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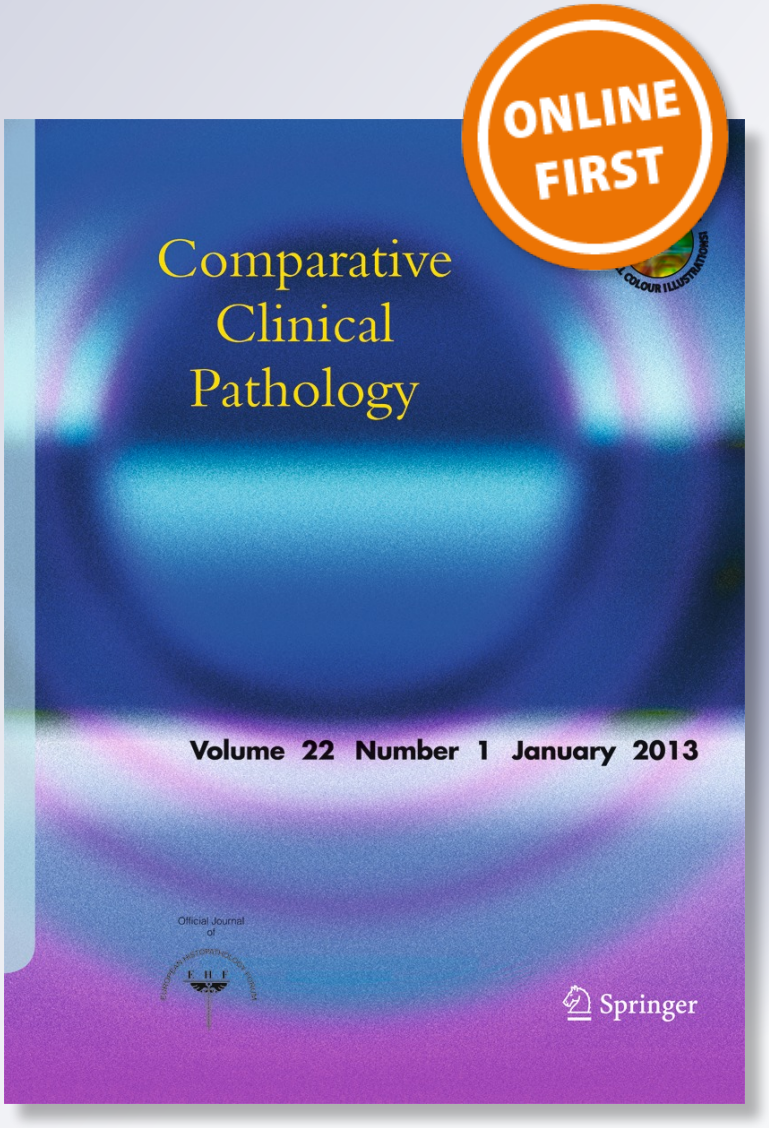
Comparative Clinical Pathology

ISSN 1618-5641

Comp Clin Pathol
DOI 10.1007/s00580-013-1707-7



Springer

Comp Clin Pathol
DOI 10.1007/s00580-013-1707-7

ORIGINAL ARTICLE

An ultrastructural investigation of the blood neutrophils in camel (*Camelus dromedarius*)

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Received: 10 November 2012 / Accepted: 7 February 2013

Abstract Evaluation of camel neutrophil's structure and ultrastructure is essential for fundamental and applied research on innate immunobiology. There is little information on camel neutrophil's ultrastrucure. Healthy camel blood neutrophils were isolated, prepared, and stained for light, scanning, transmission electron microscopy, and organelles' biometry. We observed that (1) the nuclei of healthy camel neutrophils were highly lobulated, predominantly ≥5 lobules/nucleus, with euchromatin and hetrochromatin portions and very low ratio of nucleus to cytoplasm, (2) surface of neutrophils contained many psuedopods, and (3) the cytoplasm contained enormous granules with different sizes and forms and high density at the center, as well as with many different organelles such as abundant mitochondria, rough endoplasmic reticulum, microtubules, phagolysosome, vacuoles, and Golgi apparatus. Biometric analyses of key organelles in neutrophils also shed some new lights on the features of camel neutrophils. This ultrastructural study emphasizes the notion that camel neutrophils are highly equipped with the cytoskeletal machinery for efficient organelle movement, phagocytosis, and microbicidal activities. The ultrastructural features of camel neutrophils observed in this study would be outstandingly comparable to the neutrophils of other ruminants and needs further detailed comparative study.

Keywords Camel · Electronic microscope · Neutrophils · Nuclei · Organelles · Ultrastructure

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Introduction

As the main sources of antimicrobial substances to protect host, neutrophils (PMNs) are the most abundant blood leukocytes in animals with short life-span and little proliferative potential (Burvenich et al. 2003; Mehrzad et al. 2001, 2009, 2011; Mehrzad 2012). Blood neutrophils are formed in bone marrow by hematopoietic pluripotent stem cells through the multi-step process of granulopoiesis, from the colony-forming unit of granulocytes to myeloblasts, promyelocytes, myelocytes, metamyelocytes, band cells, and terminally differentiated neutrophils (Bainton et al. 1971; Borregaard et al. 1990). Precursors of neutrophils undergo substantial morphologic and functional changes during granulocytic maturation (Smits et al. 1996; Van Merris et al. 2002; Burvenich et al. 2003; Mehrzad 2012). The ultrastructure of neutrophils has been exclusively demonstrated in classic studies in cows (Paape et al. 2002; Mehrzad 2012), but rarely have the camel neutrophils' ultrastructure been investigated, except work published by Ali et al. (1989) focusing mainly on functional aspects of neutrophil granules. With ~50 % neutrophils, peripheral blood of healthy adult bovine and camelids contains about 4,500–12,000 leukocytes/μl (Mehrzad et al. 2001, 2009; Nathan 2006; Mehrzad 2012; and plenty more). Almost similar to human, a healthy adult cow and/or camel produces ~10^{11} neutrophils each day each of which survives about 6–8 h in the circulation (Burvenich et al. 2003; Mehrzad 2012). There is little information on immunobiological aspects of this very exceptionally resistant, biomedicinally pivotal and nutritionally promising animal, the camel. Classical study on the ultrastructure, mechanomics, and biophysics of this pivotal circulating innate immune cell, neutrophil, in camel is therefore warranted in biomedical research.

Structurally, neutrophils are delineated by a plasma membrane that has a number of functionally crucial molecules, receptors, and organelles that contribute to efficient diapedesis, phagolysosome formation, and quick removal of

Published online: 21 February 2013

invading pathogens (Ali et al. 1989; Paape et al. 2002; Burvenich et al. 2003; Stevens et al. 2011). Based on the cell size, cytoplasm, organelle, and nuclear morphology during granulopoiesis, in the final (polymorphonuclear) stage of neutrophil development, further condensation of the nuclear chromatin is observed. Among the most remarkable morphological characteristics is multi-lobes connected with filamentous strands are seen as the nucleus. Also, abundance of specific granules causes the cytoplasm to appear faintly pink due to stain with a reagent. The antimicrobial molecules are tightly packed in granules which are released when activated. Initially and based on their affinity for dye, two predominant types of granules are distinguished in neutrophils: azurophil (primary) granules, which are larger, denser, and take up the basic dye azure A and specific (secondary) granules, which are smaller, less dense, and do not take up the azure A (Borregaard and Cowland 1997; Ali et al. 1989). Furthermore, a clear distinction between the two types of granules can be established since identification of myeloperoxidase (MPO), only present in azurophil granules (Bainton et al. 1971; Ali et al. 1989), is simply feasible by a peroxidase staining-based method of electron microscopy (EM). In addition, high-resolution EM also reveals the existence of other granules, vacuoles, and secretory vesicles that appear at the final stage of neutrophil maturation (Ali et al. 1989; Borregaard et al. 1990). Generally, granules begin to form at the stage of neutrophil maturation marked by transition from myeloblast to promyelocyte (Bainton et al. 1971). It is believed that formation of granules is achieved by aggregation of immature transport vesicles that bud off from the trans-Golgi network (Sossin et al. 1990).

Within the cytoplasm, there are plenty of functionally pivotal organelles, e.g., granules, phagolysosome, microtubules, and mitochondria that make the neutrophils professional mobile phagocytes. During differentiation into a mature neutrophils, the nucleus undergoes a series of changes, from large, round, and euchromatic (bright) to band-shaped and less euchromatic to a small, heterochromatic (dark) nucleus with three to five lobes even more than five in ruminants. In the mature neutrophils, prominent Golgi and the rough endoplasmic reticulum (RER) remains active for production and packaging of secondary granules. Granules plus vacuoles are critical for digestion and ingestion of invading pathogens (Ali et al. 1989; Reeves et al. 2002; Mehrzad 2012). How those key organelles appear in camel neutrophils remains deeply unnoticed.

The main purpose of this study was to provide a basic cytology of camel neutrophils by electron microscopy and to bring some new concepts in order to accelerate more fundamental and applied comparative research on morphobiological aspects of neutrophils in camelids. The detailed ultrastrucure of camel neutrophils was described, and a number of biometric analyses with particular focus on the biophysicochemical characteristics of the cytoplasmic granules and vacuoles of camel neutrophils were determined.

Materials and methods

Camel blood neutrophil isolation, preparation, identification, and enumeration

A group of 15 clinically healthy one-humped female camels (*Camelus dromedaries*; age 4–6 years) were used as a source of neutrophils for the experiments. Blood samples were aseptically collected from the external jugular vein into heparinized vacutainer tubes (Becton Dickinson). Total number of circulating PMN was determined with a coulter counter. Differentiation of nucleated blood cells was performed microscopically on whole blood smears (Mehrzad et al. 2001, 2009). Isolation of PMN from peripheral blood was performed by hypotonic lysis of erythrocytes (Carlson and Kaneko 1973; Mehrzad et al. 2001; 2009; 2011). The isolation procedure of blood PMN yielded >95 % neutrophils with a viability of >98 %, evaluated microscopically with trypan blue exclusion method. PMN suspensions were adjusted to ~10×10^6 viable PMN/ml with Dulbecco's phosphate-buffered saline (DPBS; Sigma-Aldrich, Deisenhofen, Germany).

Microscopic analysis of camel neutrophils

Thirty milliliters of venous blood samples were obtained heparinized from each camel. Neutrophils were isolated according to Mehrzad et al. (2001; 2011). Isolated neutrophils were seeded for the indicated time and fixed before processing for light microscopy (LM), scanning electron microscopy (SEM), and transmission electron microscopy (TEM) for detailed structural and ultrastructural characterization. Before isolation, a drop of the heparinized blood was placed on a glass slide, and a thin blood film was made. The whole blood smear was fixed with 100 % methanol and stained with Eosin–Giemsa accordingly (Mehrzad et al. 2001, 2011). The isolated blood neutrophil film was also fixed with 70 % acetone and stained with Eosin–Giemsa. Further, randomly 100 isolated neutrophils per sample were evaluated for nuclear lobation according to Mehrzad et al. (2001; 2011).

Scanning electron microscopy

Immediately after PMN isolation, the pure neutrophils (2×10^7 neutrophils) for SEM and TEM observation were first pre-fixed in 2.5 % glutaraldehyde buffer (pH=7.2) for 24 h. The pre-fixed neutrophils were then post-fixed with 1 % osmium tetroxide solution. After dehydration using

increasing graded ethanol series, the neutrophils were dried at room temperature and coated with platinum and gold with sputter coating (SC7620), as described in other cells in camel (Raji and Naserpour 2007); the mounted neutrophils specimens were then observed by scanning electron microscope (LEO 1450VP) at accelerating voltages of 20.0 kV. Each stage of centrifugation was (400×*g*, 5 min, 4 °C).

Transmission electron microscopy

The fixed pellets of isolated pure neutrophils (2×10^7 neutrophils) for TEM preparation were gently washed in PBS (400×*g*, 5 min, 4 °C). They were then dehydrated in increasing graded ethanol and immersed in Araldite 502 resin kit (Taab Laboratories equipments Ltd, England). Selected fragments of the resin blocks were sectioned with ultra microtome and double-stained with uranyl acetate and lead citrate (Dykstra 1992; Raji and Naserpour 2007). Sections were observed by transmission electron microscope (LEO 912AB). Each stage of centrifugation was 400×*g*, 5 min, and at 4 °C.

Biometric and statistical analyses of neutrophils' organelles

To further analyze ultrastructure of neutrophils and to get more insight into the camel neutrophils' organelles, biometric analyses of the neutrophils, granules, and mitochondria were done using scale bars of microscopic images. The mean±SEM number of nuclear lobes in neutrophils with LM was assessed in 15 different isolated neutrophils' slides, and the average of 100 neutrophils per slide were counted and analyzed. Furthermore, in 15 different cytoplasms of different neutrophils, the average size and number of primary and secondary granules per square namometer of the cytoplasm as well as the average number of the small and large vacuoles and/or lysosomes and average diameters of mitochondria were analyzed in the cytoplasm of the neutrophils with TEM. The magnifications of LM and scale bars of the SEM and TEM were inserted on the images for further biometric information to readers. The biometrics results were presented as mean±SEM, and statistical analyses were performed using Student's *t* test. A value of $P<0.05$ was considered statistically significant.

Results

LM analyses of camel neutrophils

The slides of isolated pure blood neutrophils stained with hematoxylin–eosin showed the presence of highly multilobulated and/or segmented, dark-bluish stained nuclei with very low ratio of nucleus to cytosol in mature neutrophils (Fig. 1a–c). Biometric analyses of neutrophils in LM images revealed varying nuclear lobation, 3–4, 4–5, and more than 5 lobes (Fig. 1d). A significant percentage of camel neutrophils had more than 5 nuclear lobes (Fig. 1d; $p<0.001$).

SEM analyses of camel neutrophils

SEM images of camel blood neutrophils revealed that their neutrophils had very high convulsed cell membrane that forms protruding pseudopods (PsPs) with pronounced ruffling, and the surface of the resting neutrophils was completely covered by PsPs (Fig. 2). The size of the camel PMN in SEM, intensity, distribution, and length of PsPs on the surface in SEM can also be seen (Fig. 1).

TEM analyses of camel neutrophils

TEM images of camel neutrophils showed the presence of numerous granules and vacuoles in the cytoplasm of the neutrophils; the images further confirmed a remarkable change in nuclear, cytosolic, and plasma membrane morphology (Fig. 3). Within the cytoplasm of camel neutrophils, there were plenty of functionally pivotal granules. There were two predominantly distinct granule populations (Fig. 3), azurophilic (primary) granules, which were large and appeared as electron-dense granules, and specific (secondary) granules, which were small and appeared as light staining granules on TEM images (Fig. 3). Apart from the primary and secondary granules in cytoplasm of camel neutrophils, they also contained third novel granules that were larger, denser, and more numerous than the other two granules (Fig. 3). Two another different kind of granules in camel neutrophils were observed: one was inside dense and outside brighter and the other was very *surprisingly* lamellar granule (Fig. 3). Also, the lysosomal granules, different number, size, and shape (oval, lamellar, circular, but mainly circular) of the granules were also predominant (Fig. 3). Multivesicular vacuoles/bodies (Fig. 3), attachment of the lysosomal granules to vacuoles, which is called junction (asterisks in Fig. 3 middle), were other observations in the TEM images. Further detailed TEM images of camel neutrophils showed clear microtubules (MTs) and rough endoplasmic reticulum (RER), Golgi apparatus, perinuclear space (PnS), nuclear pore (NP), mitochondria, and other fine organelles. ERs in camel neutrophils were mainly RER, and they were pointed with ribosomes attached on them (Fig. 3). The MTs in camel neutrophils were highly dense (Fig. 3).

Biometric analyses of camel neutrophils

Biometric results from TEM images of camel PMNs revealed: (1) number of large and small granules/square

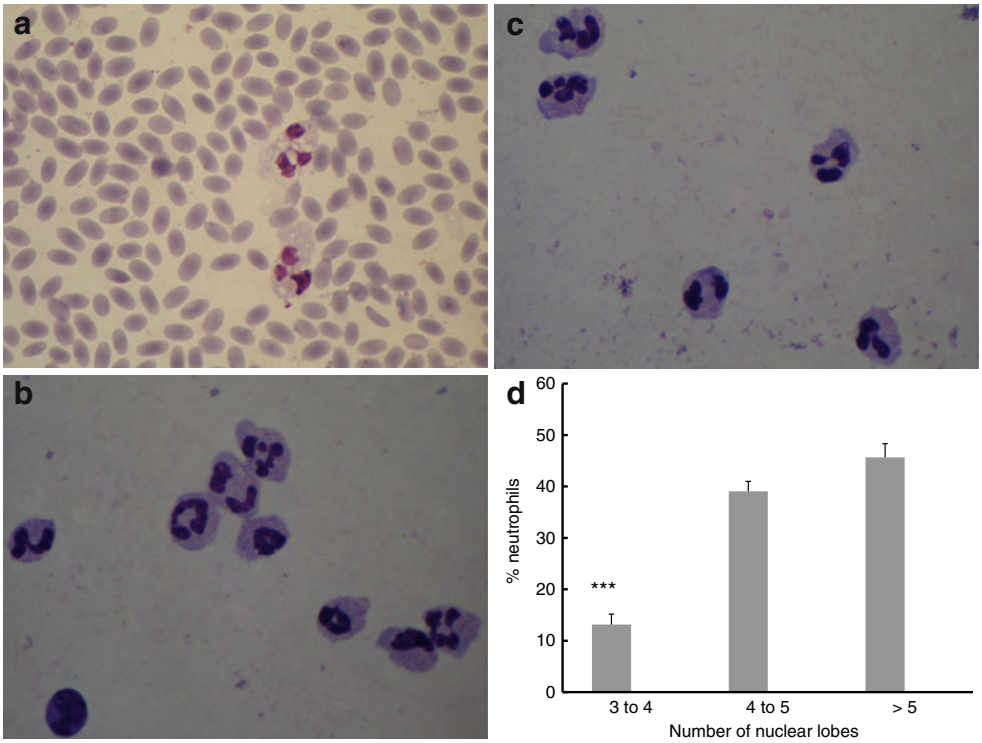

Fig. 1 Representative images of light microscopy (LM) assessment of healthy camel whole blood (**a**) and isolated (**b**, **c**) neutrophils (PMN), in which the presence of mature neutrophils is visible. The ratio of nucleus to cytosole in mature neutrophils is very low (×1,000). The histogram (**d**) shows average number of lobules (the mean±SEM of 15 different isolated neutrophils' slide, and each slides 100 neutrophils were evaluated) with LM; the number of nuclear lobes is remarkably enormous with predominant form of >5 lobes-nuclei. Significant differences are marked with *asterisks* (***$P<0.001$)

nanometer in camel neutrophils were 17.6±1.3 and 27.4±1.6/nm^2, respectively, (2) the average size of primary and secondary granules were 587.4±55.1 and 638±70.8 nm. Furthermore, apart from the status and situations of granules to the vacuoles, the sizes of small and large vacuoles were 118±6.1 and 357±21.8 nm, respectively. In 2,500 nm^2 of camel neutrophils' cytoplasm, ~46 granules plus several vacuoles were observed (Figs. 3 and 4). Biometric analyses of mitochondria showed their different sizes with large and small diameters of 580.7±22.9 and 263.3±19.6 nm, respectively (Figs. 3 and 4); it also revealed many more fine organelles in the cytosol of neutrophils (see Fig. 3).

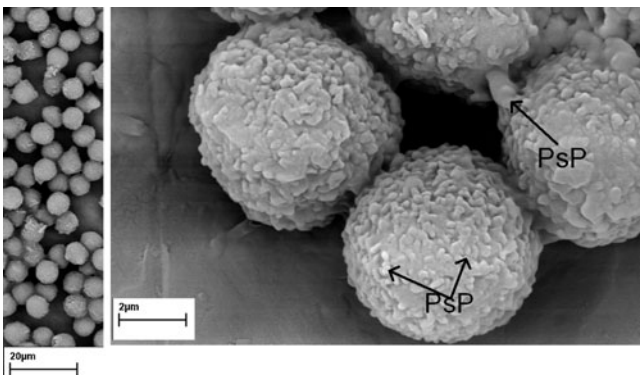

Fig. 2 Scanning electron micrographs (SEM) with *scale bars* of neutrophils isolated from blood of healthy camel. Protruding pseudopods (PsPs) needed for phagocytosis in neutrophils, which is distinguishable in intact/ resting neutrophils; enormous convulsed cell membrane and evenly distribution of PsP on the surface of the neutrophils are visible

Discussion

The information and topic about camel neutrophils is novel and has been poorly documented before; this is more special for camel neutrophils' fine structure, except one interesting work done very long time before (Ali et al. 1989). This novel study is directed at the population of neutrophilic granulocytes in the peripheral circulating blood of the one-humped camel. Structural and ultrastructural analyses of camel blood neutrophils' nuclei and cytosols showed morphological changes in the nucleus and cytoplasm, and the cytoplasm was large and contained numerous lysosomes. There were dark-bluish stained nuclei and plenty of PsPs and membrane receptors. The multi-lobulated nucleus is important because it allows the PMN to line up its nuclear lobes in a thin line, permitting rapid migration between endothelial cells. A significant percentage of camel neutrophils had more than five nuclear lobes; this is an indication of maturity and suggests that the bone marrow is releasing neutrophils in blood circulation only when the nuclei are completely segmented. Such bone marrow response in healthy camel is usually normal for replenishing of lost neutrophils. According to our experience in bovine model, grouping of healthy camel blood netrophils in this study, i.e., number of nuclear lobes per neutrophil, was done for the first time to evaluate as a criterion for neutrophil maturity and

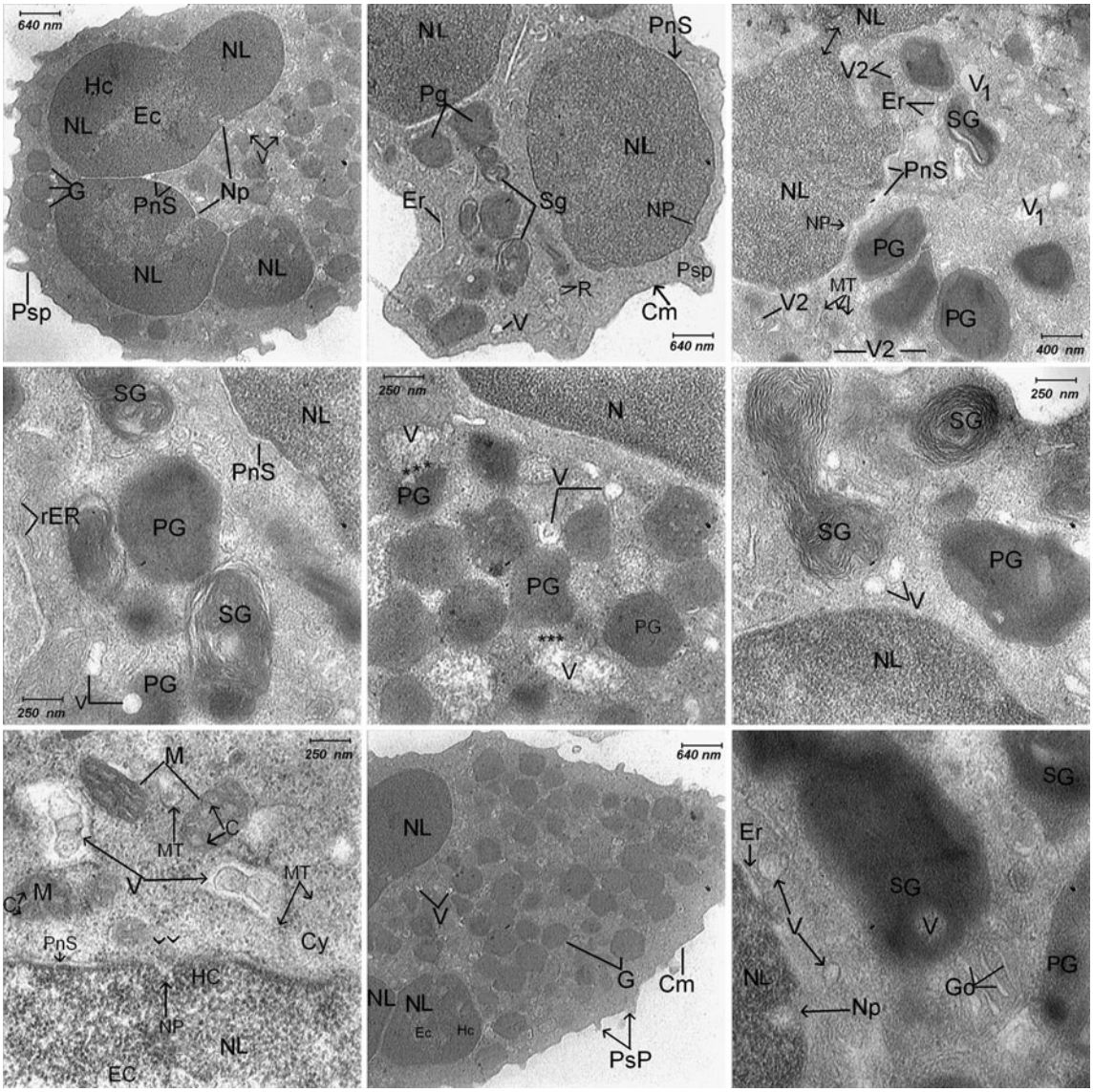

Fig. 3 Transmission electron micrographs (TEM) with scale bars of camel blood neutrophils. Many different forms of predominant granules (*G*) and different sizes of vacuoles (*V*), perinuclear space (*PnS*), highly folded cell membrane (*Cm*), pseudopods (*PsP*), mitochondria (*M*), cristae (*C*) of M, cytosole (*Cy*), highly dense microtubules (*MT*), endoplasmic reticulum (*Er*), rough endoplasmic reticulum (*rER*), ribosome (*R*), Golgi apparatus (*Go*), primary granule (*PG*), secondary granule (*SG*), fusion of vacuoles with PG and formation of SG and fusion of two SGs and PG with Vs (*asterisks* in lower-middle image) and formation of larger granules (PGs/ SGs plus vacuoles). The nuclei are shown with nuclear pore (*NP*) and nuclear lobes (*NL*) contained both with dark heterchromatic (*HC*), bright euchromatic (*EC*), and connection/bridge between two NL (*double arrow* in *upper right image*); the *arrowheads* (*left bottom*) show the movement of biomolecules

clinical implication; this would also encourage researchers to conduct deep research in the area of cellular and molecular aspects of hematology, pathology, and oncology. The nuclei in camel neutrophils were always clearly segmented with having both heterochromatic and euchromatic regions; the euchromatin is attached from the NP. When high proportion of the nucleus is euchromatic in neutrophils, it can be likely that the cell is in its mature stage (Cross and Mercer 1993; Johnson et al. 1999).

The PsPs and/or microvilli on the surface of the resting neutrophils, observed in the SEM images of camel blood neutrophils, are pivotal for their professional trapping of invading microbes and phagocytosis. The size of the PMN in SEM, number and length of PsPs in relation to cell surface in SEM and whether the PsPs are evenly distributed on the surface are other immunobiological points to be taken into consideration. Number and length of cross section is also important to study in SEM, and "whether they are branch or not" is another new kind of research topic in cytoskeletal ultrastructure.

Question may arise concerning the adverse effects of centrifugation before SEM and TEM on neutrophils' integrity

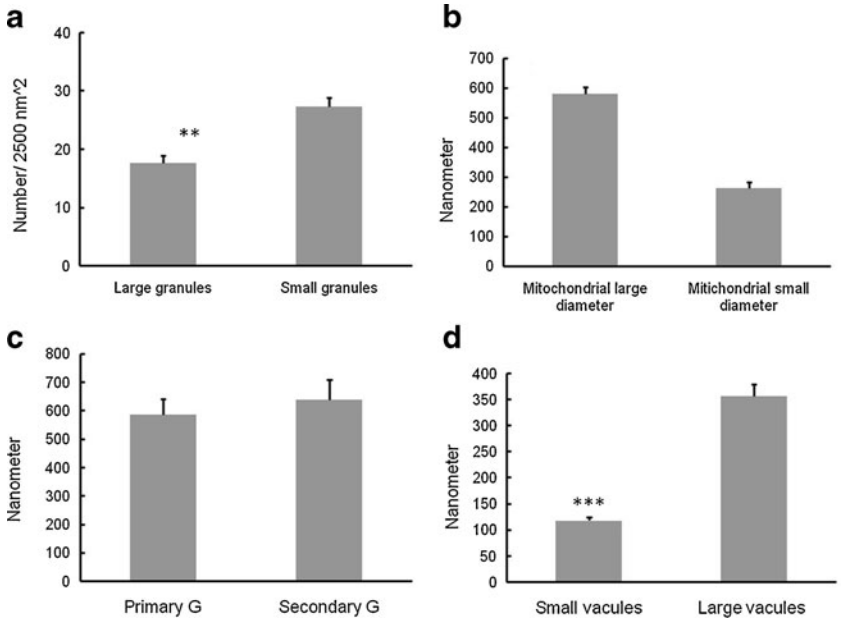

Fig. 4 Biometric analyses of some key organelles in camel neutrophils using scale bars of TEM images. **a** The average number of large and small granules/square nanometer (mean±SEM of 15 different slides in different neutrophils). **b** The average diameters of mitochondria (mean±SEM of 15 different mitochondria in different neutrophils). **c** The average diameters of primary granules (PGs) and secondary granules (SGs) (mean±SEM of 15 different PGs, SGs in different neutrophils) and **d** the average diameters of the large and small vacuoles (mean±SEM of 15 different large and small Vs in different neutrophils). Significant differences are marked with *asterisks* (**$P<0.01$; ***$P<0.001$)

(nuclei, cytoplasm, and organelles). Technically, there would be little problem for those concerns, because firstly, the isolated neutrophils for EM evaluation in our study were immediately fixed and then used for EM procedure. Secondly, we always used very low ×*g* and minimal time for centrifugation steps. Indeed, there is no way other than centrifugation to make neutrophils pellets.

TEM images of camel neurophils confirmed the presence of plenty functionally pivotal granules and vacuoles and a remarkable change in nuclear, cytosolic, and plasma membrane morphology, making them professional mobile phagocytes. In immature neutrophils, primary granules are more abundant than secondary granules (Ali et al. 1989; Borregaard et al. 2007). The cytoplasmic granules are the exclusive store of many antimicrobial substances like lactoferrin, highly cationic proteins, and powerful oxygen-dependent-and-independent microbicidal compounds specially MPO–H_2O_2–halide system (Ali et al. 1989; Gennaro et al. 1983; Heyneman et al. 1990; Mehrzad et al. 2001; 2009; 2010). The most important antibacterial mechanism derived from azurophilic granules is the MPO–H_2O_2 system. The functionality of these antimicrobial granules might be altered during physiological and pathological conditions of camel and should therefore be further investigated.

To further pinpoint the characteristics of granules, substantially high number with high density of the granules, surprisingly novel lamellar granule, in camel neutrophils was observed; to the best of our knowledge, lamellar granules have never been observed in bovine, ovine, canine, and human neutrophils, though they are normally typical for camel eosinophils (Johnson et al. 1999). Observation of lamellar granules in camel neutrophils is very interesting and first ever reported. Information from TEM images in resting PMNs is essential for fundamental and applied research on innate immunobiology.

Biometric results further emphasized the point that camel neutrophils are armed with an array of highly microbicidal weapons in their cytoplasm. As it can simply and precisely be done with TEM analyses, mechanistically, the average size and number of the granules and vacuoles are very important to report. Large granules (granules plus vacuoles) are critical for digestion and ingestion of the engulfed pathogens after attachment of the vacuoles to lysosomal granules formation of phagolysosome, in which interactions between PMN and microbe occur. Although the ultrastructure of neutrophils may be now more scientifically understood, we nonetheless know little on how camelid neutrophil's phagosome and lysosome behave when encounter to an invading pathogen, where extremely large amounts of oxidants and granule constituents are released (Ali et al. 1989; Mayer et al. 1989; Hampton et al. 1998; Mehrzad et al. 2001; Reeves et al. 2002; Mehrzad et al. 2009; 2010; Paape et al. 2002; Burvenich et al. 2003; Brinkmann et al. 2004). Observation of different and plenty granules in the cytoplasm of camel neutrophils with very large granules and

vacuoles inside with highly dense and condensed center and whitish surrounding area is not surprising, and similar pattern can be seen in human, monkey, and bovine neutrophils (Paape et al. 2002; Burvenich et al. 2003; Mehrzad et al. 2009, 2010).

Existence of MTs, RER, Golgi apparatus, perinuclear space (PnS), and NP in camel neutrophils are very important for protein and granule synthesis in neutrophils, not only for protection of structure and function of PMN, but also for transportation of biomolecules in the cytoplasm; also, the dynamic of those organelles would be as an appropriate road for cytoskeletal machinery, movement and dynamic of granules, trapping, vacuole, and phagosome formation and fusion of phagosome to lysosome and cytodynamics of organelles. RERs with ribosomes in camel neutrophils observed in TEM are important for protein synthesis and more activation. The highly dense MTs in camel neutrophls is a sign of highly functional PMN cytoplasm and inside–outside cytoplasm movement not only for protein synthesis but also for efficient function of PMN like trapping and mechanomics as well. Structurally and functionally, MTs are different from ER; ultrastructurally, inside of MTs is filled but inside of ER is empty (Cross and Mercer 1993). Medicinally, targeting the MTs is important for challenging cancer, because the MTs are pivotal for mitosis, dock formation, and cell division, thus very applicable in cancer research; this is more special for the terminally differentiated cells, PMNs. Also, the approach of structure–activity relationship model can be helpful in the area of drug discovery to see the organelle toxicity and injuries as well as the populations of organelles those are warranted for fundamental comparative studies.

In short, both high populated cytoplasmic granules and high euchromatic and multi-lobulated feature of nucleus (~5 lobes/nucleus) of neutrophils with extremely low ratio of nuclei to cytoplasm clearly revealed the notion that circulating resting mature neutrophils in healthy camels are highly armed with microbicidal weaponries in their cytoplasm. Though the ratio of nucleus to cytoplasm was addressed in accordance to our precise LM observation, but quantitatively how numerically low this ratio is, would be an open area of research. Biometry data of camel neutrophils with EM techniques reveal some novel features of camel neutrophils, which can be applicable in camel innate immunobiology. Further detailed studies on cellular and molecular aspects of camel neutrophils are in progress in our laboratories to explain these findings.

Acknowledgment This work was supported by the grant (number 2902, 20/11/2010) Ferdowsi University of Mashhad, Iran. The authors thank Mrs Pesyan and Mrs Sadegian for their technical assistance for SEM and TEM imaging analyses.

References

Ali AM, Abdo MS, Hassanain MM, Prentis PF (1989) Fine structure of the camel neutrophilic granulocytes with special references to its function. Z Mikrosk Anat Forsch 103:985–992

Bainton DF, Ullyot JL, Farquhar MG (1971) The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. J Exp Med 134:907–934

Borregaard N, Cowland JB (1997) Granules of the human neutrophilic polymorphonuclear leukocyte. Blood 89:3503–3521

Borregaard NL, Christensen OW, Bejerrum OW, Birgens HS, Clemmensen I (1990) Identification of a highly mobilizable subset of human neutrophil intracellular vesicles that contains tetranectin and latent alkaline phosphatase. J Clin Invest 85:408–416

Borregaard N, Sørensen OE, Theilgaard-Mönch K (2007) Neutrophil granules: a library of innate immunity proteins. Trends Immunol 28:340–345

Brinkmann VU, Reichard C, Goosmann B, Fauler Y, Uhlemann DS, Weiss YW, Zychlinsky A (2004) Neutrophil extracellular traps kill bacteria. Science 303:1532–1535

Burvenich C, Van Merris V, Mehrzad J, Diez-Fraile A, Duchateau L (2003) Severity of *E. coli* mastitis is mainly determined by cow factors. Vet Res 34:521–562

Carlson GP, Kaneko JJ (1973) Isolation of leukocytes from bovine peripheral blood. Proc Soc Exp Biol Med 142:853–856

Cross PC, Mercer KL (1993) Cell and Ultrastructure. WH Freeman and company, New York, pp 304–316

Dykstra MJ (1992) Theory, techniques and troubleshooting. In Biological electron microscopy. New York: Plenum press 175–180

Gennaro RB, Dewald B, Horisberger U, Gubler HU, Baggiolini MA (1983) A novel type of cytoplasmic granule in bovine neutrophils. J Cell Biol 96:1651–1661

Hampton MB, Kettle AJ, Winterbourn CC (1998) Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. Blood 92:3007–3017

Heyneman R, Burvenich C, Vercauteren R (1990) Interaction between the respiratory burst activity of neutrophil leukocytes and experimentally induced *E. coli* mastitis in cows. J Dairy Sci 73:985–994

Johnson EH, Muirhead DE, al-Busaidy R, Musa BE (1999) The ultrastructural morphology of the camel eosinophil. Vet J 157:79–84

Mayer SJ, Keen PM, Craven N, Bourn FJ (1989) Regulation of phagolysosome pH in bovine and human neutrophils: the role of NADPH oxidase activity and an Na+/H+antiporter. J Leukoc Biol 45:239–248

Mehrzad J (2012) Molecular aspects of neutrophils as pivotal circulating cellular innate immune systems to protect mammary gland from pathogens. In: Kanwar J.R. (Ed.), Recent Advances in Immunology to Target Cancer, Inflammation and Infections. ISBN: 978-953-51-0592-3. InTech Publishing Inc. pp. 385–422

Mehrzad J, Dosogne H, Meyer E, Heyneman R, Burvenich C (2001) Respiratory burst activity of blood and milk neutrophils in dairy cows during different stages of lactation. J Dairy Res 68:399–415

Mehrzad J, Duchateau L, Burvenich C (2009) Phagocytic and bactericidal activity of blood and milk-resident neutrophils against *Staphylococcus aureus* in primiparous and multiparous cows during early lactation. Vet Microbiol 134:106–112

Mehrzad J, Burvenich C, Paape M (2010) Role of neutrophils in protection of udder from infection in high yielding dairy cows. Iran J Vet Res 31:102–118

Mehrzad J, Klein G, Kamphues J, Wolf P, Grabowski N, Schuberth HJ (2011) In vitro effects of very low levels of aflatoxin B1

on free radicals production and bactericidal activity of bovine blood neutrophils. Vet Immunol Immunopathol 141:16–25

Nathan C (2006) Neutrophils and immunity: challenges and opportunities. Nat Rev Immunol 6:173–182

Paape MJ, Mehrzad J, Zhao X, Detileux J, Burvenich C (2002) Defense of the bovine mammary gland by polymorphonuclear neutrophil leukocytes. J Mammary Gland Biol Neoplasia 7:109–121

Raji AR, Naserpour M (2007) Light and electron microscopic studies of the trachea in the one-humped camel (*Camelus dromedarius*). Anat Histol Embryol 36:10–13

Reeves EP, Lu H, Jacobs HL, Messina CGM, Bolsover S, Gabella G, Potma EO, Warley A, Roes J, Segal AW (2002) Killing activity of neutrophils is mediated through activation of proteases by K+ flux. Nature (London) 416:291–297

Smits E, Cifrian E, Guidry AJ, Rainard P, Burvenich C, Paape MJ (1996) Cell culture system for studying bovine neutrophil diapedesis. J Dairy Sci 79:1353–1360

Sossin WS, Fisher JM, Scheller RH (1990) Sorting within the regulated secretory pathway occurs in the trans-Golgi network. J Cell Biol 110:1–12

Stevens MG, Van Poucke M, Peelman LJ, Rainard P, De Spiegeleer B, Rogiers C, Van de Walle GR, Duchateau L, Burvenich C (2011) Anaphylatoxin C5a-induced tolllike receptor 4 signaling in bovine neutrophils. J Dairy Sci 94:152–164

Van Merris V, Meyer E, Burvenich C (2002) Functional maturation during bovine granulopoiesis. J Dairy Sci 85:2859–2868