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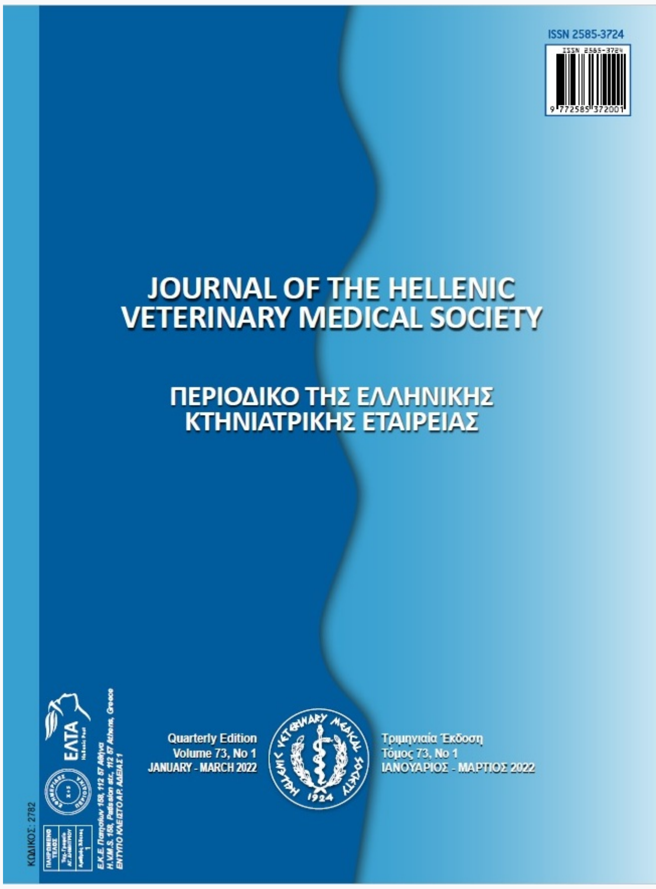

Relationships between glucose-6-phosphate dehydrogenase, glutathione peroxidase, reduced nicotinamide adenine dinucleotide phosphate, total protein, malondialdehyde, total glutathione and vitamin C parameters in goat milk cells

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Research article
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Relationships between glucose-6-phosphate dehydrogenase, glutathione peroxidase, reduced nicotinamide adenine dinucleotide phosphate, total protein, malondialdehyde, total glutathione and vitamin C parameters in goat milkcells

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ABSTRACT: In this study, to reveal the antioxidant potential of goat milk cells, the activities of glucose-6-phosphate dehydrogenase (G6PD), glutathione peroxidase (GPx) and the levels of reduced nicotinamide adenine dinucleotide phosphate (NADPH), total glutathione (tGSH), malondialdehyde (MDA), vitamin C (Vit C) and total protein (TP) in goat milk cells were determined and correlations between these parameters were evaluated. Milk samples were collected from 19 clinically healthy goats from a private goat farm. Briefly, milk cells were collected from milk by centrifugation and than they were sonicated. Supernatant G6PD, GPx activities and NADPH, tGSH, MDA, Vit C and total protein levels were determined by spectrophotometric methods. As regards correlations: milk cell MDA levels were positively correlated with milk cell tGSH ($r=0, 725$, $p<0, 01$), milk cell Vit C ($r=0.622$, $P<0.01$) and milk cell NADPH ($r=0.763$, $P<0.01$) levels. There was a positive correlation between milk cell GPx activity and milk cell NADPH levels ($r=0.659$, $P<0.01$). Milk cell tGSH levels were positively correlated with milk cell Vit C ($r=0.615$, $P<0.05$) and milk cell NADPH ($r=0.846$, $P<0.01$) levels. Milk cell NADPH levels was positively correlated with milk cell Vit C levels ($r=0.791$, $P<0.01$). As a conclusion, the antioxidant potential of goat milk cells were evaluated and discusses.

Keywords: Goat milk cell, GPx, G6PD, NADPH, tGSH

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INTRODUCTION

Though goat milk meets about 2% of the world's total annual milk supply, its contribution to human nutrition and economic well-being is extremely important in many parts of the world, particularly in the Mediterranean countries and the Middle East (Park 1994a, Park 1994b, Park and Haenlein 2007). Aleppo (Damascus) (Barıtçı and Adıgüzel, 2017) and Kilis goats (Keskin and Tüney, 2015) are two breeds in the Mediterranean and Southeastern Anatolia regions. Aleppo goat is a native breed of Syria and other Near East countries. It has been improved for over 40 years through genetic selection for milk and meat production and it has been given a high priority by the Food and Agriculture Organization (Mavrogenis et al., 2006). Kilis goat possesses crutial advantages in terms of high milk yield and reproductive traits with good toleration for warm environments and drought under extensive or semiintensive management conditions in arid and hot climates (Keskin et al., 2017). There is also an increasing demand for goat milk and its products in developed countries because people demand healthy food and also people who are allergic or have intolerance to cow milk turn towards goat milk and its products (Park, 1990; Park, 1994a; Park, 1994b; Chandan et al., 1992; Tziboula-Clarke, 2003). Although the general composition of goat milk is similar to cow milk, it is differentiated from cow milk with some features; goat milk contains on average 3.8% fat, 3.5% protein, 4.1% lactose and 0.8% ash; that is, more fat, protein and ash and less lactose than cow milk (Haenlein and Caccese, 1984). As goat milk contains a higher percentage of small-diameter fat globules than cow milk, and because of the qualitative and quantitative differences in milk proteins, especially in α-1 casein, it is easier to digest goat milk. Goat milk is rich in short and medium- chain mono and polyunsaturated fatty acids (caproic, caprylic, capric) (Djordjevic et al., 2019).

Healthy goat's milk has a higher number of somatic cells (270-2000×10^3 cells/ml, Souza et al 2012) than cow's milkdoes (<200 ±10^3 cells/ml, Dang et al., 2008).The content of the somatic cells also vary between goat and cow's milk. While 40% to 87% of somatic cells in goat's milk are formed by neutrophils from PMNs (Souza et al., 2012; Shah et al., 2017), the rate of neutrophils in cow milk cells varies between 5% and 20% (Sarıkaya, 2006; Souza et al., 2012) and macrophages are the predominant cell type in healthy cow milk (Osstensson 1993).

Goats are reported to be more resistant to infectious conditions such as mastitis, than other species, presumebly as they have a higher number of neutrophils (Tian et al., 2005; Souza et al., 2012). Besides, antioxidants such as reduced nicotinamide adenine dinucleotide phosphate (NADPH), reduced glutathione (rGSH), glucose-6-dehydrogenase (G6PD), glutathione peroxidase (GPx) and vitamin C (Vit C), have important roles in a cell's defence mechanism (Ralat et al., 2006; Stincone et al., 2015; Carole et al., 2007; Ighodaro and Akinloye, 2018).In a previous study (Akalın et al., 2019), we revealed the antioxidant potential of Holstein-Friesian cow milk cells by determining the levels of NADPH, GSH, GPx and G6PD and the relation of these parameters with mastitis, for the first time.Since it is thought that the antioxidant potential of the cells will change as the somatic cell count and somatic cell component change, we thought that revealing the antioxidant potential of goat milk cells will offer an insight to understand the goat milk cell defence mechanism.

Therefore, in order to investigate the antioxidant potential of goat milk cells, in the current study, it wasaimed to determine G6PD and GPx activities and NADPH, tGSH, MDA, Vit C and total proteinlevels in the cells of milks obtained from clinically healthy goats' udders and to investigate therelationships between the related parameters.

MATERIALS AND METHODS

Milk samples were collected from 19 healthy goats (10 Aleppo, 9 Kilis goats) aged between 1-4 and were fed in the same care and nutritional conditions in a private goat farm in April, inHatay region (36° 11' 56" North, 36° 9' 38" East). Milk was obtained while the owner was milking the goats returned from thepasture in the morning. Milk was taken from the right lobe of clinically healthy udders. During milking, the first 2-3 squeezes of milk were thrown away after the teat was wiped with 70%alcohol cotton, and milk samples were tested by California Mastitis Test (CMT) and CMT negative samples were included in the study. Collected milk samples were brought to the biochemistry laboratory in the cold chain and stored at -20°C until the treatment. Milk samples (14 ml each) were thawed and centrifuged at 600×g for 10 min at 4°C. After centrifugation, the supernatant was eliminated by removing the upper layer of fat with a cotton pad; the remaining cell pellet was washed twice with cold phosphate-buffered saline (PBS) and centrifuged at600×g for 10 min at +4°C. Finally, the supernatant was removed, and the remaining pellet wascompleted to 2 ml with PBS and sonicated (Bandelin Sonopuls HD 2070, Germany) (Akalın etal., 2016) for 5 repetitions of 10 sec each, with a 30 sec cooling period (on ice) between each repetition. By this process, milk cells in 14 ml of milk was concentrated in 2 ml PBS. After sonication, the homogenates were centrifuged at 13, 000×g for 15 min at +4°C. Milk cell supernatant was used for the further analysis.

Determination of G6PD activity

Milk cell G6PD activity was determined by using the method developed by Beutler (1971) and calculated by the spectrophotometric measurement of the absorbance difference in optical density caused by the conversion of NADP+ to NADPH at 340 nm (UV 2100 UV-VIS Recording Spectrophotometer Shimadzu, Japan). Results are presented as IU/g protein.

Determination of GPx activity

Determination of GPx activity in milk cells was done according to the method described by Beutler (1975). According to this method, GPx catalyzes the conversion of rGSH to oxidizedglutathione (GSSG) in the presence of hydrogen peroxide (H_2O_2). Oxidized glutathione, formed by GPx in anenvironment where H_2O_2 is present, is converted back to GSH with the help of glutathionereductase and NADPH. The activity was calculated by the spectrophotometric measurement of the absorbance difference in optical density caused by the conversion of NADPH to NADP+ at340 nm. Results are presented as IU/mg protein.

Determination of NADPH Levels

NADPH levels in cell supernatants were determined spectrophotometrically using a commercial kit (Sigma MAK038). The reaction principle is based on the spectrophotometric (with ELISA reader, BioTek Instruments, µQuant, U.S.A) analysis of the reduction of formazan dye by NADPH at 565 nm, which is synthesized enzymatically in the pentose phosphate pathway. Results are presented as nmol/mg protein.

Determination of tGSH Levels

Total glutathione (GSSG+rGSH) levels in cell supernatants and milk serum were calculated using a commercial kit (Sigma CS0260). It is a kinetic method based on the principle of thereduction of 5.5'-dithiobis (2-nitrobenzoic) acid to trinitrobenzoat (TNB) by glutathione. When oxidized glutathione is regenerated by glutathione reductase and NADPH, TNB absorbance at412 nm can be measured by spectrophotometry. Results are presented as nmol/mg protein.

Determination of MDA Levels

Tissue MDA levels were determined spectrophotometrically according to the method proposed by Ohkawa et al. (1979). Principle: Tissue MDA determination; it is based on spectrophotometric measurement at 532 nm of the pink complex formed by MDA with TBA, which is the secondary product of lipid peroxidation, as a result of incubation of tissue homogenate in a boiling water bath for one hour under aerobic conditions and at pH:3.5. Results are presented as nmol/mg protein.

Determination of Vit C Levels

Milk vitamin C levels were calculated according to the manual spectrophotometric method of Haag (1985). Principle: Ascorbic acid (Vitamin C) is converted to dehydro ascorbic acid with mild oxidizing agents, dehydro ascorbic acid slowly converts to diketogulonic acid in mild acid solutions. Dehydro-ascorbic acid and diketogulonic acid react with 2.4-dinitrophenylhydrazine (DNPH) to form bis 2.4-dinitrophenylhydrazone. The results are given as mg/milk cells of 1 ml milk.

Determination of Total Protein Levels

Total protein levels in milk cell supernatants were determined by the Bradford (1976) method (Coomassie Brilliant Blue G, Sigma 27815-100 G). Protein concentration was determined spectrophotometrically by determining the absorbance at 595 nm. Bovine serum albumin (Merck 112018) was used as a standard. The results are given as mg/milk cells of 1 ml milk.

Statistics

The values obtained were evaluated by SPSS 22.0 program and descriptives (Mean ± Standard Error) were evaluated. Pearson correlation was performed for correlation analysis and $P<0.05$ indicated as statistically significant.

RESULTS

As shown in Table 1, goat milk cell G6PD and GPx activities were determined to be 1.81±0.48IU/g protein and 0.38±0.02 IU/mg protein, respectively. NADPH levels were 1.18±0.23 nmol/mg protein, tGSH levels were 101.73±25.50 nmol/mg protein, MDA levels were 1.43±0.35 nmol/mg protein, Vit C levels were 0.45±0.09 µg/milk cell of 1 ml milk and TP levels were 0.09±0.001 mg/milk cell of 1 ml milk.

Table 1. Some Biochemical Parameters in Goat Milk Cells

	Mean	SE	n
G6PD (IU/g protein)	1.81	0.48	19
GPx (IU/mg protein)	0.38	0.02	19
NADPH (nmol/mg protein)	1.18	0.23	17
tGSH (nmol/mg protein)	101.73	25.50	14
MDA (nmol/mg protein)	1.43	0.35	19
Vit C (µg/ milk cell of 1 ml milk)	0.45	0.09	19
TP (mg/ milk cell of 1 ml milk)	0.09	0.001	19

G6PD:Glucose-6-phosphate dehydrogenase, GPx: Glutathione peroxidase, NADPH:Reduced nicotinamideadenin dinucleotide, tGSH: Total Glutathione, VitC: Vitamin C, TP: Total Protein

Table 2. Correlations between the parameters (Spearman's correlation test)

	TP mg/ml	GPx U/ml	tGSH nmol/ml	Vit C µg/ml	NADPH pmol/ml	G6PD U/ml
MDA nmol/ml	0,454	0,408	0,725**	0,622**	0,763**	0,226
TP mg/ml		0,392	-0,150	0,063	0,377	-0,228
GPx U/ml			0,412	0,426	0,659**	-0,344
tGSH nmol/ml				0,615*	0,846**	0,266
Vit C µg/ml					0,791**	0,065
NADPH pmol/ml						-0,032

G6PD: Glucose-6-phosphate dehydrogenase, GPx: Glutathione peroxidase, NADPH: Reduced nicotinamideadenin dinucleotide, tGSH: Total Glutathione, VitC: Vitamin C, TP: Total Protein *P<0.05,**P<0.01.

As shown in Table 2, milk cell MDA levels were positively correlated with milk cell tGSH (r=0, 725, p<0, 01), milk cell Vit C (r=0.622, P<0.01) and milk cell NADPH (r=0.763, P<0.01) levels. There was a positive correlation between milk cell GPx activity and milk cell NADPH levels (r=0.659, P<0.01). Milk cell tGSH levels were positively correlated with milk cell Vit C (r=0.615, P<0.05) and milk cell NADPH (r=0.846, P<0.01) levels. Milk cell NADPH levels were positively correlated with milk cell Vit C levels (r=0.791, P<0.01).

DISCUSSION

Milk cells are composed of epithelial cells interfused to the milk from the breast tissue and leukocytes (neutrophils, lymphocytes and macrophages) that pass from the blood to the mammary gland and then into the milk. The content and the number of somatic cells in healthy goat milk differ from that in cow milk (Sarıkaya, 2006; Dang et al., 2008; Souza et al., 2012; Shah et al., 2017). Studies determining the protein levels of milk cells are quite limited. In the study conducted on cow milk somatic cells (Akalın et al., 2019), the total protein levels were determined as 0.374mg/1 ml cell supernatant in the cell pellet obtained by taking 50 ml of cow milk and concentrating it into 2 ml PBS. When the total protein levels in the present study were calculated with dilution coefficients (14 ml milk concentrated with 2 ml PBS: 0.09 mg/1 ml cellsupernatant), they were found to be slightly lower than the levels in the cow milk (approximately 0.321 mg/1 ml cell supernatant). No study investigating total protein levels of goat milk somaticcells has been found. The major component of goat milk somatic cell, unlike cow's milk is PMN. While 40%to 87% of somatic cells in goat milk (Souza et al., 2012; Shah et al., 2017) are formed by neutrophils from PMNs, the rate of neutrophils in cow milk cells varies between 5% and 20% (Sarıkaya, 2006; Souza et al., 2012). Moreover, 15% to 41% of somatic cells in healthy udder consist of macrophages whereas 9% to 20% of them consist of lymphocytes and 1% to 6% of them consist of epithelial cells (Paape et al., 2001). Determination of low total protein levels in goat milk cell compared to cow milk may be related to the difference in milk cell components.

The main goal of the pentose phosphate pathway is to produce NADPH, which has reducing power, and ribose-5-phosphate, which is the building block of DNA and RNA. NADPH is produced in the pentose phosphate pathway by glucose-6-phosphogluconate dehydrogenase (6PGD) and G6PD, from

NADP+ (oxidized NADPH) (Reuter et al., 1990; Stincone et al., 2015). While NADPH levels in cow milk cell were 4.24 nmol/mg protein (Akalın et al., 2019) and 5.99 nmol/mg protein (Akın et al. 2019), in the current study, the levels in goat milk (1.18 nmol / mg protein) were found to be lower than in the cow milk. As reported by Akın et al. (2019) and Akalın et al. (2019), a significant positive correlation was determined between cow milk cell NADPH levels and G6PD activity. However, in the current study no correlation was observed between these parameters in goat's milk. It can be speculated that NADPH is not controlled only by G6PD but also by 6PGD in the pentose phosphate pathway in the goat milk cell. It is suggested to evaluate 6PDG levels in goat milk cells to reveal the NADPH synthesis.

While positive correlations of milk leukocyte NADP+reductase activity (Ritter et al., 1977) and milk somatic cell G6PD activity (Akalın et al., 2019) with milk cell total protein levels in cows were reported, no correlation was found between total protein levels and G6PD activity in the current study. Glutathione protects cells against free radicals, reactive oxygen species, endogenous and exogenous toxic compounds. During the reduction, oxidized glutathione (GSSG) is formed and this molecule is reduced by GR enzyme which uses NADPH.While GPx provides detoxification of H_2O_2 in the cells, it allows conversion ofrGSH to GSSG (Ighodaro and Akinloye, 2018). In the current study, tGSH levels (101.73±25.5 nmol/mg protein) were found to be slightly lower than the levels reported for cow milk cells (122.88±13.08 and 142.16±37.06 nmol/mg protein) (Akalın et al., 2019; Akın et al., 2019, respectively). Also, goat milk cell NADPH levels were positively correlated with milk cell tGSH levels and GPx activity. While Akın et al. (2019) reported no significant correlation of NADPH with rGSH and GPx activity in cowmilk cells, Akalın et al. (2019) reported only a weak positive correlation between NADPH levels and GPx activity, and they thought that GSH levels and GPx activity in cow milk cells were also related to other mechanisms, and these parameters were not directly dependent on NADPH and G6PD. In goat milk cell, it can be concluded that NADPH is related to tGSH and GPx, unlike cow milk cell. While in their study, Akalın et al. (2019) determined GPx activity as 4.44±0.36 IU/mg protein incow milk cells, GPx activity was measured as 0.38±0.02 IU/mg protein in the current study conducted with goat milk cells. Just as in the cow milk cells (Akalın et al., 2019; Akın et al., 2019), no correlation was reported between total protein and GPx activity in the goat milk cells.

Vitamin C (ascorbic acid, ascorbate) is a water-soluble vitamin that causes the reduction of compounds such as molecular oxygen, nitrate, cytochrome a and c and is capable of reacting with free radicals in the aqueous environment. It reacts with superoxide and hydroxyl radicals and forms the first antioxidant defence against oxidant agents (Carole et al., 2007). In the current study, a significant positive correlation was determined between Vit C levels and tGSH levels. To our knowledge, no study was reported regarding correlation of milk cell Vit C and GSH levels. In a study conducted on lymphocytes, which were isolated from human blood, a significant and high level of positive correlation was found between tGSH and ascorbate (Lenton et al., 2000). Reduced glutathione is used to reduce oxidized Vit C (dehydroascorbic acid) to reduced form (ascorbic acid, ascorbate) (Winkler et al., 1994; May et al., 1997). The direct correlation between Vit C and GSH suggests that these two antioxidant agents may work synergistically with each other in the cell (Lenton et al., 2000). In the current study, a significant and high level of correlation was determined between Vit C and NADPH levels. There was no literature on the correlation between Vit C and NADPH levels in milk cells. On the other hand, ex vivo in human neutrophils, it has been reported that the activity of NADPH-oxidase enzyme, which uses NADPH as a substrate and enables the formation of superoxide radicals from molecular oxygen (Lassegue et al., 2012), is inhibited by the lipophilic derivative of ascorbic acid, thereby reducing the formation of superoxide radicals (Schmid et al., 1994). In the current study, the existence of a positive correlation between Vit C and NADPH in the absence of a correlation between Vit C and G6PD activity suggests that this might be due to NADPH-oxidase. It has been reported that thioredoxin reductase enzyme, which enables the conversion of dehydroascorbic acid to ascorbat in rat liver, uses NADPH as a reducing agent (May et al., 1997). Also, the positive correlation between Vit C and NADPH may be different in healthy and inflammative environment. Malondialdehyde is the final peroxidation product of fatty acids with multiple double bonds found in cell and organelle membranes. Increasing peroxidation of lipids by free radicals inmembranes causes an increase in MDA level. Malondialdehyde and other lipidperoxides can react with DNA or proteins and disrupt their structure (Gawel et al., 2004).The level MDA is served as a reliable biomarker of lipid peroxidation (LPO) and usually served as a marker of LPO (Acaroz et al., 2018). Nostudy on MDA levels in milk cells was found in the literature review. Goat milk cell MDA levels were determined as 1.43±0.35 nmol/mg protein. In addition, positive correlations of goat milk cell MDA levels with reducing agents such as tGSH, Vit C and NADPH were revealed. As MDA levels increase in goat milk cells, the increase seen in antioxidant molecules may indicate that these molecules play a role in the defence mechanism against oxidation and against the formation ofMDA.

CONCLUSION

In conclusion, positive correlations of milk cell MDA levels with milk cell tGSH, Vit C and NADPH levels, positive correlations of milk cell NADPH levels with milk cell tGSH, GPx and Vit C levels, positive correlation between milk cell Vit C and milk cell tGSH levels suggest that goat milk cells have an efficient antioxidant mechanism in healthy conditions which is some different from cow milk cells.

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CONFLICT OF INTEREST

None declared by the authors.

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