



Research article

Camel platelet aggregation responses and the antiplatelet effect of camel urine: comparison between black and white camels



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ARTICLE INFO

Keywords:

Camel platelets
Camel urine
Black camels
White camels
Camel hemostasis
Physiology
Internal medicine
Laboratory medicine
Clinical research

ABSTRACT

Human black and white-skinned races exhibited differences in platelet aggregation. However, no similar differences were described on white and black camels. This study aims to find out whether black and white camel skin color is associated with differences in camel platelet aggregation responses or the platelet inhibitory activity of their urine. Platelet aggregometry was undertaken in black and white camels, in response to adenosine diphosphate (ADP), Arachidonic acid (AA), Epinephrine (EPN), collagen, and Ristocetin. Platelet aggregometry was also done in human PRP after the addition of raw and serially diluted (1:2, 1:4 and 1:8) white and black camel urines. In black camels, platelet aggregation in response to ADP, AA, EPN and Collagen were slightly higher than in white camels. The addition of raw camel urine collected from mixed population of black and white camels to human platelets resulted in inhibition of platelet aggregation. Serial dilutions of camel urine (1:2, 1:4, 1:8) resulted initially in loss of the inhibitory action followed by enhancement of human platelet aggregation responses to ADP and AA. The neat and serially diluted white camel urines caused more inhibition of the human platelet aggregation responses than the black camel urines. This study uncovered a new biological feature in the camels. The camel skin color seems to be associated with different platelet aggregation responses as well as different anti-platelet activity of the camel urine; white camel urine was found to cause more platelet inhibition than black camel urine.

1. Introduction

Camel platelets display markedly inhibited platelet aggregation responses to the commonly used aggregating agents; ADP and collagen (Col) and no aggregation responses to Epinephrine (EPN), Arachidonic acid (AA) and Ristocetin, when compared to human platelets (Gader et al., 2006; Al-Ghumlas et al., 2008; Al-Ghumlas and Gader, 2013). This suggested that camel plasma has platelet inhibitory activity (Al-Ghumlas, 2009) especially when subsequent study have shown that this inhibitor is filtered into camel urine (Alhaidar et al., 2011; Alyahya et al., 2016). All these studies were performed in mixed population of black and white camels.

Information on the haemostatic system and its variations in the camels is scarce and none of the few reports looked into the color of the camel skin as a factor that may be associated with variations in platelet function or urine platelet inhibitory activity, similar to what was documented in human black and white-skinned races (Buchanan et al., 1981; Mackie et al., 1982; Meade et al., 1985; Gader et al., 1991; Otahbachi et al., 2010). For example, the human black ethnic group exhibits

markedly inhibited aggregation responses especially to Ristocetin, when compared to whites (Buchanan et al., 1981). This encouraged us to look into the association between the black and white camels and the aggregation activity, in view of the recently reported difference in the genetic makeup between black and white camels (Almathen et al., 2018). Therefore, our current study aims to find out whether the difference in the camel skin color (black and white) is associated with either different camel platelet aggregation responses or different inhibitory activity of their urine.

2. Materials and methods

A total of forty-one healthy lactating female camels (*Camelus dromedaries*) 20 Mejhem (black) and 21 Wadeh (white) were studied. The camels' ages ranged from 2 to 10 years. These animals were sampled from a private farm near Riyadh. The camels were kept under intensive management and veterinary care and they had free access to food and water. Human blood samples were collected from blood donors. The Control group referees to PRP that is separated from healthy human

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subjects and exposed to aggregating agonist only without adding camel urines.

This study was approved by the Institutional Review Board (IRB) of The College of Medicine, King Saud University (Approval number, E-13-896; Approval date, 24 March 2013).

3. Blood and urine collection and processing

3.1. Camel blood collection

Venous blood samples (30–40 ml) were collected from the Jugular Vein in the neck directly into vacutainer tubes containing Sodium Citrate (0.129M) to give a blood: citrate ratio of 9:1 (Terumu Co. Japan). Proper mixing of blood and anticoagulant was attained by gentle inversion and the samples were transported without delay (within 2 h of collection) to the Coagulation Laboratory, College of Medicine.

3.2. Camel urine collection

Urine was collected from 35 healthy camels, 17 black and 18 white camels from which the blood samples were also collected. It was undertaken by experienced camel attendants, as detailed previously (Alhaidar et al., 2011).

4. Laboratory procedures

4.1. Camel blood aggregation studies

The blood samples were centrifuged at 1000 rpm for 7min, at room temperature, to separate platelet rich plasma (PRP), which was used for platelet aggregation studies. The PRP was removed, and the remaining sample was centrifuged at 3000 rpm for 10 min to obtain platelet-poor plasma (PPP), which was used as standard for the aggregation studies.

Aggregation was measured in response to ADP (20 $\mu\text{mol/l}$), COL (0.19 g/l), AA (1.64 mmol/l), EPN (100 $\mu\text{mol/l}$) and Ristocetin (1.5 g/l) (Bio/Data, Corp. USA). All these concentrations of agonists represent the final concentrations obtained by adding 20 μl of the aggregating agent to 180 μl of PRP. Aggregation was recorded in the Aggregation Profiler, PAP4 (Bio/Data, USA), which registers the results of the aggregation responses as maximum aggregation percentage (MA %) as well as slopes (S) of the aggregation curves.

4.2. Effect of camel urine on human platelets

We preincubated human PRP with 0.05 ml of raw camel urine for two min. Then, 20 μl of the aggregating agent was added and the recording started. Dose response aggregation studies were performed by preparation of serial dilutions of camel urine (1:2, 1:4 and 1:8) which were prepared by using saline diluent. Each of the dilutions was added to the 180 μl human PRP, mixed, and incubated for 2 min at room temperature before adding 20 $\mu\text{mol/l}$ of ADP and 1.64 mmol/l of AA.

4.3. Statistical methods

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) version 22.0 software (SPSS Inc., Chicago, IL, USA). We calculated mean \pm standard deviation (SD) for all numerical variables (measurable variables). Student t-test was used for two independent groups to compare between black and white camels with respect to all numerical variables. A P-value of 0.05 or less was considered statistically significant. In addition, a general Linear Model (GLM) was fitted to compare the different measurements of the four samples of human PRP that were mixed with diluted camel urine (Neat, 1:2, 1:4, and 1:8). Estimated marginal means plot was constructed to discern differences. Significant difference was assessed at 5% level of significance using Wilk's lambda.

5. Results

5.1. Camel platelet aggregation in black and white camels

The results obtained showed that in black camels, the average MA% of platelet aggregation in response to ADP, AA, EPN and collagen (Mean \pm S.D: 31.5 \pm 11.3, 10.3 \pm 10.3, 12.7 \pm 6.4 and 9.5 \pm 7.2) were slightly higher in comparison to white camels (Mean \pm S.D: 28.5 \pm 9.7, 7.2 \pm 8.9, 10.5 \pm 5.4 and 7.4 \pm 5.8) but the difference was not statistically significant. In contrast the aggregation response to Ristocetin was slightly lower in black camels as compared to white camels (Mean \pm S.D: 3.6 \pm 7.3 and 4.9 \pm 5.3) respectively. This difference was not statistically significant (Figure 1).

5.2. The effect of neat camel urine collected from mixed population of black and white camels on human platelet aggregation in response to ADP and AA

The addition of raw urine collected from mixed population of black and white camels (n = 35) resulted in significant inhibition of human platelet aggregation in responses to ADP (Figure 2); the average MA% of human platelet aggregation response to ADP was: (Mean \pm S.D: 35.8 \pm 18.7), which is significantly lower than the control: (Mean \pm S.D: 56.2 \pm 9.8); P-value < 0.05. Similarly, the human platelet aggregation responses to AA decreased after the addition of raw camel urine (Mean \pm S.D: 53.3 \pm 22.0) as compared to the controls (Mean \pm S.D: 62.2 \pm 13.6) but this decrease was not statistically significant (Figure 2).

5.3. Dose response aggregation studies of urine collected from mixed population of black and white camels on human platelet aggregation in response to ADP and AA

It was noted with great interest that when serial dilutions of urine (1:2, 1:4, 1:8) which were collected from mixed population of black and white camels were added to human PRP before ADP addition, it resulted initially in loss of the inhibitory action at (1:2) 50% dilution (MA%: mean \pm S.D: 56.2 \pm 19.8). This is followed astonishingly by enhanced responses and activation of human platelet in response to ADP at (1:4) 25% and (1:8) 12.5% dilutions (MA%: mean \pm S.D: 63.3 \pm 18.9 and 80.40 \pm 18.9) respectively as compared to the control (MA%: mean \pm S.D: 56.2 \pm 9.8) (Figure 2). The last dilution (1:8) 12.5% was the only dilution that triggered statistically significant aggregation response when compared to the control; P value < 0.05.

Similar results were obtained for AA. Interestingly, adding diluted urine to human PRP like the aggregation response to ADP triggered enhanced aggregation responses of human platelet rather than inhibition (Figure 2). The results of platelet aggregation after diluting the urine with (1:2, 1:4, and 1:8 was: MA%: mean \pm S.D: 66.7 \pm 14.1, 74.3 \pm 17 and 86.2 \pm 18) respectively. These values were higher than the control (Mean \pm S.D: 62.2 \pm 13.6). Only the last dilution attains statistical significance when compared to the control, P value < 0.05.

5.4. Studies of the effects of black and white camel urines, each on its own, on human platelet aggregation in response to ADP and AA

5.4.1. Dose response effect of black and white camel urines on human platelet aggregation in response to ADP

As seen in the dose response aggregation studies in mixed camel population, the dose response results obtained after the addition of serial dilutions of black and white camel urines showed a similar pattern of response to ADP. We obtained gradual increase in human platelets aggregation responses. In fact, the human platelet aggregation in response to ADP with 25% (1:4) and 12.5% (1:8) urine dilutions exceeded the control value.

Comparison between the effects of the serially diluted camel urines of the black and white camels on human PRP in response to ADP showed

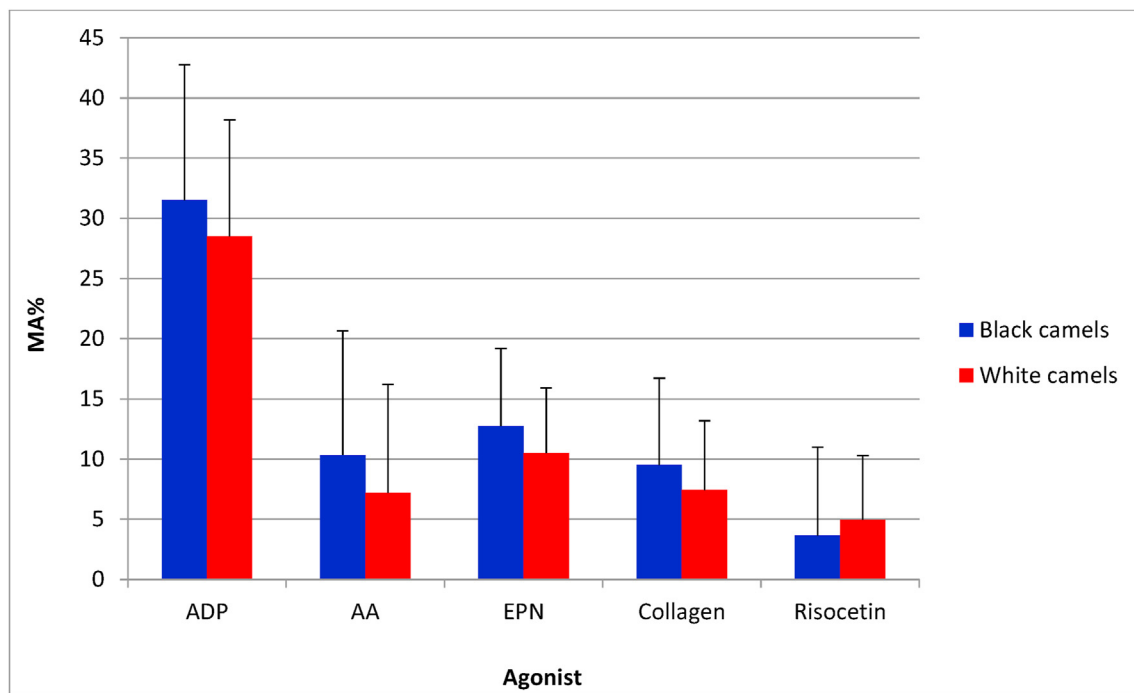


Figure 1. Comparison of the platelet aggregation responses (expressed as MA%) between black and white camels. Results are expressed as mean \pm S.D. No significant differences were found. A P-value of 0.05 or less was considered statistically significant.

that the inhibition of the human aggregation responses in the presence of neat black camel urines was less than the inhibition triggered by neat white camel urines. This difference was statistically significant, $P < 0.05$. Similarly, diluted white camel urines (1:2, 1:4, 1:8) resulted in more inhibition of the human platelet responses than the diluted black camel urines and this difference was statistically significant in all dilutions P value < 0.05 (Figure 3).

5.4.2. Dose response effect of the black and white camel urine on human platelet aggregation in response to AA

The aggregation response of human platelets to AA decreased after the addition of neat black and white camel urine to human PRP but this did not reach statistical significance.

Similar to the aggregation response to ADP, the addition of serial dilutions of camel urines (1:2, 1:4, 1:8) to human PRP resulted in loss of the urine inhibitory action in response to AA (Figure 4). White camel urine caused more inhibition of human PRP than black camel urine does. However, the difference was not statistically significant.

6. Discussion

In humans, ethnic differences are known to be associated with variable platelet aggregation in response to various aggregating agents especially ADP, AA, EPN and Col (Meade et al., 1985). Earlier studies in our laboratory (Gader et al., 1991) and others (Otahbachi et al., 2010) confirmed this observation when comparing Africans, Westerners, Arabs and Asians. For example, Saudi Arabs and Westerners (Europeans/Americans) showed more aggregation responses to ADP than Asians and Africans. In addition, the aggregation response to Col was more pronounced in Saudis and Africans than in Westerners and Asians. Also, 40% of the Asians did not respond to AA. On the other hand, 74% of Africans have very inhibited responses to Ristocetin when compared to other races and this is in line with early reports (Buchanan et al., 1981, Mackie et al., 1982, Otahbachi et al., 2010). These differences in platelet aggregability between ethnic groups have been explained by genetic variations, dietary and many other factors (Renaud et al., 1981; Salo et al., 1985). Comparative hemostasis between humans and camels

particularly platelets physiology remains rarely studied. Thus, we also wondered if black and white camels skin color display differences in platelet aggregation responses similar to what has been reported in humans.

In the present study, we first tested black and white camel platelets aggregation responses to ADP, AA, EPN, Col and Ristocetin using light transmission aggregometry. We found that the aggregation responses to be less in white camels as compared to black camels. This was taken to indicate that the aggregation response of the camel platelets may be associated with the camel skin color as in humans (Gader et al., 1991; Otahbachi et al., 2010). Obviously, all the platelet responses in black and white camels were significantly less than those of human platelet responses and this result agrees with our previous reports on mixed population of black and white camels (Gader et al., 2006; Al Ghumlas et al., 2008; Al Ghumlas and Gader, 2013; Alhaidar et al., 2011).

On the other hand, when we incubated human PRP with raw white or black camel urines, we obtained significant inhibition of the human platelet aggregation response to ADP. This inhibitory action is similar to that of adding the antiplatelet drug; Clopidogril to human platelets (Al Ghumlas and Gader, 2013). As mentioned earlier, this was taken to indicate that this inhibitor in the camel urine, like Clopidogrel, competes with ADP to the binding site for the ADP receptor P2Y₁₂ on the platelet surface membrane, thereby inducing irreversible inhibition of ADP-induced aggregation.

In contrast, incubating raw white or black camel urine with human PRP resulted in variable aggregation responses to AA. This finding strongly suggested that the platelet inhibitory action of the AA in the camel urine is not always present. This inhibitory action may be related to different factors that are transient and changeable especially the food consumed by the camels which is mainly desert plants, but these factors could not be easily determined in this study. The possibility that an AA inhibitor when present will cause inhibition of the cyclooxygenase pathway and/or AA receptor blocking can't be excluded in the human platelet samples that were inhibited by camel urine in response to AA. Similarly, in response to AA, white camel urine has resulted in more inhibition of human PRP than the black camel urine. Although the difference did not reach statistical

significance, the result is suggestive of the existence of similar cyclooxygenase pathway in camels as it is in human platelets. So far, no study is available in the literature that compared the effect of black and white camel urine on human platelet aggregation.

It is clear from the above discussion that the neat white and black camel urine studies did not uncover clear cut differences in their inhibitory properties. Therefore, we resorted to dose response aggregometry using doubling dilutions (1:2, 1:4 and 1:8) of the neat camel urines and we repeated the human platelet aggregation in response to ADP and AA. This approach uncovered two interesting findings; firstly: The gradual reduction in the concentration of black and white camel urines resulted in the expected loss of the urine inhibitory action and the gradual recovery of the human platelet aggregation responses to both ADP and AA. When comparing the two dose response curves, it is clear that white camel urine resulted in significantly more inhibition of human platelets aggregability than the black camel urine (Figure 3). This strongly indicates that the potency of the inhibitor in the white camel urine is more than that of the black camel urine.

Secondly: The highest human platelet aggregation responses were obtained using the lowest camel urine dilution of 1:8. Interestingly the two urine dilutions 1:4 (25%) and 1:8 (12.5%) resulted in human platelet aggregation responses that surprisingly superseded the response to the neat camel urine. The mechanism for this unexpected finding is open for speculations. It is possible that when using camel urine at low doses, the camel urine inhibitor will be diluted, and the obvious result is a gradual enhanced response of the platelet aggregation as a result of this decrease in the camel urine concentration.

It is also possible that these responses resulted in modification of the platelet membrane receptors i.e. the more the dilution of the urine the

more will be the exposure of individual receptor sites to interact with the agonists. It is also feasible that these receptors increase in numbers after the dilution of the urine thereby resulting in more enhanced aggregation responses. Previous observations on platelet aggregation in children with nephrotic syndrome from our laboratory using doubling dilutions of AA have shown a similar phenomenon; platelets from nephrotic children showed higher responses to lower doses of AA but failed to respond to higher doses or even to the neat AA (Al-Mugeiren et al., 1995).

A careful search to find any differences in the biology between black and white camels identified a recent study by (Almathen et al., 2018) who uncovered genetic difference between black and white camels (Almathen et al., 2018). These authors detected the sequence variations at the MC1R and ASIP genes. MC1R was linked to the white skin color and ASIP was associated with black skin color. In this respect, it has also been proposed that the skin color variation may influence the performance of animals under heat stress, heat tolerance and their adaptation capacity to hot environments (Finch et al., 1984). However, the connection between camel skin color and other physiological functions has not been explored further.

Our findings of differences in the platelet inhibitor in both white and black camel urines could also give some support to the practice of camel urine therapy in the treatment of wide range of diseases (Gader and Alhaidar, 2016) including cancer. Previously we pointed out that the Aspirin-like action (inhibitor of the AA aggregation) could give support to the claimed anti-malignant action of camel urine by inhibiting platelet activity and subsequent clot formation, thereby delaying dissemination of cancer. Also, Aspirin itself is known to have direct anti-malignant action (Alhaidar et al., 2011). Urine therapy practiced widely among camel breeders and local alternative medicine practitioners who use it as

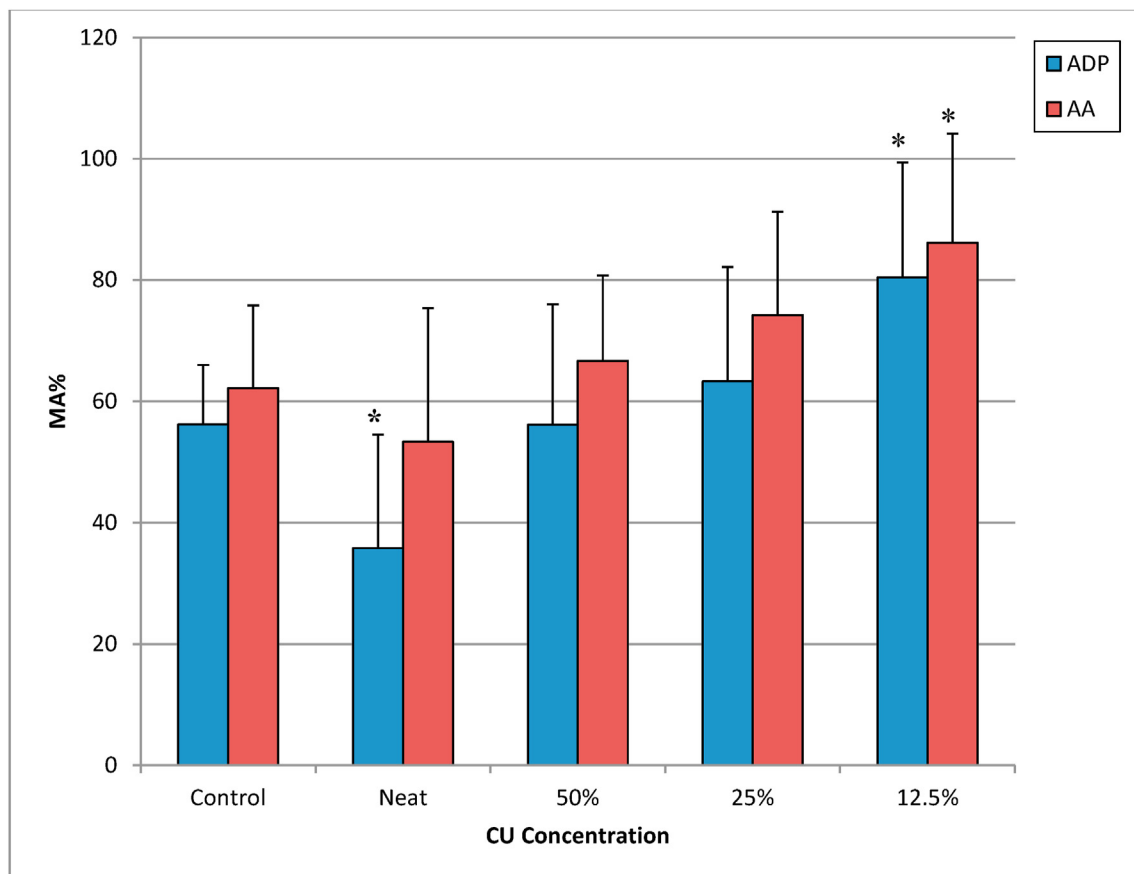


Figure 2. Dose response effect of mixed population of (black and white) camel urines (CU) on the ADP and AA-induced platelet aggregation responses (expressed as MA%) of human PRP. Results are expressed as mean \pm S.D. A P-value of 0.05 or less was considered statistically significant. (* designates statistical significance as compared to the control response).

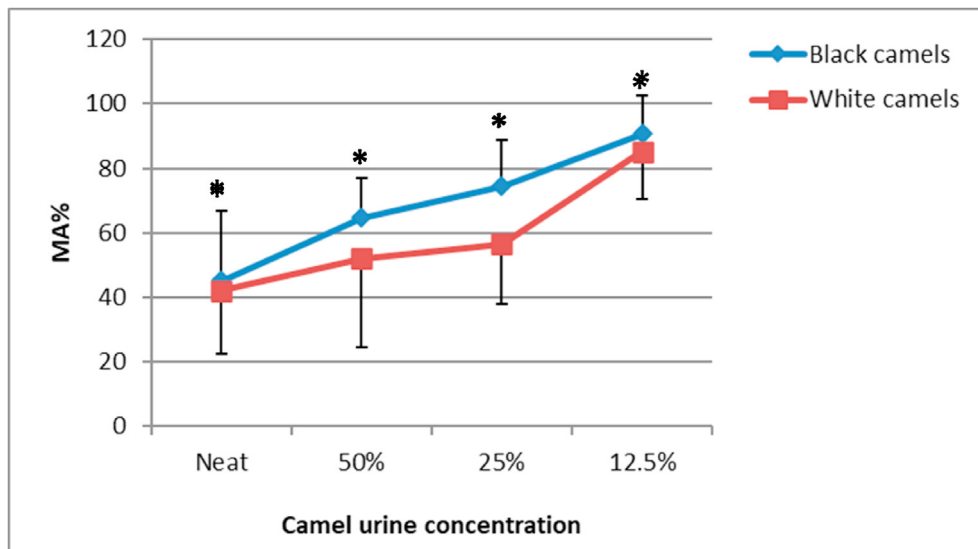


Figure 3. Comparison between the effects of black and white camel urines on the ADP-induced platelet aggregation responses (MA%) of human PRP. (* designates statistical significance of human platelet response after addition of white camel urine as compared to human platelet response after addition of black camel urine). Results are expressed as mean \pm S.D. A P-value of 0.05 or less was considered statistically significant.

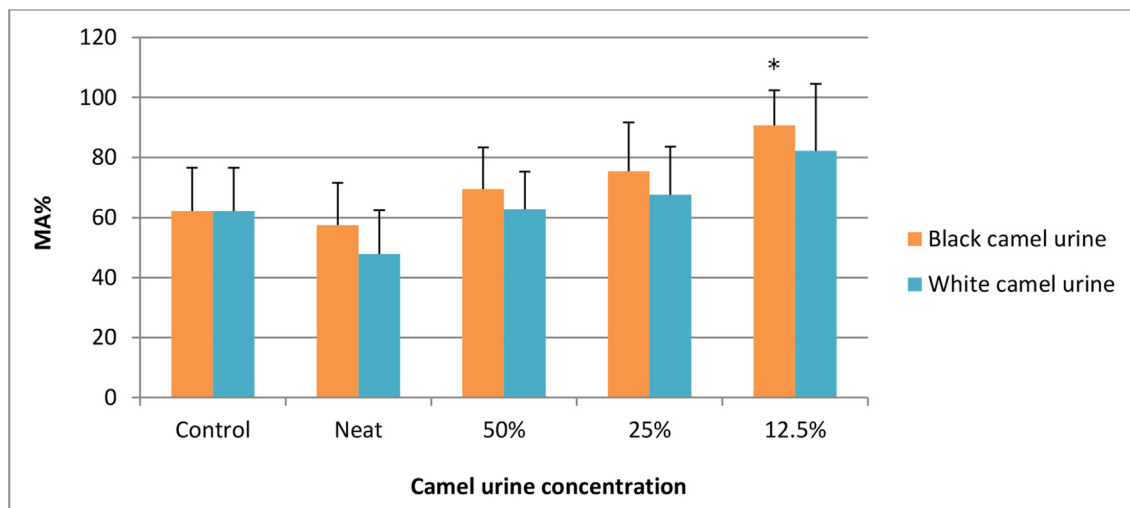


Figure 4. Dose response effect of serial dilutions of black and white camel urines on the AA-induced platelet aggregation responses (MA%) of human PRP. Results are expressed as mean \pm S.D. A P-value of 0.05 or less was considered statistically significant. (* designates statistical significance as compared to the control response).

therapy without dilution. Thus, we can assume that the more the dilution of the urine the less effective will be its claimed therapeutic action.

Lastly, we attempted to draw a physiological benefit to the camels from the presence of a platelet inhibitor in the plasma and urine of the camels. It is possible that this platelet inhibitor regardless of the biological difference in camel color will help to sustain months of very hot summer without any significant risk for thrombotic diseases, bearing in mind that camels which lived and survived in very hot environment have also very high platelets count as compared to humans (Gader et al., 2006). It is known that when humans exposed to very high environmental temperature, they are liable to develop heat stroke which involves activation of human platelet and subsequent consumption coagulopathy (Gader et al., 1990). In this way, the presence of such inhibitor in the camel plasma and urine would serve to prevent platelet activation and subsequent thrombotic complications. In addition, the characterization of the platelet inhibitor in the urine may eventually open the door for the development of therapeutic agents from camel urine that block the ADP

and AA platelet membrane glycoprotein receptors without affecting the fluid phase of the coagulation system.

The findings of this study add new information on the difference between black and white camels related to platelet function of similar nature to what was reported in black and white humans. One of the limitations of this study is the number of the black camels studied was small because they are not kept in the farms in the vicinity of the capital city, Riyadh. Moreover, the study could not be extended further to test the platelet inhibitory activity of camel urine in another biological setup to see if it can prevent thrombus formation. Also, we don't have the analytic method to determine the nature of the platelet inhibitory activity of camel urine as this needs a specialized laboratory facility.

7. Conclusion

The results of the present study add a new camel biological feature that is related to their skin color in addition to the already reported

genetic makeup in a similar way as it has been reported in white and black ethnic groups. The camel skin color seems to be associated with different platelet aggregation responses as well as different antiplatelet activity of their camel urines; white camel urines were found to cause more inhibition than black camel urines. This opens the door for further studies to clarify the nature of this inhibitor and its possible future use as antiplatelet agent that could help in the management of thrombotic disease.

Declarations

Author contribution statement

Abeer Khalid Al-Ghumlas: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Funding statement

This work was supported by College of Medicine Research Centre, Deanship of Scientific Research, King Saud University, Riyadh, Saudi Arabia.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

Acknowledgements

I would like to thank Mr. Lugman El-Sid for his technical assistance. Special thanks to Mr. AbdulAziz Al Muhaideb, who provided the camel samples for this research.

References

- Al-Ghumlas, A.K., Abdel Gader, A.G.M., Hussein, M.F., Al Haidary, A., White, J.G., 2008. Effects of heat on camel platelet structure and function—a comparative study with humans. *Platelets* 19, 163–171.
- Al-Ghumlas, A., 2009. Morphological and Functional Characterization of Camel Blood Platelets: A Comparative Study between Camel Platelets and Human platelets. Thesis, Ph-D of Physiology. King Saud University, Saudi Arabia.
- Al-Ghumlas, A.K., Gader, A.G.M.A., 2013. Characterization of the aggregation responses of camel platelets. *Vet. Clin. Pathol.* 42 (3), 307–313.
- Alhaidar, A., Abdel Gader, A.G.M., Mousa, S.A., 2011. The antiplatelet activity of camel urine. *J. Alternative Compl. Med.* 17, 803–808.
- Almathen, F., Elbir, H., Bahbahani, H., Mwacharo, J., Hanotte, O., 2018. Polymorphisms in MC1R and ASIP genes are associated with coat color variation in the arabian camel. *J. Hered.* 109, 700–706.
- Al-Mugeiren, M.M., Gader, A.M.A., Al-Rasheed, S.A., Bahakim, H.M., Al-Momen, A.K., Al-Salloum, A., 1995. Platelet aggregometry-dose-related responses to Arachidonic acid in childhood nephrotic syndrome. *Platelets* 6, 71–74.
- Alyahya, A.M., Gader, A.G.M.A., Alhaider, A.A., 2016. Characterization of inhibitory activity of camel urine on human platelet function. *J. Taibah Univ. Med. Sci.* 11, 26–31.
- Buchanan, G.R., Holtkamp, C.A., Levy, E.N., 1981. Racial differences in ristocetin-induced platelet aggregation. *Br. J. Haematol.* 49, 455–464.
- Finch, V.A., Bennett, I.L., Holmes, C.R., 1984. Coat colour in cattle: effect on thermal balance, behaviour and growth, and relationship with coat type. *J. Agric. Sci.* 102, 141–147.
- Gader, A.M., Al Ghumlas, A.K., Hussain, M.F., Al Haidary, A.I., 2006. Platelet aggregation and platelet function analyzer 100 (PFA-100) closure time in camels—a comparative study with humans. *Comp. Clin. Pathol.* 15, 31–37.
- Gader, A.G.M.A., Alhaider, A.A., 2016. The unique medicinal properties of camel products: a review of the scientific evidence. *J. Taibah Univ. Med. Sci.* 11, 98–103.
- Gader, A.M.A., Bahakim, H.M., Malaika, S.S., 1991. Ethnic variations in platelet aggregation—comparison between Saudi Arabs, Westerners (Europeans and Americans), Asians and Africans. *Platelets* 2, 197–201.
- Gader, A.M.A., Al-Mashhadani, S.A., Al-Harthy, S.S., 1990. Direct activation of platelets by heat is the possible trigger of the coagulopathy of heat stroke. *Br. J. Haematol.* 74, 86–92.
- Mackie, I., Bull, H., Kennedy, J., Brozović, M., 1982. Ristocetin-induced platelet agglutination in Afro-Caribbean and Caucasian people. *Br. J. Haematol.* 50, 171–173.
- Meade, T.W., Vickers, M.V., Thompson, S.G., Stirling, Y., Haines, A.P., Miller, G.J., 1985. Epidemiological characteristics of platelet aggregability. *Br. Med. J. (Clin. Res. Ed.)* 290, 428–432.
- Otaibachi, M., Simoni, J., Simoni, G., Moeller, J.F., Cevik, C., Meyerrose, G.E., Roongsritong, C., 2010. Gender differences in platelet aggregation in healthy individuals. *J. Thromb. Thrombolysis* 30, 184–191.
- Renaud, S., Morazain, R., Godsey, F., Dumont, E., Symington, I.S., Gillanders, E.M., O'Brien, J.R., 1981. Platelet functions in relation to diet and serum lipids in British farmers. *Heart* 46, 562–570.
- Salo, M.K., Vartiainen, E., Puska, P., Nikkari, T., 1985. Platelet aggregation in Finnish men and its relation to fatty acids in platelets, plasma and adipose tissue. *Thromb. Haemostasis* 53, 563–569.