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Investigating Relationships between Catalase, Reduced Glutathione, Malondialdehyde, Vitamin C, and Total Protein Levels in Simmental Cow's Milk and Milk Cells

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Abstract: In this study, to evaluate some antioxidant parameters of Simmental dairy cow's milk and milk cells, the catalase (CAT), reduced glutathione (GSH), malondialdehyde (MDA), vitamin C (Vit C), and total protein (TP) were determined, and correlations between these parameters were revealed. The milk samples, collected from 28 clinically healthy cows from a private farm, were tested by CMT. Furthermore, CMT negative samples were included in the study. Briefly, milk cells were isolated from 15 mL of milk by centrifugation, and then they were sonicated. Milk and milk cell CAT activities, GSH, MDA, Vit C, and TP levels were determined by spectrophotometric methods. TP levels were 0.043 ± 0.008 mg in milk cell of 1 mL milk and 34.28 ± 0.656 mg/mL in milk. GSH levels were 21.19 ± 1.834 nmol/mg protein in milk cells and 25.78 ± 3.054 nmol/mL in milk. CAT activities were 0.13 ± 0.017 U/mg protein in milk cells and 2.391 ± 0.277 U/mL in milk. MDA levels were 2.27 ± 0.180 nmol/mL and Vit C levels were 68.89 ± 4.226 µg/mL in milk. As regards correlations: Milk cell GSH and milk GSH levels were negatively correlated with milk cell TP levels ($p<0.01$). Milk cell GSH levels were positively correlated with milk GSH levels ($p<0.05$). Milk Vit C levels were positively correlated with milk TP levels ($p<0.01$). Although it was weak, there was a positive correlation between milk CAT activities and milk Vit C levels ($p=0.05$). In conclusion, some biochemical parameters (CAT, GSH, MDA, Vit C, and TP) of Simmental cow's milk and milk cells were evaluated and discussed in the present study. It is thought that udder health will be positively affected by increasing the antioxidant capacity of milk cells.
Keywords: *CAT, GSH, MDA, Simmental milk cell, Vit C.*

Simental İnek Sütü ve Süt Hücrelerinde Katalaz, İndirgenmiş Glutatyon, Malondialdehit, Vitamin C ve Total Protein Düzeyleri Arasındaki İlişkilerin Araştırılması

Özet: Bu çalışmada, Simmental inek sütü ve süt hücrelerinin bazı antioksidan parametreleri, katalaz (CAT) aktiviteleri, indirgenmiş glutatyon (GSH), malondialdehit (MDA), vitamin C (Vit C) ve total protein düzeylerinin belirlenmesi ve bu parametreler arasındaki korelasyonların ortaya çıkarılması amaçlanmıştır. Özel bir çiftlikten klinik olarak sağlıklı 28 inekten toplanan süt örnekleri (her biri 15 mL), California Mastitis Testi (CMT) ile test edilmiş ve CMT negatif örnekler çalışmaya dahil edilmiştir. Kısaca, süt hücreleri santrifüjleme ile 15 mL sütten izole edildi ve ardından sonikasyona tabi tutuldu. Süt ve süt hücresi CAT aktiviteleri, GSH, MDA, Vit C ve TP seviyeleri spektrofotometrik yöntemlerle belirlendi. TP seviyeleri 1 mL sütün süt hücresinde 0.043 ± 0.008 mg ve sütte 34.28 ± 0.656 mg/mL belirlendi. GSH seviyeleri süt hücrelerinde 21.19 ± 1.834 nmol/mg protein, sütte 25.78 ± 3.054 nmol/mL ölçüldü. CAT aktiviteleri süt hücrelerinde 0.13 ± 0.017 U/mg protein ve sütte 2.391 ± 0.277 U/mL bulundu. Sütte MDA seviyeleri 2.27 ± 0.180 nmol/mL, Vit C seviyeleri sütte 68.89 ± 4.226 µg/mL belirlendi. Korelasyonlar açısından: Süt hücresi GSH ve süt GSH seviyeleri, süt hücresi TP düzeyleri ile negatif korelasyon gösterdi ($p<0.01$). Süt hücresi GSH seviyeleri, süt GSH seviyeleri ile pozitif korelasyon gösterdi ($p<0.05$). Süt Vit C seviyeleri ile süt TP seviyeleri arasında pozitif korelasyon vardı ($p<0.01$). Zayıf olmasına rağmen süt CAT aktiviteleri ile süt Vit C düzeyleri arasında pozitif korelasyon vardı ($p=0.05$). Sonuç olarak, sunulan çalışmada Simmental inek sütü ve süt hücrelerinin bazı biyokimyasal parametreleri (CAT, GSH, MDA, VitC ve TP) değerlendirildi ve tartışıldı. Süt hücrelerinin antioksidan kapasitelerinin artırılabilmesi sayesinde meme sağlığının olumlu etkileneceği düşünülmektedir.
Anahtar Kelimeler: *CAT, GSH, MDA, Simmental süt hücresi, Vit C.*

Introduction

Nowadays, it has been mentioned that oxidized flavor in milk proceeds a common problem affecting the dairy industry due to its adverse effect on milk acceptability (Gutierrez et al., 2018). Milk is considered a precious element of a complete diet and a rich nutrient source including, bioactive peptides, protein, conjugated linoleic acid, omega-3 fatty acid, vitamins, calcium, and selenium. These components are available in milk, play an essential role in physiological activities, and act as anti-cancer, anti-inflammatory, antimicrobial, and antioxidant (Khan et al., 2019).

The antioxidant capacity of milk is mainly due to non-enzymatic antioxidants such as glutathione,

vitamins (vitamin A, C, E), carotenoids, and also antioxidant enzymes that include catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) (Grażyna et al., 2017; Zivkovic et al., 2015). Lipid peroxidation negatively affects the quality of milk. The end products of lipid peroxidation as a biological lipid (omega-3 and omega-6 fatty acids) oxidation are reactive aldehydes, such as malondialdehyde (MDA) (Kapusta et al., 2018). The presence of antioxidants in milk may inhibit the free radical mechanism by donating the proton and thus prevent the onset of autoxidation (Khan et al., 2019). Reduced glutathione (GSH), a key component of the antioxidant system, converts dehydroascorbate to ascorbate and supports antioxidant defenses and detoxification (Szarka et al., 2012). Vitamin C (Vit C) is the primary and most essential water-soluble antioxidant present in milk that is readily oxidized at the pH of milk (Zivkovic et al., 2015) and possesses a strong affinity to scavenge free radicals such as superoxide, iron oxide, nitric oxide, and alkoxyl radicals (Gutierrez et al., 2018). One of the most active milk enzymes, CAT, a heme protein, converts hydrogen peroxide to water and oxygen. Synergistic interactions among antioxidants impart high antioxidant potential to milk and efficaciously protect milk fat against oxidation (Grażyna et al., 2017). Shortly, milk includes plenty of enzymatic and non-enzymatic antioxidant compounds (Zivkovic et al., 2015).

All types of milk contain a specific level of somatic cells (Hamed et al., 2008). Milk somatic cells play a protective role opposite to diseases in the mammary gland (Kehrli and Shuster, 1994). Somatic cells count in milk changes depending on the calving season, and also, it is thought that somatic cell count in milk is a poor indicator of the milk quality (Khastayeva et al., 2021). Hamed et al. (2008) found the relationship between milk somatic cell counts and antioxidant enzymes in bovine milk. Akalın et al. (2019) determined the antioxidant potential of Holstein cow milk cells by determining the levels of antioxidants. Moreover, milk protein is an essential indicator of milk quality (Khastayeva et al., 2021). Nevertheless, the oxidant-antioxidant potential of Simmental cow milk cells by determining the levels of MDA, Vit C, GSH, and CAT activity has not yet been fully considered in Simmental cows. The main aim of the present study was to evaluate MDA levels and antioxidant capacities of Simmental dairy cow's milk and milk cells. Therefore, CAT activity, the levels of GSH, MDA, Vit C, and total protein (TP) of milk and milk cells were determined and correlations between these parameters were evaluated in the present study.

Material and Methods

Milk samples were collected from 28 clinically healthy Simmental cows aged between 4 and 5 (in 2. and 3. lactation period). They were fed in the same care and nutritional conditions in a private intensive breeding farm in January in the Hatay region (36° 11' 56" North, 36° 9' 38" East). The commercial feed used in the farms is mainly corn silage and contains complementary milk feed, beet pulp, alfalfa hay, barley flakes, corn flakes, and hay (Table 1). The nutrient composition of complementary bovine milk (Simmental special feed, CP 9730, Turkey) feed is given in Table 2.

Table 1. The foodstuffs of the daily ration.

Foodstuffs	Quantity kg/day
Corn silage	14
Complementary milk feed	7
Beet pulp	6
Alfalfa hay(dried)	5.75
Barley flakes	1.5
Corn flakes (69 %starch)	1.5
Hay	1.5

Table 2. The nutrient composition of bovine complementary milk feed.

Crude protein	19%
Crude ash	7%
Crude cellulose	7%
Crude fat	3.2%
Calcium	1%
Phosphorus	0.7%
Sodium	0.4%
Vitamin A	20.000 IU/kg
Vitamin D3	3.000 IU/kg
Vitamin E	80 mg/kg

Milk samples were collected from the right-front lobe of clinically healthy udders between 09:00 and10:00 am. During milking, the first 2-3 squeezes of milk were thrown away after the teat was wiped with 70% alcohol cotton. The milk samples were tested by California Mastitis Test (CMT), and CMT negative samples were included in the study.

Collected milk samples (15 mL each) were brought to the biochemistry laboratory in the cold chain and centrifuged at 2300 rpm for 10 minutes at 4°C. After centrifugation, skimmed milk (supernatant) was collected by removing the upper layer of fat with a scraper and used in the assays. The cell pellet was washed twice with cold phosphate-buffered saline (PBS) and centrifuged at 2300 rpm for 10 minutes at +4°C. Finally, the supernatant was removed, and the remaining pellet was completed to 1.5 mL with PBS and sonicated (Bandelin Sonopuls HD 2070, Germany) (Akalın et al., 2016) for four repetitions of 10 seconds each, with a 30 seconds cooling period (on ice) between each repetition. By this process, milk cells in 15 mL of milk were concentrated in 1.5 mL PBS (10 times concentrated). After sonication, the homogenates were centrifuged at 7500 rpm for 20 minutes at +4°C. Milk cell supernatant was used for further analysis. Skimmed milk and milk cell CAT activity, GSH, MDA, Vit C, and TP levels were determined by spectrophotometric methods (UV 2100 UV–VIS Recording Spectrophotometer Shimadzu, Japan).

Determination of Total Protein (TP) Levels: Total protein levels were assayed by Lowry's (1951) method spectrophotometrically by determining the absorbance at 700 nm. Bovine serum albumin (Merck 112018, Germany) was used as a standard. The results are given as mg/milk cells of 1 mL milk for milk cell and mg/mL for milk.

Determination of Reduced Glutathione (GSH) Levels: Reduced glutathione levels were analyzed according to Ellman's (1959) method. It is a kinetic method based on the principle of the reduction of 5.5'-dithiobis (2-nitrobenzoic) acid to trinitrobenzene by glutathione. The optical density of the reduced disulfide compound absorbance at 412 nm can be measured by spectrophotometry. The results are presented as nmol/mg protein for milk cells and nmol/mL for milk.

Determination of Catalase (CAT) Activities: Catalase activities were determined using the method developed by Aebi (1984). Catalase is an enzyme that dissociates hydrogen peroxide into molecular oxygen and water. Catalase activities are usually proportional to the amount of dissociation of hydrogen peroxide. The activity measurement was monitored by the decrease in absorption at 240 nm. The results are presented as U/mg protein for milk cells and U/mL for milk.

Determination of Malondialdehyde (MDA) Levels: Malondialdehyde levels were determined spectrophotometrically according to the method proposed by Ohkawa et al. (1979). 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 sample in a boiling water bath for one hour under aerobic conditions and at pH:3.5. The results are presented as nmol/mL for milk.

Determination of Vitamin C (Vit C) Levels: Vitamin C levels were calculated according to the manual spectrophotometric method of Haag (1985). Ascorbic acid (Vitamin C) is converted to dehydroascorbic acid with mild oxidizing agents, and dehydroascorbic acid slowly converts to diketogulonic acid in mild acid solutions. Dehydroascorbic acid and diketogulonic acid react with 2.4-dinitrophenylhydrazine (DNPH) to form bis 2.4-dinitrophenylhydrazone. The results are given as µg/mL for milk.

Statistics: The values obtained were evaluated by Windows Statistical Package for the Social Sciences program (IBM SPSS 22 version, USA), and descriptives (Mean and Standard Error (SE)) were evaluated. Because some data were not distributed normally, Spearsman's correlation was performed for correlation analysis, and $P<0.05$ indicated statistical significance.

Results

Some biochemical parameters in milk cells (Table 3) and milk (Table 4) were presented. Total protein levels were 0.04 ± 0.008 mg in milk cells of 1 mL milk and 34.28 ± 0.656 mg/mL in milk. GSH levels were 21.19 ± 1.834 nmol/mg protein in milk cells and 25.78 ± 3.054 nmol/mL in milk. CAT activities were 0.13 ± 0.017 U/mg protein in milk cells and 2.391 ± 0.277 U/mL in milk. MDA levels were 2.27 ± 0.180 nmol/mL in milk, and Vit C levels were 68.89 ± 4.226 µg/mL in milk. MDA and Vit C levels were under the determination limit in supernatants of milk cells obtained from 15 mL of milk. Dairy milk yield was 24.61 ± 0.28 L.

Table 3. Some biochemical parameters in Simmental dairy cow's milk cells.

Parameters	Mean	SE	n
TP (mg/milk cells of 1 mL milk)	0.043	0.008	28
GSH (nmol/mg protein)	21.19	1.83	28
CAT (U/mg protein)	0.13	0.02	28

Table 4. Some biochemical parameters in Simmental dairy cow's milk.

Parameters	Mean	SE	n
TP (mg/mL)	34.28	0.66	28
GSH (nmol/mL)	25.78	3.05	28
CAT (U/mL)	2.391	0.28	28
Vit C (µg/mL)	68.89	4.23	28
MDA (nmol/mL)	2.27	0.18	28

TP: Total protein, GSH: Reduced glutathione, CAT: Catalase Vit C: Vitamin C, MDA: Malondialdehyde.

The correlations between the biochemical parameters are presented in Table 5. As regards correlations: Milk cell GSH (r=-0.684, p<0.01) and milk GSH (r=-0.487, p<0.01) levels were negatively correlated with milk cell TP levels. Milk cell GSH levels were positively correlated with milk GSH levels (r=0.475, p<0.05). Milk Vit C levels were positively correlated with milk TP levels (r=0.509, p<0.01). Although it was weak, there was a positive correlation between milk CAT activity and milk Vit C levels (r=0.374, p=0.05).

Table 5. Correlations between the parameters (Spearsman's correlation test).

	Milk TP (mg/mL)	Milk cell GSH (nmol/mg protein)	Milk GSH (nmol/mL)	Milk Cell CAT (U/mg protein)	Milk CAT (U/mL)	Milk MDA (nmol/mL)	Milk Vit C (µg/mL)
Milk Cell TP (mg/milk cells of 1 mL milk)	0.150	-0.684**	-0.487**	-0.256	0.029	0,000	-0.170
Milk TP (mg/mL)		0.040	-0.026	-0.021	0.301	0.094	0.509**
Milk Cell GSH (nmol/mg protein)			0.475*	0.289	0.181	0.018	0.174
Milk GSH (nmol/mL)				0.112	0.134	0.095	0.083
Milk cell CAT (U/mg protein)					-0.024	0.334	-0.102
Milk CAT (U/mL)						0.200	0.374*
Milk MDA (nmol/mL)							0.211

TP: Total protein, GSH: Reduced glutathione, CAT: Catalase Vit C: Vitamin C, MDA: Malondialdehyde. *P=0.05, **P<0.01.

Discussion

Milk protein is an essential indicator of the quality of milk (Khastayeva et al., 2021). Total protein levels were 34.28 mg/mL in milk in this study. Studies determining the TP levels of milk cells are pretty limited. In the study conducted on Holstein cow milk cells (Akalın et al., 2019), the TP levels were determined as 0.374 mg/1 mL cell supernatant in the cell pellet obtained by taking 50 mL of cow milk and concentrating it into 2 mL PBS. In the present study, the TP levels were calculated at 0.043 mg/1 mL cell supernatant in 15 mL milk concentrated with 1.5 mL PBS. Simmental cow milk was found to be approximately threefold lower than the levels in Holstein cow milk. The determination of low total protein levels in Simmental cow milk cells compared to Holstein cow milk may be related to the difference in milk cell components due to some age, lactation period, and breed properties. Reduced glutathione (GSH) protects cells against free radicals, reactive oxygen species, endogenous and exogenous toxic compounds (Meister and Anderson, 1983). In the current study, GSH levels were 25.78 ± 3.05 nmol/mL in milk. GSH levels (21.19 ± 1.83 nmol/mg protein) were found to be lower than the levels reported for Holstein cow milk cells (142.16±37.06 nmol/mg protein) (Akın et al., 2019). Milk cell GSH levels were positively correlated with milk GSH levels (r=0.475, p<0.05). No study on the correlation between GSH levels in milk cells and milk was found in the literature review. While Akın et al. (2019) reported no significant correlation of TP levels with GSH levels in Holstein cow milk cells, in this study milk cell TP levels were negatively correlated with milk cell GSH (r=-0.684, p<0.01) and milk GSH (r=-0.487, p<0.01) levels. There was no literature regarding the relation between total protein and GSH levels in biological fluids.

Catalase has been reported to play a central role in milk redox control (Silanikove et al., 2005). One of the most active milk enzymes, CAT, a heme protein, converts hydrogen peroxide to water and oxygen (Grażyna et al., 2017). In the present study, CAT activities were 0.13 ± 0.017 U/mg protein in milk cells and 2.39 ± 0.28 U/mL in milk. A few studies (Hamed et al., 2008; Kitchen et al., 1970) reported a positive correlation between the CAT activity and the milk somatic cell counts, whereas no relationship was observed in a study (Phillips and Griffiths, 1987) between those two parameters.

Vitamin C 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 aqueous environments. It reacts with superoxide and hydroxyl radicals and forms the first antioxidant defence against oxidant agents (Khan et al., 2019). In

the current study, lactating Simmental cow Vit C levels (68.89 ± 4.23 µg/mL) were found to be higher than the levels reported for lactating Holstein cows (23.78±0.55 and 22.87±1.20 µg/mL) (Akalın et al., 2016; Weiss et al., 2004). In the study conducted on Holstein cow milk cells (Akalın et al., 2016), the Vit C levels were determined as 1.18 µg/mL milk cell obtained by taking 50 mL of cow milk and concentrating it into 2 mL PBS. Vit C levels were under the determination limit in supernatants of milk cells obtained from 15 mL milk Simmental cow milk and concentrated into 1.5 mL PBS. The difference in Vit C level determination in Simmental cow milk cell compared to Holstein cow milk cell may be related to either amount of milk for measurement or some properties such as breed.

Milk antioxidants interact effectively by forming an antioxidant network (Grażyna et al., 2017). We found a weak positive correlation between milk CAT activity and milk Vit C levels (r=0.374, p=0.056). The appearance of ROS in milk initiates enzymatic protective and repair mechanisms (Grażyna et al., 2017). Antioxidants enter into particular interactions, boosting their activities and supporting mutual regeneration (Skibsted, 2012). It is thought that mentioned interactions between CAT and Vit C can effectively protect milk fat against oxidation and also diseases in the Simmental dairy cow mammary gland. Also, milk Vit C levels were positively correlated with milk TP levels (r=0.509, p<0.01). Sufficient literature information was not found to explain the positive correlation between milk Vit C and TP levels in the study.

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 in membranes causes an increase in MDA levels. Malondialdehyde and other lipid peroxides can react with DNA or proteins and disrupt their structure (Gaweł et al., 2004). Lipid peroxidation negatively affects the quality of milk. The end products of lipid peroxidation as a biological lipid (omega-3 and omega-6 fatty acids) oxidation are reactive aldehydes, such as MDA (Kapusta et al., 2018). In our previous study (Kazak et al., 2021), goat milk cell MDA levels were determined as 1.43±0.35 nmol/mg protein, whereas Simmental milk cell MDA levels were not determined in this study. This situation may be because healthy goat milk has a higher number of somatic cells than cow milk (Podhorecká et al., 2021). Moreover, no study on MDA levels in cow milk cells was found in the literature review. In the present study, milk MDA level was measured as 2.27 ± 0.18 nmol/mL. There was no correlation between MDA levels and other parameters in the study.

Conclusion

In conclusion, some of the antioxidant parameters of Simmental cow milk cells were evaluated for the first time. This study suggests that the milk Vit C-milk CAT and milk cell GSH-milk GSH levels are the essential coherent pathways for reactive oxygen metabolite neutralization in the milk of Simmental dairy cows. This study suggested that revealing the antioxidant potential of Simmental cow milk may be beneficial to understanding the Simmental milk cell defense mechanism. It can also be suggested that increasing the antioxidant potential of milk cells may protect dairy cows in terms of breast health. Lastly, further studies are required to clarify the oxidant-antioxidant mechanism of milk by revealing the interaction between the antioxidant capacities of the milk and milk cells in different dairy bovine species.

Conflict of Interest

The authors stated that they did not have anyreal, potential or perceived conflict of interest.

Ethical Approval

This study is not subject to HADYEK permission in accordance with Article 8 (k) of the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees".

Similarity Rate

We declare that the similarity rate of the article is 15% as stated in the report uploaded to the system.

Explanation

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Author Contributions

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