathway	ET up	ET+CM up	P Value
map04710	Circadian rhythm	2	1	4.47×10^{-4}
map04350	TGF-beta signaling pathway	2	3	2.54×10^{-4}
map05206	MicroRNAs in cancer	4	2	9.89×10^{-4}
map04978	Mineral absorption	3	0	9.32×10^{-3}
map04550	Signaling pathways regulating pluripotency of stem cells	2	2	8.23×10^{-3}
map04931	Insulin resistance	2	1	1.88×10^{-2}
map04390	Hippo signaling pathway	2	3	1.52×10^{-2}
map00590	Arachidonic acid metabolism	2	1	1.81×10^{-2}
map04933	AGE-RAGE signaling pathway in diabetic complications	1	2	2.33×10^{-2}
map04010	MAPK signaling pathway	3	2	2.70×10^{-2}
map04150	mTOR signaling pathway	5	0	3.35×10^{-2}
map04152	AMPK signaling pathway	3	0	3.69×10^{-2}
map04068	FoxO signaling pathway	2	1	4.72×10^{-2}
map00140	Steroid hormone biosynthesis	1	2	3.34×10^{-2}
map04210	Apoptosis	2	2	4.68×10^{-2}
map04920	Adipocytokine signaling pathway	1	1	4.62×10^{-2}

ET up: the DEGs which were up-regulated in ethanol group, ET+CM up: the DEGs which were up-regulated in ethanol plus astaxanthin group.

The development of alcoholic liver disease (ALD) is a complex process. The increase of oxidative stress and the activation of the innate immune system are essential elements in the pathophysiology of ALD. The oxidative stress of exposure to ethanol is due to the increased production of reactive oxygen species. The antioxidant activity of liver cells is reduced, and

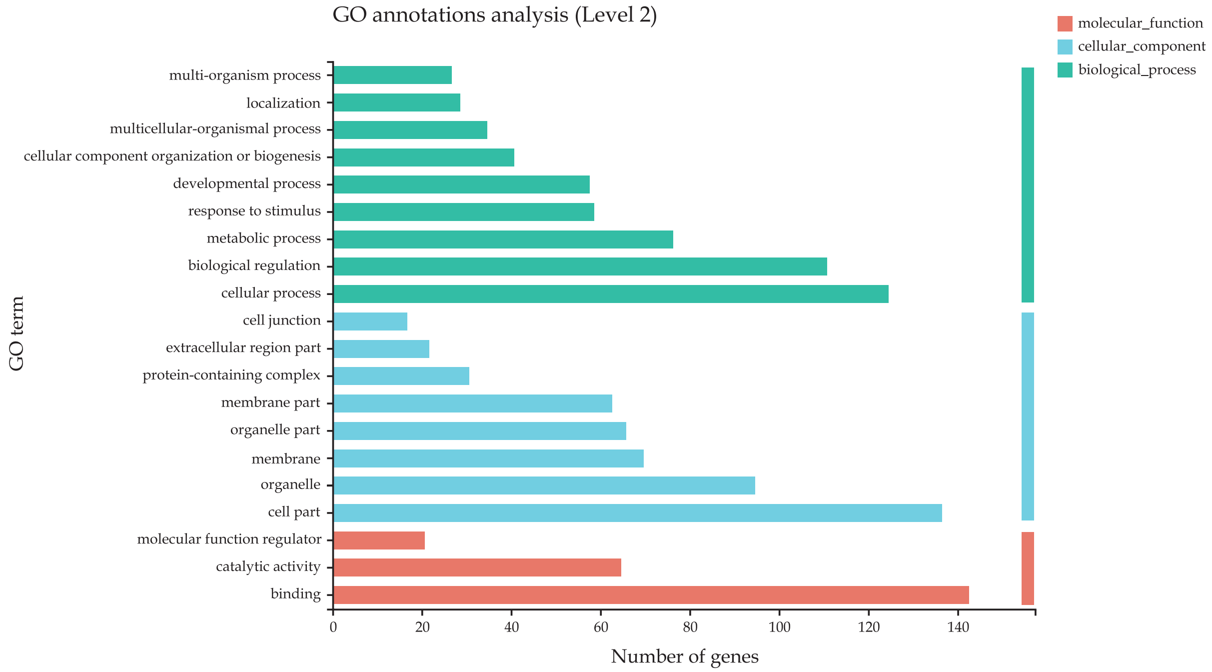

Fig S1. Histogram presentation of gene distribution in Gene Ontology (GO) functional classification. The x-axis represents level to GO terms; the left y-axis represents gene numbers in each GO term. Genes were further classified into sub-groups in biological process, cellular component and molecular function.

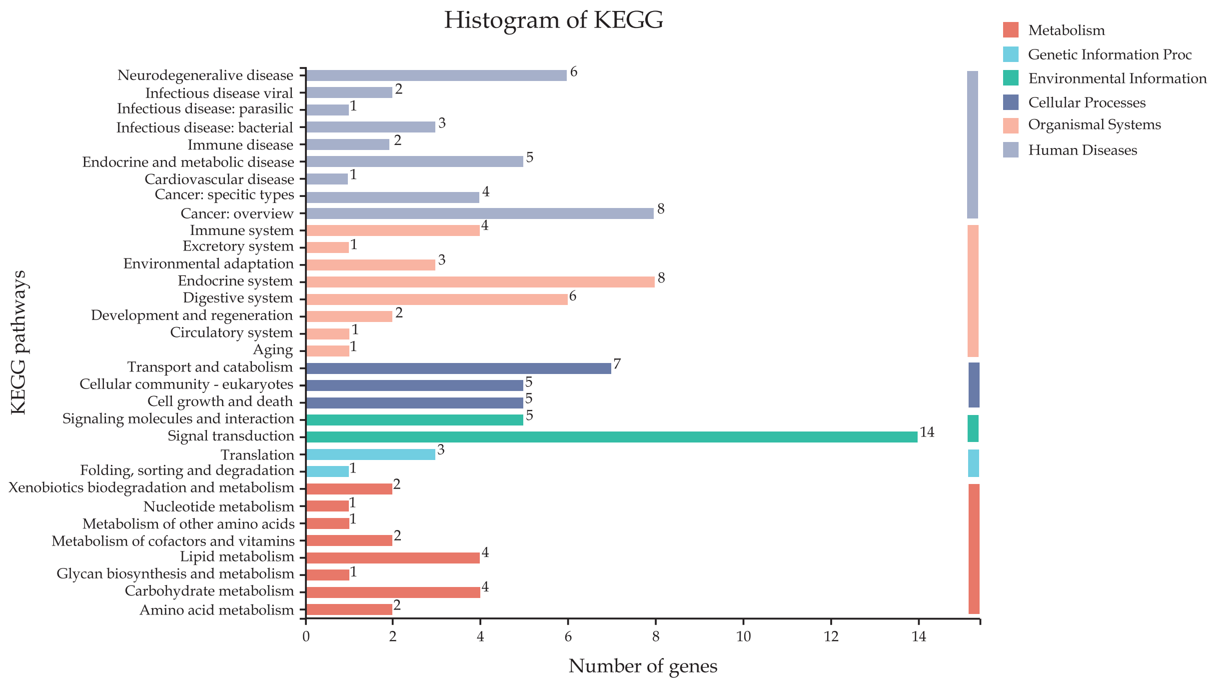

Fig S2. Histogram presentation of gene distribution in KEGG classification. The x-axis represents level to KEGG terms; the left y-axis represents gene numbers in each term. Genes were further classified into sub-groups in metabolism, signal transduction, human diseases and cell process.

the cells and circulating components of the innate immune system are activated by exposure to ethanol, thereby exacerbating ethanol-induced liver damage (Cohen *et al*, 2011). Previous studies have shown that camel milk can enhance the body's immune system (Khan, 2017), reduce the risk of cancer (Badawy *et al*, 2018) and lower blood sugar and anti-thrombotic effects (Korish *et al*, 2020). In addition, camel milk also has a potential protective effect on liver injury, by inhibiting lipid peroxidation, enhancing the

Table S2. Statistics on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment of DEGs between NC and ET.

Pathway ID	Pathway	NC up	ET up	P Value
map00830	Retinol metabolism	4	14	1.31×10^{-13}
map00140	Steroid hormone biosynthesis	1	15	9.86×10^{-11}
map05204	Chemical carcinogenesis	2	13	2.76×10^{-9}
map04640	Hematopoietic cell lineage	18	0	1.76×10^{-8}
map05140	Leishmaniasis	16	1	1.63×10^{-7}
map05169	Epstein-Barr virus infection	22	3	1.13×10^{-6}
map00590	Arachidonic acid metabolism	1	9	2.83×10^{-6}
map05310	Asthma	14	1	1.24×10^{-5}
map05152	Tuberculosis	18	0	1.13×10^{-5}
map05323	Rheumatoid arthritis	16	1	1.67×10^{-5}
map04621	NOD-like receptor signaling pathway	14	1	2.61×10^{-5}
map00591	Linoleic acid metabolism	0	7	2.57×10^{-5}
map04659	Th17 cell differentiation	10	3	2.48×10^{-5}
map04145	Phagosome	18	1	3.80×10^{-5}
map04672	Intestinal immune network for IgA production	14	0	7.85×10^{-5}
map05330	Allograft rejection	15	0	8.61×10^{-5}
map04650	Natural killer cell mediated cytotoxicity	11	2	1.25×10^{-4}
map05320	Autoimmune thyroid disease	15	0	1.39×10^{-4}
map04750	Inflammatory mediator regulation of TRP channels	3	6	1.80×10^{-4}
map00100	Steroid biosynthesis	4	0	1.73×10^{-4}
map05164	Influenza A	13	1	2.08×10^{-4}
map04662	B cell receptor signaling pathway	10	2	2.24×10^{-4}
map05146	Amoebiasis	11	2	2.54×10^{-4}
map05416	Viral myocarditis	16	0	2.93×10^{-4}
map04658	Th1 and Th2 cell differentiation	9	1	3.45×10^{-4}
map05321	Inflammatory bowel disease (IBD)	7	2	3.63×10^{-4}
map00982	Drug metabolism - cytochrome P450	2	5	4.19×10^{-4}
map04664	Fc epsilon RI signaling pathway	10	1	4.38×10^{-4}
map04620	Toll-like receptor signaling pathway	5	2	5.08×10^{-4}
map04623	Cytosolic DNA-sensing pathway	5	1	5.57×10^{-4}
map05150	*Staphylococcus aureus* infection	15	0	5.79×10^{-4}
map04666	Fc gamma R-mediated phagocytosis	11	1	8.99×10^{-4}
map05340	Primary immunodeficiency	11	0	9.71×10^{-4}
map00980	Metabolism of xenobiotics by cytochrome P450	2	5	1.11×10^{-3}
map05322	Systemic lupus erythematosus	15	1	1.74×10^{-3}
map05143	African trypanosomosis	9	0	1.72×10^{-3}
map04060	Cytokine-cytokine receptor interaction	10	1	2.13×10^{-3}
map04010	MAPK signaling pathway	6	6	2.29×10^{-3}
map04380	Osteoclast differentiation	4	3	2.25×10^{-3}
map04062	Chemokine signaling pathway	8	1	2.97×10^{-3}
map05166	Human T-cell leukemia virus 1 infection	10	4	2.90×10^{-3}
map05414	Dilated cardiomyopathy (DCM)	11	0	3.23×10^{-3}
map00030	Pentose phosphate pathway	0	4	3.73×10^{-3}
map04931	Insulin resistance	0	6	4.33×10^{-3}
map04072	Phospholipase D signaling pathway	10	1	4.91×10^{-3}
map00983	Drug metabolism - other enzymes	2	5	5.05×10^{-3}
map05145	Toxoplasmosis	7	0	5.49×10^{-3}
map04933	AGE-RAGE signaling pathway in diabetic complications	3	3	6.45×10^{-3}
map05160	Hepatitis C	7	2	7.10×10^{-3}
map04726	Serotonergic synapse	0	7	7.75×10^{-3}
map04710	Circadian rhythm	1	2	7.63×10^{-3}
map04668	TNF signaling pathway	5	1	8.46×10^{-3}
map00072	Synthesis and degradation of ketone bodies	2	0	9.93×10^{-3}

NC up: the DEGs which were up-regulated in control group, ET up: the DEGs which were up-regulated in ethanol group.

antioxidant defence system, inhibiting cell apoptosis and liver inflammation, and protecting the liver from ethanol-induced liver injury (Hamed *et al,* 2019 and Ming *et al,* 2020). This study mainly discussed the *in vivo* experiment of feeding the mouse model of chronic alcoholic liver injury with camel milk, and performed the transcriptomics analysis on the liver samples of ALD mice. Based on the selected DEGs, many significantly changed GO functions and KEGG pathway enrichment were found. At the same time, it was found that most of the enriched KEGG pathway is related to the immune system and oxidative stress. The study further confirmed that camel milk can effectively prevent liver damage caused by ethanol.

Alcoholic liver disease (ALD) caused by alcohol abuse is the main cause of acute and chronic liver damage (Lamas-Paz *et al,* 2018). In previous studies, it was found that alcohol can damage the immune system-related NOD-like receptor signaling pathway (Liu *et al,* 2019) and Toll-like receptor signaling pathway (Saikia *et al,* 2017) and cause alcoholic liver damage. The NOD-like receptor signaling pathway is involved in the occurrence of inflammatory diseases. Ethanol can destroy the NOD-like receptor signaling pathway in the body and significantly aggravate liver steatosis, inflammation and fibrosis. At the same time, the levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in mice also increase (Ji *et al,* 2020). Improper stimulation of Toll-like receptor signaling pathway plays a key role in inflammation and autoimmunity (Chen and Sun, 2011). Data showed that alcoholic steatohepatitis is not only caused by liver cell damage and reactive oxygen stress, but also by increased binding of specific ligands to their receptors, includes lipopolysaccharide bound to toll-like receptors. Therefore, Toll-like receptor signaling pathway is closely related to alcoholic liver injury (Byun *et al,* 2013). The study found that, the DEGs related to NOD-like receptor signaling pathway and Toll-like receptor signaling pathway were significantly down-regulated in the ET group compared with the NC group. This result may be due to alcohol destroying the NOD-like receptor signaling pathway and Toll-like receptor signaling pathway causing alcoholic liver damage in mice.

Previous studies have also shown that alcohol activates the MAPK signaling pathway (Cui *et al,* 2019) and the mTOR signaling pathway (Chen *et al,* 2018) causes liver damage. Mitogen activated protein kinase (MAPK) pathways are the main signal transduction pathway that controlled cell life and death. The activation of MAPKs is related to oxidative stress in the liver of mice. MAPKs play significant role in a myriad of pathophysiological pathways (Sadek *et al,* 2018). In recent years, a large number of reports have shown that the MAPKs signaling pathway plays a vital role in liver injury-related diseases (Li *et al,* 2017 and Morio *et al,* 2013). This study proved that camel milk can down-regulate the activation of the MAPK pathway and effectively inhibit the activation of MAPKs in a mouse model of alcoholic liver injury. The mTOR signaling pathway is the key to the regulation and treatment of liver injury (Wang *et al,* 2019). More studies have found that inhibition of mTOR signaling pathway can reduce liver fibrosis (Zhang *et al,* 2019), prevent alcoholic liver disease (Tedesco *et al,* 2018) and treat liver damage (Wang *et al,* 2019). The results of the study showed that compared with ET mice, DEGs related to mTOR signaling pathway were significantly down-regulated in ET+CM mice. This result might be because camel milk effectively inhibited the mTOR signaling pathway and interfered with liver damage caused by alcohol intake in mice.

According to this study, we can infer that in ALD mice or patients, camel milk can prevent further liver damage caused by long-term alcohol intake by inhibiting MAPK signaling pathway and mTOR signaling pathway.

Acknowledgements

This work was supported by grants from the Science and Technology Project of the College of Food Science and Engineering (SPKJ201901), and the Inner Mongolia Natural Science Foundation Project (2018BS03017).

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DOI : 10.5958/2277-8934.2021.00004.7 Vol 28 No 1, p 25-32

ER-α EXPRESSION IN THE HYPOTHALAMUS-PITUITARY-GONAD AXIS OF THE BACTRIAN CAMEL (*Camelus bactrianus*)

Jianlin Wang[1], Libaihe Jing[1], Yuanzhang Zheng[1], Peng He[1], Yan Zhang[1] and Yingjie Zhou[2]
[1]Department of Zoology and Biomedical Science, School of Life Sciences, Lanzhou University, Lanzhou, 730000, Gansu, China
[2]Gansu Protection Centre of Endangered Animals in Wuwei, 733000, Gansu, China

ABSTRACT

The aim of this study was to examine the expression of estrogen receptor (ER-α) in the Bactrian camel's hypothalamus-pituitary-ovary (HPO) axis and its significance. Immunohistochemistry (SABC) and Image-Pro Plus 6.0 were used to study the expression of Estrogen Receptor (ER-α). Immunohistochemical analysis revealed that the expression of the ER-α was found in all of three organs in the hypothalamus-pituitary-ovary (HPO) axis. The ER-α immunopositive neurons were found in the main hypothalamus nuclei, which were stained in various degrees. A lot of ER-α immunopositive cells were observed in the pars intermedia of the adenohypophysis. Meanwhile, a small amount of ER-α immunopositive cells were found in the pars distalis near the pars intermedia. In contrast, no ER-α expression was observed in the neurohypophysis. The ER-α immunopositive production was detected in the follicular granules, interstitial gland, corpus luteum and mesenchyme of the ovary. These results suggested that estrogen of the camel acted not only on the sexual gland, but on the various areas of the central nervous system. Thus, we speculated that ER-α took part in the regulation of reproduction, endocrine and cognition in the brain.

Key words: Bactrian camel, estrogen receptor (ER), hypothalamus-pituitary-ovary (HPO) axis

Animals mainly regulate reproduction by Hypothalamus-pituitary-gonadal axis (HPG axis) (McGowan *et al,* 2008). As one of important hormones of regulating animal reproduction on the HPG axis, estrogen acts on target organs by means of estrogen receptor to regulate functions of target organs. It is proved that ER positive product existed in the wide ranges of some nucleus in the hypothalamus, hypophysis and some peripheric organs, such as ovary, spermary, vascular endothelium, smooth muscles, digestive tract, bone tissue, prostate, uterine, oviduct and so on (Gao *et al,* 2008; Takashi *et al,* 2007; Pedram *et al,* 2010; Elvira *et al,* 2009; Qian *et al,* 2011). Estrogen receptor has two subtypes: ER-α and ER-β, distributing different organs and playing different roles. ER-α mainly takes part in reproductive regulation, highly expressed in hypothalamus and brains regions of regulating reproduction, such as bed nucleus of the stria terminalis (BNST). (Corina *et al,* 2007; Deepak *et al,* 2010; Heather, 2007; Liu *et al,* 2008). So far, the study of the ER-α expression on the HPG axis in camels has not been reported. In this paper, we shall review the data obtained in our laboratory regarding the localisation of ER-α on the HPG axis, using the highly-sensitive streptavidin-biotin-peroxidase complex method (SABC). By mean of these results, the connection of different exsit of ER-α on the hypothalamus, pituitary and ovaries is detected. The study offers the morphological basis for the further study of the mechanism of ER-α action, partial rationale for the research of livestock reproductive physiology, animal reproduction, and thermatology.

Materials and Methods

Six aged female Bactrian camels, similar weight and health in clinic, were sampled from Allashan Right Banner of Inner Mongolia in China.

Sampling and preparing histotomy

After arteria carotis communis being chopped up, the heads of the camels were cut open to remove brains. Then the brains were cut sagittally, hypothalamuses were disported. The pituitary glands were taken out and were fixed in the 4% PFA. Ovaries were also removed and fixed in the 4% PFA. After having been fixed for 12h, tissues were fixed in the new 4% PFA 48h. Then tissues were dehydrated using graded ethanol, vitrificated by dimethylbenzene,

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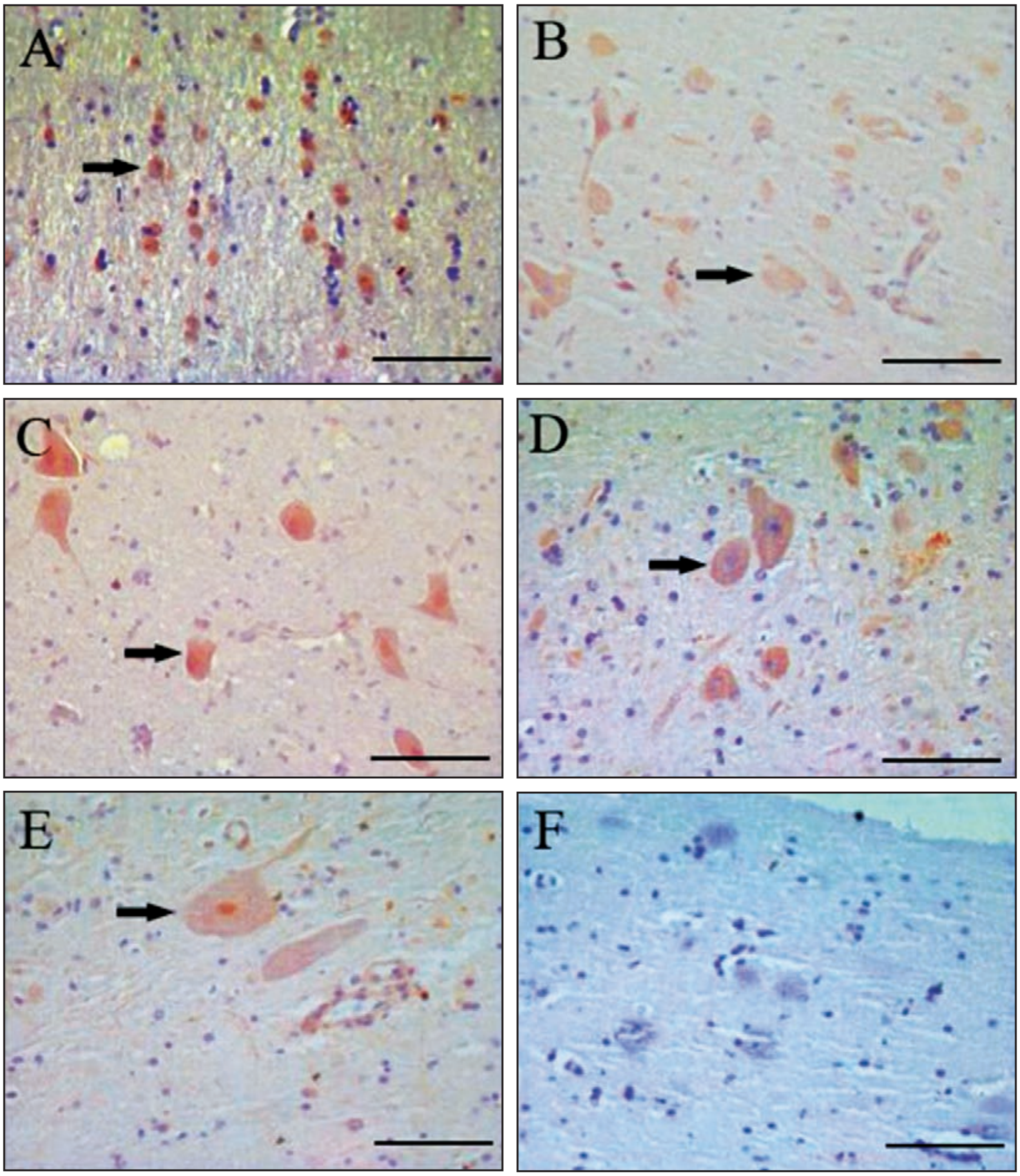


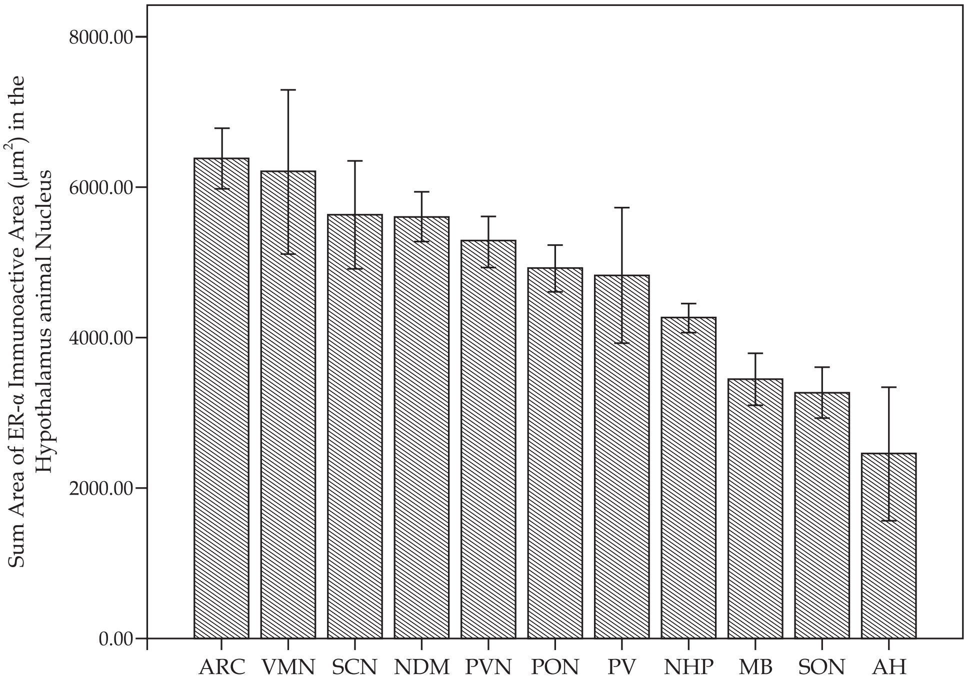
Fig 1. Immunohistochemical localisation of ER-α on the principal Nucleus in the hypothalamus of the camel (400×). **A.** ER-α immunoactive neurons in suprachiasmatic nucleus. **B.** ER-α immunoactive neurons in arcuate nucleus. **C.** ER-α immunoactive neurons in preoptic nucleus. **D.** ER-α immunoactive neurons in ventromedial nucleus. **E.** ER-α immunoactive neurons in posterior nucleus. **F.** None ER-α immunoreactivity neurons in control group. Scale bars, 100μm.

embedded in paraffin, cut into successive slices which were 5 μm thick.

Three suits of slices were attained from these three tissues of every camel for different experiments, including immunohistochemistry, Nissle staining (the hypothalamus nuclei positioning) and HE staining (the histological structure observation to pituitary and ovary), negative control. The hypothalamus nuclei positioning was based on relative atlas in the Systematic Anatomy (Blechman *et al*, 2007; Shimogori *et al*, 2010).

Immunohistochemistry procedures

(1) Sections were deparaffinised in xylene and dehydrated in graded ethanol. (2) Antigen retrieval: after being washed by PBS, the sections were boiled in citrate buffer (10 mM, pH 6.0) for 15 min. Then the buffer was boiled again, followed by a period of cooling. (3) Endogenous peroxidase was blocked by incubation in 0.3% hydrogen peroxide. (4) Following washes in PBS three times for 5 min, the sections were blocked for 1h with 1% bovine serum albumin. (5) The sections were incubated with



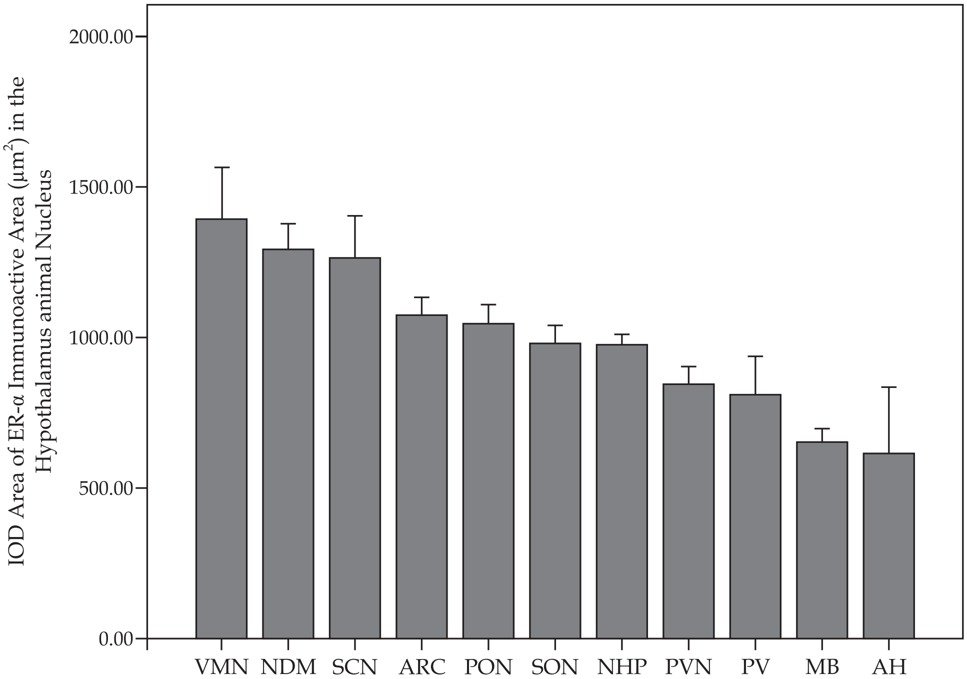


Fig 2. Sum area and IOD of ER-α Immunoactive area (μm^2) in the hypothalamus nucleus.

the primary antibody (ER-α monoclonal antibody, 1:200). (6) Following washes in PBS three times for 5 min, sections were incubated in the biotinylated goat anti-rabbit secondary antibody (1:200) for 2h at room temperature. (7) Following washes in PBS three times for 5 min, sections were incubated in the streptomycin-biotin-HRP complex for 2h at room temperature. (8) Following washes in PBS three times for 5 min, the 3, 3- diaminobenzidine (DAB) were used as chromogen. (9) Sections were then counterstained, dehydrated and coverslipped. For the negative control, sections were incubated in PBS (pH=7.4) instead of the primary antibody (Xu *et al,* 2010).

Observation and Statistical Analysis

The sections of hypothalamus, pituitary and ovary were viewed using the Olympus microscope. 5

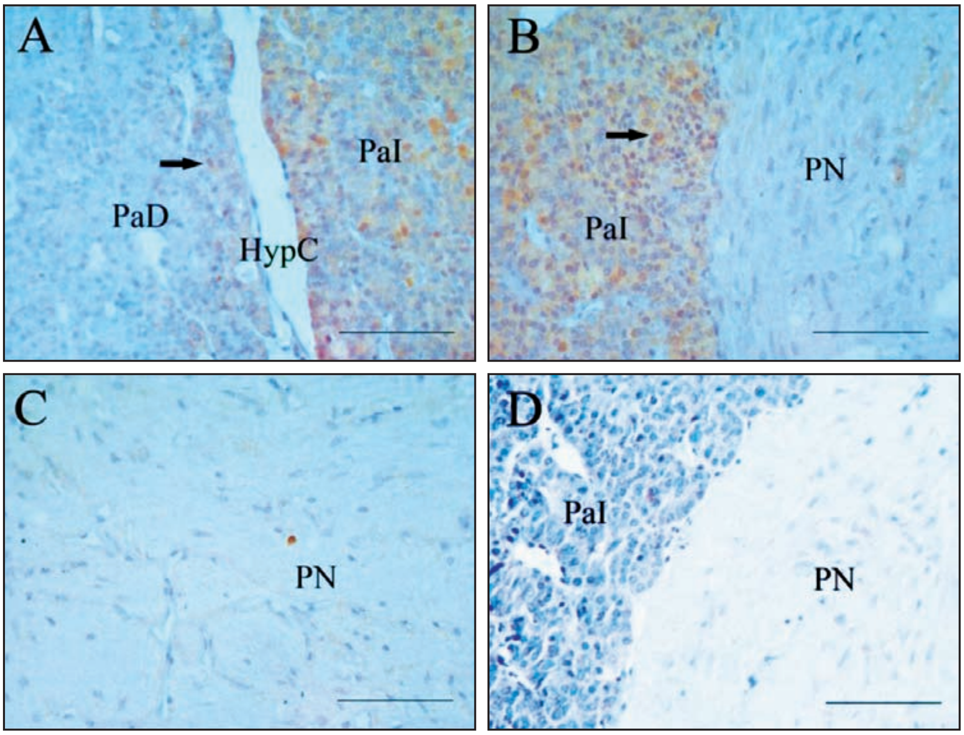


Fig 3. Immunohistochemical localisation of ER-α on the pituitary of the camel. **A.** ER-α immune active in pars distails and pars intermedia; **B.** ER-α immune active in neurohypophysis and pituitary pars intermedia; **C.** ER-α immune active in neurohypophysis; **D.** Negative control. PaD: Pars distails, PN: Neurohypophysis; PaI: Pituitary pars intermedia. HypC : hypophysial cleft, Scale bars=100μm.

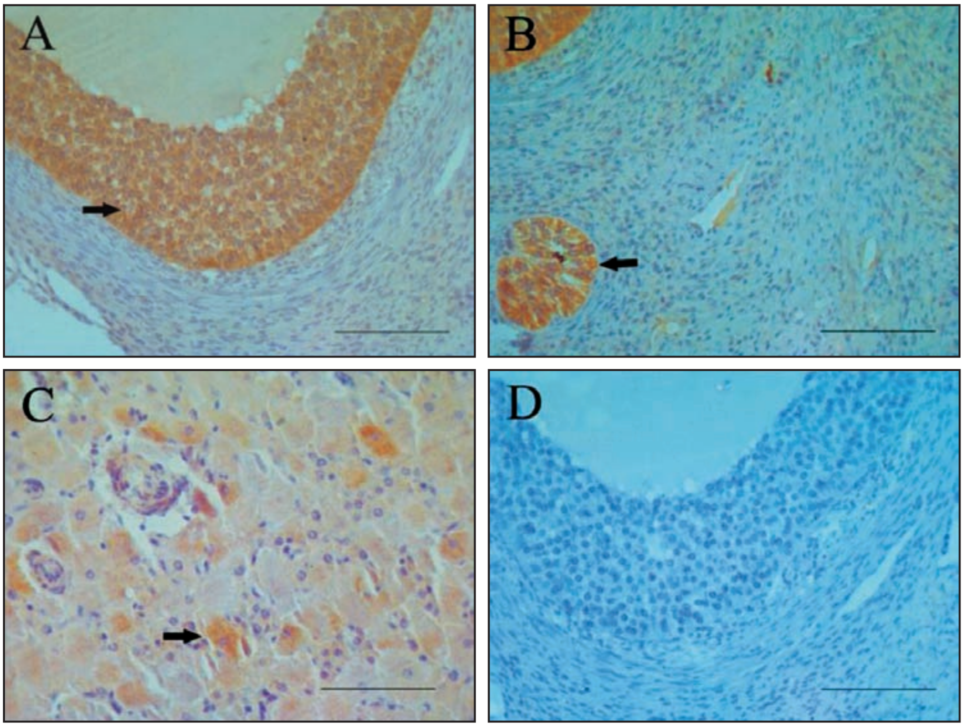


Fig 4. Immunohistochemical localisation of ER-α on the ovary of the camel. **A.** ER-α immune active production in ovarian follicle; **B.** ER-α immune active production in interstitial gland; **C.** ER-α immune active production in corpus luteum verum; **D.** None ER-α immune active production in ovarian follicle. Scale bars=100μm.

fields of vision from two different sections in different areas of each sample using Motic electric microscope. The optical density of ER positive product in the 60 pictures from different areas of 6 camels were analysed by Image-Pro Plus 6.0. All data were assessed for statistically significant differences via a one-way ANOVA and t test. All statistical analyses were carried out using SPSS16. 0.

Results

Results showed that ER-α expression was observed in hypothalamus, pituitary and ovary, but varying in different areas. Especially, the expressing areas and integral optical density (IOD) of ER positive product in the hypothalamus nuclei had more obvious difference.

The distribution of ER-α positive neurons and nerve fibres in the hypothalamus

Examination of Immunohistochemistrical sections (Sagittal plane) revealed that the neurons were unequal positive for ER-α expression in the nucleus, cytoplast and protuberance, various from brown to brownish yellow. In addition, the background was colourless or light brown. On the basis of different nucleius, the styles of neurons were different, including multipolar neurons and two polar neurons. These neurons existed together or dispersedly, having the irregularly round, oval, spindle, triangle somas, etc. ER-α positive nerve fibres were shallow brown filaments or beads in shape between neurons. In accordance with staining intensity of reaction particles in the cytoplasm, the ER-α expression was divided into three grades-strong, moderate and weak expressions. The cytoplasm of strong positive cells was stained in nigger-brown, because of brownish yellow particles fusing together. As for the weakly positive cells, they were slightly stained, approaching to the background. The profiles of these cells were unclear. Of course, the features of moderately positive cells were somewhere in-between. According to the result that the negative controls were stained in blue and had no positive products, the method used in the study was reliable.

In the hypothalamus, ER-α positive neurons existed in the eleven nuclei, such as anterior hypothalamic nucleus (AH), mamillary nucleus (MB), ventromedial nucleus (VMN), arcuate nucleus (ARC), periventricular nucleus (PVN), dorsomedial nucleus (NDM), posterior nucleus (NHP), suprachiasmatic nucleus (SCN), supraoptic nucleus (SON), paraventricular nucleus (PV) and preoptic nucleus (PON). Remarkably, the level of expressions in the different nuclei was variable (Fig 1). Optical density analysis and multiple comparison data analysis revealed that the expressed areas and integral optical density (IOD) of ER positive product of ARC, NDM, SCN and VMN were larger than other nuclei ($P < 0.001$) (Fig 2).

ER-α expression in pituitary

In the pars intermedia of the adenohypophysis, the most gland cells were stained in nigger-brown, being strong positive. In the positive cells, most of which were round, the reaction particles mainly were observed in the cytoplasm (Fig 3A and B). It was analysed that the positive area was 13,072 μm^2 and IOD was 2,798. A small amount of ER-α immunopositive cells were found in the pars distalis of the adenohypophysis. One part of these positive cells existed round the sinusoid capillary and another part unevenly scattered. In contrast, no ER-α expression was observed in the neurohypophysis (Fig 3B and C).

ER-α expression in ovary

ER-α immunopositive production was detected in the follicular granules, interstitial gland, corpus luteum and mesenchyme of the ovary. In the granular cell layers, a mass of ER-α positive cells were found, which mainly were round and round to oval. Majority of these positive cells were strong positive and the reaction particles mainly were observed in the cytoplasm (Fig 4A). According to the statistic data, the positive area had amounted to 19,576μm^2. Meanwhile, the IOD had also reached to 6,514. A lot of positive cells were also examined in the interstitial gland (Fig 4B), whose positive area was 4858μm2 and IOD was 1,679. In the case of corpus luteum, ER-α strongly positive cells were observed in the corpus luteum peripheral. However, weakly positive cells were found uniform distribution in the whole corpus luteum (Fig 4C). Besides, some positive cells were detected in the mesenchyme of the ovary (Fig 4).

Discussion

The (ER-α) expression was found in all the three tissues in the hypothalamus-pituitary-ovary (HPO) axis.

According to this study, ER-α positive neurons existed in the eleven nuclei. ER positive cells of ARC, NDM, SCN, SON, PON and VMN were more than other nuclei, by means of optical density analysis (the expressed areas and integral optical density) and multiple comparison data analysis. Hence, it is construed that ER-α neurons of ARC, NDM and SCN may play a dominant role in regulating reproduction.

The ER-α expression in PVN and SON suggested that ER-α partly mediated the secretion of oxytocin neurons and pitressin neurons activities in PVN and SON, which was in accordance with the result from Zhao and Qing (2005). The fact that ER-α positive cells existed widely in the female hypothalamus indicated some relative conclusions. The hypothalamus was one of the important target organs of estrogen. ER-α participated in or regulated neuroendocrine activities of the hypothalamus. Estrogen played a role in neuron growing development and differentiation, hormone secretion, neurotransmitter synthesis and release and sexual behaviour and so on (Sergei *et al,* 2007). ER-α positive product existed both in cell nucleus and cytoplasm, which was in accordance with the results from Blaustein *et al* (1992) and Mei and Zhang (2009).

A lot of ER-α immunopositive cells were observed in the pars intermedia of the adenohypophysis. Meanwhile, a small amount of ER-α immunopositive cells were found in the pars distalis near the pars intermedia. In contrast, no ER-α expression was observed in the neurohypophysis. Eosinophil was main cells around the pars intermedia. Those Eosinophil cells just right were cellgen of prolactin cells and ER-α immunopositive cells existed around prolactin cells. It was the same with the result from Zhang and Cai (2010) that ER-α play a main role in regulating the lactation central in the hypophysis. In addition, Pelltier and Liao (1988) revealed that ER was relative to the estrogen feedback inhibition of gonadotropin secretion and affected the development of anterior pituitary by mediating estrogen.

ER-α was also found in the ovaries, especially in the granular cells and interstitial gland. Besides, the expression in the corpus luteum was similar with interstitial cells. Above results agreed with the studies from Greene *et al* (1984) in the mammal. Estrogen was mainly produced by the granular cells (Zhan *et al,* 2005) from which the conjecture could be got that ER-α which was highly expressed in the granular cells was involved in the estrogen production. Similarly, on the basis of the facts that the interstitial gland had strongly positive reaction products and could secrete estrogen (Salvetti *et al,* 2009; John *et al,* 2006; Xiong *et al,* 2012), it could be guessed that ER-α from the interstitial gland also took a part in the estrogen production. A small amount of positive cells were observed in the mesenchyme. John *et al* (2006) found that estrogen inhibited the development and functions of the mesenchymale cells via ER-α.

The corpus luteum can synthetise corpus luteum hormone, oxytocin and norepinephrine and so on. In turn, those hormones affect the function of corpus luteum by with the feedback mechanism. Especially, oxytocin directly acts on luteal cells by means of oxytocin receptors on the luteal cellular membranes (Okuda *et al,* 1992). Mature corpus luteum may regulate the synthesis of thyroxine and luteal hormone through autocrine and paracrine (Mutinati *et al,* 2010). Hence, it was envisaged that in the corpus luteum estrogen interacted with ER-α and cooperated with oxytocin, norepinephrine and luteal hormone to regulate hormonal balance and maintain internal environment homeostasis.

Earlier studies indicated that female ER-α gene knockout mice were sterile. The reason was that the development of follicles would stop in preovulation stage and then what happened was not ovulation, but atresia or haemorrhagic cyst (Judith *et al,* 2005; John *et al,* 2004). ER-α expression in the hypothalamus and hypophysis, possibly resulted in ER-α gene deletion and disappearance of negative feedback E2 on the hypothalamus – hypophysis axis. Hence, LH was promoted to release, which led to the ascent of LH in the serum. The increasing secretion of LH would further trigger the ovulation obstacles (John *et al,* 2004; Zhan *et al,* 2018). Thus, it can be guessed that ER-α regulated ovulation by means of negative feedback loop.

The ER-α expression was observed in all the three tissues in the hypothalamus-pituitary-ovary (HPO) axis. The result suggested that estrogen of the camel not only acted on the sexual gland, but also on the various areas of the central nervous system. Thus, it was construed that ER-α took part in the regulation of reproduction, endocrine and cognition in the brain.

The female camel is a kind of induced ovulation animal, and the ovulation inducing factor (OIF) in the sperm of the male camel (Chen and Yun, 1980). The preliminary research of the mechanism of the OIF in the female camel was done by Pan Guangwu using the radiation mark. The result suggested that OIF of the camel acted on the pituitary (Pan and Chen, 2002; Pan and Xie, 2003), but the particular pathway of the OIF regulating the pituitary to control the ovulation remain unclear. According to this study, ER-α immune active production existing on the Hypothalamus-Pituitary-Gonad Axis of Camel was similar with other non-induced ovulation animals such as goats, so clarifying the reproductive mechanism of the female camel depends on a deep research (Zhu *et al,* 2019; Mijidodorj *et al,* 2012; Zhou *et al,* 2019).

Acknowledgements

This study received financial support from the Gansu Natural Fund Project (17YF1NH084). The authors thank Mrs Haiyan Li for her technical assistance and Mr Jizhong Ma for sample collection assistance.

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DOI : 10.5958/2277-8934.2021.00005.9 Vol 28 No 1, p 33-37

IMMUNOREACTIVITY OF ALPHA SMOOTH MUSCLE ACTIN IN THE EPIDIDYMIS OF THE DROMEDARY CAMEL: IMPACT OF THE SEXUAL MATURITY AND THE BREEDING SEASONALITY

Mohamed Alkafafy
Department of Biotechnology, College of Science, Taif University, P.O. Box 11099, Taif 21944, Saudi Arabia

ABSTRACT

This study was carried out to highlight the seasonal variation of the immunoreactivity of the protein alpha smooth muscle actin (α-SMA), within the epididymal duct of the male dromedaries. Immunostaining for detection of α-SMA was applied on paraffin-embedded sections taken from different regions of the epididymal duct from both juvenile and adult dromedaries, during the different seasons of the year. Immunoreactivity (IR) was mainly confined to the smooth muscle cells (SMCs) within the peritubular muscle coat and in the walls of the blood vessels. The intensity of IR displayed a remarkable seasonal variation in adults. The strongest IR has been recorded in all epididymal regions (head, body and tail), during the breeding season (corresponds to winter months). In the season of breeding inactivity (corresponds to summer months), the α-SMA-IR exhibited the lowest intensity. During the period of transition from activity to inactivity (corresponds to spring months) and from inactivity to activity (corresponds to autumn months), a moderate α-SMA-IR has been reported. On the other hand, a weak to moderate immunoreactivity for α-SMA appeared mainly in innermost layers of the peritubular cells surrounding the epididymal duct of the juvenile dromedaries throughout the year. In conclusion, both of the sexual maturity and the breeding seasonality demonstrated a clear impact on the immunostaining for α-SMA in male dromedaries. This may be relevant to the physiological alterations that are linked to hormonal control throughout the year.

Key words: Alpha smooth muscle actin, dromedary camel, epididymis, immunohistochemistry

There are many conflicts and contradictions on the breeding seasonality in dromedary camels (Al Eknah, 2000). Camels may retain their reproductive potency throughout the year. Though male dromedaries show a minimal reduction in the process of spermatogenesis during non-rut season, they still do it throughout the year. Thus, they may be recognised by many authors as atypical seasonal breeders (Zayed *et al,* 1995). Accordingly, the epididymis of the camel show minor seasonal differences both in morphometric and histological features (Zayed *et al,* 2012; Ibrahim and Abdel-Maksoud, 2019).

Actin isoforms are reliable differentiation markers (Skalli *et al,* 1986). α-SMA is principally expressed in contractile cells and is a characteristic isoform and a specific marker for both of SMCs and myoepithelial cells (Skalli *et al,* 1986; Moustafa, 2012). α-SMA is highly expressed in the SMCs in the walls of blood vessels (Skalli *et al,* 1989) and in the peritubular SMCs (Alkafafy and Sinowatz, 2012; Helal *et al,* 2013; Ibrahim *et al,* 2017; Marettova and Maretta, 2018). Immunohistochemical studies showed that the application of a monoclonal antibody, raised against α-SMA, has been reported to be a powerful probe in the study of SMCs differentiation (Francavilla *et al,* 1987).

In a continuing series of studies on the epididymal duct in male dromedaries, the present study has been conducted to use immunohistochemistry (IHC) to underline the impact of breeding seasonality and sexual maturity on the immunostaining for α-SMA within the different regions of the epididymal duct and to interpret their potential morpho-functional correlates.

Materials and Methods

Animals and tissues

Epididymal tissue specimens were obtained from both juvenile (age of 2 years; n =20) and adult (age of 5 years; n =20) clinically healthy, dromedary

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camels (*C. dromedarius*) slaughtered at the central abattoir of Cairo, Egypt. The specimens were collected from a total of 5 animals for each age group during each season, immediately after slaughter. Each epididymis was divided into three parts: head, body and tail.

Chemicals and methods

Specimens were fixed in Bouin's fluid, dehydrated in a graded series of ethanol, cleared in xylene, embedded in paraffine wax and sectioned at 5µm thickness. Tissue sections were mounted on positively charged and coated slides.

Immunohistological techniques

Dewaxed and rehydrated sections were subjected to inactivation of endogenous peroxidases by incubation in 1% hydrogen peroxide (H_2O_2) for 15 minutes. Antigen has been retrieved from sections placed in 0.01 mol/L citrate buffer (pH 6) by heating in a microwave oven (700 watt) for 10 minutes. The sections were blocked by phosphate buffered saline (PBS) containing 5% bovine serum albumin (BSA) for an hour, and then each section was incubated in humidified chamber with the mouse primary antibody (Dako, Hamburg, Germany) at a dilution rate of 1:200, for 1 hr, at room temperature. The sections were washed by PBS for 5 minutes 3 times and incubated with biotinylated rabbit anti-mouse secondary antibody (Dako, Hamburg, Germany) at a dilution rate of 1:300, for 30 minutes at room temperature. The sections were washed by PBS for 10 minutes. Then the secondary antibody was detected with Vectastain ABC kit (Vector Laboratories Inc., USA) firstly each section is covered with 100 x dilution of A and B reagent in PBS (1 µl reagent A+ 1 µl reagent B + 98 µl PBS), then washed by PBS for 10 minutes 3 times and the colour was developed using DAB reagent (Sigma-Aldrich, St. Louis, MO, USA). Sections were counterstained with hematoxylin for 30 seconds, washed in water, dehydrated through graded ethanol, cleared in xylene and mounted with DPX permanent mounting media.

Positive and negative controls

Immunohistochemical negative controls, where each primary or secondary antiserum or the ABC reagent was omitted, gave no positive staining. Positive controls were used according to the instructions provided by the manufacturers of the primary antibodies.

Labelling assessment and photomicrography

The intensity of immunolabelling has been evaluated using a semi-quantitative subjective scoring by three independent observers. A digital imaging system (DM LB light microscope and EC3 digital camera, Leica Microsystems, Wetzlar, Germany) has been used to capture the photomicrographs.

Results

Effect of sexual maturity

In the present work the cytoplasm both of peritubular and vascular SMCs in the epididymal sections from adult dromedaries (Fig 1) showed a distinct α-SMA-immunoreaction when compared to those from juvenile dromedaries (Fig 2). Yet, this immunoreactivity is season-dependent in adults. On the other hand, a weak to moderate immunoreactivity for α-SMA appeared in innermost layers of the peritubular cells surrounding the epididymal duct of the juvenile dromedaries. The peripheral layers of the peritubular SMCs in the same sections displayed a negative to weak immunoreaction. The immunoreactivity was distinct both in head and tail regions but less distinct in the body region. The vascular SMCs were almost strongly reactive especially in the larger vessels.

Effect of the season

The cytoplasm in both peritubular and vascular SMCs in the epididymal sections from adult dromedaries showed a strong positive α-SMA-immunoreaction in the winter (Fig 1 A-C). This reactivity has been markedly declined in the epididymal sections from adult dromedaries in the summer (Fig 1 G-I). The sections taken during spring (Fig 1 D-F) and autumn (Fig 1 J-L) displayed a weak to moderate immunoreactivity. In general, the immunoreactivity was more distinct both in the head and tail regions than in the body region throughout the year. In the juvenile dromedaries, the influence of the season either on the intensity or on the pattern of immunostaining was insignificant (Fig 2). The vascular SMCs were almost strongly reactive especially in the larger vessels throughout the year.

Discussion

A distinct immunostaining for α-SMA has been reported within the cytoplasm of the SMCs both in epididymal tubules and in the walls of the blood vessels in epididymal sections from adult dromedaries during the breeding months. This is in agreement with the findings reported in the testis

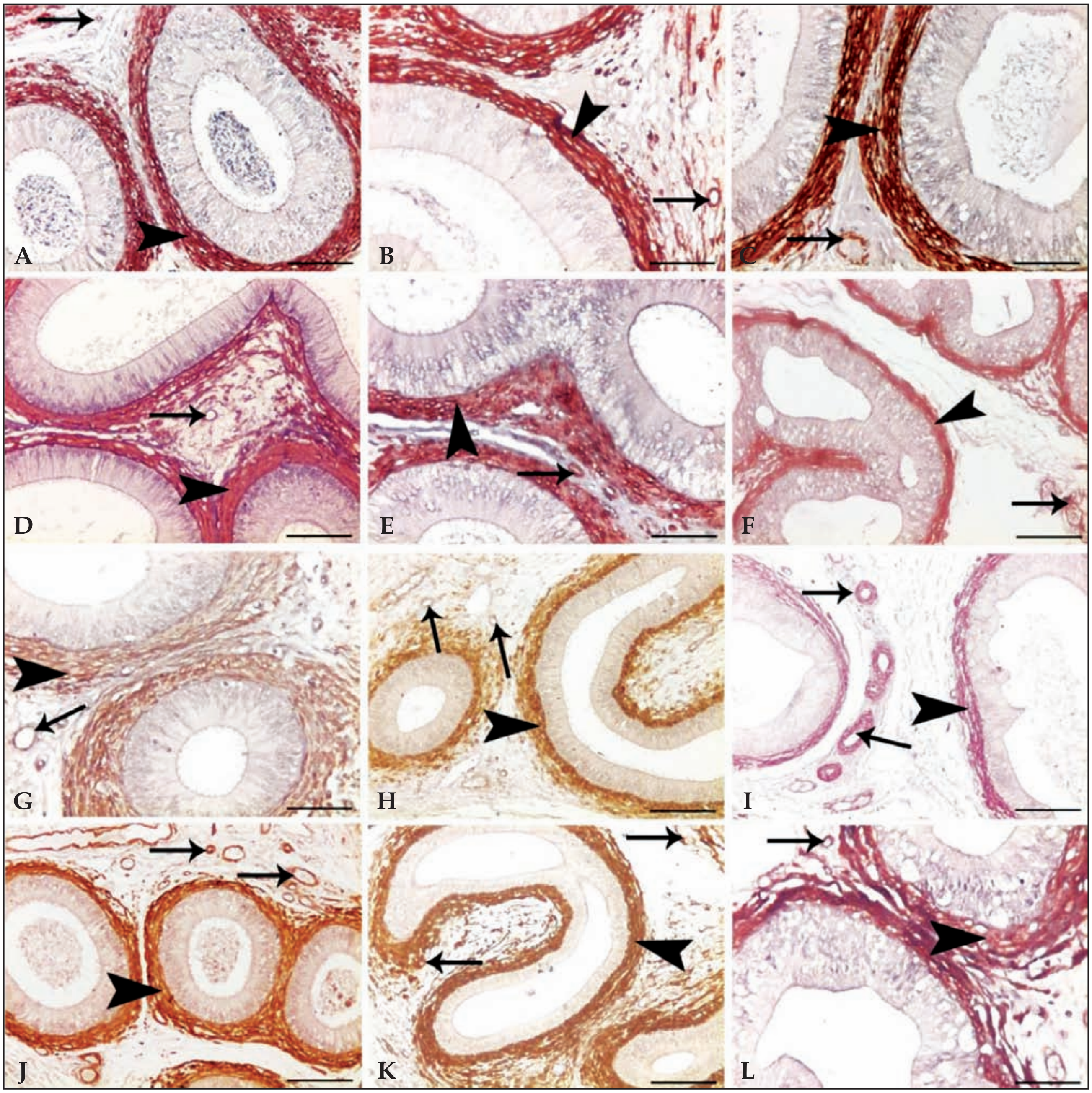

Fig 1. Alpha smooth muscle actin-immunostained adult camel epididymal sections showing Head, body and tail (arranged from the left to the right, respectively); during winter (A, B and C), spring (D, E and F), Summer (G, H and I) and autumn (J, K and L).. The intensity of immunoreaction was season-dependent as shown in the periductal (arrowheads) and the vascular (arrows) SMCs. The strongest intensity was seen in sections during winter and the weakest one was in sections during summer. Scale bars: 100 µm (A, B, C, E, G, I and L) and 200 µm (D, H, J and K).

(Schlatt *et al,* 1993; Moustafa, 2012; El-Azab and El-Mahalaway, 2019), efferent ductules (Alkafafy and Sinowatz, 2012; Ibrahim, 2015); epididymis (Abd-Elmaksoud, 2009; Alkafafy, 2009; Alkafafy *et al,* 2011; Alkafafy and Sinowatz, 2012; Ibrahim *et al,* 2017), ductus deferens (Alkafafy *et al,* 2010; Marettova and Maretta, 2018) and mammary gland (Helal *et al,* 2013) from different animal species.

The differentiation of SMCs has been previously studied using immunolocalisation of α-SMA both in normal and disease conditions (Skalli *et al,* 1989). The cellular differentiation of the peritubular SMCs is related to the emergence of contractile filaments within their cytoplasm. This synchronises with the progressive increase of α-SMA-immunoreaction (Francavilla *et al,* 1987). Our findings in the juvenile

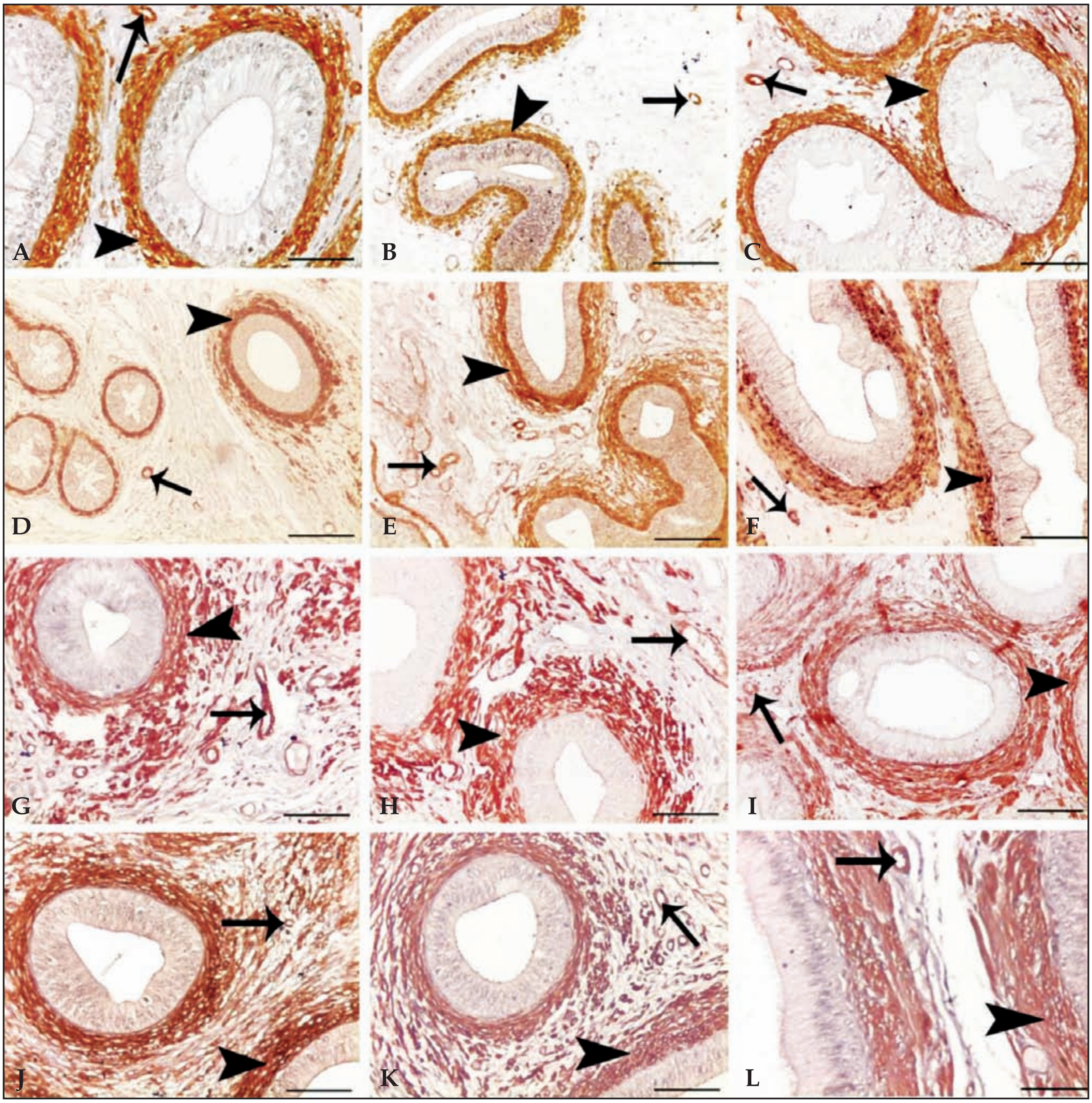

Fig 2. Alpha smooth muscle actin-immunostained juvenile camel epididymal sections showing head, body and tail (arranged from the left to the right, respectively); during winter (A, B and C), spring (D, E and F), Summer (G, H and I) and autumn (J, K and L). The intensity of immunoreaction displayed in the periductal (arrowheads) and the vascular (arrows) SMCs, was weak to moderate during all seasons with minimal season-dependent variations. Scale bars: 50 µm (L), 100 µm (A, G, H, I, J and K) and 200 µm (B, C, D, E and F).

dromedaries go in line with those reported prenatally in the epididymal sections from the bovine foetus (Alkafafy and Sinowatz, 2012) and postnatally in the epididymal sections from new-born rats (Francavilla *et al*, 1987). Additionally, this differentiation may subject to variations during developmental stages (Schlatt *et al*, 1993; Alkafafy and Sinowatz, 2012), breeding seasonality (Ibrahim, 2015; Ibrahim *et al*, 2020) or cyclic functional activity (Helal *et al*, 2013). Thus, the differentiation of the peritubular cells (testis, efferent ductules, epididymis and ductus deferens) during sexual development is a hormone-dependent process and is mainly regulated by androgens (Schlatt *et al*, 1993; Ibrahim, 2015). A similar notion has been suggested in cases of perialveolar and periductal myoepithelial cells in the mammary gland (Helal *et* *al,* 2013) and in the poll gland (Ibrahim *et al,* 2020) glands in female and male camels, respectively. The distinct α-SMA-immunoreactivity in the camel epididymis during breeding season may correlate to the propulsive capacity of the epididymal duct, which is mainly derived from the contractility of the peritubular SMCs (Hinton, 2010).

In conclusion, the spatial distribution of α-SMA was dependent both on season and sexual maturity. Distinct binding sites to α-SMA were consistently evident in the peritubular SMCs throughout the whole length of the duct in adult camels, especially during the months of breeding season. This seasonal variation may be relevant to the hormone-dependent physiological alterations throughout the year.

Acknowledgements

Taif University Researchers Supporting Project number (TURSP-2020/57), Taif University, P.O. Box 11099, Taif 21944, Saudi Arabia

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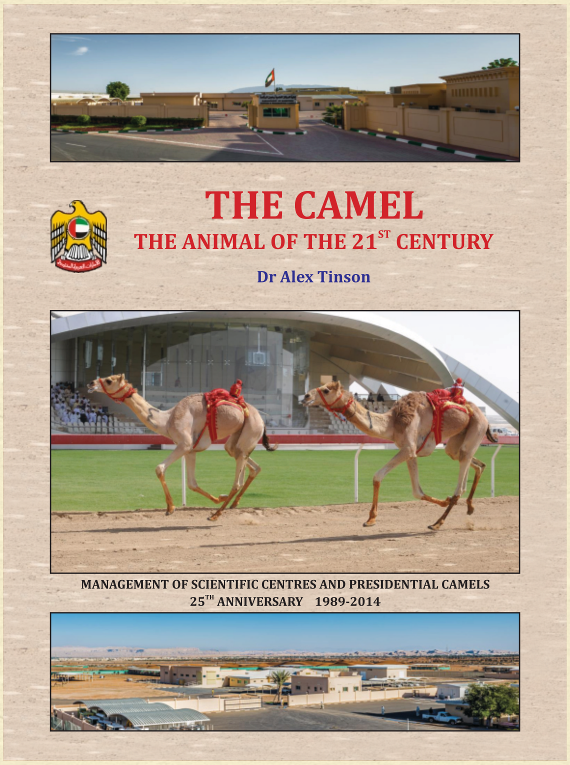
THE CAMEL

THE ANIMAL OF THE 21ST CENTURY

This book authored by Dr Alex Tinson is an acknowledgement to the support and inspiration that His Highness Sheikh Khalifa Bin Zayed Al Nahyan has provided to the centre and to research in general. The last 25 years has been an incredible adventure for us, the noble camel and the people of the U.A.E. Dr Tinson has been involved with many world first's since moving to Abu Dhabi 25 yrs ago. First there was the establishment of pioneering centres in exercise physiology and assisted reproduction. The establishment of the Hilli Embryo Transfer Centre led to five world firsts in reproduction. The world's first successful embryo transfer calf birth in 1990, followed by frozen embryo transfer births in 1994, twin split calves in 1999, pre-sexed embryo births in 2001 and world's first calf born from A.I. of frozen semen in 2013. The hard bound book is spread in 288 pages with 5 chapters. The first chapter involves early history of the centre, world's firsts, world press releases, history of domestication and distribution, evolution of camel racing in the U.A.E. and historical photos the early days. Second chapter comprises camel in health and disease and it involves cardiovascular, haemopoetic, digestive, musculoskeletal, reproductive, respiratory, urinary and nervous systems in addition to the description of special senses. This chapter describes infectious, parasitic and skin diseases in addition to the nutrition. The third chapter is based on Examination and Differential Diagnosis. The fourth chapter is based on special technologies bearing description of anaesthesia and pain management in camels, diagnostic ultrasound and X-Ray, assisted reproduction in camels, drug and DNA testing and surgery. The last chapter entailed future scope of current research.



Author
Dr Alex Tinson

First Edition : 2017

Publisher:
Camel Publishing House
67, Gandhi Nagar West, Near Lalgarh Palace
Bikaner-334001, india
Email : tkcamelvet@yahoo.com

Website:
www.camelsandcamelids.com
www.tkgahlotcamelvet.com

ISBN : 81-903140-5-X
Printed in India

DOI : 10.5958/2277-8934.2021.00006.0 Vol 28 No 1, p 39-45

EVALUATION OF TRANSTRACHEAL WASH (TTW) AND TRACHEAL WASH (TW) IN DROMEDARY CAMELS WITH RESPIRATORY DISORDERS

Turke Shawaf[1], Babiker, H.A.E[1], Ahmad Al Aiyan[2] and Fadlelmula A[3]

[1]Department of Clinical Studies, College of Veterinary Medicine and Animal Resources, King Faisal University, Saudi Arabia
[2]Department of Veterinary Medicine, College of Food and Agriculture, United Arab Emirates University, Al Ain, UAE
[3]Department of Microbiology, College of Veterinary Medicine and Animal Resources, King Faisal University, Saudi Arabia

ABSTRACT

This study aims to analyse Transtracheal Wash (TTW) and Tracheal Wash (TW) samples cytologically from healthy camels and those affected by respiratory disorders. Endoscopy was used to examine the lower respiratory tract and to take TW samples, while TTW was done through using a special needle and catheter. Cytological analysis of TTW and TW fluid were analysed for fifteen camels, six healthy camels and nine camels affected by respiratory disorders. Oral cells were found in the TW sample due to contamination while inserting the endoscope. The TTW procedure is easier, quicker and without the use of an endoscope, compared to the TW procedure. The concentration of neutrophils in the TTW and TW samples of affected camels were higher, compared to the concentration in samples from healthy camels. A lower concentration of macrophages was present in the TTW and TW samples from affected animals, compared to the samples from healthy camels. The cytological analysis of the TTW and TW samples indicated that there was no significant difference between healthy and affected camels.

Key words: Camel, cytology, endoscope, respiratory, tracheal wash, transtracheal

Dromedary camel is predisposed to various respiratory pathogens, such as viral, bacterial and fungal pathogens (Al-Ruwaili *et al,* 2012; Gebru *et al,* 2018; Li *et al,* 2017; Scaglione *et al,* 2017). Respiratory disease in dromedary has received little consideration in the past, although it is an emerging problem, causing significant losses in production and high mortality rates (Alnaeem *et al,* 2020; Bekele, 1999; Fassi-Fehri, 1987). The involvements of camels in racing, during the past three decades, has caused an increase in the prevalence of respiratory problems, which results from contact between animals, transportation of animals and stress during racing events (Elgioushy *et al,* 2020). Tracheal wash (TW) samples are valuable mirrors of the tracheal lumen (Doyle *et al,* 2017; Malikides *et al,* 2003). Transtracheal wash (TTW) is a minimally invasive procedure used to sample the larger airways by enabling exploration of the lower respiratory tract (Doyle *et al,* 2017; Pravettoni *et al,* 2020). Cytological analysis of tracheal aspiration samples has been extensively used in veterinary medicine since the 1970s (Beech, 1975). Cytological and microbiological analysis of TTW and TW samples provide veterinarians essential information about the respiratory tract and associated pathology (Angen *et al,* 2009; Cooper and Brodersen, 2010; Fulton and Confer, 2012). TTW and TW samples are useful in providing an understanding of the stage and severity of the inflammatory reaction in the respiratory tract and detecting subclinical respiratory diseases (Beech, 1975; Caldow, 2001). TTW can deliver samples for a more comprehensive diagnostic approach, compared to that of nasopharyngeal swabs (Cooper and Brodersen, 2010; Doyle *et al,* 2017). Generally, there is limited practical information with regards to the tools which can be utilised for TTW procedure (Pravettoni *et al,* 2020). For the TTW procedure in large animals, trocars or angiocatheters can be used through which a small urinary catheter can be introduced, while for the TW procedure an endoscope can be used (Angen *et al,* 2009; Fulton and Confer, 2012; Shawaf, 2019). There is a lack of research conducted on the cytological analysis of TTW and TW in healthy and respiratory-diseased dromedaries. This study aims to analyse the TTW and TW cytology, comparatively in healthy and respiratory-diseased dromedaries.

SEND REPRINT REQUEST TO TURKE SHAWAF email: tshawaf@kfu.edu.sa

Materials and Methods

2.1 Animals

This study examined a total of 15 camels out of which 6 males and 9 females were aged 2-18 years (median age ± SEM; 9 ± 5.5). Six camels were healthy and 9 were affected by respiratory disease. The healthy camels were selected from a herd stationed at the Camel Research Centre, King Faisal University, Al-Al-Ahsa, Saudi Arabia. A physical and clinical examination was conducted on each camel in the healthy group to ensure that they were free from any apparent disorders. TW was collected from the healthy group and after four weeks, TTW was collected from the same group of animals. Camels with respiratory disorders were randomly selected from camels brought to Veterinary Teaching Hospital, College of Veterinary Medicine, King Faisal University. The main criteria used to distinguish between healthy and diseased camels was clinical history and clinical examination of the respiratory tract. The animals manifesting symptoms such as coughing, dyspnoea and abnormal respiratory sounds, were considered into the respiratory diseased group. TW was collected from 4 affected camels and TTW was collected from the other five affected camels. This study was conducted from August 2019 to September 2020.

2.2 Bronchoscopy and tracheal wash (TW) sampling

Tracheal wash samples were collected through bronchoscopy from all healthy camels and four affected animals while being restrained in the sternal recumbency position. All animals received mild sedation through administering Xylazine 2% (Rompun; Bayer Health Care) @ 0.1 mg/kg body weight. Due to the narrowness of the nasal passage of the camel, the bronchoscope and TW samples were taken via the oral cavity using a mouth gauge specifically developed for camels (Fig 1A). A flexible endoscope (EVIS Olympus, OLYMPUS AUSTRIA Ges.m.b.H., Vienna) with a 12 mm diameter, 300 cm length and supported with an insufflation system, alongwith a light source and irrigation system was used for the bronchoscopy procedure during the TW collection. The endoscope was passed via an opened oral cavity, along the pharynx, through the rima glottidis into the tracheal lumen, up until the bifurcation was reached (Fig 1D). A catheter (EQUIVET; 2.3 mm x 350 cm) was advanced to the trachea through the biopsy channel of the endoscope, 10 mL of sterile saline, at room temperature, was injected through the catheter. The fluid was immediately aspirated back into the syringe from tracheal lumen (Fig 1E). TW samples were submitted to the laboratory for analysis and processed within 15 minutes of collection.

2.3 Transtracheal wash (TTW) sampling

TTW samples were collected from all camels in the healthy group and from 5 camels in the affected group. An EQUIVET IV catheter 14G x 10 cm (BBraun, Milan, Italy) and a 4FG 1.3 mm OD x 50 cm dog urinary catheter (SMI AG, Steinberg, BELGIUM) was used to collect samples from the tracheal lumen. The camels were sedated through intravenous injection of Xylazine @ 0.1 mg/kg. The camels were positioned in sternal recumbency and head was lifted while the neck was extended by an assistant (Fig 1B). A skin surface area of 10 x 10 cm was prepared aseptically on the ventral surface of trachea between the middle and distal third part of the neck. The prepared surface area was locally blocked by subcutaneous infiltration with 5 mL 2% Lidocaine. The trachea was held between the fingers of the operator while a hypodermic needle (14 G x 10 cm) was inserted into the trachea between cartilaginous rings (Fig 1B & C). Two different techniques were used during the insertion of the cannula. The first technique, which was performed on 3 healthy and 2 diseased camels, the cannula was inserted into the tracheal lumen and directed towards the thoracic inlet and was stopped at a point in the middle of the lumen (Fig 1B). During the second technique, which was performed on 3 healthy and 2 diseased animals, the same procedure was followed, but the cannula was kept directed towards the larynx (Fig 1C). During both techniques, the cannula was completely inserted up to the point where the needle grip made contact with the skin surface. A 50 cm catheter was inserted through the cannula into the tracheal lumen. A 50 mL sterile syringe was connected to the catheter and 30 mL of sterile saline solution, at room temperature was injected into the tracheal lumen and immediately aspirated out using the same syringe (Fig 1B & C). Atleast 10 mL of washing fluid was aspirated out. During the procedure, the camel's head was gradually returned to a horizontal position. At the completion of the procedure, the catheter and the cannula were removed. The time spent to perform the complete procedure ranged between 10 and 20 minutes. There were no reported cases of post-procedure complications. The samples were transferred into a sterile single-use tube and immediately transported to the laboratory.

2.4 Cytological analysis of TW and TTW samples

Slides from the TW and TTW samples were prepared for differential cell counts, through centrifugation of 10 minutes at 300 g of undiluted sample. Smears were made from the sample pellet after removal of the supernatant. The air-dried smears were stained with the Diff-Quick stain. The slides were examined under a microscope for mucus cells, bacteria, red blood cells and white blood cells. The differential cell count was performed under oil immersion (X1000) in order to accentuate the specific morphologic characteristics of each cell. The differential counts for 400 cells of macrophages (MAC), lymphocytes (LYM), neutrophils (NEU), mast cells (MAST), eosinophils (EOS) and epithelial cells (EPITH) were counted from each TW and TTW slide. The analysis results for each cell type was expressed as a percentage of total cells.

2.5 Statistical analysis

The obtained data were analysed by using Student's t-test in order to determine the significant difference. It was done through using Graph Pad Prism 7 software in order to determine the range, mean and standard error of the mean. In addition, values normal distribution was evaluated by D'Agostino & Pearson omnibus normality test.

Results

There was no adverse effect on the camels during or after the TTW and TW procedures. Two different techniques were used for the TTW procedure based on the direction of the catheter during the insertion phases (Fig 1B & C). When the catheter was inserted towards the direction of the thoracic inlet (Fig 1B), no red blood cells were detected in the samples but these were seen in the method during

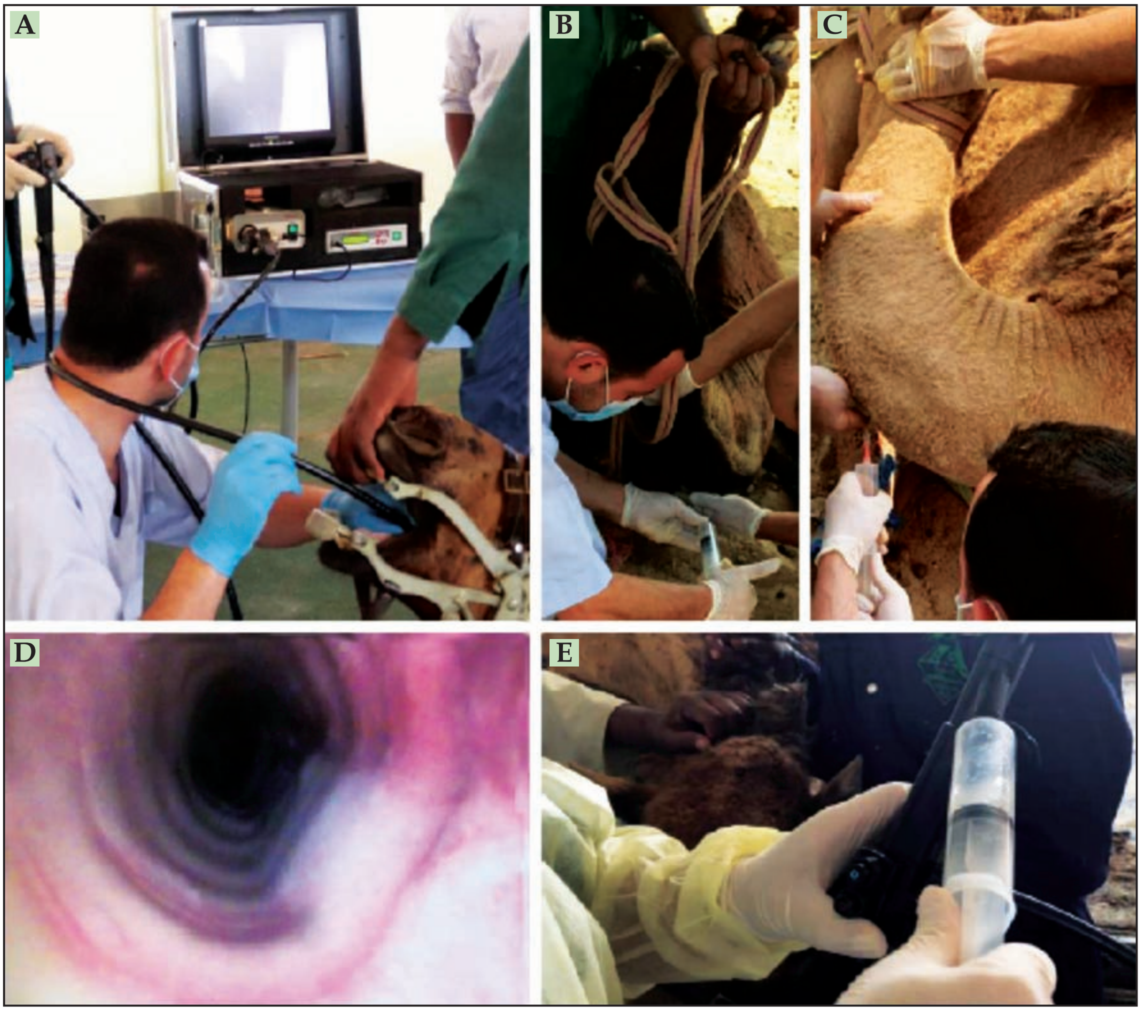


Fig 1. **A:** Bronchoscopic procedure of the lower respiratory tract via the oral cavity using a mouth gauge. **B:** Transtracheal wash (TTW) procedure using a 12G needle and a dog urinary catheter directed towards the lungs. **C:** Transtracheal wash (TTW) procedure in the larynx direction. **D:** Endoscopic image from a diseased camel showing moderate mucopurulent exudate in the thoracic trachea. **E:** Aspiration of a tracheal wash sample (TW) using a bronchoscope.

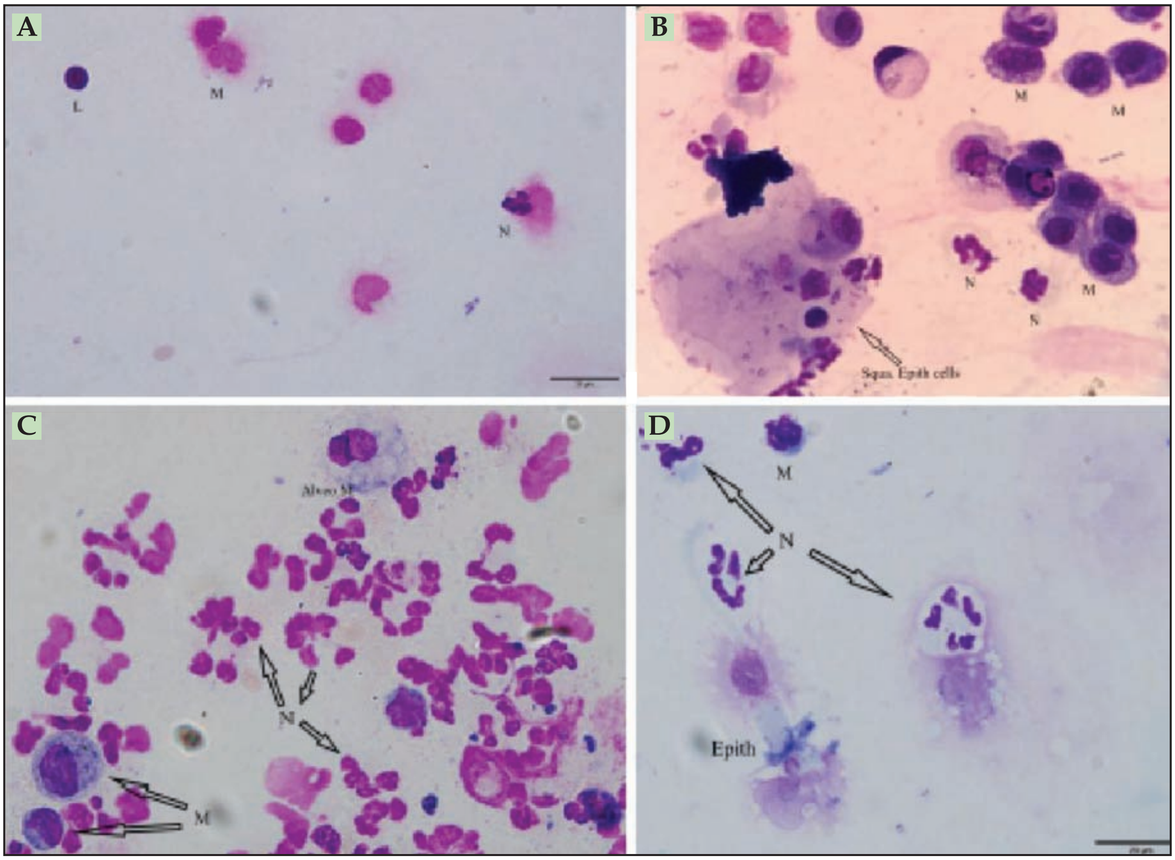


Fig 2. Cytological slides of tracheal and transtracheal wash from camels. **A:** Cell composition of a TTW sample from a healthy camel where lymphocyte (L); macrophage (M); neutrophil (N). **B:** Squamous epithelial cell (Squa. Epith. Cells), several macrophages and neutrophils in a TW sample, contaminated with oral/pharyngeal material from a healthy camel. **C:** Degenerated neutrophils, alveolar macrophage (Alveo. M) and macrophages (M) and pollen particle in a TTW sample from a camel with respiratory disorders. **D:** Neutrophils (N), macrophage (M) and ciliated epithelial cell (Epith) in a TW from a camel with respiratory disorders.

which when the catheter was inserted in the direction of the larynx in 3 samples (one healthy and two affected camels) (Fig 1C). The presence of mucous within the TTW samples were higher when catheter was directed towards the thoracic inlet as compared to the TTW samples collected when directed towards the larynx. Samples obtained from the TW procedure had a higher count of oral epithelial cells and bacteria, compared to the TTW samples (Fig 2D). The cytological analysis results from the TWW and TW samples, for both healthy and affected camels, are summarised in Table 1 and Fig 1, 2, 3 and 4. There was a significantly higher concentration of neutrophils in the TWW (63.6±3.7% P<0.0092) and TW samples (74.4±7.86% P<0.008) of affected camels, compared to the TTW and TW samples from healthy camels (31±4.79%, 26±6.92%).

The concentration of macrophages was lower in the TWW (20.2±1.74% P<0.0097) and TW (22.5±7.98% P<0.032) samples from affected camels compared to healthy camels (51.25±4.87%, 55.4±7.25%) (Fig 3A & B). The lymphocyte cells in the TW samples from affected camels were lower (2.8±0.91% P<0.0038), compared to the samples from healthy camels. There was no significant difference in the mast, eosinophils and epithelial cells in the TTW or TW samples from healthy and diseased camels. There was no significant difference in the cell population for the TTW samples compared to the TW samples from either healthy or affected animals (Fig 3C & D), except for lymphocytes. The concentration of lymphocyte cells was higher in TTW samples as compared to TW samples from affected animals (7.6±1.43%, 2.8±0.91%).

Discussion

The results in the study were compared to the studies of other livestock species because limited information was available about TTW and TW in camels. The TW procedure in camels was found more complicated compared to the the same procedure in

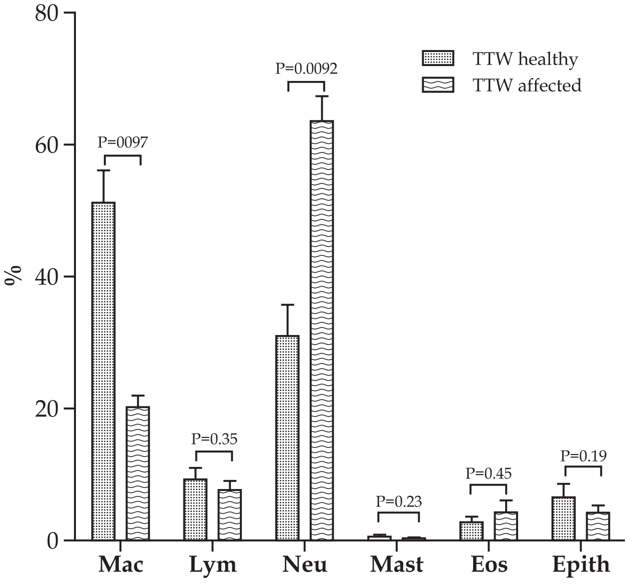


Fig 3a. The differential cell counts of transtracheal wash (TTW) in healthy and affected camels.

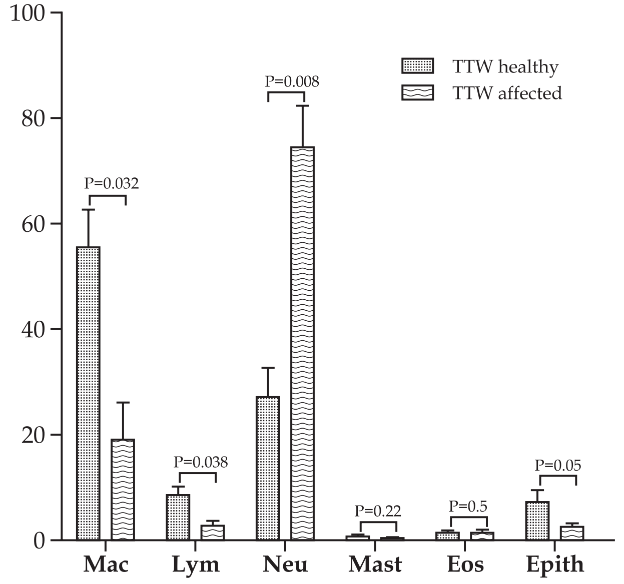


Fig 3b. The differential cell counts of tracheal wash (TW) in healthy and affected camels.

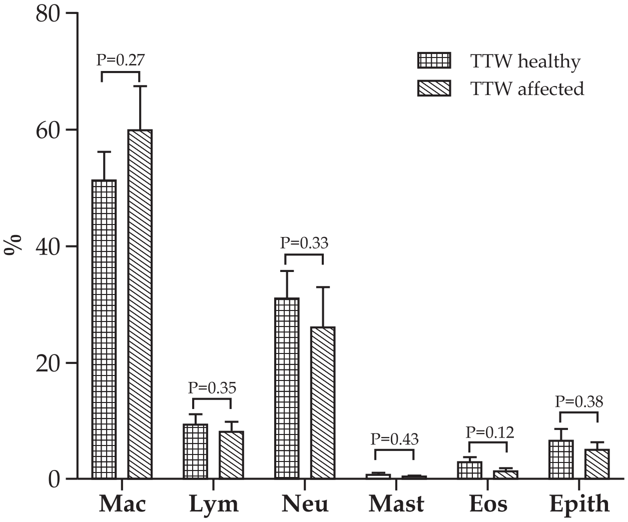


Fig 3c. The differential cells of transtracheal wash (TTW) and tracheal wash (TW) in affected camels.

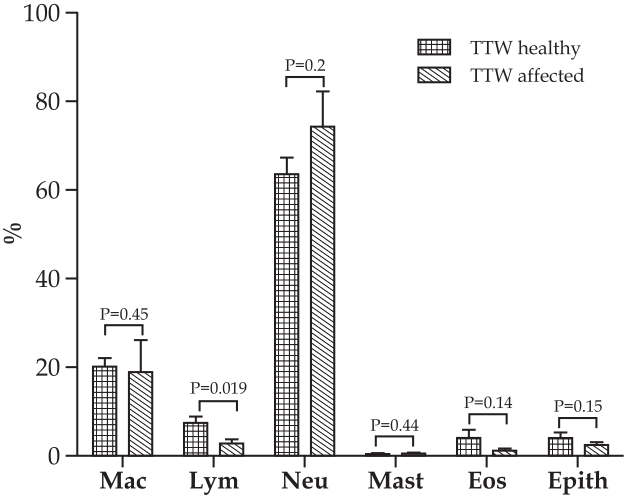


Fig 3d. The differential cells of transtracheal wash (TTW) and tracheal wash (TW) in healthy camels.

Table 1. Differential cell counts (Mean ± SEM and range) of transtracheal wash (TTW) and tracheal wash (TW) samples from healthy and affected camels.

Blood cells (Per cent)	Transtracheal wash (TTW)				Tracheal wash (TW)			
	Healthy		Affected		Healthy		Affected	
	Mean±SEM	Range	Mean±SEM	Range	Mean±SEM	Range	Mean±SEM	Range
Macrophages	51.25±4.87	38-61	20.2±1.74	14-24	55.4±7.25	38-72	22.5±7.98	6-39
Lymphocytes	9.25±1.8	6-14	7.6±1.43	3-11	8±1.83	4-12	2.8±0.91	1-5
Neutrophils	31±4.79	23-42	63.6±3.7	52-72	26±6.92	12-45	74.4±7.86	52-92
Mast cells	0.5±0.29	0-1	0.2±0.2	0-1	0.5±0.29	0-1	0.2±0.2	0-1
Eosinophils	2.75±0.85	1-5	p4.2±1.88	0-10	1.25±0.48	0-2	1.2±0.58	0-3
Epithelia cells	6.5±2.1	1-11	4.2±1.16	1-8	5.25±1.13	3-8	2.4±0.81	0-5

other livestock species, due to difficulties in insert endoscope through the oral cavity possibly due to the anatomical and physiological differences in camels compared to other livestock species (Burger *et al,* 2019; Faye, 2016). Microscopic examination of the TW samples revealed that the samples were contaminated during the TW procedure with squamous epithelial cells and with oral/pharyngeal material from the oral cavity (Fig 2B), which complicates the bacterial analysis (Smith, 2019). On the other hand, the process of aspiring the TTW samples was easier, less complicated, require fewer instruments and was completed in less time compared to the TW procedure. TTW sample collected while the catheter was directed towards the larynx contained red blood cells due to haemorrhage during sampling. No blood was observed in the TTW samples when the catheter was directed towards the lungs. This study agreed with Doyle's statement that each method of sample collection from the lower respiratory tract had its own advantages and disadvantages (Doyle *et al,* 2017). However, Doyle *et al* (2017) reported that the TTW procedure was more effective than the TW procedure to detect pathogenesis in bovines. Prevalence of most abundant macrophage cells in the TTW and TW samples from healthy camel were in agreement with previously published data (Chemuturi *et al,* 2005; Couetil and Thompson, 2020; Rossi *et al,* 2018; Shawaf, 2019; Vaught *et al,* 2018). The decreased cell count of macrophages in the TTW and TW samples from affected camels in the study was in agreement with the results of previous studies (Doyle *et al,* 2017; Rossi *et al,* 2018; Shawaf, 2019; Vaught *et al,* 2018). The lower cell count of macrophages in samples from affected camels was associated with the elevation of other cells, such as neutrophils (Smith, 2019). The lymphocyte cell population of the TTW and TW samples from healthy and affected camel were lower than that reported for other livestock species (Angen *et al,* 2009; Chemuturi *et al,* 2005; Rossi *et al,* 2018; Shawaf, 2019). Contrary to previously published data on other livestock species (Brazzell *et al,* 2006; Rossi *et al,* 2018; Shawaf, 2019; Vaught *et al,* 2018), we found higher levels of neutrophils in the TTW and TW samples from healthy camels. The increased values of neutrophils in TTW and TW samples in healthy camels can be explained by the higher levels of neutrophils in the blood of camels as compared to other species (Hussen *et al,* 2017). The increase of neutrophils in both the TTW and TW samples from affected camels compared to the samples from healthy camels in present study was in agreements with previous studies (Brazzell *et al,* 2006; Couetil and Thompson, 2020; Rossi *et al,* 2018; Shawaf, 2019; Smith, 2019; Vaught *et al,* 2018). The lower count of mast cells and eosinophils in the TTW and TW samples from healthy and affected camel was in agreement with other researchers (Rossi *et al,* 2018; Shawaf, 2019), who stated that these cell populations played a more critical role in bronchoalveolar lavage (BAL) than in TW. However, Shawaf (2019) reported a higher eosinophils cell count in TW samples from healthy and affected donkeys, when compared to the results of present study. The TTW and TW epithelial cell count in this present study was lower for affected camel compared to the same cell count for healthy camels, which was in contradiction to previous studies (Riihimaki *et al,* 2008; Wysocka and Klucinski, 2015).

In conclusion, TTW and TW were helpful techniques in diagnosing lower respiratory tract diseases in camels. No significant difference was found between TTW and TW in camels. The TTW procedure was more practical and did not require an endoscope in comparison to the TW procedure.

Acknowledgements

The authors acknowledge the Deanship of Scientific Research at king Faisal University for the financial support under Nasher Track (Grant No. 206142). The authors appreciate Dr. Mohamed Al Ali to assist with samples collection.

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DOI : 10.5958/2277-8934.2021.00007.2 Vol 28 No 1, p 47-51

MOLECULAR CHARACTERISATION OF GROWTH HORMONE (GH) GENE IN INDIAN DROMEDARY AND BACTRIAN CAMEL

Ved Prakash, Basanti Jyotsana, Shalini Suthar, Kashi Nath, Rakesh Ranjan and R.K. Sawal

ICAR- National research Centre on Camel, Bikaner-334001, India

ABSTRACT

Molecular characterisation of 613 bp long growth hormone (GH) gene fragment spanning partial exon-1, intron-1, exon-2 and partial intron-2 was done in Indian (one humped) dromedary and Indian (double humped) bactrian camel through PCR amplification, sequencing and bioinformatics analysis. The sequence variations within and between single and double humped Indian camels were observed. In the Indian single humped camels of Mewari, Kachchhi and Bikaneri breed at position 264 C>T variation was seen. At this particular locus animals with single and double peaks in the sequence chromatograms were observed. Accordingly, two allele (C, T) and three genotype (CC, CT, and TT) were identified in the Indian dromedary camels. In Indian Bactrian camel at position 264 only C allele and CC genotype was identified. In double humped camel at position 242, A>G and at position 469 G>A transition variation was observed compared to single humped camel. The different sequences obtained were submitted to NCBI and Gen Bank Accession No. MT478653 for C allele Indian Dromedary, MT478654 for T allele Indian Dromedary and MT478655 for Indian Bactrian camel were obtained. Similarity ranging from 98% to 100 % was observed with available GH Sequences of camel at GenBank. Camel sequence was found to have close similarity with other Camelidae family members like *Lama pacos* (97.07%) and *Lama glama* (96.58%). The evolutionary relationship between sequences showed close relationship between dromedary and bactrian camel followed by vicugna and llama. The domesticated species like cattle, buffalo, sheep, goat, yak and mithun were distantly related to camel. The present study showed close similarity between GH gene sequence pattern of Indian one and double humped camel except transition variation at two positions in bactrian camel.

Key words: Bactrian, camel, dromedary, growth hormone (GH) gene, sequence

The efforts to improve the productivity of camels can be accelerated by supplementing the conventional genetic improvement programmes by molecular genetic techniques. Camel presents a unique case where success of conventional breeding methods and constrained by the biological and economical limitations on the applying desired selection intensity, as a result of which, response is affected, so is the selection process. However, with the advancement in molecular genetics technology, the identification of molecular markers and their subsequent utilisation in the breeding programme has become possible. To maximise the benefits of the camel production potential, improved understanding of the genetics underlying their unique biology is needed. Till date, there are relatively few published studies in the area of camel genetics and genomics. The candidate gene strategy involves study of genes that are supposed to be responsible for a considerable amount of the genetic variation for the traits of interest based on their known physiological function (Moioli *et al,* 2007). In farm animals, promising candidate genes for many traits are located in the growth hormone axis. The growth hormone is a polypeptide hormone with diverse biological activities. It is necessary to select genotypes with high growth and meat quality for more contribution of camels to the agricultural economy (Ramadan and Inoue-Murayama, 2017). Growth hormone (GH) is an anabolic hormone which plays an important role in postnatal longitudinal growth, tissue growth, lactation, reproduction as well as protein, lipid and carbohydrate metabolism (Dybus, 2002; Daverio *et al,* 2012). Among the several candidate genes, growth hormone (GH) gene structure and its role in farm animals like cattle, buffalo, sheep and goat production is widely studied. But only few reports on growth hormone gene in camels are available (Maniou *et al,* 2001; Shah, 2006; Ishag *et al,* 2010; Afifi *et al,* 2014; Abdel Aziem *et al,* 2015’ Shawki *et al,* 2015;

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El-Kholy *et al,* 2016). The camel growth hormone (GH) gene extends over about 1900 bp, and like other mammalian GH genes; it splits into 5 exons and 4 introns (Maniou *et al,* 2001). The molecular structure of GH gene in Indian dromedary and bactrian camels are not available. Hence, the present study was undertaken to characterise the growth hormone (GH) gene in Indian dromedary and bactrian camel.

Materials and Methods

Blood samples were collected from 5 camels each from Bikaneri, Mewari, Kachchhi breeds maintained at ICAR-National Research Centre on Camel Farm at Bikaner and 5 double humped camels from farmer's herd at Nubra valley, Ladakh in 10 ml vacutainer tubes containing EDTA. The DNA was extracted from blood cells using standard phenol-chloroform extraction protocol (Sambrook *et al,* 1989). PCR amplification of 613 bp GH gene fragment was done utilising primers reported by Abdel Aziem *et al* (2015). The amplified GH gene fragment covered partial exon-1, intron-1, exon-2 and partial intron-2 Primers were synthesised from Eurofins genomics. The PCR reaction was carried out in 25µl of total volume, containing ready to use Go taq Green master mix-12.5 µl (Promega, USA), 1µl of each primer with concentration of 10 pM, 1µl of 80-100 ng camel genomic DNA and nuclease free water (Promega, USA) to make total volume up to 25µl. Amplification was performed in Mastercycler® Gradient (Eppendorf AG, Hamburg, Germany) programmed for initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 57°C for 45 s, extension at 72°C for 45 s, and final extension at 72°C for 10 min. PCR products were checked for amplification by electrophoresis on 2.0% agarose gel (Himedia), in parallel with 100 bp DNA marker (Thermoscientific). After purification of the amplified fragment bidirectional sequencing using forward and reverse primers was done using Sanger Dideoxy Chain termination method (Eurofins Genomics). The forward and reverse sequences obtained for each animal were edited using Codon Code Aligner software (USA) and different sequences patterns were generated. The pair wise and multiple alignment of the different sequence pattern was done to analyse the differences and relationship between Indian camels GH gene sequences. The pairwise and multiple alignment of identified Indian camel GH gene sequences were done with other reported dromedary and bactrian camels as well as other related and domesticated species GH sequences available at National Centre for Biotechnology Information (NCBI) database using BLAST software program (http://www. ncbi.nlm.nih.gov/) to study the sequence variation and relationship. The estimation of evolutionary relationship between different species and sequences obtained were inferred by neighbour joining method using Molecular Evolutionary Genetics Analysis software (MEGA 7.0).

Results and Discussion

The annealing temperature of 57° C was found optimal for amplification of the target GH gene fragment. A single clear band was observed when the PCR products were checked for amplification by electrophoresis on 2.0 % agarose gel in parallel with 100 bp DNA marker (Fig 1). After bidirectional sequencing of PCR products and its editing using Codon Code aligner software, 613 base pair GH gene fragment's genetic variant were identified (Table 1). The different sequence pattern thus obtained were submitted to NCBI with GenBank Accession No. MT478653 for Indian dromedary (C allele), MT 478654 for Indian dromedary (T allele) and MT478655 for Indian bactrian. The sequence variations within and between single and double humped camels are depicted in table 1. In the 613 bp long GH gene fragment sequence, the nucleotides were conserved at all positions except 3 position in Indian dromedary and bactrian camel. Sequence variation was seen at position 264 C>T in Indian one humped camels of Mewari, Kachchhi and Bikaneri breed. At this particular locus, the dromedary camels with one and two peaks in the sequence chromatograms were observed. Accordingly, 2 allele (C, T) and 3 genotype (CC, CT, and TT) were identified in the Indian dromedary camels (Fig 2). In the Indian bactrian camel at position 264, only C allele and CC genotype were identified (Fig 2). In Indian double humped camel, transition variation was present at position 242, A>G and 469 G>A as compared to single humped camel. All the observed variation were in the intronic region. The exon sequences were similar in double hump and single hump camels and similar amino acids were coded. The sequences

Table 1. Sequence variation between Indian dromedary and Bactrian camel GH gene.

Name	Accession No.	Nucleotide position		
		242	264	469
Indian Dromedary C allele	MT478653	A	C	G
Indian Dromedary T allele	MT478654	A	T	G
Indian Bactrian	MT478655	G	C	A

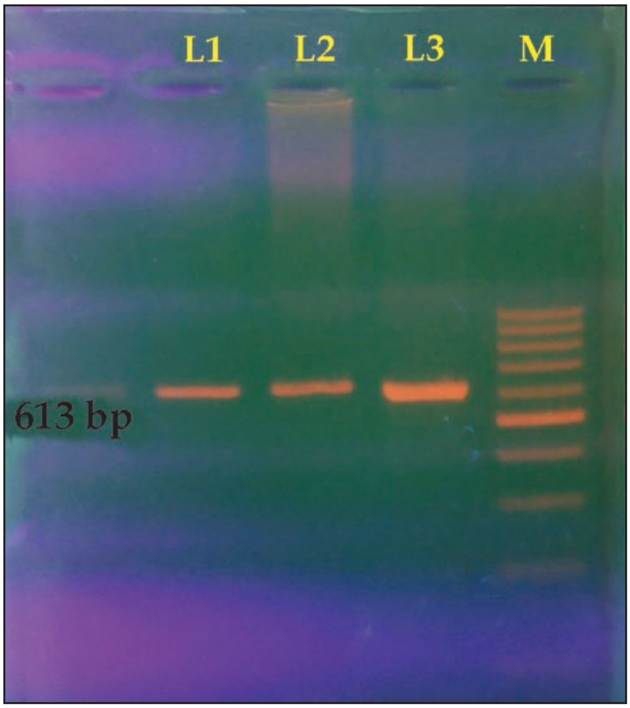

Fig 1. PCR amplification of Growth Hormone (GH) gene resolved on 2.0% agarose gel M marker 100 bp DNA ladder, L1, L2, L3 GH gene product.

obtained was similar to finding of Shah (2006); Ishag *et al* (2010); Abdel Aziem *et al* (2015); Shawki *et al* (2015) in different Asian and African camel breeds. The sequence identity matrix of 3 identified

Table 2. Sequence Identity (above diagonal) and Genetic distance (below diagonal) between Indian dromedary and Bactrian camel GH gene.

	Indian Dromedary C allele	C allele Dromedary T allele	Indian Bactrian
Indian Dromedary C allele	1	0.998	0.996
Indian Dromedary T allele	0.0016	1	0.995
Indian Bactrian	0.0033	0.0049	1

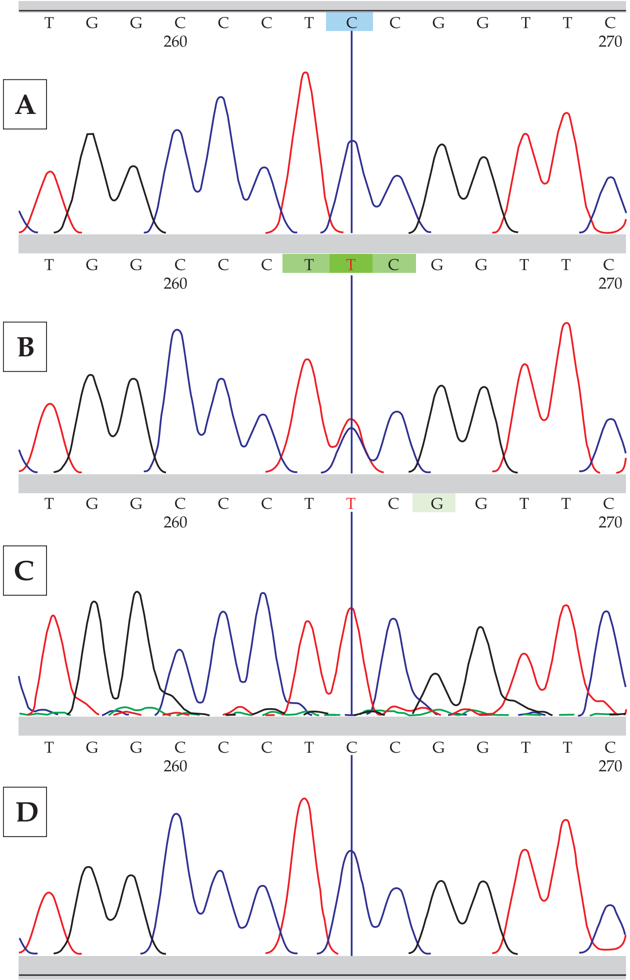

Fig 2. Sequence variation at position 264 in GH gene sequence, A depicts CC, B depicts CT, C depicts TT genotype of Indian dromedary camel and D depicts CC genotype in Indian bactrian camel.

sequences showed more than 99 percent identity and the average genetic distance between 3 sequences were 0.0032 between Indian dromedary GH alleles and bactrian camel GH genes (Table 2). The sequence variation and percent similarity determined on the basis of pairwise nucleotide BLAST of Indian

Table 3. Variation in the camel GH gene sequences relative to Indian dromedary GH sequence (Accession No. MT478653).

Accession No	Total sequence differences	Position and type of variation	Insertion	Deletion	% Identity
AJ575419.1	0	-	-	-	100
J X891650.1	0	-	-	-	100
KP1435181.1	1	480 (G/T)	-	-	99.84
JX891651.1	2	242 (A/G), 469 (G/A)	-	-	99.67
KP143517.1	1	264 (C/T)	-	-	99.67
MK986663.1	3	264 (C/T), 490 (A/G), 491 C/T	-	-	99.42
KR902744.1	7	62 (C/T), 63 (T/A), 225 (G/A), 513 (G/A)	516 (GA)	508 (A)	98.66
KR902745.1	7	12 (A/G), 264 (C/T), 492 (C/T), 510 (T/G), 514 (A/G)	454(A)	508 (A)	98.65

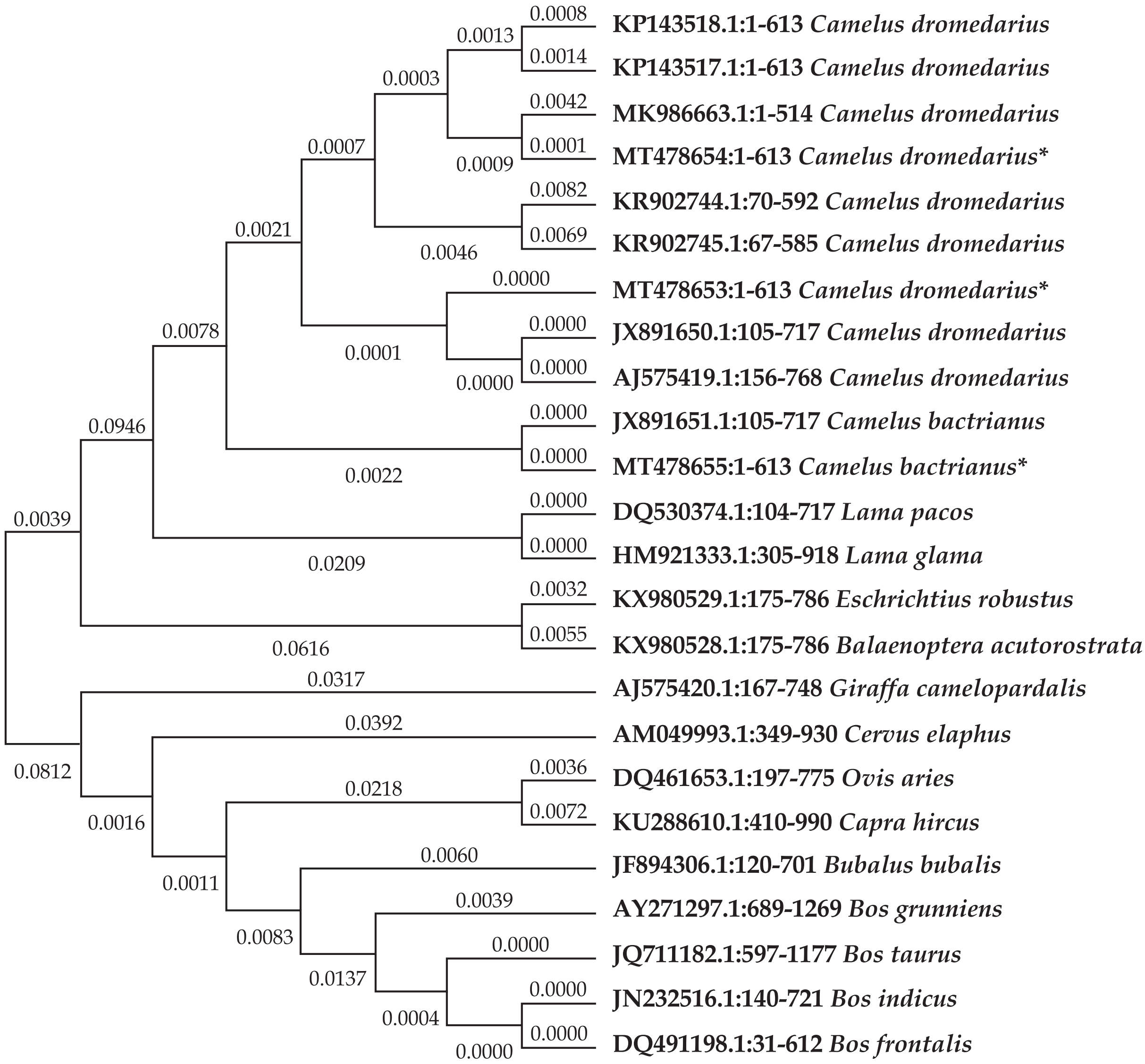

Fig 3. Phylogenetic tree between GH gene sequences from different species. Sequences with * asterisk sign show sequence under study, at extreme right of Fig Sequence name is denoted as accession number, sequence range and zoological name of species.

dromedary GH gene (C allele) with published camel GH gene sequences at GenBank repository is given in table 3. Similarity ranging from 98% to 100 % was obtained with available GenBank camel GH sequences. The GH gene (C allele) sequences also differed at few position to other camel GH Gen bank sequences. The differences were due to transition, transversion, insertion and deletion (Table 3). Camel sequences has close similarity with other camelids family members *Lama pacos* (97.07%) and *Lama glama* (96.58%). With other species the sequence identity varied from 83.65% (*Eschrichtius robustus*) to 78.63% (*Capra hircus*). The evolutionary relationship between GH genes of different species was inferred using Neighbour joining method by analysing 24 nucleotide sequences including three consensus sequences generated in the present study. The sum of the branch length of optimal phylogeny tree (Fig 2) was 0.4507. The evolutionary relationship between sequences showed close relationship between dromedary and Bactrian camel species followed by vicugna and llama. The domesticated species like cattle, buffalo, sheep, goat, yak and mithun were distantly related to camel. Thus, present study showed close similarity between GH gene sequence pattern of Indian Single and double humped camel. Further Indian camels share GH gene structure similar to its Asian and African counterparts. The variation observed at different locus need to be investigated in larger population for gene and genotype frequency and possible association of GH genotypes with performance traits.

Acknowledgement

Authors deeply acknowledge the contribution and support of all staff of Animal Genetics and Breeding Unit, Camel Dairy and LFS for assistance in blood collection and laboratory work and Director, ICAR-NRCC, Bikaner for successful execution of the project.

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SELECTED RESEARCH ON
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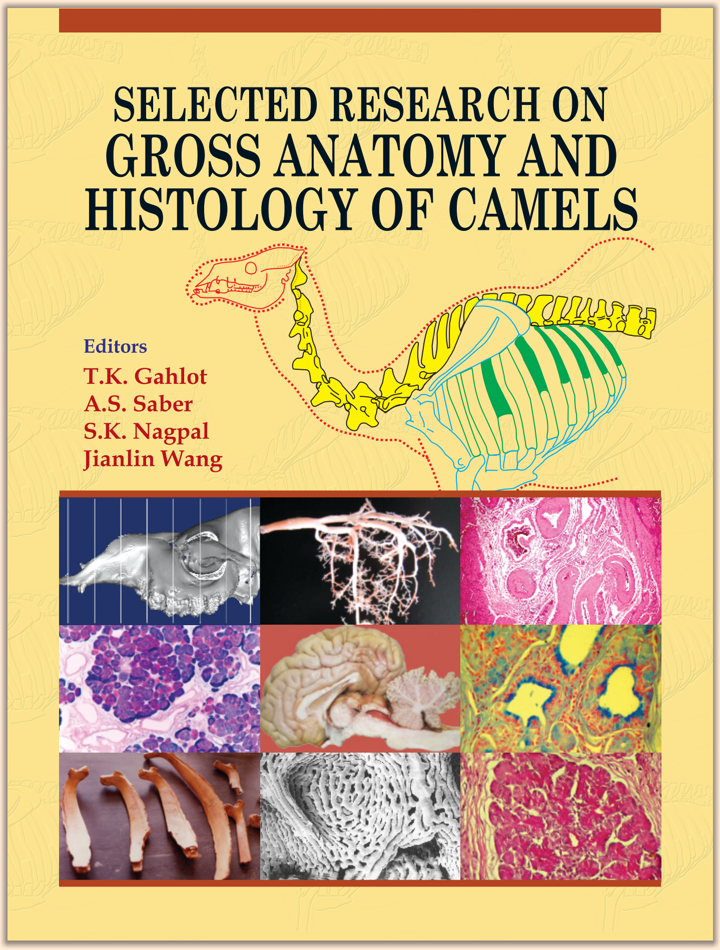
Selected Research on Gross Anatomy and Histology of Camels is a unique reference book on anatomy of dromedary and bactrian camels. This book contains a first ever wide spectrum of histological description of various organs of camels which is depicted by special stains and scanning electronmicroscopy in addition to the gross anatomy, histochemical and immunohistochemical studies. The book has 92 manuscripts in 9 sections, e.g. radiographic anatomy, anatomy of various systems (skeletal, digestive, respiratory, circulatory, urogenital and nervous), common integument and miscellaneous. These manuscripts were published by 158 authors working in 37 laboratories or colleges or institutions from 14 countries in the Journal of Camel Practice and Research between June 1994 to June 2010. Bactrian camel anatomy research was exclusively contributed by the researchers of China. The countries involved in camel anatomy research were China, Egypt, India, Iran, Saudi Arabia, Iraq, Jordan, Japan, Pakistan, Sweden, United Arab Emirates, United States of America, France and Germany. Camel Publishing House has taken a step forward to compile this knowledge in form of a book and this herculian task was accomplished by its dedicated editors, viz. T.K. Gahlot (India), S.K. Nagpal (India), A.S. Saber (Egypt) and Jianlin Wang (China). This classic reference book will serve as a one stop resource for scientific information on gross anatomy and histology of camels.



Editors:
T.K. Gahlot, A.S. Saber, S.K. Nagpal and Jianlin Wang

Edition: 2011

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Price: US $ 375 (Abroad)
INR 7500 (India)

ISBN: 81-903140-1-7

DOI : 10.5958/2277-8934.2021.00008.4 Vol 28 No 1, p 53-58

FATALITIES IN DROMEDARY CAMELS ACROSS THE ARABIAN PENINSULA CAUSED BY PLASTIC WASTE

Ulrich Wernery[1], Renate Wernery[1], David Wernery[1], Amy Lusher[2], Marcus Eriksen[3] and Mia Nixon[3]

[3]5 Gyres Institute, 3131 Olympic Blvd #302, Santa Monica, CA, 90404, USA, [2]Norwegian Institute for Water Research (NIVA), Oslo, Norway, [1]Central Veterinary Research Laboratory, Dubai, United Arab Emirates

ABSTRACT

Ecological impacts of widespread, plastic pollution and subsequent ingestion of anthropogenic waste, primarily plastic bags and ropes by dromedary camels (*Camelus dromedarius*) in the United Arab Emirates (UAE) and across the Arabian Peninsula is reported here. The ingested waste is turned into a collection of tightly packed indigestible materials which can include plastics, ropes, other litter and salt deposits trapped in the stomach or digestive tract forming a large stone-like mass termed as plastic gastroliths or polybezoars. Central Veterinary Research Laboratory (CVRL), Dubai, UAE evaluated more than 30,000 camels since 2008, there have been 300 documented deaths contributed to polybezoars in the stomach. Here, we analyse a subset of five gastroliths extracted from dessicated camel skeletons found in the desert, weighing from 6.2-53.6 kg. Two random samples of anthropogenic material, primarily plastic bags and synthetic ropes, from each of these five polybezoars were analysed for polymer content, showing predominantly polyethylene and polypropylene. Gastrointestinal blockages were caused by these polybezoars, leading to sepsis from multiplying populations of gut anaerobes, and dehydration and malnutrition due to limited available space for food and water in the gut, which leads to a false sense of satiation. The frequency of these impacts result in a population-level effect of an estimated 1% mortality rate for camels living in the region. The force of high winds and the open desert environment possibly lead to escape of plastic bags and other thin, film-like packaging easily force open waste bins and landfills, travelling long distances from waste management services, therefore, alternative systems are urgently required for package and deliver goods to replace plastic bags throughout the region of Arabian peninsula.

Key words: Arabian peninsula, dromedary camel, gastroliths, plastic pollution, polybezoar, UAE

Plastic pollution poses significant environmental problems around the world. Plastic pollution of the global environment has been dominated by reports of ecological impacts on marine organisms, including evidence of entanglement and ingestion in over 637 species that interacted with plastic pollution (Gall and Thompson, 2015). Yet, emissions of plastic to the terrestrial environment may be 4-23 times higher than inputs to the marine environment (Horton *et al,* 2017).

Plastics have been observed in digestive tracts of cattle (Jebessa *et al,* 2018), sheep and goats (Tiruneh and Yesuwork, 2010), Arabian oryx (Anajariyya *et al,* 2008), camel calves (Ahmed, 2011) and adult camels (Wernery *et al,* 2014). Most of the ingested items were plastic bags and film. Plastic materials cannot be digested and may take a long time to pass through the digestive tract or be retained indefinitely when caught in complex digestive tracts. Consequences of plastic ingestion include ruminal impaction, where indigestible plastic foreign bodies accumulate in the stomach compartments, which leads to indigestion, the formation of gastroliths or polybezoars, traumas, poor body condition, immune suppression, reduced health status, and mortality (Hailat *et al,* 1997; Jebessa *et al,* 2018; Priyanka and Dey, 2018).

Grazing and scavenging animals such as ruminants, feed indiscriminately on plastic pollution in the environment. Animals ingest plastic waste due to erratic feeding behaviour, and confusing plastic with food when trying to eat leftover feed materials in plastic wrappings (Priyanka and Dey, 2018). Plastic waste accumulated in the rumen may release dioxins, phthalates, polychlorinated biphenyls (Vanitha *et al,* 2010), and heavy metals (Osuga *et al,* 2013). Ingested plastic materials in the rumen slowly release chemicals to the rumen fluid, which may enter the food chain through milk and meat products (Kunisue *et al,* 2004).

In the region surrounding the Arabian Gulf, camels are the dominant foraging ruminants, existing

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in all countries bordering the gulf. In the UAE alone, populations of camels have been estimated at over 390,000 (FAO, 2019). Camels are browsing animals with up to 37% of their time in a 24-hour period spent grazing. This feeding behaviour predisposes them to plastic pollution ingestion. Although camels have been identified as versatile animals, capable of surviving and performing in arid and semiarid regions (Iqbal and Khan, 2001), as individuals, they are not able to cope with ingested plastic pollution. Plastic pollution in the form of thin film products and packaging, like balloons and plastic bags, is increasingly abundant in deserts worldwide (Zylstra, 2013) (Fig 1).

Adverse effects on camels (*Camelus dromdarius*) due to the ingestion of anthropogenic material, consisting of primarily plastic bags, but also ropes and textiles, has been widely observed. Of 156 camels evaluated post-mortem in Jordan, foreign-body accumulation within the first and second stomach compartments was the predominant gastrointestinal disease of slaughtered adult camels (22%), including plastic (65%), rope and leather (23.5%), or all three (11.5%) (Al-Rawashdeh *et al,* 2000). A recent study of eight juvenile camels sent to a veterinary clinic in Saudi Arabia with obstructions of the oesophagus caused by plastic bags (75%) and pieces of cloth (25%) (Shawaf *et al,* 2017), and an earlier study in the same region found six juvenile camels with obstructions in the oesophagus due to plastic bags in five and cloth in one (Ahmed, 2011).

In the Central Veterinary Research Laboratory (CVRL) in Dubai, UAE, over 30,000 camels have been observed since 2008, with 300 cases of mortality due to ingesting anthropogenic waste, primarily plastic bags and ropes (Wernery *et al,* 2014). They have been observed to die for several reasons :

- Sudden death caused by complete obstruction of the intestine by a plastic bag, or incomplete obstruction accompanied by a secondary clostridial enterotoxemia, a bacterial infection, due to plastic ingestion. In the later cases, lesions are observed and toxin-producing anaerobes are abundant and isolated where the plastic mass nears the tissues.

- Death within two to three weeks due to organ failure. In these cases, the ingested plastic rubbish releases toxins into the circulatory system, which causes the liver values (glutamate oxalaetate transaminase - GOT [AST], gammaglutamyl transferase [y-GT], glutamate-pyrovatetransminase-GPT [ALT]) and kidney values (blood urea nitrogen-BUN, creatine) to increase steadily, culminating in organ failure (Wernery *et al,* 2014).

- Slow death due to starvation. Plastic bags, parts of plastic bottles and caps, plastic ropes used to hold hay bales together, and other plastic utensils accumulate, most probably over weeks, months, and years, in camels' stomach compartments. When in the stomach, they start to calcify, forming a solid plastic mass, which may fill and take the shape of the first compartment in the stomach. This plastic mass, or polybezoar, can affect feeding behaviour, resulting in camels eating less until they stop eating completely, as the camel always feels full, resulting in a false sense of satiation.

Harm to individual ruminants from plastic ingestion can be straightforward, such as mechanical obstructions, perforations of the intestinal tract, and abscessed or ulcerated intestinal linings. These impacts can lead to stomach volume displacement, false-satiation and slow malnourishment, dehydration and toxification from leached compounds from the plastics themselves or sepsis from high bacterial loads living in the folds of plastic film. This vulnerability may contribute to immuno-suppression, liver damage, and clostridium. These observations show clear harm to individual animals, but the extent of harm to entire populations has not been fully explored yet.

Therefore, the aim of this study was to document the occurrence, abundance, and composition of ingested anthropogenic matter in the stomachs of camels, introduce polybezoar as a distinct nomenclature to describe these observations, and suggest mitigations to address the problem.

Materials and Methods

Between 2008 and 2017, five bezoars of anthropogenic material (Fig 2) were recovered during post-mortem from desiccated skeletons of camels found near Dubai, UAE in a distance not more than 100 km south and 50 km east to the foothills of the Al-Hajar mountains. The polybezoars were brought to the Central Veterinary Research Laboratory (CVRL) in Dubai, whereby they were brushed and shaken to dislodge loose sediment, and were suspended outside the laboratory facility for display until gathered for this study. The five polybezoars were brought into the CVRL and weighed using a digital scale to the nearest 10 grams. Volume was ascertained by putting the bezoar inside a vacuum sealed bag, filling a large bin with water and submerging the polybeozar beneath the water surface. The volume of displaced

Fig 1. Camel (*Camelus dromdarius*) foraging on plastic waste in the UAE desert. Photo: Ulrich Wernery.

water was collected and measured to the nearest 0.1 litres. Fourier Transform Infrared Spectroscopy (FT-IR) was used for polymer identification using two instruments with different libraries. In each polybezoar, the two largest items externally visible were sampled by cutting away a small fragment of the material. Each sample, two from each of the 5 polybezoars, (n=10) was analysed on two different FT-IR instruments to get comparative results. The samples were cleaned with isopropanol to remove as much calcification and dirt as possible before analysis. First, plastic pieces were tested using an Agilent Cary 630 FT-IR spectrometer with a diamond ATR accessory followed by a Perkin Elmer Spectrum Two FT-IR with a diamond ATR accessory. Separate library searchers were performed using the Agilent Polymers ATR library. Best matches were calculated based on the library software of each instrument. Each match reported was above 90%.

Fig 2. Polybezoars collected from camel skeletons (a, b), bezoar split to expose an interior of compacted plastic film (d), polybezoars were composed of synthetic material polyethylene as per FTIR analysis (c).

Results

Plastic was clearly present in each polybezoar, with two dominated by rope fragments and the other three dominated by plastic bags, based on external evaluation.

Polybezoar was sawn in half to expose the centre, which revealed plastic film throughout the entire mass, primarily plastic bags, with no calcification internally (Fig 2). External calcification on one polybezoar was minimal, but polybezoars calcification of the rope fragments into a hardened mass was seen (Fig 2).

Discussion

Of 300 cases of mortality due to ingesting anthropogenic waste, a subset of five polybezoars, collected by the Central Veterinary Research Laboratory (CVRL) in Dubai, UAE were evaluated in this study. Using simple descriptive techniques to understand the weight and contents of each one, this study revealed the dominance of polyethylene plastic bags, with polypropylene rope second in abundance. Evidence of harm from plastic ingestion has been observed in hundreds of camels evaluated live and post-mortem by the CVRL and other veterinary clinics in the region over the past several decades. To mitigate the harm from anthropogenic plastic waste on camels, we must understand the significance of plastic waste impacts to individual animals and whether it suggests population-level harm, the exposure of animals to plastic waste in the region, and lastly the types of mitigation strategies available to reduce exposure. The literature on harm caused by plastic ingestion or entanglement is dominated by studies of marine organisms, and is largely focused on field observations of individual organisms or laboratory studies showing impact. What is missing in the literature are studies of populations of organisms at ecologically relevant concentrations of plastic waste. While field studies of population-level effects are low, the perception that population-level harm is high (Rochman *et al,* 2016). For example, in a recent risk analysis of seabird species, of 135 species between 1962 and 2012, 59% had ingested plastic waste.

By standardising the data, the authors estimated the ingestion rate would increase to 90% by 2015 (Wilcox *et al,* 2015). The authors reported "Although evidence of population level impacts from plastic pollution is still emerging, our results suggest that this threat is geographically widespread, pervasive, and rapidly increasing". Here we observe a population-level effect. The total dromedary camel population in the UAE region is estimated to be approximately 390,000 animals. The CVRL has evaluated over 30,000 camels since 2008, with over 300 documented deaths contributed to polybezoars in the stomach, representing a 1% mortality rate among camels evaluated. Similarly, 100% of the camels that contained plastic waste in their guts, and were also evaluated for toxicity, were found with elevated levels of liver and kidney enzymes, indicating toxification.

Reducing exposure to plastic waste exposure to plastic waste is abundant in the desert regions surrounding the Arabian Gulf. In the case of camels in the UAE, animals are roaming the desert in small groups that forage in acacia forests, roadsides, and in landfills. The exposure to thin film plastic bags and packaging is common in these areas, as plastics escape waste bins or dumpsters, or are littered, resulting in wind-borne macroplastics travelling long distances. FTIR analysis of 10 fragments of synthetic material, two from each of the five polybezoars make a spectrum of colours of polymer fragment image raging film blue-white-green grey-black. Profound hypophosphatemia and hypochloremia was seen in cattle with nutritional disorders known as 'Pica' where cattle and other farm animal eat unusual objects including indigestible waste like rope, cloth, polythene etc (Nikvand *et al*, 2018; Elshahawg *et al*, 2016).

Gameel *et al* (2000) surveyed 337 camel in an abattoir study and found that 40.4% investigated camel had foreign bodies, i.e. bones, trichobezoars, strings, ropes, plastics, rags, canvas and calcified bodies. In present study some similar composition foreign bodies were seen.

Desert recreation from campers, hunters, and falconers are responsible for significant loss of plastic waste. In a study of Arabian oryx in the fenced Mahazat as-Sayd Protected Area in Saudi Arabia, there is a 70km highway connecting Riyadh to Khurma City. Thirty oryx were captured and contained in the fenced protected area. Within one year, seven died of plastic waste ingestion, whereby roadside litter trapped against the fence was the primary exposure to waste that was eaten (Anajariyya *et al,* 2008). This prompted public education campaigns and waste management to recover plastic waste along the fenceline. Municipal solid waste (MSW) management is rapidly developing throughout countries surrounding the Arabian Gulf. MSW management alternatives include landfilling, incineration, and recycling. The option of landfilling is declining in most developed countries as soil, water, and air contamination, increased potential for human health risks, and the scarcity of locations near urban developments increases (Paleologos *et al,* 2016). The MSW component of the General Waste stream in the UAE has increased from 1,523,822 tonnes in 2003 to 2,689,808 in 2011. According to the waste composition analysis conducted in 2012, 35% of the General Waste stream was organic waste, 24% paper, and 24% plastic (Saifaie and Municipality of Dubai, 2013). A recent survey of public attitudes in the UAE shows a high level of interest in rapidly addressing plastic waste (Hammami *et al,* 2017).

In recent years, as the UAE and other countries surrounding the Arabian Gulf experience a rise in GDP and population size, which correlate to increased consumption and waste generation, new models of waste management beyond landfilling have been considered. These countries are considering waste to energy as the dominant disposal option for the foreseeable future (Paleologos *et al,* 2016). In the cities of Dubai, Abu Dhabi, and Sharjah, large waste to energy facilities are currently operational or soon to become operational, meeting the goal of 75% diversion of MSW away from landfill by 2021 (United Arab Emirates, 2019). Regardless of these mitigation strategies, including common devices called "BinStraps" used to secure lids on waste bins so the force of wind cannot open them, plastic film and bags continue to escape urban developments into the environment as often people do not close the lids or they are opened by clever dromedaries. Plastic bag bans are increasing in municipalities across the globe (Xanthos and Walker, 2017). The Dubai Municipality launched the "Say No to Plastic Bags" campaign in 2013, aimed to reduce plastic bag consumption by 20% in the first year, to tackle the annual 2.9 billion plastic bag consumption rate across the UAE (Pandy, 2016). Today, efforts to eliminate plastic bags from the UAE are primarily conducted in the private sector, as shopping malls and grocery stores voluntarily eliminate plastic bags or charge a fee for bags to disincentivise their use. While these actions are noteworthy, they will not curtail the loss of plastic waste to the environment. Finally, in the absence of significant single-use plastic reduction measures, and the continued loss of plastic bags and film to the environment, it becomes the responsibility of animal husbandry and every person to reduce exposure of animals to plastic waste. Good animal husbandry, by providing adequate feed, water, shelter and mineral supplements, as well as establishing grazing centres and water facilities will deter the straying of animals to roadsides and landfills in search of sustenance (Priyanka and Dey, 2018).

Recommendations for use of plastic bags, their disposal and adverse effects on environment should be made similar to that done in Ethiopia (Adane *et al,* 2011).

Camels, while they are prized in competitive breeding, racing and are utilised in cultural events, such as weddings and political parades, they are significantly harmed by the abundance of plastic waste, especially single-use plastics and bags blowing across deserts and escaping even the most efficiently designed waste management systems. Therefore, it is essential that careful consideration be placed on the role of single-use plastics, their current use, and eventual elimination from modern societies.

Acknowledgements

The authors wish to thank the Central Veterinary Research Laboratory (CVRL) for providing the polybezoars for this study and Olivia Turner Smith from the School of Ocean Sciences, Bangor University, Wales, United Kingdom for her assistance conducting lab analysis of plastic samples.

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DOI : 10.5958/2277-8934.2021.00009.6 Vol 28 No 1, p 59-67

IMMUNOMODULATORY EFFECT OF *Escherichia coli* LIPOPOLYSACCHARIDE ON PHENOTYPE AND FUNCTION OF BLOOD MONOCYTES IN CAMELS

Jamal Hussen[1*], Khaled R. Alkharsah[2], Ibrahim Hairul-Islam M[3] and Naser Abdallah Al Humam[1]

[1]Department of Microbiology, College of Veterinary Medicine, [3]Biological Sciences Department, College of Science, King Faisal University, Al-Ahsa, Saudi Arabia

[2]Department of Microbiology, College of Medicine, Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia

ABSTRACT

The objective of the current study was to investigate the effect of lipopolysaccharide (LPS) from *E. coli* on the phenotype and the function of the camel monocytes. Flow cytometry was used to analyse the expression of different myeloid markers and cell adhesion molecules on camel monocytes and to evaluate the ability of monocytes to engulf bacteria and to generate reactive oxygen species (ROS). In LPS-stimulated blood, monocytes showed shifting toward inflammatory macrophage-1 (M1) profile by enhancing the expression of high levels of MHCII molecules and reduced levels of CD163. Furthermore, LPS-stimulated monocytes upregulated the expression of the adhesion molecules CD62L and CD11b while downregulated the expression of CD18. Functionally, stimulation with LPS reduced the phagocytosis capability of monocytes but enhanced their ability to produce ROS. These results suggest a modulating effect of LPS on the phenotype, adhesion, and phagocytic functions of the camel blood monocytes and propose a possible new immune evasion mechanism.

Key words: Adhesion molecules, camel, innate immunity, monocytes, lipopolysaccharide, phagocytosis, ROS

Escherichia coli (*E. coli*) is a gram-negative bacterium, which causes several diseases in the dromedary camel. This includes mastitis and metritis in adult female camels and septicemia in the newborn camel calf leading to high mortality rates early in life (Aljumaah *et al,* 2011; Al-Ruwaili *et al,* 2012).

Monocytes are circulating immune cells with a key role in innate immunity to bacterial pathogens. In addition to their ability to ingest and kill bacteria, monocytes constitute the main source of tissue macrophages upon migration from the bloodstream to tissues (Soehnlein and Lindbom, 2010; Jakubzick *et al,* 2017; Pomeroy *et al,* 2017). The immunophenotype of blood monocytes is characteristic of their functional subtype. Depending on the type of the activating signal, monocytes undergo different phenotypic and functional changes. The expression of the monocytic markers CD172a, CD14, CD163, and MHCII are good indicators for the functional subtype of monocytes during their differentiation into macrophages (Schwartz and Svistelnik, 2012; Thawer *et al,* 2013; Hussen *et al,* 2014; Hussen and Schuberth, 2017). CD172a, which is known as the signal-regulatory protein alpha (SIRPa), is glycosylated cell surface receptor expressed on myeloid cells and functions as a regulatory receptor that inhibits cell signaling (Hussen *et al,* 2013). In camels, monocyte subsets I and II show higher abundance of CD14 than monocyte subset III (Hussen *et al,* 2020). Due to the low expression of CD14 and CD16, mouse monocytes are identified based on the expression of Ly6C and CD43 (Zawada *et al,* 2012).

Lipopolysaccharide is an important component of the gram-negative bacterial outer membrane and is considered a powerful activator of the innate immune response. The impact of LPS stimulation on the phenotype and function of camel monocytes has not been yet studied. The aim of the current study was to evaluate the immunomodulating effect, in terms of phenotype and function, of *E. coli*-lipopolysaccharide stimulation on camel blood monocytes *in vitro*.

Materials and Methods

Blood sampling

Blood samples were collected from 7 healthy dromedary camels (*Camelus dromedarius*) aged between 6 and 9 years by venipuncture of the vena jugularis externa into EDTA-containing vacutainer tubes (Becton Dickinson, Heidelberg, Germany).

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LPS whole blood stimulation

Whole blood stimulation was performed as described previously. Blood from healthy camels was stimulated with 1 µg/ml Lipopolysaccharide purified from *E. coli* O55:B5 (Sigma-Aldrich, Germany) at 37°C in 5% CO_2 or left without stimulation. After incubation for 4 h, blood samples were diluted with phosphate buffer saline (1:1) and centrifuged at 4°C for 10 min at 1000xg. After removing the supernatant, the cell pellet was resuspended in PBS.

Leukocytes separation

Separation of whole leukocytes from camel blood samples was performed with hypotonic lysis of blood erythrocytes (Hussen *et al,* 2013). Briefly, blood cells were suspended in distilled water for 20 sec. Later, double-concentrated PBS was added to restore tonicity. This step was repeated at least twice or until complete erythrolysis. The remaining cells were finally resuspended in MIF (Membrane Immunofluorescence) buffer composed of PBS containing 5 g/l of bovine serum albumin and 0.1 g/l of NaN_3 at a concentration of 5 x 10^6 cells/ml. The mean viability of the separated leukocytes was determined by the dye exclusion method using 2 µg/ml of propidium iodide (Calbiochem, Germany). The mean leukocyte viability in our experiments was above 95%.

Membrane immunofluorescence and flow cytometry

The expression of monocytic markers and cell adhesion molecules was analysed using membrane immunofluorescence test (Eger *et al,* 2015; Hussen *et al,* 2017). For blocking of FC receptor binding, separated camel blood leukocytes (4 x 10^5) were incubated with MIF buffer containing 5% autologous camel serum for 20 min at 4°C in 96 well round-bottom microtitre plates. After two times washing with MIF buffer (300 xg for 3 min at 4°C), cells were incubated with monoclonal antibodies (mAbs) specific for the monocytic markers CD172a, CD14, CD163, and MHCII and the cell adhesion molecules CD18, CD11a, CD11b, and CD62L cross-reactive with homologous camel molecules (0.2 µg of each mAb in 100 µl MIF buffer/well) (Hussen *et al,* 2017). After incubation for 15 min at 4°C, cells were washed with MIF buffer twice and incubated with mouse fluorochrome-labeled secondary antibodies (IgG1, IgG2a; 0.2 µg in 100 µl MIF buffer/well; Invitrogen) or with mouse isotype control antibodies (0.2 µg of each mAb in 100 µl MIF buffer/well; Becton Dickinson Biosciences, USA). After washing, the cells were analysed on a Becton Dickinson FACSCalibur flow cytometer (Becton Dickinson Biosciences, California, USA). Data of 10^5 cells were collected and analysed with the flow cytometric software FlowJo (FLOWJO LLC). After the exclusion of dead cells (PI-negative cells), forward and sideward scatter were used to gate for monocytes. The median fluorescence intensity (MFI) for the selected CD marker was measured (Fig 1).

Phagocytosis Assay

Heat-killed *Staphylococcus aureus* (*S. aureus*) (Merck, Nottingham, UK) was labeled with fluorescein isothiocyanate (FITC) (Sigma-Aldrich, Missouri, USA). Leukocytes were separated from LPS-stimulated (4 h) or un-stimulated camel blood. Separated leukocytes were plated in 96-well plates at a density of 10^6 cells per well and incubated with the heat-killed FITC-labeled *S. aureus* (50 bacterial cells per leukocyte) for 30 minutes at 37°C in a 5% CO_2 incubator. Additionally, leukocytes, which were neither induced with LPS nor incubated with bacteria, were used as control. After incubation, propidium iodide (PI) (2 µg/ml final) was added to exclude dead cells and samples were analysed by flow cytometry. Phagocytic activity of monocytes was calculated as the percentage of cells expressing green fluorescence among all viable monocytes. The mean green fluorescence intensity (MFI) of phagocytosis-positive monocytes was measured as an indicator for the number of the phagocytosed bacteria by each monocyte.

Generation of ROS

The ROS-generation was measured as previously described (Hussen *et al,* 2016). LPS-stimulated or uon-stimulated camel leukocytes (1×10^6/well) were incubated without or with heat-killed non-opsonised (50 bacteria/cell) *S. aureus* (Pansorbin, Calbiochem, Merck, Nottingham, UK) for 20 min (37°C, 5% CO_2). For the detection of ROS, dihydrorhodamine (DHR123) (Mobitec, Goettingen, Germany) was added to the cells at a final concentration of 750 ng/ml. Later, the cells were washed with MIF buffer and the relative amount of the generated ROS was determined by the median green fluorescence intensity of gated monocytes.

Study ethics

This study obtained ethical approval from the Ethics Committee at King Faisal University, Saudi Arabia (Permission number: KFU-REC/2019-10-01).

Statistical Analyses

Statistical analysis was performed with the software Prism (GraphPad). Results were presented

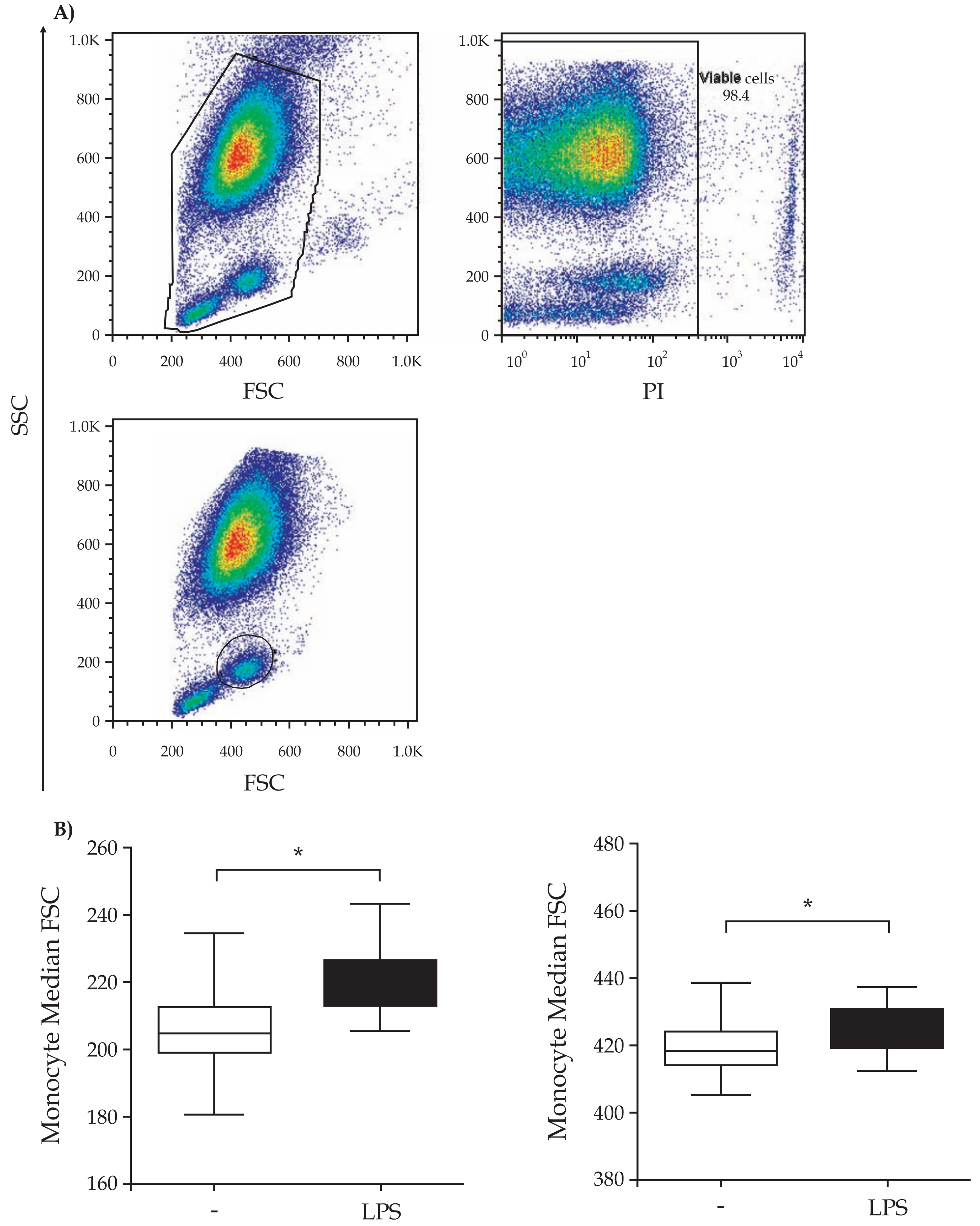

Fig 1. A) Gating strategy for flow cytometric analysis of the expression of myeloid markers and cell adhesion molecules on camel blood monocytes. After setting a gate on total leukocytes in an SSC/FSC dot plot, dead cells were excluded based on their positive staining with propidium iodide. In a SSC/FSC dot plot, monocytes were gated based on their forward and side scatter properties. The mean SSC and FSC of gated monocytes were measured and presented for unstimulated and stimulated cells (* = $p<0.05$).

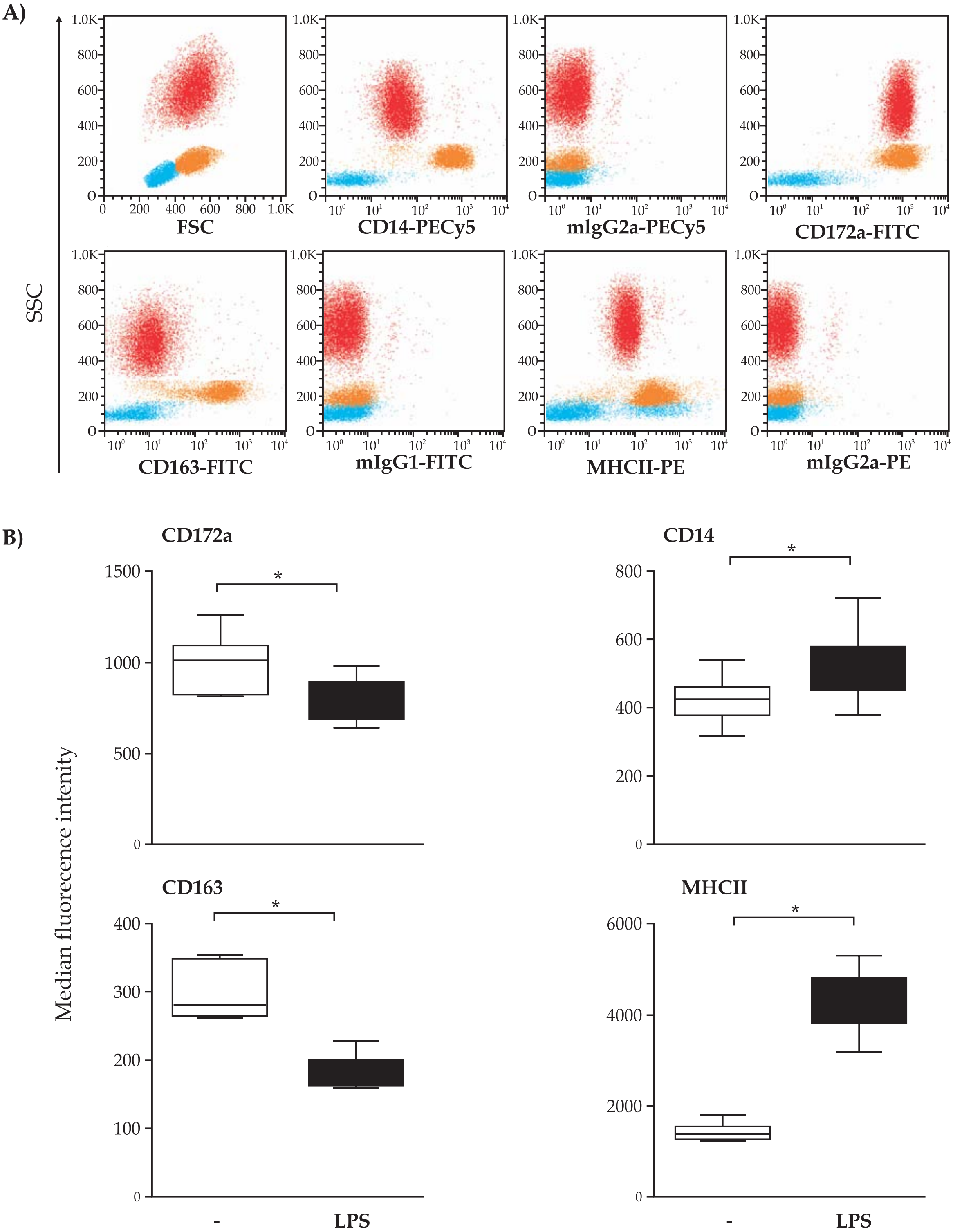

Fig 2. **A)** The staining pattern of camel leukocyte populations with monoclonal antibodies to CD172a, CD14, CD163, and MHCII. In an FSC against SSC dot plot, camel granulocytes, monocytes, and lymphocytes were identified based on their FSC and SSC characteristics. After setting gates on granulocytes (in red color), monocytes (in orange color), and lymphocytes (in blue colour), the staining patterns of different leukocyte populations with the used monoclonal antibodies were shown in separate dot plots. **B)** The impact of LPS-stimulation on the expression of the myeloid markers CD172a, CD14, CD163, and MHCII on camel blood monocytes. Camel's blood was stimulated with LPS for 4 h. After hypotonic lysis of erythrocytes, leukocytes were labeled with monoclonal antibodies to CD172a, CD14, CD163, and MHCII molecules. Labeled cells were analysed by flow cytometry. After setting a gate on monocytes, the main fluorescence intensities of labeled cells were calculated and presented as means ± SEM. (* = $p<0.05$).

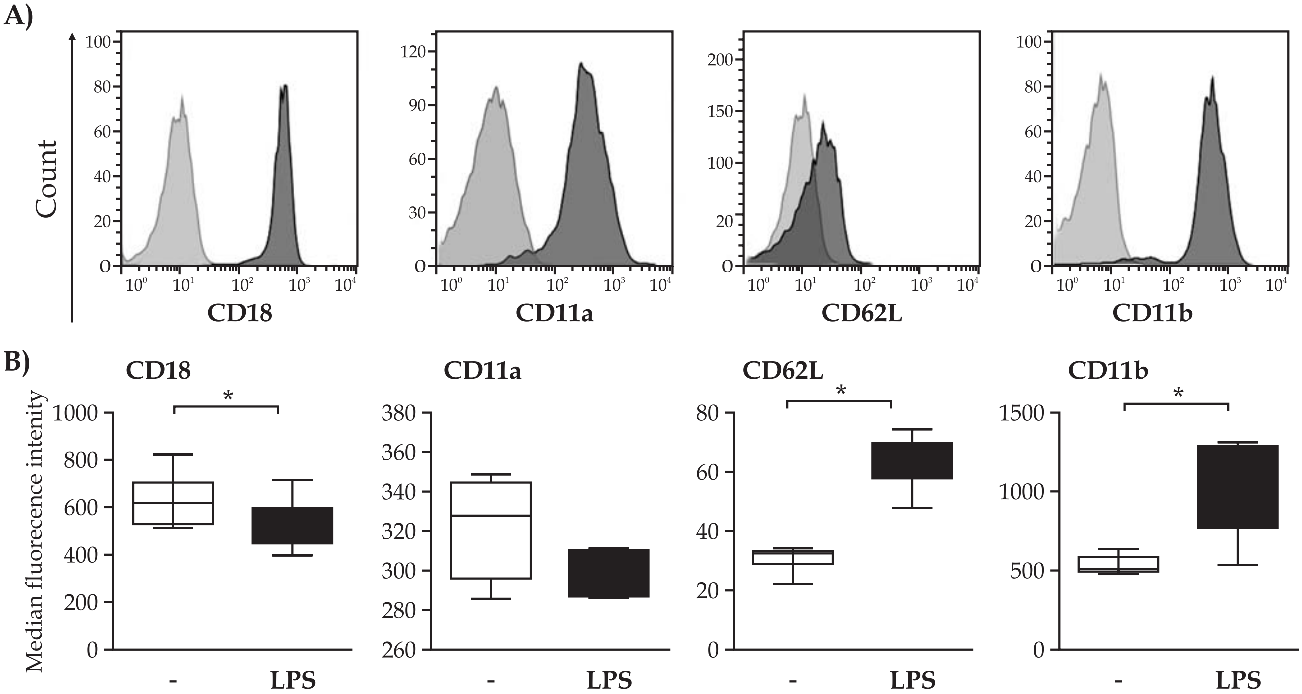

Fig 3. Influence of LPS-stimulation on adhesion molecules expression on blood monocytes. Camel blood was stimulated with LPS for 4 h. After hypotonic lysis of erythrocytes, separated leukocytes were labeled with monoclonal antibodies to CD18, CD11a, CD11b, and CD62L. Labeled cells were analysed by flow cytometry. A) Monocytes were gated based on their FSC and SSC properties. The staining of monocytes with monoclonal antibodies to CD18, CD11a, CD11b, and CD62L or with mouse isotype controls was shown as histograms. **B)** After setting a gate on monocytes, median fluorescence intensities of labeled cells for CD18, CD11a, CD11b, and CD62L were calculated and presented as means ± SEM. (* = p<0.05).

as means ± S.E. of the mean (SEM). The t-test (two groups) was used to test the difference between means. For the comparison between more than two groups (The impact of LPS on ROS production in monocytes with or without bacteria), the one-factorial analysis of variance (ANOVA) was used. A p-value of less than 0.05 was considered significant.

Results

LPS-stimulation modulates the expression of monocytic markers

Stimulation with LPS induced monocyte activation as measured by the increased median FSC and SSC (Fig 1).

In LPS-stimulated blood, monocytes changed the expression of different monocytic markers. The median fluorescence intensities (MFI) of the molecules CD172a (390 ± 16 versus 495 ± 22) and CD163 (112 ± 6 versus 182 ± 11) on monocytes were significantly reduced in LPS-stimulated blood in comparison to unstimulated blood. In contrary to this, LPS-stimulated blood showed higher MFI values for monocyte CD14 (345 ± 10 versus 285 ± 7) and MHCII molecules (4164 ± 117 versus 1455 ± 47) (Fig 2).

Effects of LPS-stimulation on the expression pattern of cell adhesion molecules on monocytes

LPS stimulation also modulated the expression of different cell adhesion molecules on blood monocytes. In comparison to unstimulated blood, the expression of CD11b (MFI 453 ± 58 versus 302 ± 15) and CD62L (MFI 69 ± 5 versus 25 ± 0.5) on monocytes was significantly increased in LPS-stimulated blood, while the expression of CD18 (MFI 372 ± 30 versus 481 ± 34) was significantly reduced. However, the expression of CD11a on monocytes did not change after stimulation with LPS (Fig 3).

Impact of LPS stimulation on phagocytosis capacity of monocytes

The capacity of the monocytes to phagocytose FITC-labelled *S. aureus ex vivo* was significantly affected by LPS-stimulation. In LPS-stimulated blood, the percentage of phagocytosis-positive monocytes was significantly lower than that in unstimulated blood (24 ± 2 versus 55 ± 9). The MFI of phagocytosis-positive monocytes, as an indicator for the number of bacteria ingested by each monocyte, was also lower in LPS-stimulated blood in comparison to unstimulated blood (598 ± 22 versus 780 ± 116) (Fig 4).

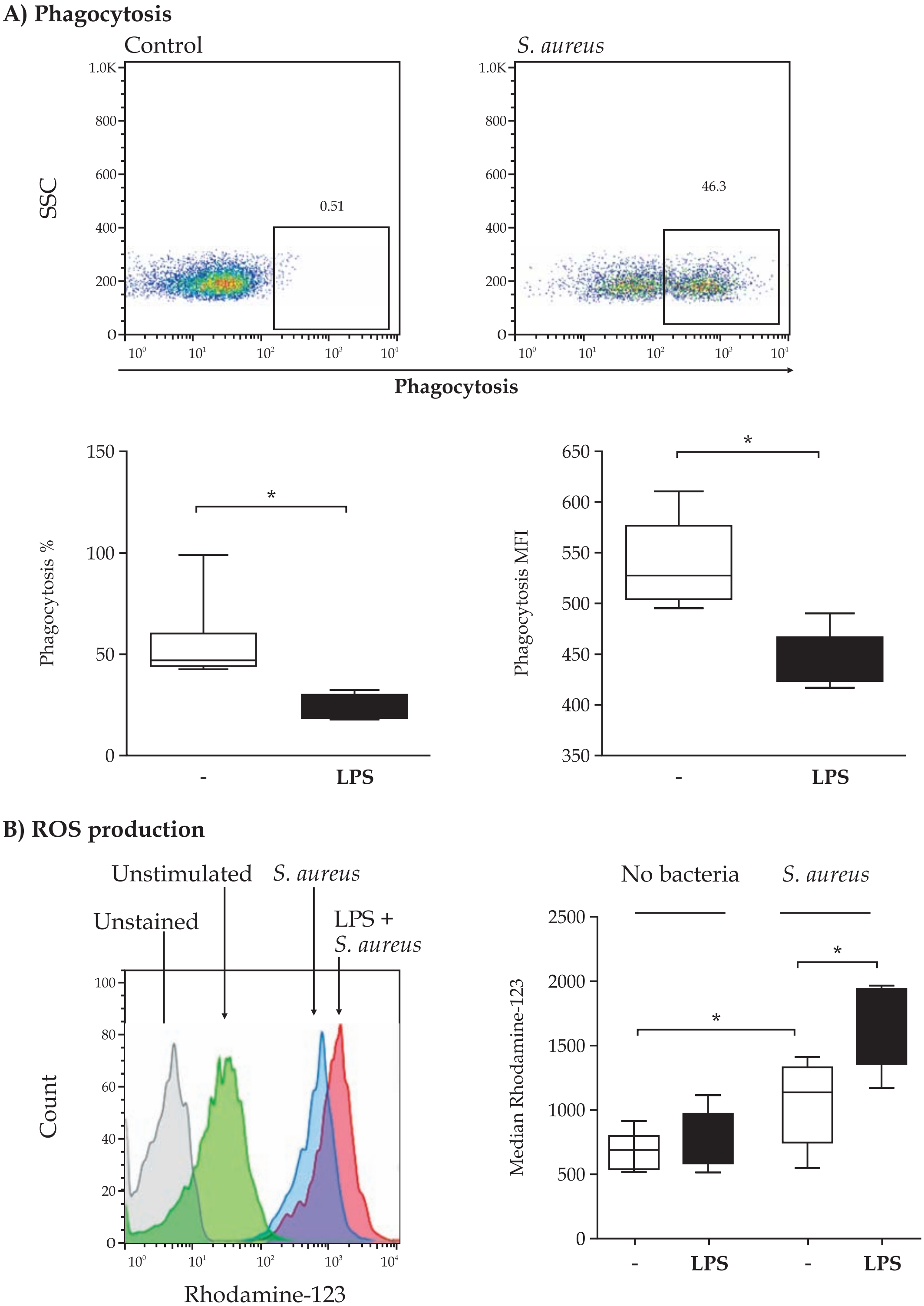

Fig 4. The impact of LPS stimulation on phagocytosis and ROS activity of the camel monocytes. Camel's blood was stimulated with LPS for 4 h or was left without stimulation (control). A) After red blood cell lysis, LPS-stimulated and un-stimulated leukocytes were incubated with FITC-labelled heat inactivated *S. aureus* and analysed by flow cytometry. After setting a gate on monocytes, phagocytosis-positive cells were defined based on their higher green fluorescence (representative results are shown in **A).** The percentage of phagocytosis and the median fluorescence intensities of green fluorescence-positive monocytes were calculated (means ±SEM). (* = $p<0.05$). **B)** LPS-stimulated and un-stimulated leukocytes were incubated with heat-inactivated *S. aureus* in the presence of the ROS-sensitive dye dihydrorhodamin 123 and labeled cells were analysed by flow cytometry (representative results are shown in B). After setting a gate on monocytes, ROS production was calculated as the median green fluorescence intensity of gated cells (means ± SEM). (* = $p<0.05$).

Impact of LPS stimulation on reactive oxygen generation in camel monocytes

Stimulation with *S. aureus* significantly induced ROS production in camel monocytes. In LPS-stimulated blood, monocytes produced significantly more ROS upon incubation with *S. aureus* when compared with monocytes from unstimulated (without LPS) blood (1654 ± 192 versus 1210 ± 67). LPS stimulation alone, however, did not induce a significant change in median ROS values of camel monocytes (Fig 4).

Discussion

Infections with the gram-negative bacterium *E. coli* are responsible for several illnesses in the dromedary camel including gastroenteritis and septicemia in camel calves and mastitis and metritis in adult she-camels (Aljumaah *et al,* 2011; Al-Ruwaili *et al,* 2012). Studies on the interaction of *E. coli* with the innate immune system of the dromedary camel are scarce. Monocytes play a key role in the antibacterial immune response through their ability to ingest and kill bacteria and to differentiate into different subtypes of tissue macrophages (Soehnlein and Lindbom, 2010; Jakubzick *et al,* 2017; Pomeroy *et al,* 2017). Depending on the type of the activating signal, monocytes undergo different phenotypic and functional changes.

To analyse the impact of the *E. coli* lipopolysaccharide (LPS) on the phenotype and the function of blood monocytes in dromedary camel, we used the whole blood stimulation model, which has the advantage of maintaining the microenvironment of immune cell interaction as it occurs *in vivo* (Gomes *et al,* 2010). LPS-stimulated camel blood monocytes showed polarisation toward the inflammatory macrophage (M1) subset as indicated by the upregulated expression of MHCII and downregulated expression of CD163 markers. The inflammatory nature of LPS-stimulated monocytes is also supported by the higher expression of the LPS-receptor CD14 and the lower expression of the signal-regulatory protein alpha (SIRPα), which functions as a regulatory receptor that inhibits cell signaling (Hussen *et al,* 2013).

Monocyte migration starts with their adhesion to endothelial cells of blood vessels, which is mediated by a set of cell adhesion molecules on monocytes and their ligands on endothelial cells (Imhof and Aurrand-Lions, 2004; Gerhardt and Ley, 2015). LPS stimulation of camel monocytes induced the upregulation of L-selectin, which is constitutively expressed on non-activated leukocytes and is rapidly shed upon chemotactic stimulation (Amulic *et al,* 2012). This indicates an inhibitory effect of LPS-stimulation on monocyte adhesion and likely transmigration. This is also supported by the LPS-induced downregulation of CD18, the beta chain of the cell adhesion molecule Mac-1 (CD11b/CD18), which mediates the subsequent firm adhesion of monocytes to the activated endothelium (Imhof and Aurrand-Lions, 2004; Gerhardt and Ley, 2015). However, the expression of CD11a was unchanged and the expression of CD11b was even enhanced on the LPS-stimulated monocytes in our study. These two molecules are essential for the adhesion of the migrating monocytes (Imhof and Aurrand-Lions, 2004; Hussen *et al,* 2013; Gerhardt and Ley, 2015; Hussen *et al,* 2016). CD11a requires to dimerize with CD18 to form the adhesion molecule LFA-1 (Roos and Law, 2001; van de Vijver *et al,* 2012). The lack of one of the heterodimer components renders this molecule nonfunctional. Similarly, the CD11b binds to CD18 to form the complement receptor 3 (CR3), which plays an important role in opsonisation and enhancing phagocytosis (Ley *et al,* 2007; Muller, 2013). Therefore, through the downregulation of CD18, LPS impairs leukocyte adhesion and phagocytosis.

Phagocytosis of bacterial pathogens and the subsequent killing of ingested bacteria are key anti-microbial effector mechanisms of monocytes during the first stages of the innate immune response (Hussen *et al,* 2013). Our data showed that LPS-stimulated monocytes have a reduced capacity to ingest *S. aureus*, but produced more ROS upon stimulation with the same bacteria. This indicates a negative effect of LPS on the antimicrobial capability and an enhancing effect on the pro-inflammatory function of monocytes.

In a previous report, we described three heterogenic subpopulations of monocytes in dromedary camels based on the expression profiles of MHCII and CD14 (Hussen *et al,* 2020). Subset one expresses high levels of CD14 and low levels of MHCII and is the most abundant monocytes. Subset two is a minor subset of monocytes, which expresses high levels of CD14 and MHCII and is considered the inflammatory monocytes with increased phagocytic activity. While subset three is another minor subpopulation of monocytes with low levels of CD14 and high levels of MHCII. LPS stimulation of camel monocytes in the current study seems to drive the monocyte population into a new subtype resembling subset two but with reduced phagocytic activity resembling subset three. This might represent a new immune evasion mechanism by which *E. coli* escapes phagocytosis. Indeed, treatment of mouse bone marrow-derived macrophages with LPS was shown to induce tolerance and impaired *E. coli* phagocytosis (Kapellos *et al,* 2016).

Conclusions

The enhanced expression of MHCII molecules and the reduced levels of CD163 on LPS-stimulated camel monocytes indicate a shifting toward inflammatory macrophage-1 (M1) profile. LPS-stimulated monocytes increased the expression of the adhesion molecules CD62L and CD11b while decreased the expression of CD18. Functionally, stimulation with LPS reduced the phagocytosis capability of monocytes but enhanced their ability to produce ROS. Collectively, these results suggest a modulating effect of LPS on the phenotype, adhesion, and phagocytic functions of camel blood monocytes and propose a possible new immune evasion mechanism. Whether these effects contribute to the pathogenesis of *E. coli* infections in dromedary camels, needs further studies. Although the current study may contribute to the understanding of the response of camel monocytes to LPS, several questions are still open in this regards, including LPS-tolerance in camels and the characterization of functional subtypes of camel monocyte-derived macrophages.

Acknowledgements

The authors extend their appreciation to the Deputyship for Research & Innovation, Ministry of Education in Saudi Arabia for funding this research work through the project number IFT20010.

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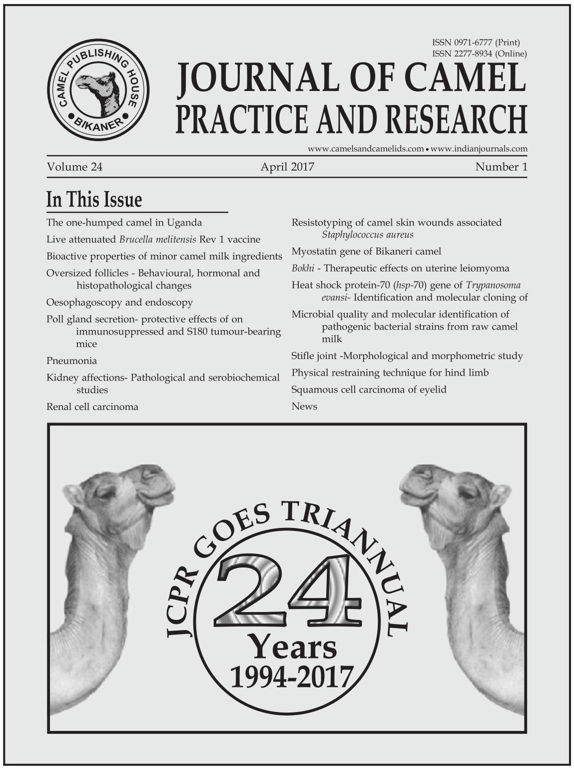

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DOI : 10.5958/2277-8934.2021.00010.2 Vol 28 No 1, p 69-73

MOLECULAR DETECTION OF *Trypanosoma evansi* IN CAMEL (*Camelus dromedarius*) USING INTERNAL TRANSCRIBED SPACER 1 OF RIBOSOMAL DNA

Vijayata Choudhary[1], Samar Kumar Ghorui[2], Abhishek Gupta[1], Sonu Yadav[1] and Monika Choudhary[1]
[1]Department of Veterinary Parasitology, College of Veterinary and Animal Sciences, RAJUVAS, Bikaner, Rajasthan, India
[2]NRCC, Jorbeer, Bikaner, Rajasthan, India

ABSTRACT

The purpose of this study was to determine clinical and subclinical prevalence of *T. evansi* among camels in Bikaner, India. In the present study, camels were examined parasitologically by blood smear examination and by Polymerase chain reaction (PCR) targeting ITS region of *T. evansi*. Blood samples of 74 camels were collected during the period from July 2019 to November 2019. The blood smear examination and molecular analysis showed 6.75 and 21.62%, respectively prevalence of *T. evansi* infection in camels, suggesting high sensitivity of diagnosis towards molecular tests. In comparison to parasitological methods, PCR proved fast, precise, sensitive detection diagnostic method for trypanosome infected camels. The adult camels possessed acute infection of 28.88% of *T. evansi* when compared to young camels with 10.34% infection, suggesting no significant difference (χ^2=1.141, P > 0.05). Hence, molecular diagnosis targeting ITS region among the infected trypanosome camels is more reliable and accurate for epidemiological survey and control programmes of trypanosomosis caused by *T. evansi*.

Key words: Camel, diagnosis, polymerase chain reaction (PCR), *Trypanosoma evansi*, trypanosomiasis

Trypanosomosis occurs both in acute and chronic form in camel showing intermittent fever, anaemia, progressive weight loss, dependent oedema, nervous symptoms, abortion and major production losses (Abdel-Rady, 2008; Desquesnes *et al,* 2013; Zangooie *et al,* 2018). The early diagnosis through parasitological technique is considered to be critical as the detection of the organism in blood is not reliable because of intermittent parasitaemia, leading to non-specific confirmatory diagnosis for *T. evansi* (Ali *et al,* 2011). Many reports suggest the diagnosis of trypanosomosis through serological assays (like card agglutination tests) and molecular techniques (like genus or species specific PCR or ITS-PCR) (Elhaig *et al,* 2016; Zongooie *et al,* 2018). However, it lacks specificity or sensitivity for serological methods that detect antibodies or antigens. Hence, molecular technologies like, polymerase chain reaction (PCR) have been developed for specific regions. In addition, PCR is a reliable diagnostic tool that detects infection with acute sensitivity, reproducibility and specificity at an early stage (Tehseen *et al,* 2015; Elhaig *et al,* 2016).

Several DNA sequences in the host blood have been examined to evaluate the sensitiveness and enzymatic amplification of animal trypanosomal DNA (Masiga *et al,* 1992). The ribosomal genetic analysis area of internal transcribed spacer 1 (ITS1) showed the insight into the diversity and epidemiological implications of trypanosome species (Lun and Desser, 1995; Lai *et al,* 2008). This locus, which has a length usually between 300-800 bp between the 18s and 5.8s, is considered to be very specific and has no effect with apicomplexan or bacterial or mammalian DNA species. The aim of the study was to utilise case assessments for the detection and confirmation of *Trypanosoma evansi* in camels at Bikaner, India by parasitological (Giemsa stained blood examination) and molecular based methods (PCR).

Materials and methods

Sample collection

A representative of 74 blood samples [males (n=55) and female camels (n=19)] was collected randomly from a wide age group of both sexes (Table 1) from July to November 2019. Jugular venepuncture was done to ollect blood samples in (3 ml of each) a clean Vacutainer with ethylene di-amine acetic acid (EDTA) and samples were taken to the laboratory

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under cold chain. Part of the collected blood samples were taken with the use of Giemsa stained blood smear for parasitological examination and the other part was preserved for PCR amplification at -20°C for the extraction of trypanosoma DNA. All the camels were further injected with suitable chemotherapeutic agent on the same day of sample collection.

Parasitological examination of blood smears

Blood smear from each camel was prepared, dried in air, fixed with methanol (99%), stained with diluted Giemsa stain. The presence of *T. evansi* in the oil microscope submersion objective was checked for a maximum magnification of 100X. Furthermore, as described by Murray *et al* (1977) and Paris *et al* (1982), trypanosome species was identified.

Molecular analysis of DNA samples

Blood DNA extraction kit spin-column was used to extract genomic DNA from the blood samples (Thermo-Fisher, US), as directed by the manufacturer. Ultraviolet spectrophotometers were used to check the concentration and integrity of the DNAs at 260 and 280 nm, diluted them into 50 ng / µl and store them at -20°C later for PCR applications. The PCR assay was carried out targeting internal transcribed spacer (ITS) region of rDNA using the primers ITS1 CF: 5′-CCG GAA GTT CAC CGA TAT TG-3′ and ITS1 BR: 5′-TTG CTG CGT TCT TCA ACG AA-3′ (Njiru *et al,* 2005). Desalted and deprotected oligoes were synthesised at 25nm scale by the commercial vendor (Integrated DNA Technologies, USA), dissolved and finally diluted to working solution of in Tris-EDTA buffer.

The total volume of the reaction was 50µl containing a 5XGoTaq® coloured reaction buffer, 3.0mM MgCl2, deoxynucleoside Triphosphates (200 µM each), 1.0 µM and 1.25 U Taq DNA (GoTaq ®, Promega Co. USA) primers. The first cycle was 94°C (for 4 minutes and then 30 cycles of denaturation at 94°C (for 15 seconds), 1 minutes at 58°C and 45s at 72°C, and 10 minutes at 72°C). In contrast to a molecular marker (1 Kb plus DNA ladder, Invitrogen, life technology, USA), the products of PCR were tested using 1.0% agarose gel with ethidium bromide (0.5 g /mL) by electrophoresis, the amplified DNA image was captured by using Alpha imager (USA) documentation system.

Statistical analysis

Two age groups of animals, animals up to 5 years (young), and animals over 5 years old (adult) were divided. A significant P value for chi - square (χ^2) test ($p < 0.05$) has determined the association between prevalence of *T. evansi* and risk factors such as sex and age.

Results and Discussion

Surra is seen in all areas of the world as a major restriction on camel health and productivity that has serious morbidity and mortality (Enwezor and Sackey, 2005; Abdel-Rady, 2008). In this study, we aimed for the confirmation of surra assessed by diagnostic techniques including microscopic detection of parasites and molecular diagnostic technique. In the middle of the Thar desert, Bikaner region (study area) has a warm desert climate with very little precipitation and extreme temperatures, according to Koppen climate classification (BWh) (Peel *et al,* 2007).

The summary of all results is presented in the Table 1 and 2. In blood examination, *T. evansi* was found in 5 camels (6.75%). The Giemsa stained blood smears microscopy revealed that *T. evansi* was monomorphic, slender thin trypomastigotes with free flagellum and a subterminal small kinetoplast thin posterior extremity (Fig 1). The undulating membrane of parasite was well developed and highly visible.

In this research, the microscopic parasite description stated to be identical as defined, irrespective of geographical location and strain origin (Desquesnes *et al,* 2013). The level of low parasites in the peripheral blood was claimed to be a diagnostic tool which is of limited value and low sensitivity for subacute or chronic cases among camels (Baticados *et al,* 2011). A low prevalence (6.75%) through microscopic examination of blood smear in the present study. As the disease advances to the chronic form, very low incidence of parasitemia is exhibited which prevents parasitological diagnosis of trypanosomes (Abdel-Rady, 2008) and possibly it is governed by the 'behavioural' nature of trypanosomes to hide itself in the hosts hence not seen in the peripheral circulation. Unsuccessful detection of trypanosomes during the chronic stage of infection was evidenced (Masake *et al,* 2002; Desquesnes, 2004). However, microscopic detection of parasites below 2,500,00 parasites/ml was not feasible, hence other methods are necessary for the diagnosis of surra disease (Desquesnes *et al,* 2013).

Molecular detection of T. evansi through ITS PCR assay

The results of ITS-PCR for *T. evansi* DNA in infected camels showed an amplicon size of ~480 bp. The obtained amplicons in different samples

Table 1. Prevalence of *Trypanosoma evansi* by conventional blood smear assay and ITS1-PCR assay.

Species	Total no. of samples	No. of positive blood smear samples	Prevalence (%) for blood smear assay	No. of ITS1-PCR amplicons	Prevalence (%) for ITS1-PCR assay
Camel	74	5	6.75%	16	21.62%

Table 2. Prevalence according to the age groups and sex of *Trypanosoma evansi* in examined camels.

Factor		No. of samples	Blood smear assay		ITS1- PCR assay	
			No. of samples	Prevalence (%)	No. of samples	Prevalence (%)
Age	Young (< 5 years)	29	1	1.35	4	10.34
	Adult (> 5 years)	45	4	8.88	12	28.88
χ^2 value			0.732		1.141	
Sex	Male	55	4	7.27	12	21.81
	Female	19	1	5.26	4	21.05
χ^2 value			0.080		0.003	

The statistical analysis for χ^2-chi square was represented in P > 0.05 and is considered as non-significant.

showed different intensity, suggesting the varying levels of infection, while control samples did not show any amplicon (Fig 2). Fig 2 shows the random amplification of certain samples among the 74 analysed. Overall, a total of 16 samples gave a positive amplification of around 480 bp.

Molecular methods are the most powerful tools in host animals and vectors to detect the *T. evansi* and have been increasingly used to detect *T. evansi* in carrier animals (Sukhumsirichart *et al*, 2000; Aboed and Faraj, 2017). In the present study ITS-PCR results revealed 21.62% prevalence of trypanosomosis in camels (Table 1). A higher incidence of *T. evansi* in camels (34.4%) in PCR than blood film examinations (3.3%) in previous studies was reported in India (Ravindran *et al*, 2008). Similarly, higher *T. evansi* prevalence was reported for PCR assay in camels (56.9%) than blood film examination (4.1%) from

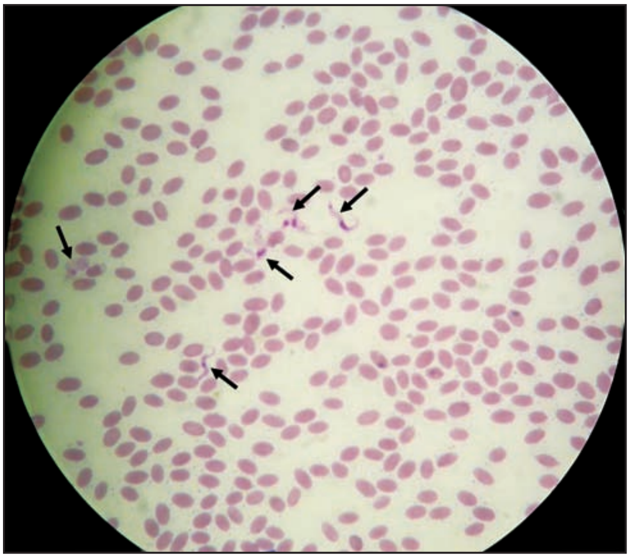

Fig 1. *Trypanosoma evansi* in thin Giemsa stained blood film from infected camel (X1000).

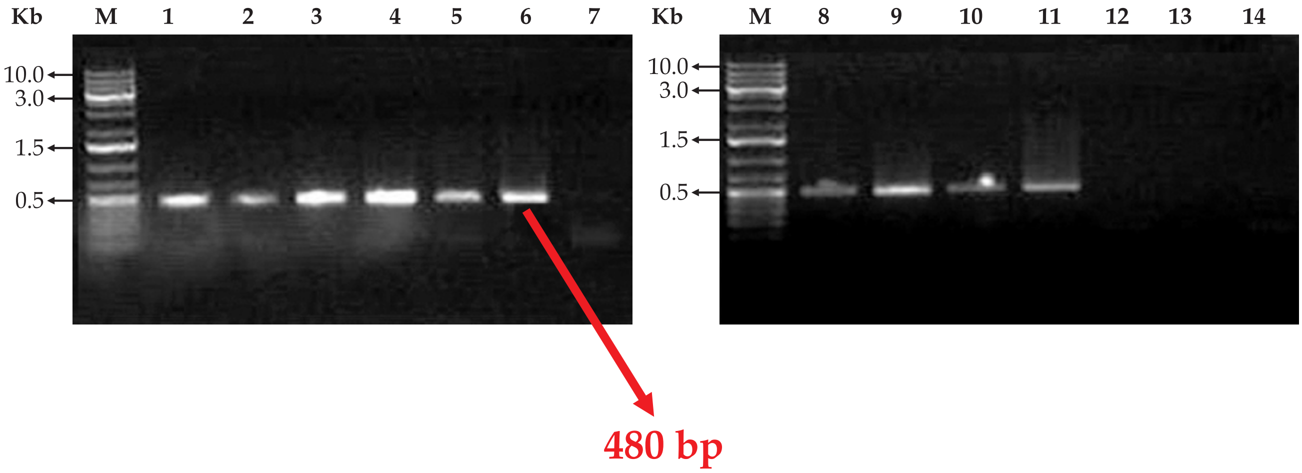

Fig 2. Detection of *T. evansi* through ITS1-PCR assay in few blood samples. The EtBr stained agarose gel image represents samples with expected amplicon size of 480 bp from Lane 1- 6, lane 8- 11, Lane M- 1 Kb DNA marker, lane 7- negative control, lane 12-14- *T. evansi* negative camel blood samples.

Egypt (Abdel-Rady, 2008), 90% and 28% from Iraq (Aboed and Faraj, 2017), 90% and 6% from Sudan (Ali *et al,* 2011), 31% with PCR and 0.7% with Giemsa stained thin smears (GST) from Pakistan (Tehseen *et al,* 2015). The samples which were not detected through blood smear examination were able to get diagnosed through PCR assay by giving a positive amplicon. It may be due to the fact that PCR is able to detect infections in blood in the very early stage. The sensitivity threshold (ranges from 0.001-0.02 parasite/μL) for trypanosomes detection by PCR is not available below this infection level (Desquesnes and Davila, 2002). Therefore, PCR is considered the more specific and sensitive method the other traditional parasitological methods or clinical signs used in this study. In this respect, the ITS rDNA region favours a standardised test, as its flank areas have remained highly conserved and the size differences between trypanosome species and their subsets have increased. The locus is made up of 100–200 copies, of 18S, 5.8S and 28S rRNA genes each isolated from two ITS regions (Desquesnes and Davila, 2002). The ITSl primer pair is used to enhance this region that reveals the amplicon size (~480bp) of trypanosomes and also in *T. evansi* in other parts of the world, in this study annealing to rDNA regions 18S and 5.8S was allowed. A similar kind of study was performed in characterising the *T. evansi* isolated from ponies, camel, donkeys and cattle from India, and suggested a clear evidence of diversity in the ITS-1 gene region (Sarkhel *et al,* 2017). When few camel samples of Iranian dromedary were analysed, the 5.8S rRNA region showed high conserved nucleotide sequence of Trypanosoma spp., where as the ITS-1 and ITS-2 regions showed genetic diversity (Pourjafar *et al*, 2013). Such PCR analysis on ITS1 region can also differentiate the *T. evansi* isolated from different geographical regions of Sudan (Salim *et al,* 2011).

Overall, 16 of the 74 camels examined were positive for *T. evansi* in present study (Table 1). The data on the prevalence of *T. evansi* in different age groups and sex as examined in camels are provided in Table 2. The *T. evansi* infection prevalence was higher (28.88%) in adults when compared with young camels (10.34%) with a lack of significant variation (χ^2=1.141, P > 0.05). The rate of *T. evansi* infection in males and females was 21.81 and 21.05%. The prevalence of *T. evansi* between male and female animals, however, was not significantly distinct (χ^2=0.003, P > 0.05).

Trypanosomosis was high in camels over the age of five (28.88%), while infections in young camels was low (10.34%). Old camels with poor management, chronic nature of illness, heat, draught, and vector preference may be more susceptible. Higher prevalence rates in adults than young once's is in more agreement with previous reports (Eshetu *et al,* 2013; Khosravi *et al,* 2015; Mirshekar *et al,* 2017; Bala *et al,* 2018). This finding is inconsistent with the studies by Singh *et al* (2004); Elbalkemy *et al* (2016), who reported that the highest prevalence was in young camels up to 5 years of age. Sex-wise prevalence and percentages reported were almost similar, in males 21.81% and females 21.05% of the positives. Present study was in agreement with Ngaira *et al* (2002), who reported no differences in *T. evansi* prevalence in both sexes. The results showed that the prevalence of *T. evansi* was influenced and statistically significant by age and sex in camels (P >0.05).

In conclusion, conventional parasitological techniques are important to understand protozoan parasites and their biology, ecology and epidemiology. However, the molecular diagnostic technique such as PCR is useful to detect the infection in very early stages. The PCR should be considered seen for the effective monitoring and supervision of trypanosomes as an additional approach and should be used in conjunction with the microscope examination method.

Ethics declaration

The study protocol was implemented with approval from the Institutional Animal Ethics Committee (IAEC). Consent for blood sampling of flock was obtained from owners. Animals were bled using conventional protocols.

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DOI : 10.5958/2277-8934.2021.00011.4 *Vol 28 No 1, p 75-77*

CRIMEAN-CONGO HAEMORRHAGIC FEVER: A SEROLOGICAL SURVEY IN DROMEDARY CAMELS

Ulrich Wernery, Rekha Raghavan, Ginu Syriac, Marina Rodriguez, Fatma Graiban Almheiri and Sunitha Joseph

Central Veterinary Research Laboratory, Dubai, UAE

ABSTRACT

Crimean-Congo Haemorrhagic Fever (CCHF) is a tick-borne viral infection caused by a tick-borne virus (Nairovirus), a negative sense, single-stranded RNA virus in the family *Bunyaviridae*. A serological survey conducted on 173 camel sera from the United Arab Emirates (UAE), Pakistan, and Kazakhstan showed a high CCHF serological prevalence of 94.2%.

Key words: Antibody ELISA survey, CCHF, dromedary camels, tick-borne viral infection

Crimean-Congo Haemorrhagic Fever (CCHF) is caused by a tick-borne virus (Nairovirus), a negative sense, single-stranded RNA virus in the family *Bunyaviridae*. It is a human viral disease that was first found in the Crimea in 1944 and therefore given the name, Crimean haemorrhagic fever. In 1969, the disease was also detected in Congo, thus resulting in the current name of the disease (Appannanavar and Mishra, 2011). The virus has been found in wide areas of South Africa, Southern Europe, Eurasia, and Western China as shown in the following map (Fig 1).

The virus replicates in the host tick as it passes from larval to adult stages (transstadial transmission) and it can also be transmitted from one generation of ticks to the next (transovarial transmission). Thus, the tick is not only the disease vector, but also a reservoir (The Merck, 2016). Thirty species of ticks, particularly the genus *Hyalomma*, the most prevalent tick in the Arabian Peninsula, is the vector of the CCHF virus, but it has also been isolated from other genera of ixodid ticks. CCHF is a severe haemorrhagic viral disease of humans acquired from tick bites, tissues of

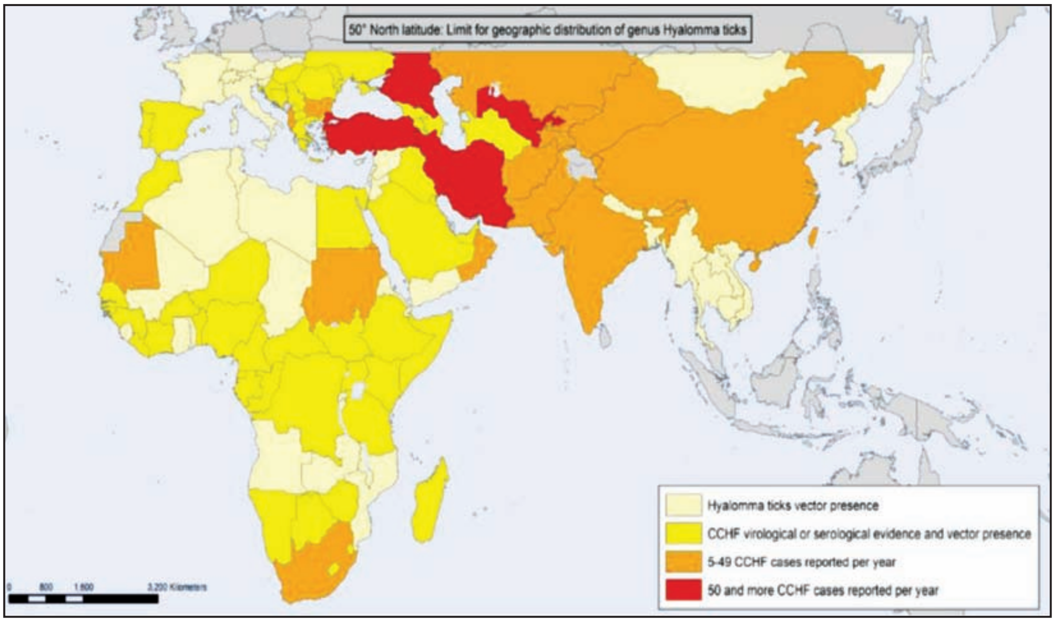

Fig 1. Geographical distribution of CCHF. Downloaded from https://www.who.int/images/default-source/health-topics/crimean-congo-haemorrhagic-fever/global-cchfrisk-2017.png?sfvrsn=4b961c4c_6 on December 13, 2020.

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infected wild or domestic animals and human patients with the disease.

It is primarily an occupational disease seen in butchers, veterinarians, animal handlers and farmers (Sahak *et al*, 2019). However, sporadic human cases have been reported in the UAE. For example, in 2010, two fatal human cases of CCHF were reported by Dabal *et al* (2016) in a hospital in Dubai, UAE.

Animals seem to be immune to the virus, but many different animal species have produced antibodies against CCHF.

A serological survey was conducted on camel sera from the UAE, Pakistan and Kazakhstan with a sandwich antibody ELISA, the results of which are reported here.

Materials and Methods

For the serological investigation of the dromedary sera, a novel double-antigen sandwich ELISA was used. It is a multispecies antibody ELISA from ID Vet, named ID Screen® CCHFV double antigen multispecies ELISA. This ELISA has a specificity of 100% and a sensitivity of 99% for the detection of CCHF antibodies. The ID Screen® uses a recombinant purified CCHF nucleoprotein antigen-HRP. The cut-off stands at 30%.

In total 173 camel sera including 8 Bactrian camels introduced from Kazakhstan and 8 hybrid camels between Bactrian and dromedary camels bred in Dubai were tested which is shown in Table 1. Also, 46 camel sera, tested from the Dubai area included 5 sera that had been collected in 2011.

Table 1. Number and origin of camel sera tested for CCHF antibodies at CVRL.

Origin	Number
Pakistan	49
Dubai	46
Fujairah Bactrians (Kazakhstan)	8
Hybrid camels	8
CVRL dromedary	11
Dubai surroundings	51
Total	**173**

Results

Results of the ID Screen® CCHF double antigen multi-species antibody ELISA are summarised in Table 2. Of the 173 camel sera tested, 10 of them were negative which is 5.7% and 94.2% were positive. Dromedary sera from Pakistan had a seroprevalence of 98%, 8 Bactrian camels introduced from Kazakhstan to Fujairah (UAE) were all positive, whereas hybrid camels raised in Dubai displayed a seroprevalence of 75%. Dromedary camels in and around Dubai showed a seroprevalence between 88 and 100%.

Table 2. Competitive CCHF antibody ELISA results of 173 camel sera of different origin.

Origin	Number	ELISA positive samples (%)
Pakistan	49	48 (98%)
Dubai	46	46 (100%)
Fujairah Bactrian (Kazakhstan)	8	8 (100%)
Hybrid Camels	8	6 (75%)
CVRL	11	10 (91%)
Dubai surroundings	51	45 (88%)
Total	**173**	**163 (94.2%)**

Discussion

CCHF is enzootic, but asymptomatic in many animal species such as cattle, sheep, goats, camels, and hares (Schwarz *et al*, 1996). Several reports deal with the detection of CCHF antibodies from different animal species as well as the isolation of this virus from animals. An overview of the literature was compiled by Wernery *et al* (2014). In experimental inoculations with the CCHF virus, sheep and cattle become infected but do not produce disease. IgG ELISAs detect life-long antibodies and antibody prevalence in adult livestock species may reach more than 50% in endemic regions (The Merck, 2016).

In a recently published paper, Camp *et al* (2020) indicated that exposure to CCHFV is common among camels in the UAE, suggesting that the virus is endemic in this country. The researchers found CCHFV ELISA antibodies in 67% of dromedary sera from the UAE, a percentage which is lower than found in our study with 88 to 100%. Interestingly, a small number of camel sera which had been collected in 2011 were all positive. Additionally, dromedaries from Pakistan and Bactrians from Kazakhstan introduced into the UAE possessed a high CCHF seroprevalence. Also, hybrids between Bactrian and dromedary camels bred in the UAE showed a 75% positivity to the CCHF virus. Both research groups used the same competitive ELISA.

Camp *et al* (2020) not only showed a high CCHF serological incidence in the UAE dromedary camels, but also obtained CCHF viral RNA from *Hyalomma dromedarii* ticks and camel sera. This showed that transmission to camels is via native infected *Hyalomma dromedarii* ticks which is the most common tick in the UAE. Interestingly, in a previous survey of the UAE livestock around 1995, camels and camel ticks were ruled out as CCHFV reservoirs (Rodriguez *et al*, 1997). Our investigation shows that over the last 25 years CCHF seroprevalence in dromedary camels has increased significantly in the UAE and poses a severe risk to people working with camels. The lack of significant clinical signs in livestock warrants no treatment considerations for animals. However, controlled strategies for human beings' infection should include the avoidance of tick bites by using insecticides when camels which harbour ticks are treated. Tick control must also be practiced before slaughtering or grooming animals.

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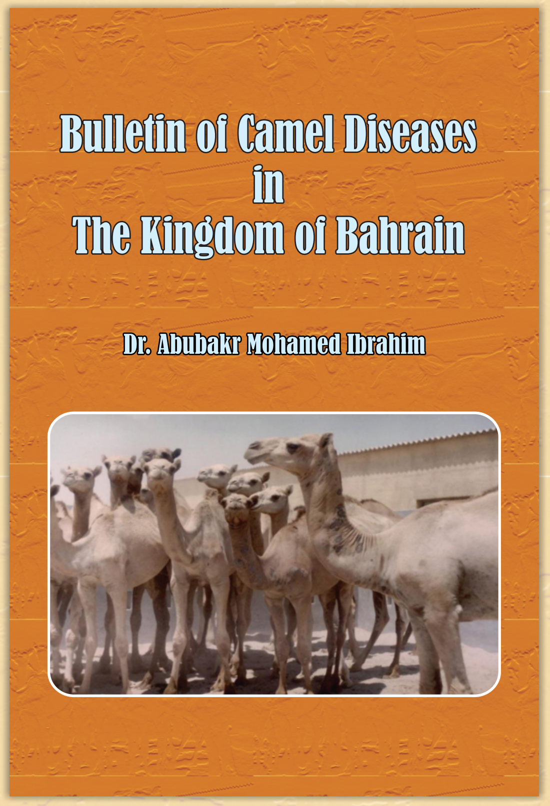
Bulletin of Camel Diseases in The Kingdom of Bahrain

This is a unique book which contains chapters on infectious and non-infectious diseases. The chapter on infectious diseases contains six sections. The section of bacterial diseases is subclassified as corynebacterium abscesses, paratuberculosis, hepatic necrobacillosis, mastitis, *Streptococcus zooepidemicus,* bacterial Infection in young camels, uterine Infection, infection of the vagina and vulva and other disorders. The section of protozoal diseases has narrations on trypanosomiasis, anaplasmosis and babesiosis. The section on parasitic infections is composed of gastrointestinal parasites in young camels, echinococcosis and mange. The section of mycotic diseases contains phycomycosis and ringworm. The section of viral diseases contains subsections on camel pox and contagious ecthyma. Edema Disease is described in miscellaneous section. The chapter on noninfectious diseases has three sections. Other section on poisoning describes pyrethroid, nitrate and toxic jaundice. The section describes zinc deficiency. The miscellaneous section describes foreign bodies, sand colic, bloat, caecal impaction, hydrocephalus, corneal opacity and osteochondroma.

About the Author

Dr. Abubakr Mohamed Ibrahim is a Veterinary Pathologist and worked for a long period as head of Royal Court Veterinary Laboratory. Kingdom of Bahrain which led to genesis of this publication out of his rich experience in diagnosing camel diseases in the Kingdom of Bahrain. This would be counted as his significant contribution and future researchers will find it easy to understand the pattern of camel diseases in this part of the world. Dr. Abubakr had majority of his publications based on camel diseases of Bahrain. Thus publication of this book would prove an important reference book for the camel practitioners and researchers.



Editor:
Dr. T.K. Gahlot

Edition: 2014

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Publisher:
Camel Publishing House
67, Gandhi Nagar West,
Near Lalgarh Palace,
Bikaner-334001 Rajasthan, India
email: tkcamelvet@yahoo.com

Website:
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Price: US $ 90
INR 1000

ISBN 81-903140-2-5

DOI : 10.5958/2277-8934.2021.00012.6 Vol 28 No 1, p 79-84

INFLUENCE OF 8 KM TRAINING ON CARDIAC BIOMARKERS ALONGSIDE HAEMATOBIOCHEMICAL PROFILES IN RACE CAMELS

Mohamed Tharwat[1,2]

[1]Department of Veterinary Medicine, College of Agriculture and Veterinary Medicine, Qassim University, P.O. Box 6622, Buraidah, 51452, Saudi Arabia

[2]Department of Animal Medicine, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt

ABSTRACT

This study was designed to investigate the effect of 8 km training on the serum concentrations of the cardiac biomarkers troponin I (cTnI) and creatine kinase myocardial band (CK-MB) in 23 healthy racing camels (*Camelus dromedarius*). From each camel, 2 blood samples were collected; before training (T0) and within 2 h after training (T1). Serum concentrations of cTnI and CK-MB, and hematobiochemical profiles were estimated. Compared to a value of 7.21±1.9 ×10^9/L pre-training, neutrophils decreased significantly to 6.2±2.2 × 10^9/L post-training (P=0.05). Similarly, haemoglobin concentration decreased from 11.1±1.1 g/dL before training to 10.3±2.0 g/dL after training (P=0.0002). The MCV showed a similar pattern where it decreased from 26.0±1.3 (fl) pre-training to 24.0±3.6 (fl) post-training (P=0.01). Other haematological variables did not show any significant changes before and after training (P>0.05). The serum activity of AST increased from 85.5±12.8 U/L before training to 91.5±8.6 U/L after training (P=0.0001). Serum concentration of TP increased also from 54.2±8.7 g/L pre-training to 59.0±3.8 g/L post-training (P=0.04). On the contrary, the serum concentration of lactic acid decreased from 3.9±0.8 (mmol/L) before training to 3.3±0.4 (mmol/L) after training (P=0.004). Other biochemical variables did not show any significant changes before and after training (P>0.05). Before training the serum concentration of cTnI was 0.03±0.03 ng/mL; a value that did not differ significantly when compared to the value of 0.04±0.02 (ng/mL) after training (P=0.60). The CK-MB value differed significantly before and after training (0.47±0.1 ng/mL before training vs 0.48±0.8 ng/mL after training; P=0.004). In conclusion, the cardiac biomarker cTnI did not change significantly after training compared to baseline levels, a result that disagrees with values in camels after race. However, the CK-MB increased significantly after training compared to pre-training serum concentrations.

Key words: Cardiac biomarkers, cTnI, CK-MB, racing camels, training

In recent years, there has been increasing interest in camel racing in the Arab countries especially Gulf region. The average speed of a camel during a race is approximately 9.5 m/sec (Snow, 1992). At the beginning of the race, most camels gallop, and they change frequently between pacing and galloping during the race. Interestingly, camels can pace almost as fast as they can gallop. Many scientific investigations have focused on the training (Evans *et al,* 1992; Snow, 1992). Although the physiological adaptations of the camel have been studied extensively, changes associated with exercise have been ignored until recently (Evans *et al,* 1992).

The diagnostic and prognostic value of the cardiac biomarkers troponin I (cTnI) and creatine kinase myocardial band (CK-MB) has been studied extensively in camels as well as in other animal species (Tharwat, 2012; Tharwat *et al,* 2012; Tharwat *et al,* 2013a,b,c,d,e; Tharwat and Al-Sobayil, 2014a,b,c; Tharwat *et al,* 2014a,b; Tharwat, 2015; Tharwat and Al-Sobayil, 2015; Tharwat, 2020). The serum concentration of cTnI elevates after acute myocardial injury because of leakage from the damaged myocardial cells (O'Brien *et al*, 2006). The cTnI has also a high sensitivity and specificity in animals with diseases of cardiac and non-cardiac origin (O'Brien *et al,* 2006. The degree of increase in cTnI has been shown to correlate with the extent of myocardial damage and with survival in humans (Stanton *et al*, 2005) and animals (Oyama and Sisson, 2004; Fonfara *et al*, 2010). In human athletes, a number of studies have shown increased cTnI concentrations following high-intensity short-duration exercise and cycle-touring events (Serrano-Ostáriz *et al,* 2009; Shave *et al,* 2010; Serrano-Ostáriz *et al,* 2011). The other cardiac biomarker CK-MB has been reported to increase

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with exercise (Mamor *et al,* 1988; Rahnama *et al,* 2011). A rise in CK-MB is not always indicative of myocardial damage; it has been elevated in patients with acute skeletal muscle trauma, dermatomyositis, polymyositis, muscular dystrophy and renal failure (Erlacher *et al,* 2001).

Recently, the cTnI and CK-MB changes in race camels following 5 km race have been determined (Tharwat *et al,* 2013c). Knowledge of the effect of racing on the concentrations of the cardiac biomarkers cTnI and CK-MB is of importance when evaluating racing camels with suspected cardiac disease after recent racing or maximal exercise. However, studies on the influence of training on the serum concentrations of the cardiac biomarkers in camels is lacking. The aim of the present study was therefore to investigate the effect of 8 km training on the serum concentrations of cTnI and CK-MB alongside haematobiochemical profiles in healthy racing camels.

Materials and Methods

Animal and blood sampling

Twenty-three healthy female racing camels (*Camelus dromedarius*) aged 7.6±2.4 years and weighed 312±61 kg that participated in 8 km training were used in another experimental design but with a different protocol (Tharwat *et al,* 2013c). These animals were ensured normal complete physical examination findings, normal cardiac auscultation, normal complete blood cell counts (VetScan HM5, Abaxis, CA, USA), normal biochemistry profiles (VS2, Abaxis, CA, USA), a continuous electrocardiography recording (Kenz-Cardio 302 Suzuken Co Ltd., Nagoya, Japan), and echocardiography (SSD-500, Aloka, Tokyo, Japan) (Tharwat *et al,* 2012). Blood samples (10 mL) were collected from the jugular vein as follows; 3 mL in EDTA tubes, 2 mL in heparinised tubes and the remaining 5 mL in plain vacutainer tubes of each, immediately prior to training (T0) and within 2 h of completion of the training (T1). Sera were harvested and were aliquotted in tubes and immediately stored at -20°C pending analysis.

Haematobiochemical profiles and cardiac biomarkers assays

Haematological examinations were carried out immediately on EDTA blood samples as shown in Table 1 using an automated analyser (VetScan HM5, Abaxis, California, USA). Heparinised blood samples were used to determine the bichemical parameters as shown in Table 2 using an automated biochemical analyser (VetScan VS2, Abaxis, California, USA). The serum samples were thawed and immediately analysed for cTnI using the point-of-care analyser according to the manufacturer's instructions. The CK-MB mass measurements were performed using the Cobas 6000 C501 assay (Roche Diagnostics, Indianapolis, Indiana, USA), with an electrochemiluminescent assay. The lower limit of detection of CK-MB for this assay was 0.1 ng/mL.

Statistical analysis

Data normality was examined using the Kolmogorov–Smirnov test. The data were presented as means ± SD, and were analysed statistically using the SPSS statistical package (2009). A Student's t-test was used for comparisons between pre- and post-training values. Significance was set at $P \leq 0.05$.

Results

Table 1 summarises the haematological variables (mean±SD) in race camels before and after 8 km training, alongside the 25^{th}, 50^{th}, 75^{th} and 95th and 99th percentiles. Compared to a value of 7.21±1.9 $\times 10^9$/L pre-training, neutrophils decreased to 6.2±2.2$\times 10^9$/L post-training (P=0.05). Similarly, haemoglobin concentration decreased from 11.1±1.1 g/dL before training to 10.3±2.0 g/dL after training (P=0.0002). The MCV showed a similar pattern where it decreased from 26.0±1.3 (fl) pre-training to 24.0±3.6 (fl) post-training (P=0.01). Other haematological variables did not show any significant changes before and after training (P>0.05).

The biochemical profiles (mean ± SD) in race camels before and after 8 km training, alongside the 25^{th}, 50^{th}, 75^{th} and, 95^{th} and 99^{th} percentiles are presented in Table 2. The serum activity of AST increased from 85.5±12.8 U/L before training to 91.5±8.6 U/L after training (P=0.0001). Serum concentration of TP increased also from 54.2±8.7 g/L pre-training to 59.0±3.8 g/L post-training (P=0.04). On the contrary, the serum concentration of LA decreased from 3.9±0.8 (mmol/L) before training to 3.3±0.4 (mmol/L) after training (P=0.004). Other biochemical variables did not show any significant changes before and after training (P>0.05).

Fig 1 illustrates the serum concentration of the cardiac biomarkers cTnI before and after training. Before training the serum concentration of cTnI was 0.03±0.03 ng/mL; a value that did not differ significantly when compared to the value of 0.04±0.02 (ng/mL) after training (P=0.60). The serum concentration of the cardiac biomarker CK-MB before and after training is illustrated in Fig 2. The CK-MB

Table 1. Haematological parameters in race camels before and after 8 km training (n=23).

Variable	Before training						After training						*P* value
	Mean ± SD	Percentile					Mean ± SD	Percentile					
		25%	50%	75%	95%	99%		25%	50%	75%	95%	99%	
WBCs ($\times10^9$/L)	12.9±3.5	9.8	12.9	14.7	16.7	16.8	11.7±2.1	10.6	11.7	14.0	15.5	15.7	0.92
Lymphocytes ($\times10^9$/L)	4.3±1.8	2.9	4.3	6.3	6.6	7.0	3.7±1.9	2.4	3.7	5.4	6.9	6.9	0.21
Monocytes ($\times10^9$/L)	0.3±0.3	0.2	0.3	0.4	0.9	1.1	0.3±0.3	0.2	0.3	0.5	0.9	1.0	0.81
Neutrophils ($\times10^9$/L)	7.21±1.9	6.4	7.2	8.5	9.4	10.0	6.2±2.2	5.1	6.1	7.8	8.1	9.0	0.05
Lymphocytes (%)	37.6±5.8	33.5	37.6	42.8	44.8	44.8	37.5±5.2	35.0	37.5	41.3	44.6	46.3	0.98
Monocytes (%)	2.3±1.7	1.9	2.3	3.1	6.3	6.7	2.1±2.1	1.8	2.1.0	4.2	7.03	7.5	0.85
Neutrophils (%)	60.3±6.8	54.3	60.3	64.0	66.5	74.0	60.0±6.4	53.3	60.0	63.1	67.8	73.4	0.96
RBCs ($\times10^{12}$/L)	9.2±1.0	8.7	9.2	10.1	10.9	11.4	8.8±2.5	8.0	8.8	10.1	12.2	15.2	0.57
Haemoglobin (g/dL)	11.1±1.1	10.7	11.1	12.6	13.4	13.7	10.3±2.0	10.0	10.3	11.4	13.4	13.5	0.0002
Hematocrit (%)	23.7±4.5	20.7	23.7	26.2	27.6	30.0	22.2±4.8	20.8	22.2	25.3	27.6	30.2	0.60
MCV(fl)	26.0±1.3	26.0	26.0	27.0	28.0	28.0	24.0±3.6	22.8	24.0	26.3	27.0	27.0	0.009
MCH (pg)	11.6±1.7	10.4	11.6	12.1	12.6	12.9	12.0±8.2	11.0	12.0	16.3	23.1	39.7	0.070
MCHC (g/dL)	44.5±7.1	40.4	44.5	47.0	49.7	53.3	48.6±25.1	42.4	48.6	58.8	80.2	128.0	0.08
Platelet count ($\times10^9$/L)	124.5±30.9	115.0	124.5	147.0	173.7	213.9	123.5±32.0	111.3	123.5	140.0	166.9	179.8	0.31

WBCs, white blood cells; RBCs, red blood cells; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration.

Table 2. Biochemical parameters in race camels before and after 8 km training (n=23).

Variable	Before training						After training						*P* value
	Mean ± SD	Percentile					Mean ± SD	Percentile					
		25%	50%	75%	95%	99%		25%	50%	75%	95%	99%	
Albumin (G/L)	54.0±9.6	50.0	54.0	60.5	68.05	68.8	58.0±3.4	57.0	58.0	61.0	64.3	68.9	0.06
ALP (U/L)	64.8±34.5	53.3	64.8	94.5	119.3	154.3	67.0±40.5	58.0	67.0	89.5	168.7	178.5	0.53
AST (U/L)	85.5±12.8	79.8	85.5	94.5	107.2	109.4	91.5±8.6	88.5	91.5	99.0	109.2	112.2	0.0001
Calcium (mmol /L)	2.1±0.4	1.9	2.1	2.5	2.7	2.8	2.3±0.2	2.3	2.3	2.3	2.6	2.7	0.16
GGT (U/L)	7.8±1.9	7.0	7.8	8.8	11.1	11.8	8.0±2.1	7.0	8.0	8.1	11.1	11.8	0.79
Total protein (G/L)	54.2±8.7	50.0	54.2	60.5	67.2	69.4	59.0±3.8	57.8	59.0	61.0	62.4	67.7	0.04
Globulin (G/L)	2.8±1.2	2.0	2.8	3.0	4.1	5.6	3.7±1.8	1.9	3.7	4.6	5.8	6.8	0.17
BUN (mmol /L)	8.1±1.3	7.7	8.1	8.9	9.8	11.2	9.0±1.4	8.6	9.0	9.5	11.1	11.8	0.08
CK (U/L)	153.5±42.1	138.5	153.5	186.3	228.0	257.6	142.0±24.5	124.8	142.0	150.5	184.2	202.4	0.13
Phosphorus (mmol/L)	1.8±0.4	1.6	1.8	2.2	2.4	2.6	1.9±0.3	1.8	1.9	2.1	2.4	2.6	0.37
Magnesium (mmol /L)	0.9±0.2	0.8	0.9	1.1	1.3	1.3	1.04±0.1	1.0	1.1	1.1	1.2	1.3	0.18
cTnI (ng/mL)	0.03±0.03	0.02	0.03	0.05	0.08	0.09	0.04±0.02	0.03	0.04	0.05	0.06	0.07	0.60
CK-MB (ng/mL)	0.47±0.1	0.29	0.47	0.50	0.53	0.54	0.48±0.8	0.42	0.48	0.73	2.50	2.55	0.02
LA (mmol/L)	3.9±0.8	3.4	3.9	4.3	5.2	5.3	3.3±0.4	3.3	3.1	3.3	3.6	3.8	0.004

ALP, alkaline phosphatase; AST, aspartate aminotransferase; GGT, γ-glutamyl transferase; BUN, blood urea nitrogen; CK, creatine kinase; cTnI, cardiac troponin I; CK-MB, creatine kinase myocardial band; LA, lactic acid.

value differed significantly before and after training (0.47±0.1 ng/mL before training vs 0.48±0.8 ng/mL after training; P=0.004).

Discussion

Significant elevations of cTnI in camel blood following racing (Tharwat *et al,* 2013c) have been observed following racing. An elevated serum concentration of cTnI has been used as a poor prognostic indicator in goats with pregnancy toxaemia (Tharwat *et al,* 2012) and in downer camels (Tharwat, 2012). In a study published recently in camels with tick infestation (Tharwat and Al-Sobayil, 2014a), it was assumed that the increased serum concentration of cTnI above 1.0 ng/ml at initial examination has a bad prognostic indicator.

Following 5 km race in dromedary camels, the serum concentration of cTnI increased significantly 2 h after race (Tharwat *et al,* 2013c). However, in present study, the serum concentration of cTnI did not change significantly before and after training (P=0.60). Results agree with a previous study in horses, where their plasma cTnI levels did not increase (P=0.48) 3-6 h after they had performed short-term high-intensity exercise for a distance of 2.0 to 2.4 km on a treadmill (Durando *et al,* 2006). The high-intensity effort of the camels during race (Tharwat *et al,* 2013c) may be a contributing factor of cTnI increase during race, but not during training. Post-exercise cTnI release and clearance were also reported in normal Standardbred racehorses. All horses experienced an increase in cTnI post-exercise, with peak occurring 2-6 h post-exercise (Rossi *et al,* 2019). In a study carried out on racing greyhounds following a 7 km race, almost all greyhounds showed increases in cTnI concentrations which were significantly higher than the pre-race concentrations (P<0.0001). However, out of the 23 racing greyhounds, only 5 showed mild increases in CK-MB concentrations but these did not significantly differ from the pre-race values (P>0.05) (Tharwat *et al,* 2013e).

In horses, increased concentrations of cTnI have been reported in association with endurance competition as well as after short-term maximal exercise on a treadmill for 2.0-2.4 km (Durando *et al,* 2006; Holbrook *et al,* 2006). In addition, serum cTnI concentrations were mildly elevated in some horses 1 to 14 h after racing (Nostell and Haggstrom, 2008).

In a study in standardbred racehorses, all animals experienced an increase in cTnI post-exercise, with peak occurring 2-6 h post-exercise (Rossi *et al,* 2019). In contrast, Phillips *et al* (2003) have reported that serum cTnI concentrations in race-training thoroughbred horses were not significantly different from those of pastured horses.

In the 5 km race in dromedary camels, the serum concentration of CK-MB value did not differ significantly (P=0.855) (Tharwat *et al,* 2013c). In the current study, the serum concentration of CK-MB increased significantly when compared to pre-training values (P=0.004). This result agrees well with other reports of CK-MB increase with exercise (Mamor *et al,* 1988; Rahnama *et al,* 2011). There are 3 isoforms

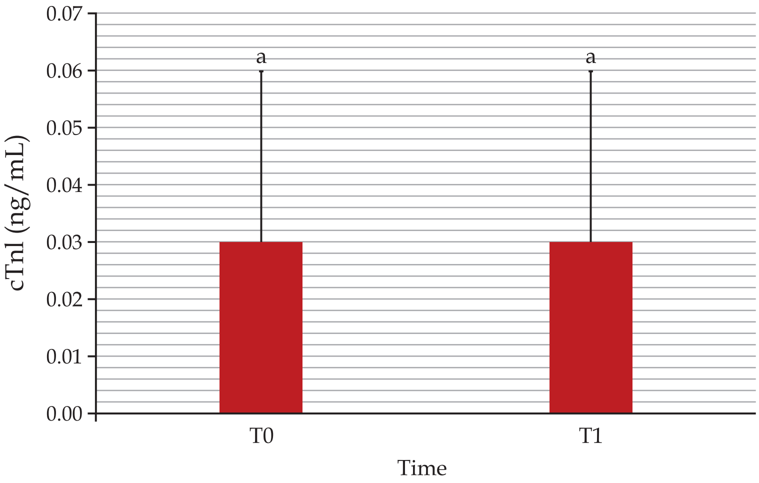

Fig 1. Cardiac troponin I (cTnI) values in camels before (T0) and 2h after 8 km training (T1). [a]Same letters did not differ significantly (P>0.05).

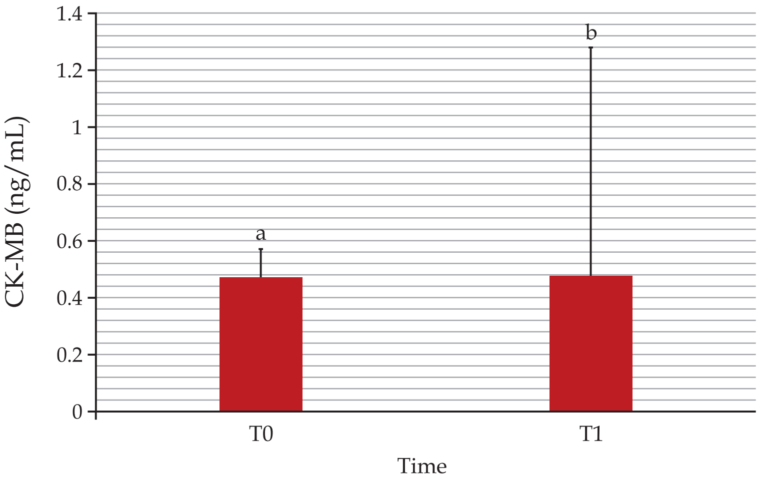

Fig 2. Creatine kinase myocardial band (CK-MB) values in camels before (T0) and 2h after 8 km training (T1). [a,b]Different letters indicate a significant difference (P<0.05).

for the enzyme CK: BB, MM, and MB. The BB isoform is found primarily in the brain. Skeletal muscles primarily contain the MM isoform, with traces of MB (estimates of 1-4% of CK activity). Cardiac muscles also contain primarily the MM isoform, but higher amounts of MB, typically around 20% of CK activity (Moss *et al*, 1994). In a study conducted by Gojanovic *et al* (2011), no changes were observed in the serum concentration of CK-MB or cTnI as a result of whole-body vibration training.

The haematological parameters decreased significantly after training included neutrophils count, haemoglobin concentration and MCV. However, the total WBCs count did not differ significantly before and after training (P=0.92). Similarly, in racing camels with 5 km race, the WBC count did not change significantly pre- and post-race (P=0.11) (Tharwat *et al,* 2013c). Concerning the biochemical parameters, the AST activity and the TP concentration increased significantly after training (P=0.0001, P=0.04, respectively). Opposite, the serum concentration of LA decreased significantly after training (P=0.004). In a similar pattern in racing camels, the serum concentration of LA decreased significantly after race (P<0.0001). In another study in camels, lactate concentration decreased, but not significantly, after transportation for a 5-h round-trip journey (Tharwat *et al*. 2013b). Lactate is known as the end product of anaerobic glycolysis, a pathway that is of key importance during normal metabolic and athletic events (Pösö, 2002). Lactate accumulation occurs when the balance between production and consumption is breached. Instead of being regarded as a waste product, LA is now seen as a valuable substrate that contributes significantly to the energy production of the heart, muscles and even the brain. It may be used as fuel by many organ systems including the heart, liver and kidneys (Pösö, 2002; Tennent-Brown 2012). Therefore, the decreased serum concentration of LA could be due to its consumption by the muscles during training. In conclusion, the cardiac biomarker cTnI did not change significantly after training compared to baseline levels, a result that disagrees with values in camels after race. However, the CK-MB increased significantly after training compared to pre-training serum concentrations.

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DOI : 10.5958/2277-8934.2021.00013.8 *Vol 28 No 1, p 85-93*

OBSTRUCTIVE UROLITHIASIS IN DROMEDARY CAMELS: CLINICAL, ULTRASONOGRAPHIC AND POSTMORTEM FINDINGS

Mohamed Tharwat[1,2]

[1]Department of Veterinary Medicine, College of Agriculture and Veterinary Medicine, Qassim University, P.O. Box 6622, Buraidah, 51452, Saudi Arabia.

[2]Department of Animal Medicine, Faculty of Veterinary Medicine, Zagazig University, 44519, Zagazig, Egypt

ABSTRACT

This article was written to evaluate camels with obstructive urolithiasis with special reference to the clinical, ultrasonographic and postmortem findings. Twenty male camels (*Camelus dromedarius*) with urolithiasis were examined. Of them, 18 had ruptured bladder and 2 had ruptured urethra. Main clinical findings included depression, anorexia and anuria. Ventral abdominal swelling was also detected in front of the penis with swelling of the prepuce. Exploratory laparotomy showed a massive amount of reddish uroperitoneum. In the camels with ruptured urethra, penile and ventral edema was detected in front of the penis with swelling of the prepuce. The urine penetrated the adjacent tissues that also yielded dark red urine on aspiration. The most important ultrasonographic findings included a collapsed urinary bladder in animals with ruptured bladder. Uroperitoneum was imaged easily where viscera were seen floating in the urine. Dilatation of the pelvic and penile urethra as a result of calculi was detected by ultrasonography. The urinary bladder wall was intact in cases before bladder perforation. Hydronephrosis with parenchymal pressure atrophy was also detected. The obstructing calculus was also seen within the penile urethra as a hyperechoic mass with distal acoustic shadowing. The perforated urinary bladder was also seen. In cases with ruptured urethra, the bladder wall was imaged intact and it contained hyperechoic sediment. Postmortem examination confirmed the ultrasonographic findings.

Key words: Camels, dromedary, imaging, ultrasonography, urolithiasis

Urinary calculi (urolithiasis, uroliths, nephrolith, bladder stone, cystolith) is common as a subclinical disorder among ruminants raised in management systems where the ration is composed primarily of grain, or where animals graze certain types of pasture (Radostits *et al,* 2007). In dromedary camels, the salt requirement is around 6–8 times than that of other domestic ruminants (Nigam, 1992). In addition, the urine of dromedaries can contain twice as much salt as sea water because of their extraordinary capacity for retention and concentration of fluids (Dorman, 1986). Therefore, small uroliths may enter the ureter or urethra and cause partial or complete obstruction of urine flow. Urinary calculi are formed in either the calices of the kidney, or more commonly in the urinary bladder. Small uroliths may enter the ureter or urethra and cause partial or complete obstruction of urine flow (Fowler, 1990; Fowler, 2000; Gutierrez *et al,* 2002; Fowler, 2008; Choudhary *et al,* 1995).

Urethral obstruction has been extensively reported in ruminant species; however, there is minimal information about its incidence in camelids. The etiology is unknown but is believed to parallel that for domestic ruminants (Smith, 1989). Previous reports of obstructive urolithiasis in llamas have suggested mineral imbalance, castration, and inflammation of the urinary tract as possible contributing factors (Kock and Fowler, 1982; Kock, 1985; McLaughlin and Evans, 1989). In two reports, the calculi contained a large proportion of calcium (Kock and Fowler, 1982; Kock, 1985), and in another report, the calculus contained necrotic inflammatory cells with no detectable mineral constituents (McLaughlin and Evans, 1989).

Rupture of the urinary bladder and subsequent uroperitoneum is a common problem in cattle, and in males, urolithiasis is the underlying cause in the majority of cases (Divers *et al,* 1982, Bertone and Smith, 1984). Uroperitoneum may be caused by trauma when the bladder is distended or from rupture of the bladder following urethral obstruction. Urine in the abdomen may not arise only from a

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single or multiple tears in the bladder wall, but also from seepage through the thinly stretched bladder wall in an over-distended bladder (Fowler, 2010). In camels, with ruptured urinary bladder, ultrasonographic examination of the abdomen simplifies the detection of either intact or perforated urinary bladder and the presence of uroperitoneum (Tharwat *et al,* 2012a; Tharwat and Al-Sobayil, 2016).

Bladder rupture leads to gradual development of ascites from uroperitoneum, ruminal stasis, constipation and depression. Finally, uraemia may take 1-2 weeks to develop to the point where euthanasia is necessary. Calculus may be identified ultrasonographically. However, it is impossible to pass a catheter in male camels because of the dorsal urethral recess and restrictive diameter of the urethra (Tharwat *et al,* 2012b; Tharwat and Al-Sobayil, 2016). A ruptured urethra has been reported in camels (Gahlot, 1992). This article reports obstructive urolithiasis in camels with special reference to the clinical, ultrasonographic and postmortem findings.

Materials and Methods

Twenty male camels (*Camelus dromedarius*) (age: 6 months until 12 years; weight 110 to 650 kg) were clinically examined at Veterinary Teaching Hospital, Qassim University, Saudi Arabia as per described procedure (Köhler-Rollefson *et al*, 2001). Animals were presented to the clinic for examination because of depression, anorexia, bloody urine and anuria. Camels were investigated during the period of 2012 to 2020. Animals were treated according to the regulations of the Laboratory *Animal Control Guidelines* of Qassim University.

Ultrasonography of the urinary tract and postmortem examination

The urinary tract was scanned by ultrasonography as recently reported (Tharwat *et al,* 2012c). The right kidney was visualised in camels at the level of the 10th and 11th intercostal spaces and the upper right flank. The left kidney was imaged from the caudal left flank. Differentiation between the renal cortex and medulla was visible; the renal cortex was relatively hyperechoic compared to the renal medulla and the renal sinus was hyperechogenic and more differentiated than the cortex and medulla. The right and left renal parenchyma were less echogenic than the neighboring hepatic and splenic parenchyma, respectively. The renal hilus was seen when the transducer was placed in the paralumbar fossa and rotated about its longitudinal axis. Ultrasonography via the so-called hepatic and splenic windows also results in good images of the right and left kidneys, respectively. The left kidney was also accessible transrectal where the entire left kidney and the cranial pole can be reached. The urinary bladder and the pelvic urethra were imaged transrectally while the penile urethra was examined transcutaneously (Tharwat *et al,* 2012b). Of the 20 male camels, 7 were euthanised and thoroughly examined at postmortem.

Results

Of the 20 male camels, 18 (90%) had ruptured bladder and the remaining 2 (10%) had ruptured urethra. One of the camels with ruptured bladder was admitted firstly with intact bladder that was ruptured 2 days later. Depression, anorexia and anuria were seen in this case. Ventral abdominal swelling was detected in front of the penis with swelling of the prepuce (Fig 1). All animals with ruptured bladder were admitted with a history of anuria. Exploratory laparotomy showed a massive amount of reddish urine (Fig 2). Fig 3 shows a male camel with anuria for the past 15 days. Abdominal paracentesis revealed blood tinged urine. Centrifugation of the abdominal fluid yielded sediment. In this camel, 2 calculi were detected within the penile body. In the two male camels with ruptured urethra, ventral abdominal subcutaneous infiltration of urine was detected in front of the penis with swelling of the prepuce (Fig 4). Fig 5 shows ruptured urethra in a male camel where severe edematous swelling at sheath was observed that yielded a massive amount of red urine on exploratory puncture. The urine also invaded gluteal muscles that also yielded dark red urine on exploratory puncture.

Ultrasonographic examination of the male camels with ruptured urinary bladder showed a collapsed urinary bladder that contained blood clots. Uroperitoneum resulted from rupture of the urinary bladder revealed floating of intestines (Fig 6). Figs 7 and 8 are showing dilated pelvic and penile urethra in a male camel with long-standing urine retention. Fig 9 was taken from a male camel with a history of cessation of micturition where transrectal ultrasonography showed distended bladder and urethra. Transcutaneous ultrasonographic examination of right kidney in the same case showed dilated renal pelvis and pressure atrophy of the renal parenchyma. A hyperechoic calculus located within the penile urethra with distal acoustic shadowing and a perforated bladder is seen in Fig 10. In cases with ruptured urethra, the bladder was imaged with intact wall and it contained hyperechoic sediment (Fig 11).

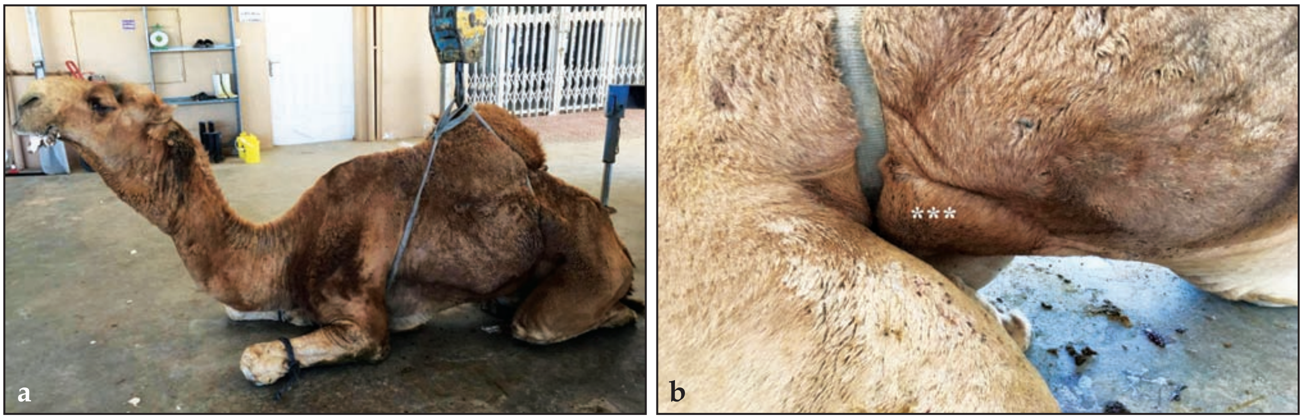

Fig 1. Ventral abdominal swelling extending up to penile sheath **(a)** and close-up view of the penile sheath and ventral swelling (stars) **(b)** in a case of retention of urine.

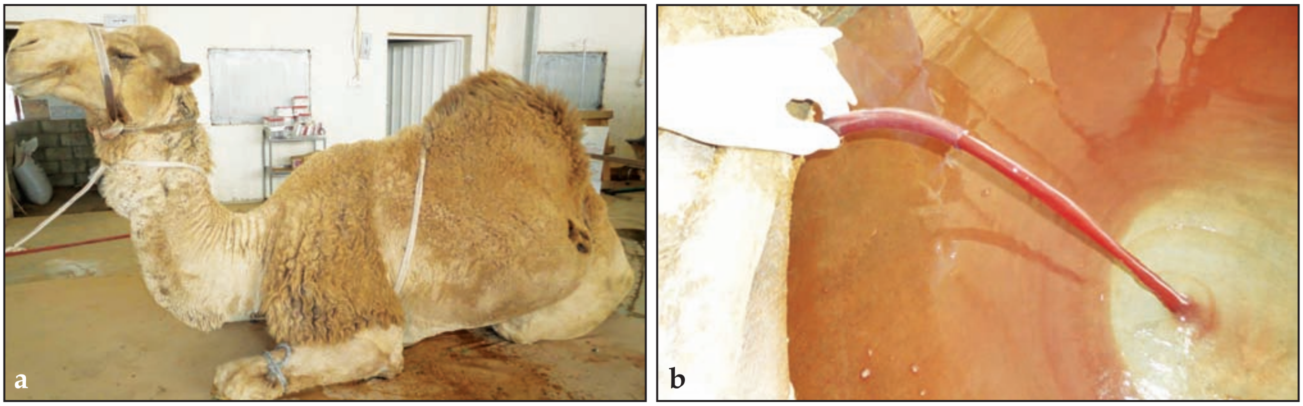

Fig 2. Depressed and dehydrated camel (**a**) and uroperitoneum was evidenced by aspirating out blood tinged urine (**b**) following exploratory laparotomy in camel with obstructive urolithiasis.

Fig 3. Abdominal paracentesis (**a**) revealed blood tinged fluid (**b**). Centrifugation of the abdominal fluid yielded sediment (**c**). Two calculi were detected within the penile body (**d**) in a camel with anuria since last 2 weeks.

Necropsy findings of a camel with ruptured bladder revealed collapsed and ruptured wall (Fig 12). The bladder contained a reddish-brown deposit that represented blood clot and it contained a rough stone. The penile body contained 4 rough stones. A 6-month old male camel calf swith ruptured urinary bladder (15 days before) had distended abdomen. Postmortem examination showed uroperitoneum, congested and haemorrhagic bladder serosa and perforated bladder (Fig 13).

Discussion

Urolithiasis appears to be more common in temperate climates, it occurs in both females and male, castrated or intact, and there appears to be no age predisposition (Kock, 1985). The diameter of the female urethra generally allows free passage of a calculus that may enter the urethra; thus, obstructive urolithiasis is rare in the female. Urolithiasis has been associated with a diet high in concentrated feeds, such as are often used in zoos. Cattle pastured on grasses

Fig 4. Ruptured urethra in a 6-month-old male camel calf. Ventral abdominal swelling was detected in front of the penile sheath (**a**) and a close-up view of the penile sheath swelling (**b**).

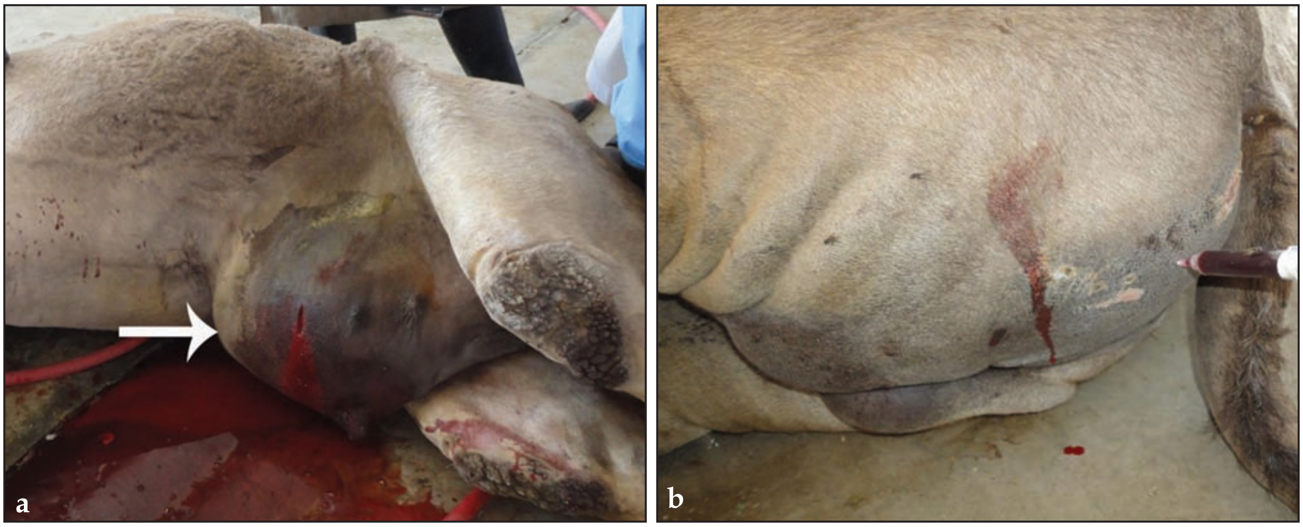

Fig 5. Ruptured urethra in a male camel. Severe sheath swelling was observed due to subcutaneous infiltration of urine which was confirmed by exploratory puncture (**a**; arrow). The urine was also infiltrated at medial thigh muscles; dark red urine on exploratory functure (**b**).

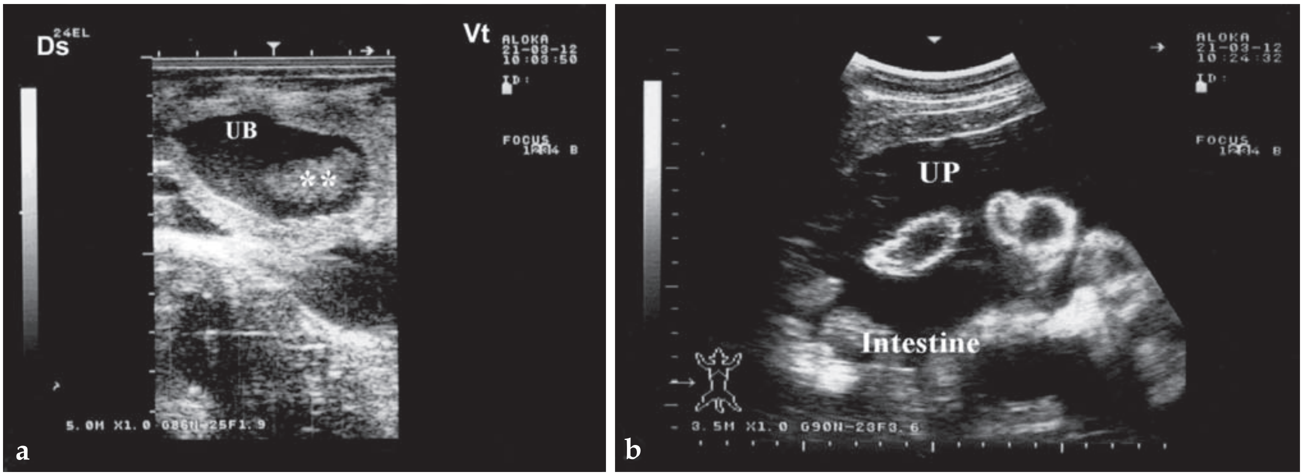

Fig 6. Ultrasonogram of a male camel with ruptured urinary bladder showing a collapsed urinary bladder (UB) and blood clot (stars) (**a**) and uroperitoneum (UP) with floating intestines (**b**).

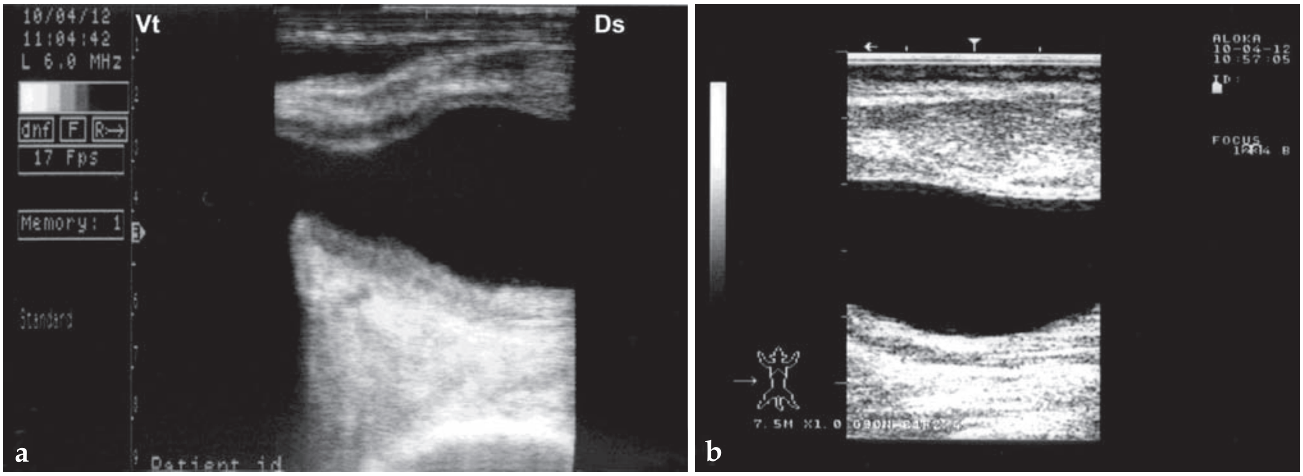

Fig 7. Ultrasonogram revealed dilated pelvic (a) and penile (b) urethra in a male camel with long-standing urine retention.

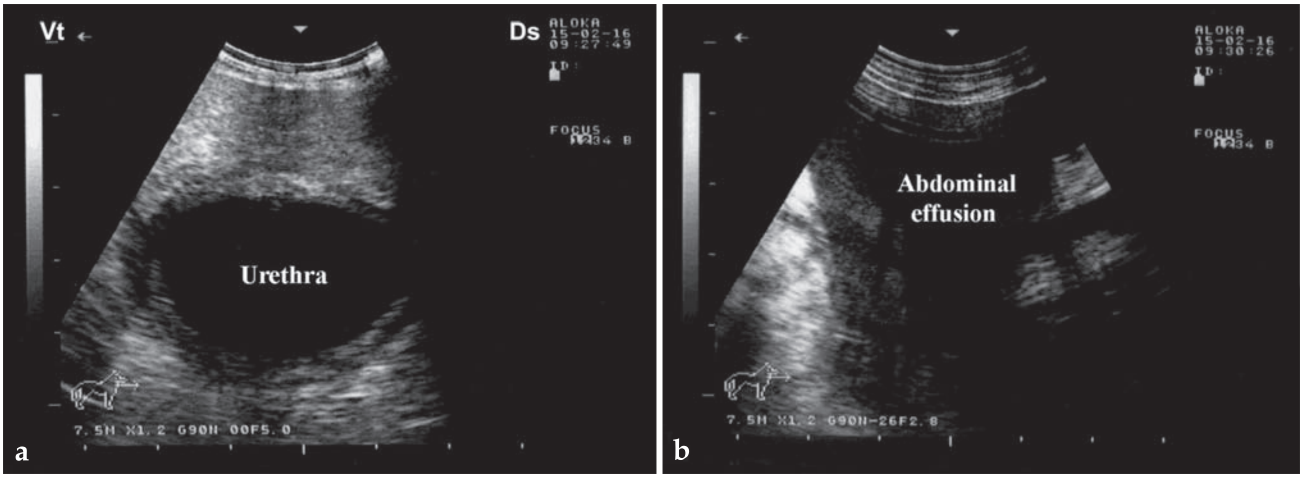

Fig 8. Ultrasonogram of the male camel with urine retention revealed dilated pelvic urethra (**a**) and uroperitoneum (**b)**.

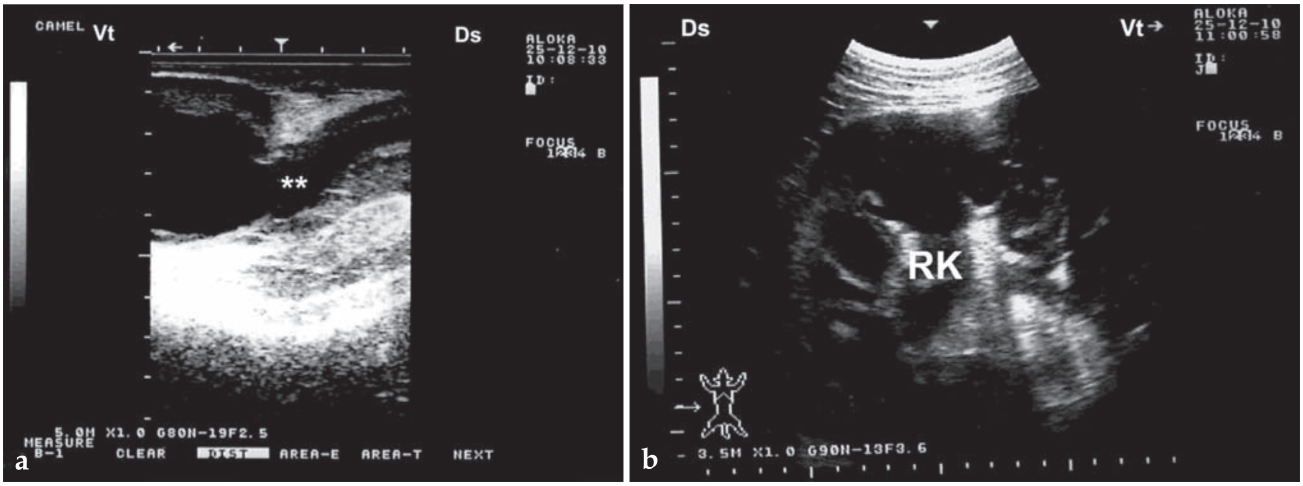

Fig 9. Transrectal ultrasonography showed distended bladder and urethra (**a**) of the male camel with a history of anuria. Transcutaneous ultrasonographic on of right kidney (RK) showed dilated renal pelvis and pressure atrophy of the renal parenchyma (b).

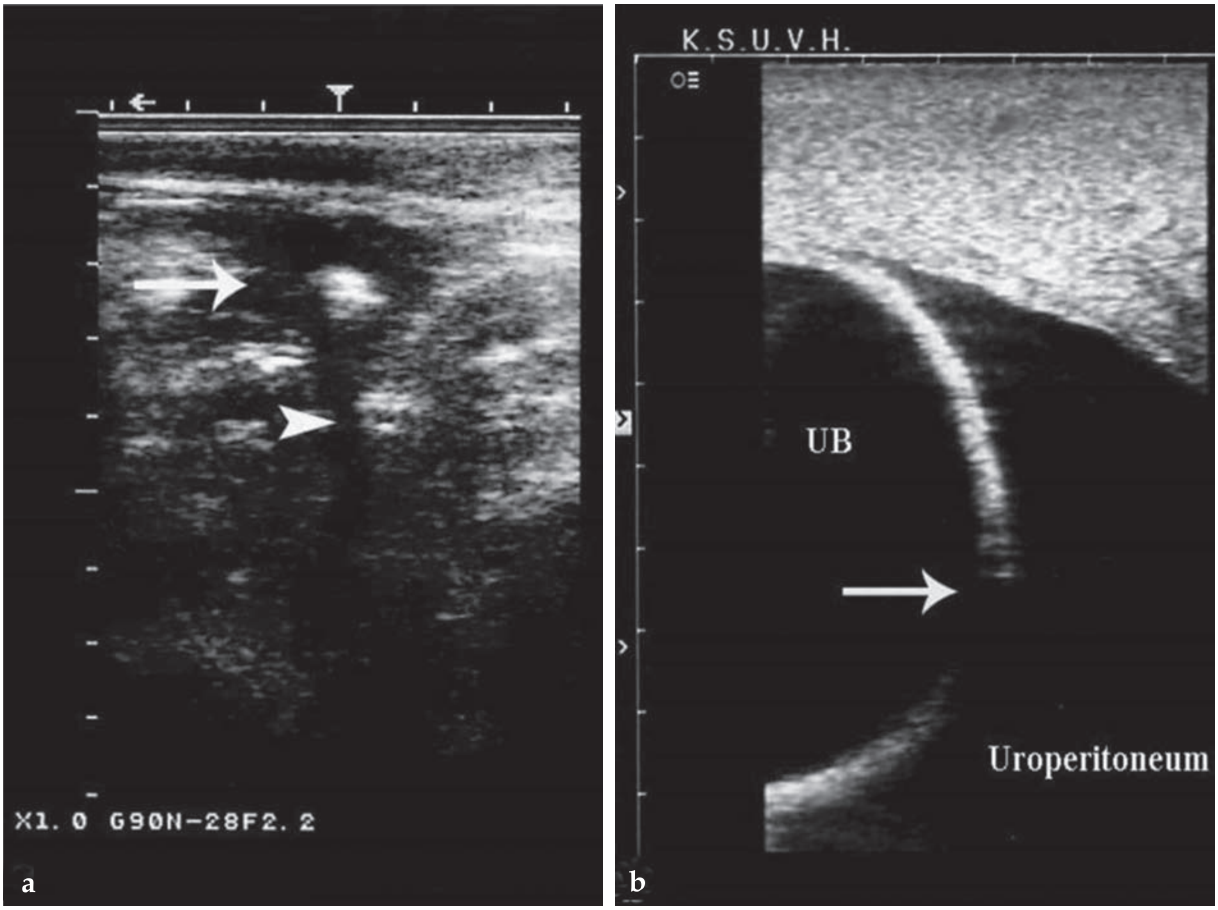

Fig 10. Ultrasonogram of a six-month old camel-calf with ruptured urinary bladder showed urinary calculi within the urethra (white arrow) with acoustic shadowing (arrowhead) (**a**) and perforated urinary bladder wall (UB) (white arrow) (**b**).

containing high levels of silicates may sometimes develop silicate urolithiasis, and presumably camelids grazing on such pastures may also be at risk (Fowler, 2010). In this study camels with urinary calculi were managed on a high concentrate feeding.

A basic understanding of the camelid urethra is required to locate sites of possible obstruction and develop approaches to management. The pelvic urethra is expansive, but at the reflection around the ischium, only a tiny orifice allows passage of urine beyond this point. The anatomy of this area is further complicated by a dorsal urethral recess, which precludes any possibility of passing a catheter into the bladder from the tip of the penis. Whereas the sigmoid flexure is the probable site of the majority of bovine urethral obstructions, this is not the case in camelids. The orifice from the pelvic urethra into the penile urethra is a common site of obstruction; another is where the penile urethra narrows as it enters the glans penis (Fowler, 2010). The penis of the camel is of the fibro-elastic type and depends primarily on its elasticity for erection and extension.

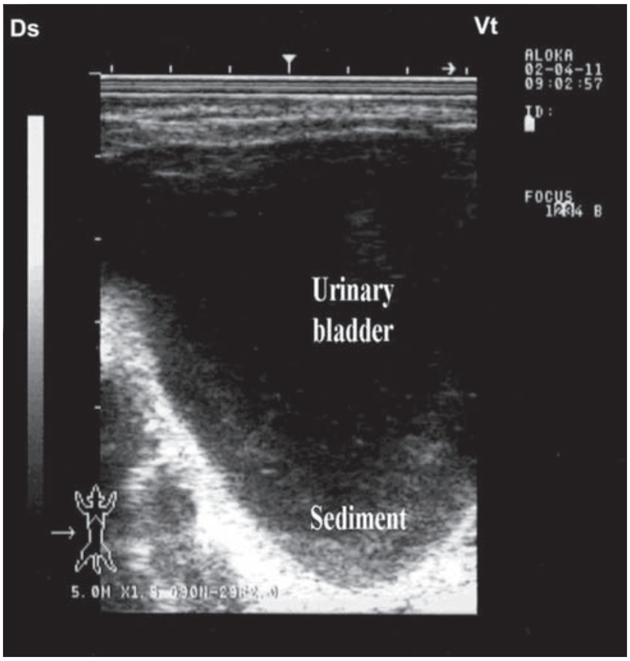

Fig 11. Ultrasonogram of a male camel calf with ruptured urethra. The bladder had an intact wall and it contained hyperechoic sediment.

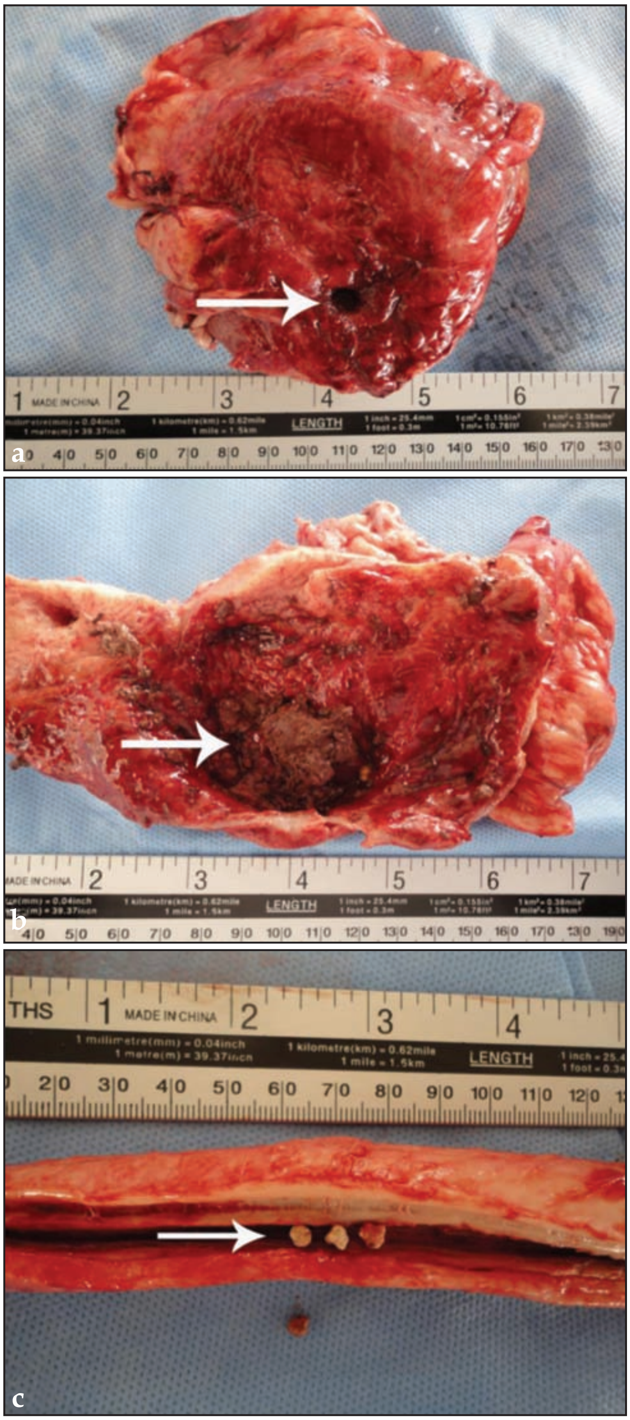

Fig 12. Necropsy findings of the camel with ruptured bladder. The bladder was collapsed and its wall was perforated (**a**). The bladder contained a reddish-brown deposit that represented blood clot and it contained a rough stone (**b**). The penile body contained rough stones (**c**).

In the absence of an erection, the penis is retracted into its sheath via a pre-scrotal sigmoid flexure not a post-scrotal sigmoid flexure, as is the case of bulls (Ali *et al,* 1996). The glans penis is curved along its vertical plane giving it a hook-shape with a definite neck between the glans and body of the penis (Mobarak *et al*, 1990; Belloa and Umarub, 2013). The penile urethra is extremely narrow and its opening is minute and the glans penis is represented by the urethral process (Smuts and Bezuidenhout, 1987).

Clinical signs of obstructive urolithiasis prior to bladder rupture include colic, straining stance to urinate, dribbling urine, blood - tinged urine, anuria, distended bladder, and possible pulsation of the urethra. Signs after bladder rupture include absence of colic, depression, anorexia, anuria, and uroperitoneum, with possible distention of the abdomen and uremia. Uroperitoneum may be caused by trauma when the bladder is distended or from rupture of the bladder following urethral obstruction. Urine in the abdomen may arise from a single or multiple tears in the bladder wall but also from seepage through the stretched - thin bladder wall (Fowler, 2010). A ruptured urethra has been reported in camels (Gahlot, 1992). Results of this study agree with these findings where depression, anorexia and anuria were the common clinical signs. Ventral abdominal swelling was also detected in penile sheath with swelling of the prepuce. Exploratory laparotomy showed a massive amount of reddish uroperitoneum that by centrifugation yielded red sediment. In the camels with ruptured urethra, penile sheath edema with ventral abdominal edema was detected. The urine in the later cases infiltrated the adjacent tissues that also yielded dark red urine on aspiration.

In this study, ultrasonographic examination of male camels with obstructive urolithiasis was valuable in evaluating diseased camels and in determining their prognosis. The most important findings included a collapsed urinary bladder in animals with ruptured bladder. Accumulation of urine within the peritoneum was also imaged easily where viscera were seen floating in the urine. Dilatation of the pelvic and penile urethra as a result of calculi was detected by ultrasonography. The urinary bladder wall was imaged intact in cases before bladder perforation. Unilateral or bilateral hydronephrosis with parenchymal pressure atrophy was also seen in camels with obstruction of the urinary tract. The obstructing calculus was also seen within the penile urethra as a hyperechoic mass with distal acoustic shadowing, and the perforated bladder was also seen. In cases with ruptured urethra, the bladder wall was imaged intact and it contained hyperechoic sediment. Postmortem examination of camels with ruptured bladder confirmed the

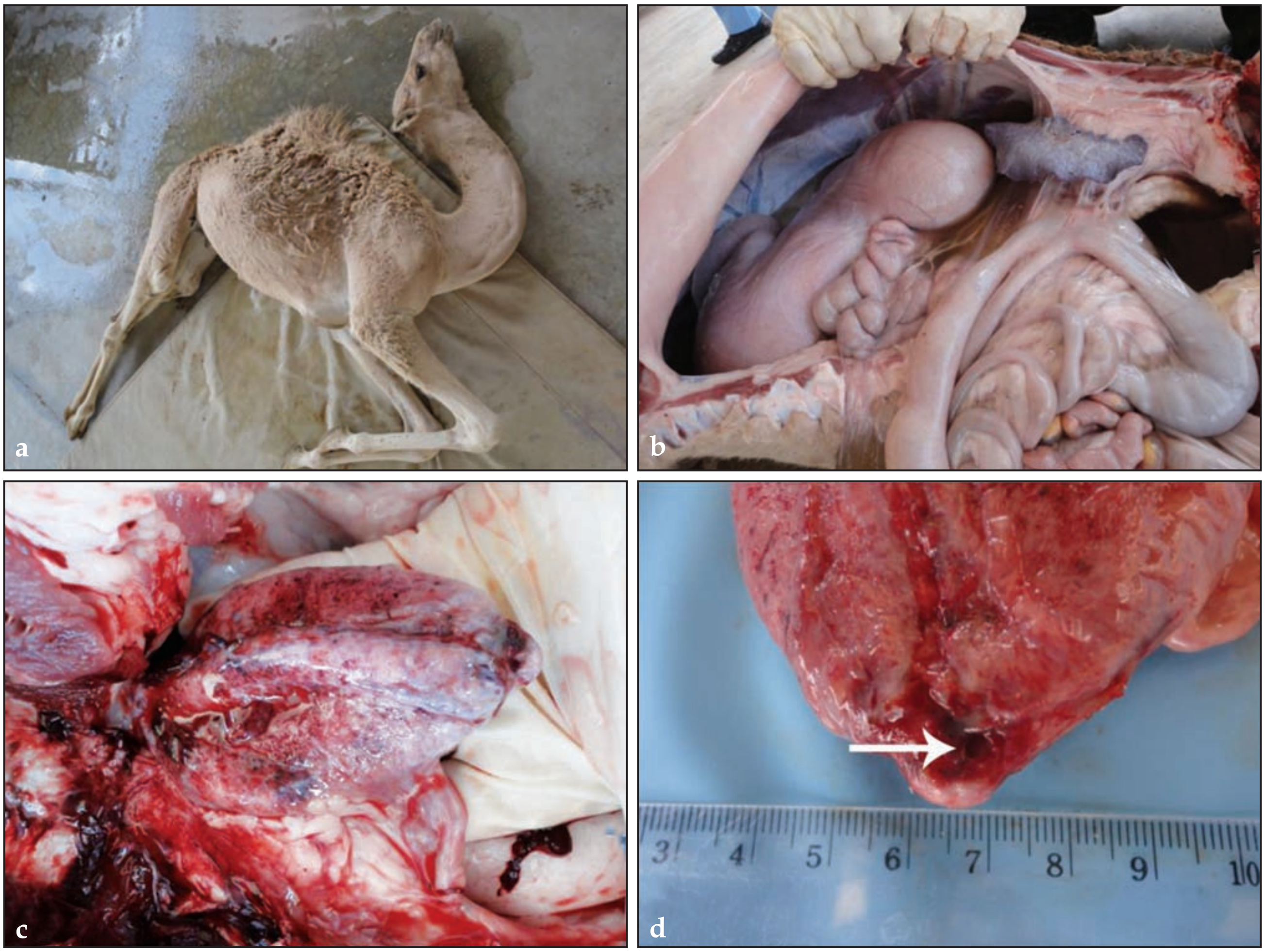

Fig 13. A 10-month old male camel calf with ruptured urinary bladder had distended abdomen (**a**). Postmortem examination showed uroperitoneum (**b**), congested and haemorrhagic bladder serosa (**c**) and perforated bladder (white arrow) (**d**).

ultrasonographic findings where the bladder was found collapsed and its wall was perforated. The bladder was found to contain blood clots and the urethra contained stones. Massive amount of uroperitoneum was detected and the bladder serosa was congested and hemorrhagic.

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DOI : 10.5958/2277-8934.2021.00014.X Vol 28 No 1, p 95-96

Short communication

PROZONE REACTION IN AN ANTIBODY ELISA OF A BRUCELLOSIS POSITIVE DROMEDARY CAMEL SERUM

U. Wernery, R. Raghavan and R. Wernery
Central Veterinary Research Laboratory, Dubai, UAE

The serological diagnosis of camel brucellosis uses routine serological tests described in details in the OIE (2018) which are mainly the Complement Fixation Test (CFT), the Serum Agglutination Test (SAT or TAT), Rose Bengal Test (RBT) and antibody ELISA. These tests were recently evaluated by Soellner *et al* (2018) for use in camelids, as each test should be validated for its fitness in the corresponding animal species (OIE, 2018). It is sometimes difficult to interpret the results as cross-reactivity with other bacterial species like *Yersinia enterocolitica,* serotype O9, may occur (Sunaga *et al*, 1983; Bisping and Amtsberg, 1988; Erdenebaatar *et al*, 2003) and with others like *Pasteurella, Campylobacter, Salmonella* and *Franciscella* (Markey *et al*, 2013). Zhulobovski and Pal'gov (1954) additionally described prozones in some sera of Bactrian camels in Russia and Nada (1984) in dromedaries in Egypt. The absence of a visual positive reaction in low serum dilutions has also been observed in 1.5% of all positive dromedary camel sera in the UAE when using Serum Agglutination Test (SAT, Wernery *et al*, 2014). The Coombs test is then necessary to verify the diagnosis of brucellosis in these cases. It is also proposed to add EDTA to the antigen which improves the test's specificity significantly (MacMillan and Cockrem, 1985). The prozone phenomenon occurs in agglutination or precipitation tests (Markey *et al*, 2013) and has until now not been described to occur with antibody ELISAs. The prozone phenomenon refers to a false negative serological response at low serum dilution due to excess antibody concentrations in the serum. The nature of this phenomenon is not entirely clear, but it is imperative that test sera be checked at several dilutions to avoid errors in reporting results. We report here a prozone phenomenon in one of 4 *Brucella*-positive dromedary camel sera with the competitive ELISA (c-ELISA) from Ingenasa, Spain (Table 1). All 4 sera were highly positive with the CFT, SAT, RBT and c-ELISA except serum number 4, which was negative in the ELISA. This serum was then diluted two-fold and it became positive in the ELISA at a dilution of 1:320. The prozone did not occur with the other 3 highly *Brucella*-positive sera and not at all in the agglutination test.

In conclusion, a dromedary camel serum which was highly positive in 3 serological tests for brucellosis, turned only positive in the antibody ELISA when it was diluted 1:320. To the knowledge of the authors, it is the first time that a prozone phenomenon was observed in a *Brucella*-positive camel serum when a c-ELISA was used.

Table 1. Serological brucellosis results of 4 dromedary camels using 4 different test methods.

ID	RBT	SAT	CFT	c-ELISA
1	+++*	1:1280	1:128 ++++*	Positive
2	+++	1:640	1:64 +++	Positive
3	++	1:320	1:4 ++++	Positive
4	++++	1:1280	1:256 ++++	Negative
				At a dilution of 1:320 positive

*= Score +++ : strong positive
++++: very strong positive

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DOI : 10.5958/2277-8934.2021.00015.1 Vol 28 No 1, p 97-101

THE IMMUNOPHENOTYPE OF CAMEL BLOOD EOSINOPHILS

Jamal Hussen[1*], Abdullah I. A. Al-Mubarak[1], Naser A. Al Humam[1], Sameer M. Alhojaily[2,3] and Ali Fadlelmula[1]

[1]Department of Microbiology, College of Veterinary Medicine, King Faisal University, Al-Ahsa, Saudi Arabia
[2]Department of Biomedical Sciences, College of Veterinary Medicine, King Faisal University, Al-Ahsa, Saudi Arabia
[3]Agricultural and Veterinary Training and Research Station, King Faisal University, Al-Ahsa, Saudi Arabia

ABSTRACT

The aim of the present study was to use staining with monoclonal antibodies to different cell surface molecules and flow cytometry to analyse the expression pattern of cell markers on camel blood eosinophils. Based on their light scatter charecterestics and green autofluorescence, camel eosinophilic granulocytes were identified as $SSC^{high}/FSC^{low}/Fl\text{-}1^{high}$ cells within the granulocyte population. In comparision to neutrophilic granulocytes, camel eosinophils showed higher abundance of the cell surface molecules CD45, CD44, and CD11a but lower abundance of the cell markers CD172a and CD14. Collectively, the findings of the current study suggests a similar phenotype of camel, human, and bovine eosinophils.

Key words: Adhesion molecules, camel, eosinophilic granulocytes, flow cytometry, immunophenotype

The immunophenotype of eosinophils has been investigated for different species (Magyar *et al,* 1995; Pelan-Mattocks *et al,* 2001; Ramirez *et al,* 2018; Hassani *et al,* 2020; Oliveira *et al,* 2020). In humans, eosinophils differ from neutrophils by higher side light scatter (SSC), lower forward light scatter (FSC), negative CD10 and CD16, and dimmer CD11b, CD11c, CD13, CD15, and CD33 (Gorczyca *et al,* 2011). Flow cytometric analysis identified porcine eosinophils as positive for LFA-1 (CD11a/CD18) and swC3, a common marker of swine monocytes, granulocytes and macrophages, with no reactivity with antibodies recognising swine CD2, CD4, CD8 or MHC class II cell surface molecules (Magyar *et al,* 1995). In the dromedary camel, little is known about the phenotype and function of eosinophils. The number of camel blood eosinophils in blood ranges between 0.38 and 1.0 cell/µl blood, with higher numbers in adult animals than in newborn calves (Gaashan *et al,* 2020).

Flow cytometry has been widely used in humans and other species for the differentiation of leukocyte subpopulations on the basis of di fferences in cell size (as measured by forward light scatter), intracellular complexity (as measured by side light scatter), and intensity of fluorescence after staining with monoclonal antibodies to different cell markers (Appay *et al,* 2008; Gorczyca *et al,* 2011; Yu *et al,* 2016; Hussen *et al,* 2019). The aim of the present study was to use flow cytometry to analyse the expression pattern of cell markers on camel blood eosinophils.

Materials and Methods

Blood was obtained by venipuncture of the vena jugularis externa into vacutainer tubes containing EDTA (Becton Dickinson, Heidelberg, Germany) from 20 adult dromedary camels (*Camelus dromedarius*) aged between 8 and 12 years. All experimental procedures and management conditions used in this study were approved by the Ethics Committee at King Faisal University, Saudi Arabia (Permission number: KFU-REC/2020-09-25).

Isolation of leukocytes from camel blood

Separation of camel leukocytes was performed after hypotonic lysis of blood erythrocytes as described previously (Hussen *et al,* 2017). Briefly, blood was suspended in distilled water for 20 sec and double concentrated PBS was added to restore tonicity. This was repeated (usually twice) until complete erythrolysis. Separated cells were finally suspended in MIF buffer (PBS containing bovine serum albumin (5 g/L) and NaN_3 (0.1 g/L)) at 5 x 10^6 cells/ml. Cell purity of separated leukocytes was assessed by flow cytometry according to their FCS/SSC properties and always exceeded 90%. The mean viability of separated cells was evaluated by dye

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exclusion (propidium iodide; 2 µg/ml, Calbiochem, Germany) and it was above 90%.

Monoclonal antibodies

Monoclonal antibodies used in this study are listed in Table 1.

Table 1. List of antibodies.

Antigen	Antibody clone	Label	Source	Isotype
CD45	LT12A	-	mIgG2a	WSU
CD44	LT41A	-	WSU	mIgG2a
CD11a	G43-25B	-	mIgG2a	BD
CD172a	DH59b	-	mIgG1	WSU
CD14	TÜK4	-	WSU	mIgG1
CD163	LND68A	-	Kingfisher	mIgG1
mIgG2a	polyclonal	PE	Invitrogen	gIgG
mIgG1	polyclonal	FITC	Invitrogen	gIgG

Ig: Immunoglobulin; m: mouse; g: goat, FITC: Fluorescein isothiocyanate, PE: Phycoerythrin.

Membrane immunofluorescence and flow cytometry

Separated leukocytes (2 x 10^5) were incubated with different combinations of unlabeled primary monoclonal antibodies (mAbs) specific for the cell markers, CD45, CD44, CD172a, CD14, CD163, and CD11a in MIF buffer [membrane immunofluorescence buffer consisting of PBS containing bovine serum albumin (5 g/L) and NaN_3 (0.1 g/L)] (Hussen and Schuberth, 2017). After incubation (15 min; 4°C), the cells were washed twice and incubated with mouse secondary antibodies (IgG1, IgG2a; Invitrogen) labeled with FITC and PE, respectively. Washed cells were analysed using the Accurie C6 flow cytometer (BD Biosciences). At least 10^5 total leukocytes were collected and analysed with the CFlow Software, Version 1.0.264.21.

Statistical Analyses

Statistical analysis was carried out using the software Prism (GraphPad software version 5). Results are expressed as mean ± S.E. of the mean (SEM). Differences between means were tested with one-factorial analysis of variance (ANOVA). Results were considered statistically significant at a p-value of less than 0.05.

Results and Discussion

Eosinophilic granulocytes are innate myeloid cells with several important roles in both innate and adaptive immunity (Jacobsen *et al,* 2012; Furuta *et al,* 2014). Especially in the immune response to parasitic infections and in allergic reactions, eosinophils represent a characteristic cell type (Ramirez *et al,* 2018). In dromedary camels, the immunophenotype of blood eosinophils has not been investigated yet. The aim of the present study was to use staining with monoclonal antibodies to different cell surface molecules and flow cytometry to analyse the expression pattern of cell markers on camel blood eosinophils.

In the present study, the comparison between camel blood eosinophils and neutrophils revealed significantly ($p < 0.05$) higher side light scatter (SSC) and lower forward light scatter (FSC) for eosinophils than neutrophils (Fig 1A and B). In addition, camel eosinophils showed a significantly ($p < 0.05$) higher autofluorescence in the green fluorescence channel (FL-1) when compared with neutrophils (Fig 1A and Fig 2). These findings indicate, that camel blood eosinophils can be identified as SSChigh/FSClow/FL-1high granulocytes, which is similar to the phenotype of human (Gorczyca *et al,* 2011) and bovine (Pelan-Mattocks *et al,* 2001) eosinophils.

CD45 is a phosphotyrosine phosphatase expressed on the surface of all leukocytes and is known to play a critical role in the regulation of both lymphoid and myeloid cell function (Liles *et al,* 1995). CD45 cross-linking on human eosinophils significantly increased ROS production response to stimulation with GM-CSF- and TNF-alpha (Liles *et al,* 1995). CD44 is a type I transmembrane glycoprotein that is expressed by most cell types, including leukocytes, and is the major cell surface receptor for hyaluronan (HA) (Wang *et al,* 2002; Senbanjo and Chellaiah, 2017). CD44 plays a central role as an essential adhesion molecule involved in the migration of human blood eosinophils to the respiratory tract in bronchial asthma (Sano *et al,* 1997). CD11a dimerises with CD18 to form the adhesion molecule lymphocyte function antigen-1 (LFA-1) expressed on all leukocytes (Roos and Law, 2001; van de Vijver *et al,* 2012). In the present study, camel eosinophils showed a significantly ($p < 0.05$) higher abundance of the pan leukocyte marker CD45 than neutrophils (Fig 2). In addition, camel eosinophils expressed the cell adhesion molecules CD44 and CD11a in a higher density than neutrophils (Fig 2). The functional importance of different expression densities of CD45, CD44, and CD11a on camel eosinophils and neutrophils needs further investigation.

CD172a, which is known as signal-regulatory protein alpha (SIRPa), is glycosylated cell surface receptor expressed on myeloid cells and functions as a regulatory receptor that inhibits cell signaling

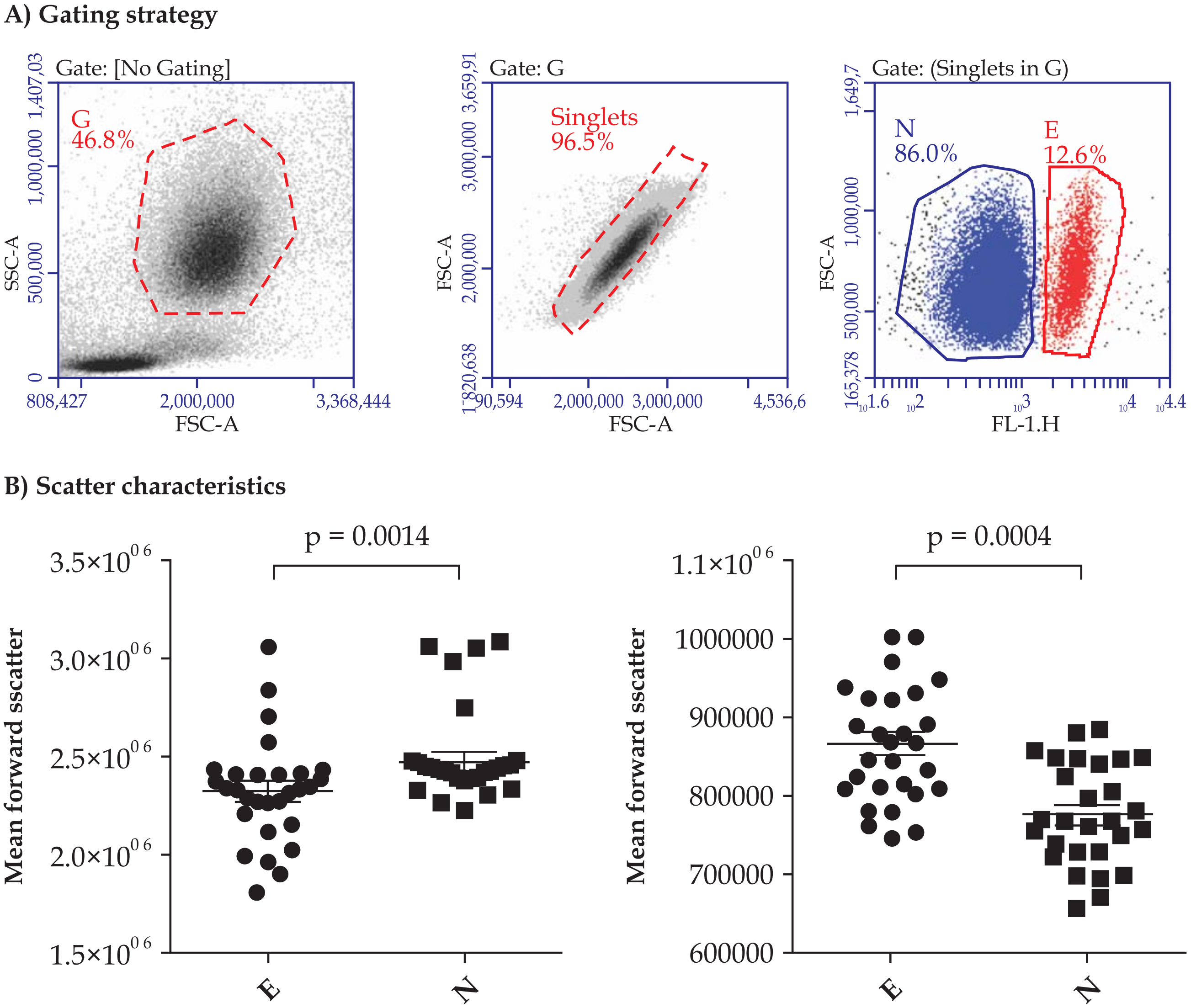

Fig 1. A) Gating strategy for camel blood eosinophils. In a forward light scatter (FSC-A) / side light scatter (SSC-A) dot plot, a gate was set on granulocytes (G) a according to their scatter characteristics. Duplets were excluded from the analysis by setting a gate on single cells in a FSC-A against FAC-H dot plot. In a SSC-A/FL-1 dot plot, eosinophils were identified within the granulocytes population by their higher green fluorescence than neutrophils in the Fl-1 channel. **B)** The mean FSC and SSC values for eosinophils and neutrophils were calculated and presented as means ± SEM.

(Hussen *et al,* 2013). In the present study, camel eosinophils showed a significantly lower abundance of CD172a when compared to neutrophils (Fig 2).

CD14 is a membrane protein which functions together with toll-like receptor 4 (TLR-4) as a bacterial pattern recognition receptor responsible for binding lipopolysaccharide (LPS) in the cell wall of gram-negative bacteria (Payne *et al,* 1993). Although it was mainly found on monocytes, camel neutrophils also show a low expression level of CD14 (Hussen, 2018). In the current work, the LPS receptor CD14 was expressed in a significantly lower intensity on camel eosinophils than neutrophils (Fig 2). Whether this can be linked to more involvement of neutrophils in sensing of LPS from gram-negative bacteria, still to be investigated.

Similar to neutrophils, camel eosinophils were found negative for the surface molecules CD163 (data not shown).

Conclusions

Based on their light scatter charecterestics and their green autofluorescence, camel eosinophilic granulocytes were identified as SSChigh/FSClow/Fl-1high cells within the granulocyte population. In comparision to neutrophilic granulocytes, camel eosinophils showed higher abundance of the cell surface molecules CD45, CD44, and CD11a but lower

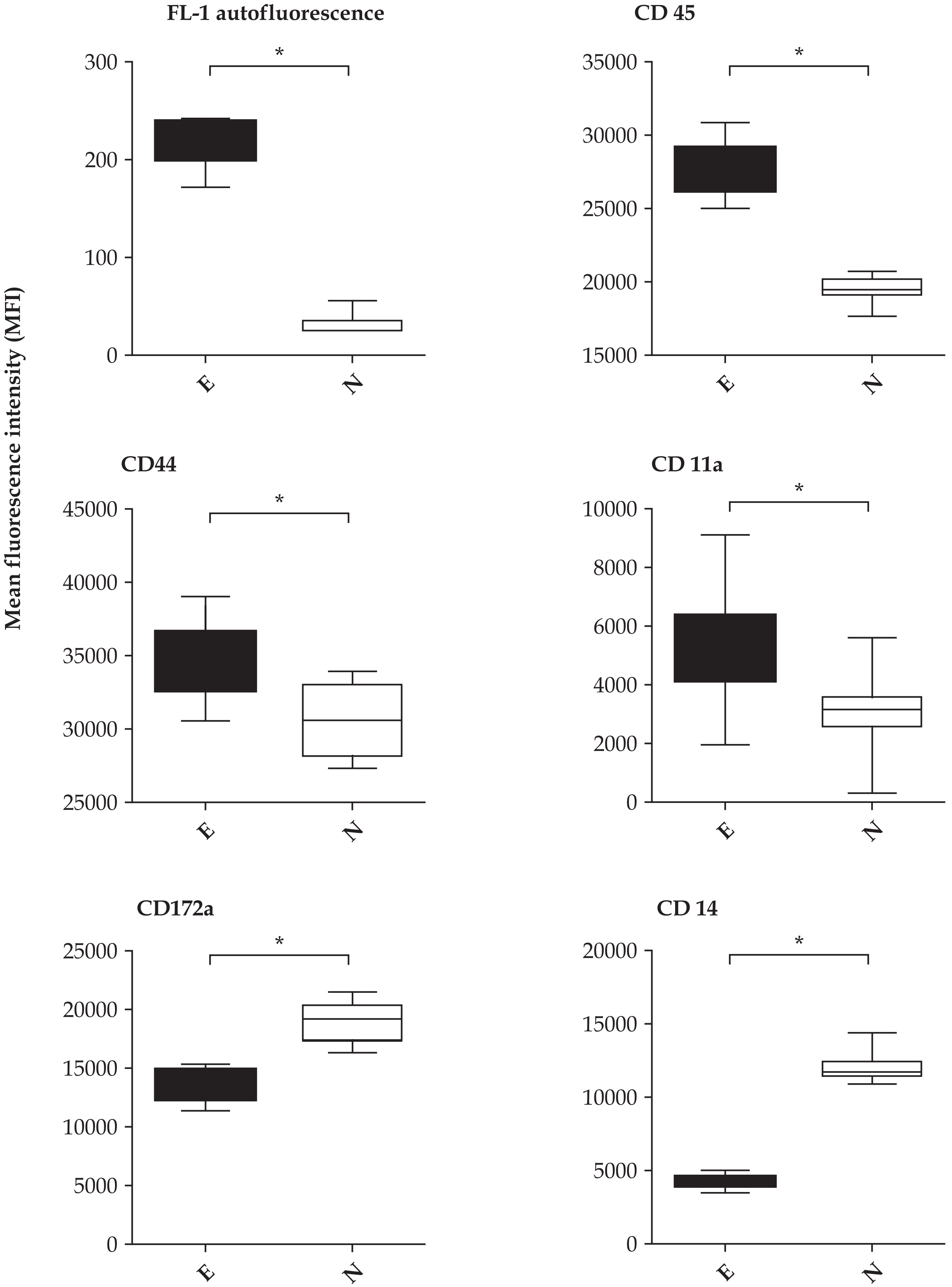

Fig 2. After gating on eosinophils (E) and neutrophils (N), the mean autofluorescence intensity in Fl-1 and the expression densities (MFI) of the cell surface molecules, CD45, CD44, CD11a, CD14, and CD172a were calculated and presented for eosinophils and neutrophils as means ± SEM. Statistical significance is indicated as * (P < 0.05.)

abundance of the cell markers CD172a and CD14. Further investigations are needed to analyse the impact of the differencies in immunophenotype between eosinophils and neutrophils on their functions in camel immunology.

Acknowledgements

The authors acknowledge the Deanship of Scientific Research at King Faisal University, Saudi Arabia for the financial support of this work under Nasher Track (grant No 00000.)

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In This Issue

ISSN 0971-6777 (Print)
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Volume 26 | December 2019 | Number 3

In This Issue

ISSN 0971-6777 (Print)
ISSN 2277-8934 (Online)

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Volume 27 | April 2020 | Number 1

In This Issue

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DOI : 10.5958/2277-8934.2021.00016.3 *Vol 28 No 1, p 103-106*

PREVALENCE OF ROTAVIRUS INFECTION IN CAMELS AND OTHER ANIMAL SPECIES

Abdelrahman Taha Hereba[1], Mohammed Soliman Shathele[1] and Maged Gomaa Hemida[1,2]
[1]Department of Microbiology, College of Veterinary Medicine, King Faisal University, Saudi Arabia
[2]Department of Virology, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt

ABSTRACT

Rotaviruses are among the main causes of enteritis in naive humans, animals, and birds. The affected animals or birds showing signs of enteritis in the form of diarrhoea, emaciation, dehydration and finally death in some cases. Little is known about its prevalence and the circulating strains of the virus is not well characterised in the eastern province of Saudi Arabia. The main objective of the current work was to detect and study the morphology of rotavirus particles in faecal suspensions from various animals from the eastern province of Saudi Arabia. To achieve these goals, we collected 140 faecal samples from dromedary camels, chicken, sheep, goat, and turkeys showing signs of diarrhoea. We processed these faecal specimen and prepared tissue suspension per each sample. We tested these faecal suspensions with the commercial available Rotavirus latex agglutination kits. Our results showed 5.17% of sheep samples positive. This represented about 2.14% of the overall tested samples from all species. We further tested these positive samples by the transmission electron microscope technique (TEM). The TEM pictures showed a typical Rotavirus shape in icosahedral in symmetry, and wheel shape. The morphometric analysis of the virus particles revealed the size of the virus ranging from 60-75 nm in diameter. These results suggested the potential roles of sheep in the transmission of rotavirus to other species of animals particularly dromedary camels living in their close proximity. There was a consistency in the results of both the latex agglutination tests and the electron microscope in the detection of rotavirus infection in faecal samples of different animals species studied.

Key words: Diarrhoea, dromedary camels, EM, enteritis, latex agglutination, rotavirus, sheep

The young children, animals and birds are usually suffering from signs of enteritis manifested clinically in the form of diarrhoea. There are many causes of enteritis in young children and animals including viruses, bacteria, parasites, toxins and many other environmental factors. Rotavirus is one of the main leading causes of enteritis in animals and birds (Ghosh and Kobayashi, 2014; Dennehy, 2015). It is well known that all children should have at least one round of rotavirus infection during the first 5 years of their life (Nguyen *et al,* 2004). Despite the presence of human vaccine against human rotaviruses, there are over 2 lacs cases of rotavirus infection reported every year particular from the developing countries (Crawford *et al,* 2017). Rotavirus belongs to the family Reovirdae that include large number of viruses affecting animals, birds as well as humans. The virus particle is non-enveloped and icosahedral in symmetry. The viral capsid composed of several layers of proteins. The virus particle is around 75 nm in diameter (Estes and Cohen, 1989). The rotavirus genome is segmented and consists of 11 segments encodes many important proteins for the virus replication (Estes and Cohen, 1989). Some human and animal strains of rotaviruses are belonging to one sero-group of the virus (Green *et al,* 1987). Detection of rotaviruses was mainly depends on the serological techniques especially the immunofluorescence, enzyme linked immunosorbent assay (ELISA) and the complement fixation test (Estes and Cohen, 1989). Several laboratory techniques were developed to detect rotavirus in clinical specimens particularly stools from human and faecal specimens from animals and birds (Al-Yousif *et al,* 2001; Xiang *et al,* 2020). New generation of the latex agglutination tests against rotavirus were developed and showed high sensitivity in the detection of the virus in faecal specimens (Dusetty *et al,* 2013). Although the electron microscope (EM) discovered long time ago, it have been still of valuable use especially in the diagnosis of the etiology of the viral gastroenteritis (Arcangeletti *et al,* 2005). The EM have great advantages in the field of diagnosing the gastroenteritis. This may be due to its ability to detect the dual infection of some common viral causes of diarrhoea such as rotavirus and Caliciviridae members. It is also useful in the

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diagnosis of some viruses that are difficult to be isolated in cell culture (Arcangeletti *et al,* 2005). Rotavirus was detected in Saudi Arabia and was responsible for gastroenteritis in young children particularly those under two years old (Kheyami and Nigel, 2006). However, there is a scarcity of research on rotaviruses from various species of animals particularly dromedary camels in Saudi Arabia. The main goal of the current study was to conduct a rotavirus surveillance among some various species of animals and birds suffering from diarrhoea in the eastern province of Saudi Arabia. To our knowledge, this is the first surveillance study among various species of animals and birds in Saudi Arabia.

Materials and Methods

All animal experiments were conducted according to the regulations of the King Abdul-Aziz City of Science and Technology, Royal Decree No. M/59, (http://www.kfsh.med.sa/KFSH_WebSite/usersuploadedfiles%5CNCBE%20Regulations%20ENGLISH.pdfAnimal ethics statement. This project was approved by the deanship of scientific reports, King Faisal University (Project No: 150050).

Samples collection and processing

A total of 140 faecal samples were collected from various species of animals and birds including (dromedary camels, sheep, goat, chicken and Turkeys) from Al-Ahsa, during late 2019 and early 2020. Each sample was collected by introducing the swabs into the cloaca of birds or the rectum of animals. The collected swabs were transferred to a sterile tube containing sterile phosphate buffered saline. Each sample was processed by centrifugation at 10000 rpm for 5 minutes. The supernatants were collected and stored at -20°C for further processing.

Latex agglutination test (LAT)

The latex agglutination test (LAT) was conducted on the faecal specimens processed above as per the manufacture's instructions. The test was conducted using the commercial available kits (Simply, Quick Stripe™ Rotavirus, Savyon® Diagnostics Ltd. 3 Habosem St. Ashdod 77610, (Catalog No. 50214)). Each strip was placed in one test tube containing the tested sample. The reaction was kept at room temperature for 10 min. Each strip has two lines, i.e. (C) designated for the control while (T) is designated for the test. Sample considered positive when two lines appear at both (C) and (T) while sample considered negative when only one line appear at (T) (Fig 1).

Processing of collected specimens for the EM testing

Processing of the faecal samples collected from various species of animals and birds for the EM technique was done as previously described (Conner *et al,* 1983). Simply, two subsequent centrifugation steps were carried out. The first cycle was done under 1ow speed 1000 RPM for 5 min then the supernatants were collected. The supernatants were subjected to a second round of centrifugation at high speed (100000 RPM) for 30 min. The supernatant were discarded then the pellets were suspended in 100 µl of distilled water.

Electron microscope (EM)

One drop of the separated faecal suspensions were placed on the Formvar coated grids for 4-5 min until drying. The grids were examined under the EM ((JOEL, JSM-5510LV, Japan) Version 5.04, JOEL Technicon's LTD, Japan) as previously described (Conner *et al,* 1983).

Results

Detection of the rotavirus in specimens from various livestock and birds in Al-Ahsa

We tested 140 faecal samples from various species of animals and birds suffering from diarrhoea. Our surveillance study showed that about 5.17% of the tested sheep samples were positive by the latex agglutination test (Table 1). This was about 2.14% of the total tested samples from all species of animals and birds. Fig 1 is showing the latex agglutination testing for specimens collected from various species of animals and birds in Al-Ahsa, Saudi Arabia in 2019-2020.

Confirmation of the detected rotavirus particles by transmission electron microscope

Fig 2 is showing the results of the transmission electron microscopy of some positive rotavirus specimens. A small virus particles ranged in size from 47-67 nm in diameter. The virus particles resembled wheel shape.

Discussion

Rotaviruses are considered among the main cause of viral diarrhoea in young animals, birds and children all over the world (Dhama *et al,* 2009; Ghosh and Kobayashi, 2014; Crawford *et al,* 2017). The virus infection induce several clinical syndromes particular in young animals, birds and children ranging from mild enteritis to severe diarrhoea, dehydration and finally death (Nguyen *et al,* 2004; Crawford *et al,* 2017). Enteritis is a multifactorial and complex syndrome in young animals and birds. The etiological agents of diarrhoea includes various types of bacteria, viruses and parasites. The Rotavirus type-A was detected in some young children and some other young domestic animals in Sudan using the antigen detection ELISA test (Ali *et al,* 2005). Rotavirus type-A was reported in camel calves in Sudan (Ali *et al,* 2005). It was also reported in sheep in India in several regions by the commercial ELISA as well as by the RT-PCR for the VP6 genes (Yilmaz *et al,* 2017). All these evidences suggested the potential roles of sheep and goats in the transmission of rotavirus to other species of animals including dromedary camels (Yilmaz *et al,* 2017). High prevalence of diarrhoea in the newborn camels in the northern region of Saudi Arabia have been reported (Al-Ruwaili *et al,* 2012). Same study reported the presence of rotavirus type-A in 14% of the tested camel calves. This is in addition to other bacterial causes of diarrhoea including bacteria such as (*E. coli*, Salmonella species and Enterococcus) (Al-Ruwaili *et al,* 2012). Recent studies showed the zoonotic potential of rotavirus infection among various species of animals and humans in Morocco where several group of animals including camels, sheep, and goat are present in close proximity of each other. It could lead to interspecies transmission of rotaviruses among various species of animals and humans (Alaoui *et al,* 2020). Detection of rotaviruses usually requires rapid, accurate, and sensitive techniques. A comparison study conducted to compare between the latex agglutination test and the EM in the detection of rotavirus in faecal specimens. Although LAT was very rapid and required less labour and time to be conducted, its sensitivity was less when compared to the EM (Moosai *et al,* 1985). This suggests initial screening with the LAT followed by further confirmation by other techniques, i.e. EM. Our results showed only 5.17% of the collected sheep faecal samples positive for rotavirus (Table 1 and Fig 1). The positive animals were young lambs suffering from diarrhoea for several days. This may postulate the high concentration of the virus particles in these collected samples. Taken in consideration that the sheep and goat usually live in close proximity of dromedary camels, they may play some roles in the transmission of rotaviruses and many other enteric pathogens to the dromedary camels. These results may be hampered by the sensitivity of the LAT and EM techniques. Further studies using some molecular based techniques are required for more wide surveillance not only for rotaviruses but also

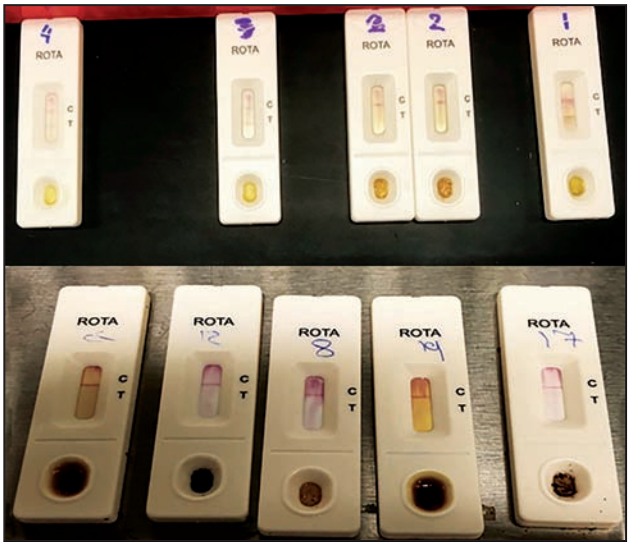

Fig 1. Results of the latex agglutination test on various faecal suspensions of some animals including camel, sheep as well as chickens. The positive results showing two lines while the negative results showing only one line. Positive and negative controls are included.

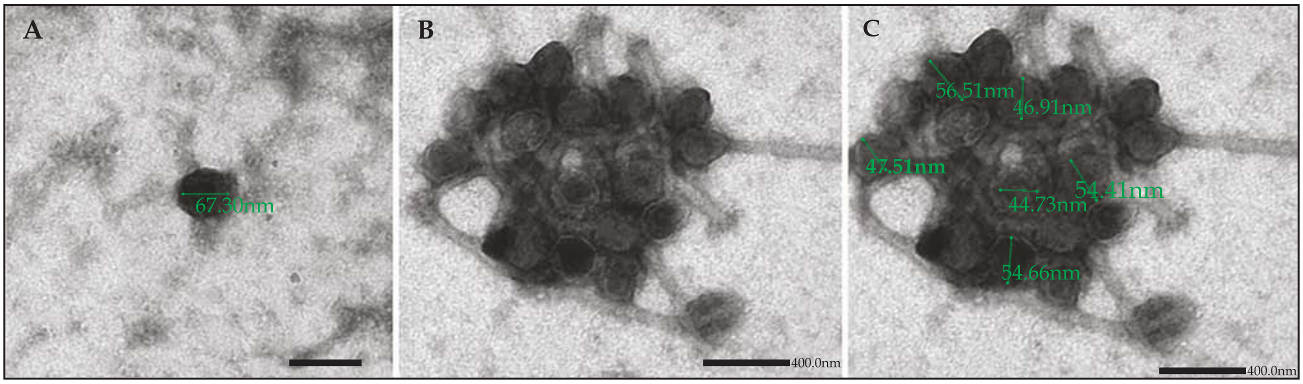

Fig 2. Detection of the rotavirus particles in faecal suspension of sheep by electron microscope. The virus particles are showing typical rotavirus shape (Wheel shape). (A) EM picture of one virus particle about 76.30 nm in diameter. (B) Clusters of rotavirus particles in the faecal suspension from sheep (C) EM picture showing the average diameter of several rotavirus particles in sheep faecal suspension.

for other viral causes of diarrhoea in various species of birds and animals.

Table 1. Summary of the surveillance of rotavirus in domestic animals and birds in Al-Ahsa, Saudi Arabia (2019-2020).

No.	Species	No of tested animals	Positive	Negative
1	Sheep	58	3	55
2	Goat	18	0	18
3	Dromedary camel	25	0	25
4	Chicken	30	0	30
5	Turkey	9	0	9
Total		140	3	137

Acknowledgement

We thank the Deanship of Scientific Research at the King Faisal University for the financial support (Grant No: 150050).

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DOI : 10.5958/2277-8934.2021.00017.5 Vol 28 No 1, p 107-109

SCANNING ELECTRON MICROSCOPY OF THE THYROID GLAND OF CAMEL (*Camelus dromedarius*)

Devendra Singh, Sanjeev Joshi, Pankaj Kumar Thanvi, Mahendra Kumar Saini and Om Prakash Choudhary[1]

Department of Veterinary Anatomy and Histology, College of Veterinary and Animal Sciences, Rajasthan University of Veterinary and Animal Sciences, Bikaner-334001, Rajasthan, India

[1]Department of Veterinary Anatomy and Histology, College of Veterinary Sciences and Animal Husbandry, Central Agricultural University (I), Selesih, Aizawl-796015, Mizoram, India

ABSTRACT

The scanning electron microscopy of the thyroid gland was done in 16 naturally dead camels (n=16) of both sexes at Veterinary Clinical Complex, RAJUVAS, Bikaner, Rajasthan. The scanning electron microscopy (SEM) of collected thyroid gland was done at Department of Veterinary Microbiology, College of Veterinary and Animal Science, Bikaner. The thyroid gland was covered by a thick fibrous connective tissue capsule and the parenchyma was made up of numerous follicles. The external forms of the follicles were mostly oval and elliptical. Some irregular follicles were observed. The size of the thyroid follicle ranged between 550-800 µm in summer and 80-350 µm in the winter season. The interfollicular or interstitial connective tissue separated the follicles. The parafollicular or "C" cells were also seen in between the thyroid follicles. The thyroid follicles were filled with gel-like round substances called colloid substances. The follicular epithelium cells of the thyroid gland were squamous to low cuboidal in shape.

Key words: Camel, colloid, follicle, SEM, thyroid gland

The thyroid gland is one of the endocrine glands that influences many organs of the body and plays an important role in the metabolism of animals (Ahmadpanahi and Yousefi, 2012). Marked variations in location, gross and histological features of the thyroid gland have been observed in different vertebrates (Dyce *et al*, 2002). The gross and histological characteristics of the thyroid gland of the dromedary camel have been described previously (Kausar and Shahid 2006; Rejeb *et al,* 2011; Ahmadpanahi and Yousefi, 2012). The functional unit of the thyroid gland is its follicle which are filled with colloid, produced by the follicular cells. The follicles are connected by interfollicular connective tissues that contain blood vessels. In the interfollicular area, there are a large number of cells, such as fibroblast and parafollicular cells (C cells), which produce calcitonin (Santos *et al,* 2013). The follicular cells produce thyroid hormones (triiodothyronine, T3, and tetraiodothyronine, T4), which have important effects on cell proliferation, differentiation, and migration as well as general growth and metabolism of embryos (Kress *et al,* 2009). A scanning electron microscope provides detailed surface information by tracing a sample in a raster pattern with an electron beam (Choudhary and Priyanka, 2017). The transmission electron microscopy of the thyroid gland of the dromedary camel has already been studied (Singh *et al,* 2021).

However, in present study scanning electron microscopic study of the thyroid gland in the dromedary camel is done.

Materials and Methods

The thyroid glands were collected from freshly dead camels (n=16) of both sexes from Veterinary Clinical Complex, College of Veterinary and Animal Sciences, Bikaner, Rajasthan. These animals were free from any pathological condition of the thyroid gland.

Processing of the samples for SEM

The scanning electron microscopy of the thyroid gland was done at the department of Veterinary Microbiology, College of Veterinary and animal Science, RAJUVAS, Bikaner. The standard protocol of AIIMS, New Delhi, was followed for scanning electron microscopy (Anonymous, 2015). For the scanning electron microscopy, 5-6 mm^2 size tissue was taken from representative areas and primarily preserved in Karnovsky's fixative (a mixture of 4% paraformaldehyde and 1% glutaraldehyde in 0.1M phosphate buffer) followed by post-fixation in 1%

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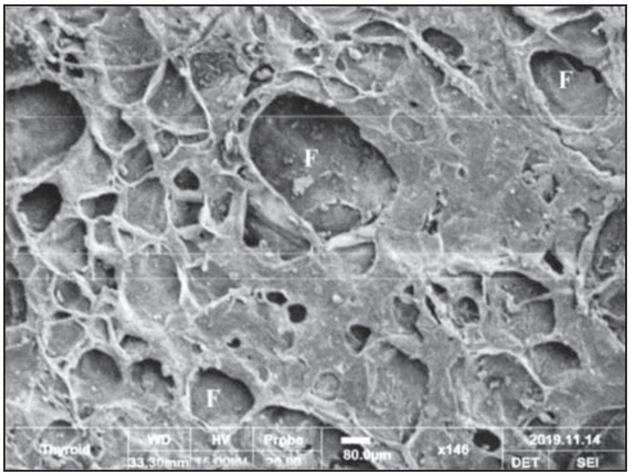

Fig 1. Scanning electron micrograph showing oval and elliptical follicles (F) in the thyroid gland of the camel (X146).

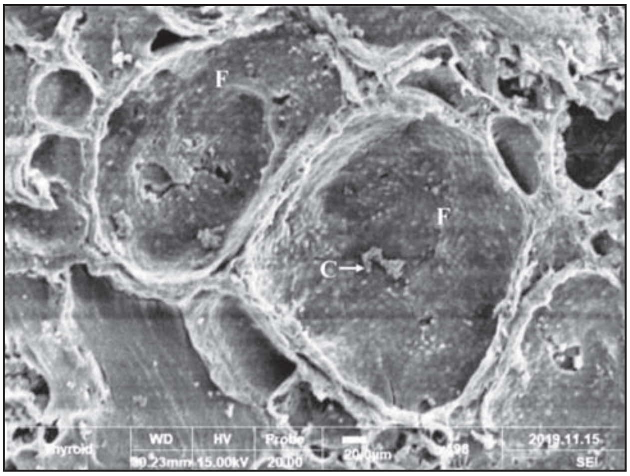

Fig 2. Scanning electron micrograph showing the internal surface of thyroid follicles (F) filled with the colloid particles (C) in the thyroid gland of the camel (X498).

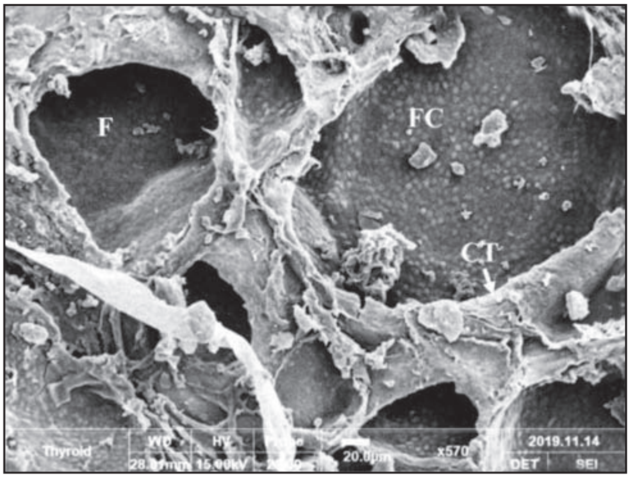

Fig 3. Scanning electron micrograph showing follicles (F), follicular cells on the internal surface of thyroid follicles (FC) and connective tissue (CT) in the thyroid gland of the camel (X570).

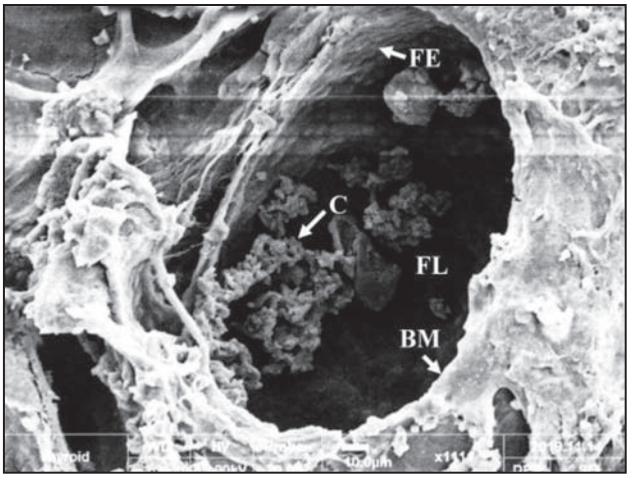

Fig 4. Scanning electron micrograph showing lumen of the follicle (F), follicular epithelium (FE), basement membrane (BM) and clumps of colloid particles (C). (X111).

solution of osmium tetraoxide and then chemical drying. All steps up to chemical drying were carried out at 4°C. Then it was followed by critical point drying (Biostag, New Delhi), mounting, metal sputter coating (Polalis, South Korea), and viewed by SEM (Genesis – 1100, Emcraft, South Korea) equipped with digital imaging and photography system.

Results and Discussion

The thyroid is an endocrine gland that secretes hormones, including thyroglobulin, triiodothyronine and thyroxin. The thyroxin hormone secreted by this gland plays an essential role in the metabolism of the body (Turner, 1966; Choudhary and Doley, 2016).

In the present study, the thyroid gland of the camel (Fig 1-4) was covered by a thick fibrous connective tissue capsule as reported previously in camel (Igwenagu *et al,* 2016). The parenchyma of the thyroid gland was made up of numerous follicles as reported in Bakerwali goat (Dar *et al,* 2018). The follicles of the thyroid gland were vascularised while these were poorly vascularised in hagfish (Suzuki and Kawabata, 1988), Jamunapari goat (Choudhary and Doley, 2016) and goat (Joshi, 2016). The external forms of the follicles were mostly oval and elliptical; however, the thyroid follicles were spherical in the thyroid gland of Muscovy (Luo and Lin, 1992). In the present study, there were some irregular follicles observed that can be due to the plane of the section of the follicles or tissue shrinkage. In another study, Rajeb *et al* (2011) reported that the activity of the thyroid gland of the dromedary was variable according to age, sex, and season. In the present study, the size of the thyroid follicle ranged between 550-800 µm, in summer and 80-350 µm in the winter season, whereas the follicle size was 300X180 µm in hagfish (Suzuki and Kawabata, 1988) and 20-90 µm in Jamunapari goat (Choudhary and Doley, 2016). The follicles were covered with membranous connective tissue (Fig 3) as reported in Jamunapari goat (Choudhary and Doley, 2016).

The large follicles were usually surrounded by smooth-surfaced cells with a large apical diameter, while the smaller follicles were surrounded by smaller cells with numerous and large microvilli, as reported in Jamunapari goat (Choudhary and Doley, 2016). The interfollicular or interstitial connective tissue separated the follicles and fibroblast and parafollicular or "C" cells were present as reported in cattle and buffaloes (Miyandad, 1973).

The lumen of the thyroid gland follicles was filled with gel-like round substances called colloid (Fig 4) as reported earlier by Kausar and Shahid (2006). The colloid particles were uniform and homogenous and size of particles was as mentioned for Jamunapari goats (Choudhary and Doley, 2016). The follicular epithelium cells of the thyroid gland were squamous to low cuboidal in shape as reported for Jamunapari goat (Choudhary and Doley, 2016), hagfish (Suzuki and Kawabata, 1988), however, the epithelium was squamous too high cuboidal in Bakerwali goat (Dar *et al,* 2018).

In conclusion, the scanning electron microscopic studies of the thyroid gland of camel did not differ from that of other studied mammalian species.

Acknowledgement

The authors are thankful to the Dean, College of Veterinary and Animal Sciences, Bikaner, RAJUVAS, Bikaner, Rajasthan for providing all the necessary facilities to carry out research work.

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Saudi Arabia sets stage for new scientific horizon to explore camel's genome

Saudi Arabia has successfully managed to lay the ground for a new scientific horizon for more world-class research to explore the camel's genome through the International Camel Organization (ICO). The fruits of such a move are widely expected to appear, within the upcoming few years, with regard to camel-related researches, especially, in the domain of its genome, administered by the International Centre for Camel Research and Studies. Camel's genome volume is estimated at about 2.38 gigabyte, containing over 20,000 genes, as some specialized scientific studies indicate that the unusual genetic composition of the camel, is the main reason behind its survival and endurance to live under such severely cruel environmental circumstances.

Its genome comprises a lot of unique differences that are verified in order to use them in treating various disorders, as a host of the camel natural products have been selected to be tested, and prescribed as an auxiliary remedy to put the evolution of as much more as possible of diseases, under control.

Saudi Arabia is aiming through the establishment of the International Centre for Camel Research and Studies, to develop and disseminate pertinent scientific studies and researches, produce experts in the domain and document their findings, in addition to setting up plans, programs, and strategies that would consolidate a scientific-cum-practical methodology and building an e-database for all aspects relating to the camel.

(Saturday January 16, 2021 / 03 , Jumada al-akhirah , 1442 Saudi Gazette BETA)

The Saudi entrepreneur selling camel milk in America

Walid Abdul-Wahab, who runs a company called Desert Farms, was brought up in Jeddah but moved to Los Angeles in 2008 to study at the University of Southern California. It was there he had the idea to introduce camel milk as an alternative dairy product to health-conscious customers. He wanted to bring something positive from back home and decided to introduce a new kind of milk that is almost 10 times better than cow’s and goat’s milk. Abdul-Wahab set up partnerships with family farms rearing camels across the US to produce the milk domestically. Abdul-Wahab said his company sells on Amazon and through the Desert Farms website as well as in regular retail stores. Along with health conscious customers, another market is selling the milk to Muslims, particularly during Ramadan and ensured that Desert Farms can provide milk to Muslims observing the holy month during the shutdown.

(Arab News: March 30, 2021)

Camel milk market to witness stunning growth

Camel milk comprehensive study was done by Application (Laban, Cheese, Ice-Cream, Yogurt, Powder, Camel Milk Infant Formulae, Flavoured Camel Milk), Distribution Channel (Supermarkets and Hypermarkets, Convenience Stores, Speciality Stores, Online), Packaging (Cartons, Bottles, Cans, Jars, Others), Process (Raw Camel Milk, Raw Camel Milk (Frozen), Raw Camel Milk Kefir, Pasteurized Camel Milk, Raw Camel Milk Colostrum) Players and Region - Global Market Outlook to 2025.

(https://www.advancemarketanalytics.com/sample-report/18440-global-camel-milk-market)

Top players in Global Camel Milk Market are Camelicious (United Arab Emirates), Al Ain Dairy (United Arab Emirates), Desert Farms (United States), Vital Camel Milk (Kenya), Tiviski Dairy, Camilk Dairy (Australia), Camel Dairy Farm Smits (Netherlands), Camel Milk Co (Australia), Camel Milk (South Africa), Amul (India)

DOI : 10.5958/2277-8934.2021.00018.7 *Vol 28 No 1, p 111-112*

Short Communication

PROMINENT PRESCAPULAR CASEOUS LYMPHADENITIS ABSCESS IN AN ADULT FEMALE DROMEDARY CAMEL: A CASE REPORT

U. Wernery and J. Kinne

Central Veterinary Research Laboratory, Dubai, UAE

Caseous lymphadenitis (CLA) or pseudotuberculosis caused by *Corynebacterium (C.) pseudotuberculosis* is one of the most important bacterial infectious diseases in livestock. It can affect sheep, goat, cattle, camelids and equids and is characterised by abscessation of one or more superficial lymph nodes and sometimes also causes severe alterations in internal organs including mammary gland (Wernery and Kinne, 2016). It is wide spread in Old World camels (OWCs) and has been reported from all camel rearing countries including in the Australian feral dromedary population. The pathogen has also been isolated from abscesses of New World camels (NWCs, Wernery *et al,* 2014), the two South American tame camel species, the llama and guanaco in their countries of origin, but also in the USA and especially in Europe, in which they were introduced as companion animals. The infection is spread by inhalation, ingestion or directly through wounds.

Morbidity of CLA may reach more than 90% in dromedaries in East African countries, whereas mortality in Bactrian camels was reported to be 28% (Chen *et al,* 1984). The mortality rate in dromedaries is unknown. Both, young and adult camels are affected by the disease.

A 14-year-old pregnant dromedary camel in poor condition weighing only 270 kg was necropsied at CVRL after it was euthanised on human grounds. It displayed a 20 cm in diameter large abscess in front of the right shoulder area (Fig 1). Multiple abscess fistulations (Fig 2) were observed which were connected to one large abscess containing necrotic material. This large abscess was most probably the primary abscess starting from the prescapular lymph node. Often different bacterial species are isolated from such multiple abscesses (Wernery *et al,* 2014). From the present case *Trueperella pyogenes, Streptococcus equi* sp. *zooepidemicus* and *C. pseudo tuberculosis* serotype 1 were cultured in high numbers from the abscesses. There were no internal lesions of CLA found.

The virulence of the pathogen is attributed to its exotoxin phospholipase D (PLD) which is produced

Fig 1. Large CLA abscess of the prescapular lymph node.

Fig 2. CLA prescapular lymph node abscess displaying multiple abscess fistulation.

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by all *C. pseudotuberculosis* strains. Two biotypes exist: ovine/caprine (serotype 1 or biotype ovis) and equine/bovine (serotype 2 or biotype bovis) and both have been identified in dromedaries using the nitrate reduction test. Hence, CLA vaccines, which were developed at CVRL (Berlin *et al,* 2015) should include both serotypes.

Acknowledgement

The authors are indebted to H.H. Sheikh Saeed bin Juma Al Maktoum who agreed to euthanise his camel and to the two vets from CVRL: F. Al Mheiri and M. Rodriguez, who performed the necropsy.

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DOI : 10.5958/2277-8934.2021.00019.9 *Vol 28 No 1, p 113-115*

A RARE CASE OF INTRAMUSCULAR MYXOMA IN AN ADULT DROMEDARY CAMEL

Shirish D. Narnaware, Rakesh Ranjan and F.C. Tuteja
ICAR- National Research Centre on Camel, Post Bag No. 07, Jorbeer, Bikaner 334001, Rajasthan, India

ABSTRACT

A rare case of intramuscular myxoma was reported in a female dromedary camel at the anterior side of hock joint of hind limb. Grossly, the tumour had 6 inch diameter and was covered by hairless dark coloured skin. The cut surface showed solid white area with oozing of blood. The histopathology of the tumour mass showed stellate to spindle shaped fibroblasts loosely arranged in an abundant myxoid matrix with few areas showing eosinophil infiltration. Cellularity was low and mitoses were rare. Based on gross and microscopic features, the neoplasm was diagnosed as an intramuscular myxoma.

Key words: Camel, gross, histopathology, myxoma

The skin and soft tissue tumours cover a wide range of tumours which are frequently reported in most of the domestic animals including camels (Gahlot, 2000; Khordadmehr *et al,* 2016). However, myxoma has been rarely reported in dromedary camels. Myxoma and myxosarcoma are tumours of fibroblast origin distinguished by their abundant myxoid matrix rich in mucopolysaccharides. The majority arise in the subcutis of the trunk or limbs. Grossly they are soft, gray-white, poorly defined masses which exude a stringy clear mucoid fluid (Meuten, 2002). They are characterised clinically by slow growth with minimal symptoms and histologically by an abundant myxoid matrix with stellate to spindle shaped fibroblasts (Stinchcombe *et al,* 2010). Present case report describes the gross and histopathological findings of an intramuscular myxoma of hind limb of a dromedary camel.

Materials and Methods

An 8 years old adult female dromedary camel of Mewari breed who died possibly of some systemic disease was presented for routine post mortem examination. Incidentally, the external examination of this carcass drew attention towards a large swelling which was observed on anterior aspect of hock joint in hind limb was measured about 6 inches in diameter and covered by hairless dark coloured skin. The cutting of this swelling revealed soild white mass resembling connective tissue with infiltration of adjacent musculature and oozing of blood (Fig 1). A tissue piece from this growth was excised and histopathology was performed by embedding in paraffin and cutting of sections of 4-μm thickness and staining with haematoxylin and eosin (HE) stain.

Results

The detailed postmortem examination of the camel did not reveal metastasis evidence in organs such as lung, liver, heart, kidneys spleen and intestines.

The histopathology of the tumour mass showed stellate to spindle shaped fibroblasts with small hyper chromatic pyknotic nuclei and scanty cytoplasm loosely arranged in an abundant myxoid matrix (Fig 2 and 3). Focal areas of hyper cellularity, thickened and hyperemic blood vessels and eosinophilic infiltration was observed occasionally. No significant mitoses and cellular or nuclear pleomorphism were observed which ruled out malignancy. These findings were found consistent with a benign intramuscular myxoma.

Discussion

Intramuscular myxoma is a very rare tumour in animals (Simundic *et al,* 2019) and to the authors knowledge their incidence in dromedary camel is not yet reported. Intramuscular myxoma is a rare benign soft tissue tumour involving the musculoskeletal system which commonly occurs in the large muscles of the thigh, shoulder, buttocks and arms (Yaligod and Ajoy, 2003; Agarwal *et al,* 2015). Laura *et al* (2017) reported that intramuscular myxoma is a rare benign soft tissue tumour of mesenchymal origin, which

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Fig 1. Solid white growth covered by dark hairless skin on anterior side of hock joint of camel.

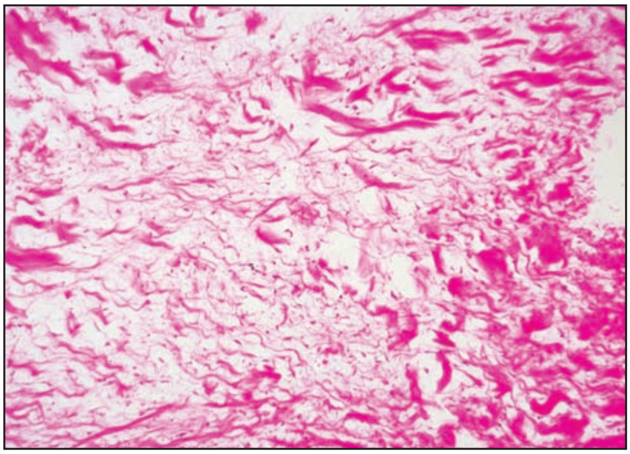

Fig 2. Histopathology of tumour growth showing stellate to spindle shaped fibroblasts. (HE X 100).

appears as a painless mass of slow growth in humans. It was emphasised that a differential diagnosis was important from soft tissue sarcoma. Computed tomography and nuclear magnetic resonance were considered the diagnostic tests of choice. The present case reported incidence of intramuscular myxoma in hind leg of a dromedary camel with infiltration of adjacent musculature. The macroscopic description as soft to solid, gray-white growth covered by dark coloured skin was consistent with earlier reports of intramuscular myxoma (Meuten, 2002; Yaligod and Ajoy, 2003). Similarly, in agreement with the present study, the myxoma was found more common in old and female patients in human and animal cases (Stinchcombe *et al*, 2010; Yaligod and Ajoy, 2013; Simundic *et al,* 2019).

The histopathological observations in the the present case such as stellate to spindle shaped fibroblasts with pyknotic nuclei and loosely arranged

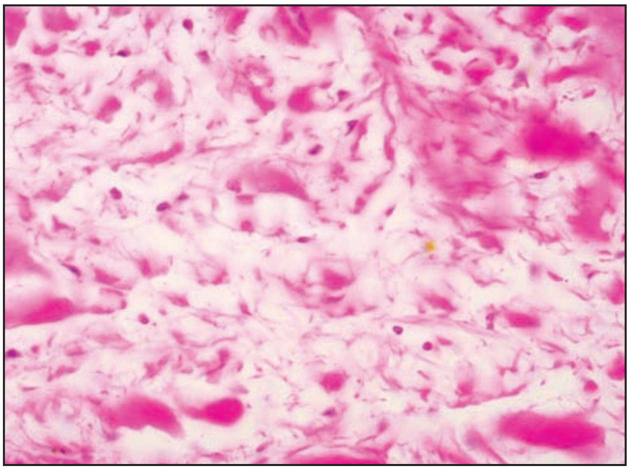

Fig 3. Stellate to spindle shaped fibroblasts with small hyper chromatic pyknotic nuclei loosely arranged in myxoid matrix. (HE X 400).

in myxoid matrix was more or less similar to the description of intramuscular myxoma in animal and human cases (Meuten, 2002; Yaligod and Ajoy, 2013; Simundic *et al,* 2019). However, in these reports relatively sparse vascular structures and hypo cellularity was observed. Ultraviolet radiation from prolonged exposure to direct sunlight is the major etiologic agent in different types of skin cancer in animals (Valentine, 2006). The camel of the present study belonged to an organised farm which was located at the thar desert where ample sunlight exposure is natural. This may underline the possible relationship between long ultraviolet exposure and incidence of cancer.

Acknowledgements

The authors were grateful to Director, ICAR National Research Centre on Camel, Bikaner, India for providing necessary facilities to carry out the research work.

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FORM IV

(See Rule 8)

1. Place of Publication	:	Camel Publishing House, 67, Gandhi Nagar (West), Near Lalgarh Palace, Bikaner-334001, Rajasthan
2. Periodicity of its publication	:	Triannual
3. Printer's Name	:	Tarun Kumar Gahlot
(Whether citizen of India)	:	Yes
Address	:	67, Gandhi Nagar (West), Near Lalgarh Palace, Bikaner-334001, Rajasthan
4. Publisher's Name	:	Tarun Kumar Gahlot
(Whether citizen of India)	:	Yes
Address	:	67, Gandhi Nagar (West), Near Lalgarh Palace, Bikaner-334001, Rajasthan
5. Editor's Name	:	Tarun Kumar Gahlot
(Whether citizen of India)	:	Yes
Address	:	67, Gandhi Nagar (West), Near Lalgarh Palace, Bikaner-334001, Rajasthan
6. Names and address of individual who own the newspaper and partners or share holders holding more than one per cent of total capital.	:	Tarun Kumar Gahlot 67, Gandhi Nagar (West), Near Lalgarh Palace, Bikaner-334001, Rajasthan

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Camel farms in canary islands

CAMELMILK EU PRIMA project:
Traditional use of camels in Lanzarote; Timanfaya park

The experience in camel farming in Canary Island has a long history (since the XIVe century) and the use of camel in the past as agricultural auxiliary was common especially in Lanzarote island. Camel population in Canary Island mainly originated from Morocco, but a certain selection has led to consider the local Majorero camel as a specific ecotype. After the years 60's the camels became touristic attraction and were used for tourists' caravans in the natural environment of the Canary Islands. Later, diversification is expected to occur on the camel production including milk and meat production as well as camel products industry. Camels are present mainly in 4 islands among the more desertic ones, i.e. Fuerteventura, Lanzarote, Tenerife and Gran Canarias. Ifin Tenerife, a small population is used for tourism only, in Gran Canarias, a big farm (Maspalomas) including around 150 camels is used for export to Europe as Canary Islands belonging to Schengen space, they are allowed for exporting to Europe. However, the largest camel population is in Fuerteventura and Lanzarote.

(Courtesy: Bernard FAYE)

CAMELMILK EU PRIMA project: Camels in farm from Lanzarote
(Canarias Islands): from Tourism to milk production?

DOI : 10.5958/2277-8934.2021.00020.5 Vol 28 No 1, p 117-121

AN OUTBREAK OF CASEOUS LYMPHADENITIS (PSEUDOTUBERCULOSIS) IN DROMEDARY CAMELS AT QASSIM REGION, SAUDI ARABIA

Salama A. Osman[1,2] and Abdullah S. Alsidrani[1]

[1]Department of Veterinary Medicine, College of Agriculture and Veterinary Medicine, Qassim University, P. O. Box 6622, Buraidah 51452, Saudi Arabia

[2]Department of Animal Medicine, Faculty of Veterinary Medicine, Kafrelsheikh University, Kafrelsheikh 33516, Egypt

ABSTRACT

This study was carried out to investigate an outbreak of lymphadenitis in dromedary camels in private farm at Qassim region, Central of Saudi Arabia. Out of 220 camels included in this study, lymphadenitis was observed in 42 camels representing morbidity rate of 19.09%. The morbidity rate did not differ significantly among different age groups ($p \leq 0.4$ and Odds Ratio = 0.7) or between different sex ($p \leq 0.1$ and Odds Ratio = 0.5). Clinically, infected camels showed enlargement and abscessation of superficial lymph nodes, emaciation in some cases with normal body temperature. *Corynebacterium pseudotuberculosis biovar ovis* was the only microorganism isolated from the pus. Haematological examination revealed significant decrease in red blood cells and packed cell volume in addition to significant increase in the total white blood cells and neutrophils in lymphadenitis-infected camels compared to healthy ones. Penicillin therapy and surgical intervention in addition to some control measures as isolation of healthy camels away from infected herd and thorough disinfection of the contaminated environment were effective measures in the control of the outbreak.

Key words: Caseous lymphadeninitis, *Corynebacterium psuedotuberculosis*, dromedary camel, pseudotuberculosis

Caseous Lymphadenitis (CLA) is an infectious bacterial disease affecting sheep, goat, cattle, camelids and equids and in rare cases humans (Peel *et al*, 1997) caused by *Corynebacterium (C.) pseudotuberculosis* and clinically characterised by abscess formation mainly in one or more of the superficial lymph nodes (superficial form) and rarely in visceral lymph nodes (internal form) and organs (Paton *et al*, 1996; Wernery and Kinne, 2016).

Caseous Lymphadenitis is transmitted through inhalation, ingestion or directly through skin abrasion or insect biting as tick infestation (*Hyalomma dromedarii*) (Wernery and Kinne, 2016).

Based on the nitrate reduction test there are two biotypes (biovars) of *Corynebacterium pseudotuberculosis*; serotype 1 (*biovar ovis*) which infect sheep and goats is negative for nitrate and serotype 2 (*biovar equi*) which infect horse and cattle is positive for nitrate reduction (Sutherland *et al*, 1996). However, Oliveira *et al* (2016) concluded that *C. pseudotuberculosis biovar ovis* is being formed from *C. pseudotuberculosis biovar equi* through anagenesis.

Prevention is better than treatment to control CLA using bacterin-toxoid vaccines due to incurable nature of the disease and low economic capacity that will disable the country to apply identification and culling policy (Oreiby *et al*, 2014).

The aim of this study was to carry out some epidemiological, clinical and control measures associated with an outbreak of caseous lymphadenitis in camel herd at Qassim region, Saudi Arabia.

Materials and Methods

Animals

Camel herd consisted of 220 dromedary camels of different ages and sex belonging to private farm in Qassim Region, Central of Saudi Arabia were used in this study. These were subjected to clinical examination according to Higgins and Kock (1984). Epidemiological data was estimated according to Martin *et al* (1987).

Bacteriological examination

Pus samples were collected from the superficial lymph nodes from each animal in sterile container by aspiration from closed ripped abscesses. All samples were taken under complete aseptic conditions and used for both direct smear and isolation of

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the causative agent by culturing onto 10% sheep blood agar, nutrient agar and MacConkey's agar plates then incubated at 37°C for 48 hours aerobically as well as in CO_2 incubator for the first isolation according to the method described by Bailey and Scott (1990). Also the ability of microorganisms to grow on Hoyle's tellurite media were done by inoculating the microorganisms onto Hoyle's tellurite lysed blood agar plates and incubated at 37°C for 48 hours according to Hoyle (1941) and Jellard (1971). Colonial and other biochemical tests were used for identification (Cruickshank *et al,* 1975).

Control measures

Control of the outbreak was done through several steps including isolation of healthy camels away from the infected ones. Treatment of infected animals using penicillin-streptomycin (Pen & Strep/NorBrook company) administered by deep intramuscular once daily for 7 consecutive days at doses of 8 mg procaine penicillin and 10 mg dihydro-streptomycin sulphate per kg bodyweight (1ml/ 25 Kg B.W). Surgical intervention was done for the ripened abscesses and irrigation using iodine in separate place away from the farm in addition to hygienic disposal of pus. Disinfection of the farm and equipments were done in adjunct with animal treatment.

Statistical analysis

The obtained data was analysed by Chi-Square and t test using the SPSS for Windows (Version 15.0, USA) statistical software program and probability (*P*-values) of less than 0.05 was considered significant.

Results

Out of the examined 220 camels of different ages and sex, 42 camels were infected with CLA representing an infection rate of 19.09%.

Concerning age predisposition, 10 camels out of 64 examined camels under three years and 32 out of 156 camels older than three years were infected representing an infection rate of 15.62 and 20.09%, respectively (Table 1).

Table 1. Prevalence of lymphadenitis in relation to camels' age.

Age	Camels examined	Infected camels	Prevalence (%)
< 3 years	64	10	15.62
> 3 years	156	32	20.51
Total	220	42	19.09

Concerning sex predisposition, 33 camels out of 189 examined female camels and 9 out of 31 male camels were infected representing infection rate of 17.46 and 29.03%, respectively (Table 2).

Table 2. Prevalence of lymphadenitis in relation to camels' sex.

Sex	Camels examined	Infected camels	Prevalence (%)
Females	189	33	17.46
Males	31	9	29.03
Total	220	42	19.09

Clinical signs observed in infected camels were in the form of enlargement of superficial lymph nodes especially submandibular and superficial cervical lymph nodes (Fig 1). Emaciation was observed in 11.9% of the affected animals. Body temperature and appetite were not affected (Table 3).

Table 3. Clinical signs in lymphadenitis infected camels.

Signs \ %	No.	Per cent
Temperature	0/42	0
Off food	0/42	0
Emaciation	5/42	11.90
Cervical LNs	37/42	88.09
Sub-mandibular LNs	35/42	83.33
Pre-femoral LNs	2/42	4.76
Pre-scapular LNs	1/42	2.38
Pre-femoral LNs	1/42	2.38
Parotid LNs	1/42	2.38

Blood examination for infected animals revealed decrease in the red blood counts and PCV in addition to increase in the white blood counts as a result of increase in the number of neutrophils and eosinophils (Table 4).

Table 4. Haemogram in healthy and lymphadenitis infected camels (mean±SD).

Variable	Healthy camels (n=10)	Infected camels (n=10)
RBCs ($10^6/\mu l$)	11.13 ± 0.81	9.61 ± 1.80*
Hb g/dl	13.12 ± 1.00	13.78 ± 2.28
PCV %	24.10 ± 1.56	20.76 ± 2.42**
WBCs ($10^3/\mu l$)	16.84 ± 2.60	28.10 ± 15.22*
Neutrophils ($10^3/\mu l$)	8.37 ± 1.85	22.15 ± 15.73*
Lymphocytes ($10^3/\mu l$)	2.33 ± 0.45	3.64 ± 1.47
Monocytes ($10^3/\mu l$)	0.15 ± 0.17	0.15 ± 0.09
Eosinophils ($10^3/\mu l$)	1.79 ± 0.90	5.02 ± 3.33**

RBC, red blood cells; WBC, white blood cells; Hb, haemoglobin concentration; PCV, packed cell volume.
* $P < 0.01$ ** $P < 0.006$

Management of the outbreak by isolation of healthy camels away from the infected ones and

Fig 1. CLA infected camel showing enlargement and abscessation in the **(A)** parotid lymph node **(B)** submandibular lymph nodes **(C)** Prescapular lymph nodes **(D)** pre-femoral lymph node.

treatment of the infected camels using penicillin in addition to surgical intervention for the ripened abscesses and irrigation using iodine in separate place away from the farm was effective in control of the outbreak and treatment of infected camels in addition to prevent other transmission.

Discussion

The prevalence of caseous lymphadenitis in this study was 19.09%. Nearly similar prevalence was recorded previously by Radwan *et al* (1989) who reported a prevalence of 15% in an outbreak of CLA in two farms in Saudi Arabia and isolated *biovar ovis* from the lesions. Lower prevalence of CLA was recorded in Egypt by Abou-Zaid *et al* (1994) who recorded a prevalence of 10% and Borham *et al* (2017) who recorded a prevalence of 10.35%. Higher prevalence was recorded previously by Borham *et al* (2016) who detected CLA in camels based on clinical and postmortem examinations in 35.4% of camels compared to seropositivity percentages of 58.06% by exotoxin ELISA and 61.29% by SWC ELISA. The variations in the disease prevalence during each study may be attributed to the number of camels in each herd in addition to the hygienic measures applied in each farm.

C. pseudotuberculosis was the only micro-organism isolated from infected animals of present study. The isolated biovar of *C. pseudotuberculosis* was negative for nitrate and this is an indication for *biovar ovis* which infect sheep and goats. This result is in agreement with the results of Hawari (2008) in Jordan and Radwan *et al* (1989) in Saudi Arabia who attributed this to the transmission of the infection from sheep and goats to camels where CLA is widespread among sheep and goats and they graze together on the same pasture (Saeed and Alharbi, 2014). Also, Borham *et al* (2017) detected serotype 1 from camel CAL. On contrary, Tejedor-Junco *et al* (2008) detected *C. pseudotuberculosis* biovar *equi* which is positive for nitrate from dromedary camels.

In agreement with our results, Braga *et al* (2006) isolated only *C pseudotuberculosis* in pure culture from closed abscesses and mixed with other pathogens as *Staphylococcus* species, *Streptococcus* species and yeasts from open abscesses from Alpacas.

Experimentally, isolates obtained from CLA infected sheep did not produce CLA in camels and produced only local abscess at the site of inoculation while isolates obtained from camels produced typical CLA in camels (Afzal *et al*, 1996).

Concerning age predisposition, no significant changes were recorded in infected camels compared to healthy ones. Similar result was observed previously by Braga *et al* (2006) who found the age has no effect on the prevalence of CLA in camels. On contrary Constable *et al* (2017) found that the prevalence of CLA increased by age and reached its maximum level in adults.

Concerning sex predisposition, no significant effect of the sex on the occurrence of CLA in camels where no differences among the disease prevalence were recorded in infected camels compared to healthy ones. On contrary, Braga *et al* (2006) recorded that the disease affected female more than male camels. Also, in a recent study in goats conducted by Yitagesu *et al* (2020) observed that the female goats were at higher risk to infection by CLA than male goats.

The main clinical signs observed in diseased camels were in the form of enlargement and abscessiation of superficial lymph nodes, emaciation in some cases with normal body temperature. Similar signs were observed previously by Radwan *et al* (1989); Tarazi and Al-Ani (2016) and Wernery and Kinne (2016). Emaciation which occurred in 11.90% of the cases of present study may be attributed to internal form of the disease. Similar observation was observed previously by Borham *et al* (2016 and 2017).

In this study all infected camels were heavily infested with ticks. This is an indication of the role of ticks in the disease occurrence. Similar observation was recorded previously in Saudi Arabia by Radwan *et al* (1989) who isolated *C. pseudotuberculosis* from ticks and mentioned the role of ticks in the disease transmission.

Blood examination revealed significant decrease in red blood counts ($P < 0.01$) and PCV ($P < 0.006$) in addition to significant increase in the number of white blood corpuscles ($P < 0.01$), neutrophil ($P < 0.01$) and eosinophil ($P < 0.006$) counts in the infected camels compared to healthy ones. Similar results were recorded previously by Tbaraka *et al* (2000) who recorded a significant decrease in the total erythrocyte counts and packed cell volume in CLA infected camels. Afzal *et al* (1996) observed no significant changes in the erythrocyte counts, haemoglobin concentration and haematocrit following experimental infection and increase in the white blood corpuscles due to neutrophilia.

Control of the outbreak by isolation of healthy camels away from the infected ones and treatment of the infected camels using penicillin in addition to surgical intervention for the ripened abscesses and irrigation using iodine in separate place away from the farm was effective in control of the outbreak. Similar results were observed by Wernery and Kaaden (2002) and Tejedor-Junco *et al* (2008) who found penicillin as effective drug in the treatment of CLA in camels.

In conclusion, *C. pseudotuberculosis* biovar *ovis* is the only biovar isolated from camels and the control of CLA outbreak can be done using several measures including isolation of healthy animals away from infected animals and pasture, treatment of infected animals, hygienic disposal of discharged pus in addition to disinfection of the environment. Moreover, tick control must be put in consideration.

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Impact of ultrasound processing on some milk-borne microorganisms and the components of camel milk

Ultrasound processing of camel milk was efficient in inactivating subsets of milk-borne pathogens without detrimental effects on camel milk fatty acids, lipid peroxides, and protein fractions. However, there were some changes in milk VC which may affect the sensory quality of milk. Inactivation of pathogenic bacteria Escherichia coli O157: H7 and *Salmonella typhimurium* in camel milk was investigated using ultrasound processing (900 W, 20 kHz, 100% power level). In addition, the effect of ultrasound treatment on raw camel milk components was studied to detect changes in fatty acid profile, lipid peroxides, protein fractions, and volatile compounds. Bacterial strains (106 CFU/ml) were added to pasteurised camel milk samples (70 ml) and transferred into a sterile aluminum container (30 mm x 120 mm, 100-ml total capacity) and then subjected to continuous ultrasound processing for 15 min in an ice water bath using a 13-mm diameter probe. The standard plate count (SPC) agar method and the in vivo imaging system (IVIS) were used to evaluate the viability of bioluminescence-transformed bacteria (E. coli O157: H7 and *S. Typhimurium*). The continuous ultrasound processing of camel milk resulted in significant ($P<0.05$) reductions in *S. Typhimurium* and *E. coli* O157: H7. Relative to unsonicated raw camel milk, the cis-9, trans-11 conjugated linoleic acid (CLA) and trans-10, cis-12 CLA contents were not affected ($P>0.05$) by the ultrasound processing. The TBAR values, a marker of lipid peroxidation, and milk protein fractions were also similar ($P>0.05$) between the sonicated and unsonicated raw camel milk. A total of 24 volatile compounds (VC) were identified including 8 aldehydes, 3 ketones, 5 acids, 5 esters, 2 aromatic hydrocarbonate, and 1 sulfo compound. Of these 24 VC, eleven VC increased ($P<0.05$) and seven decreased ($P<0.05$) after sonication.

(Dhahir, N., J. Feugang, K. Witrick, S. Park, and A. AbuGhazaleh. "Impact of Ultrasound Processing on Some Milk-Borne Microorganisms and the Components of Camel Milk". Emirates Journal of Food and Agriculture, Vol. 32, no. 4, Apr. 2020, pp. 245-54, doi:https://doi.org/10.9755/ejfa.2020.v32.i4.2088.)

Zoonotic implications of camel diseases in Iran

Approximately 60% of all human pathogens and 75% of emerging infectious diseases are zoonotic (of animal origin). Camel zoonotic diseases can be encountered in all camel-rearing countries. In this article, all studies carried out on camel zoonotic diseases in Iran are reviewed to show the importance of camels for public health in this country. More than 900 published documents were systematically searched to find relevant studies from 1,890 until late 2018. The collected articles were classified according to the aetiological agents. In this study, 19 important zoonotic diseases were reported among Iranian camels including listeriosis, leptospirosis, plague, Q fever, brucellosis, campylobacteriosis, tuberculosis, pasteurellosis, clostridiosis, salmonellosis, Escherichia coli infections, rabies, camelpox, Middle East respiratory syndrome coronavirus, Crimean-Congo haemorrhagic fever, echinococcosis, cryptosporidiosis, toxoplasmosis and dermatophytosis, most of which belong to bacterial, viral, parasitic and fungal pathogens, respectively. Results show that camels are one of the most important sources of infections and diseases in human; therefore, continuous monitoring and inspection programs are necessary to prevent the outbreak of zoonotic diseases caused by this animal in humans.

(Roya Mohammadpour Mohsen Champour Fateh Tuteja Ehsan Mostafavi
First published: 11 March 2020 https://doi.org/10.1002/vms3.239)

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