RESEARCH ARTICLE

IJVAR *International Journal of Veterinary and Animal Research*
E-ISSN: 2651-3609 6(2): 57-62, 2023
DOI: 10.5281/zenodo.8318921

Investigation of Some Antioxidant Enzyme Levels in Subclinical Mastitis in Dairy Cows at First 100 Days of Lactation

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Received: April 06, 2023
Accepted: July 13, 2023

Abstract

The purpose of this study is to investigate relationship between milk somatic cell count (SCC), total bacteria count (TBC), milk glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and serum paraoxonase (PON) levels, to identify the role of these parameters in subclinical mastitis at cows in the first 100 days of lactation. Animal material of this study consists of 100 Holstein dairy cows which were raised in a farm in Ankara province. Selected animals were hosted under same conditions and had suitable feeding regimen for appropriate period and were in the first 100 days of lactation. Cows which have less than $200x10^3$ somatic cell counts in two different analysis which performed 7-10 days apart considered as healthy (n=50), and which had $200x10^3$ somatic cell counts in two different counts performed 7-10 days apart considered as subclinical mastitis (n=50). Serum paraoxonase (PON) activity was lower in cows with subclinical mastitis than healthy ones ($p<0.001$), and GSH-Px activity was higher in cows with subclinical mastitis ($p<0.001$). It was concluded that the changes in milk SOD and GSH-Px levels are affected by the antioxidant system of the udder in subclinical mastitis, that formed oxidative stress and because of that serum PON and milk somatic cell and total bacteria have negative correlation. In conclusion, it was pointed out that serum PON activity can be used as a marker in the diagnosis of subclinical mastitis and evaluation of the antioxidant status of the udder. Also, it was stood out to importance of udder antioxidant mechanisms to understand pathogenesis of subclinical mastitis especially occurs in first 100 days of lactation.

Keywords: Dairy cow, glutathione peroxidase, oxidative stress, paraoxonase, subclinical mastitis.

INTRODUCTION

Regard of recent researches and developing technology, dairy cow farming is also changing (Hamann, 2005). In the last ten years, the individual milk yield of cows has been increasing worldwide. Producers always aim for higher milk yield in order to maintain profitability in the face of increasing costs (Baştan, 2013). While the number of enterprises and herds is decreasing worldwide, the number of animals in the existing herds is increasing. It is aimed to produce more milk from less farms and animals. Considering the 10-year animal data of the USA and Germany, it was seen that the number of herds decreased by 50%, the number of cows decreased by 10%, and the annual milk yield of a cow increased by 2% every year and approached 10 tons (Hamann, 2005). Studies show that highly productive cows are in the risk group for infectious diseases. Among these diseases, mastitis is one of the most common and costly diseases of dairy cows. Clinical and subclinical mastitis have economic effects on businesses. While calculating the economic effects of mastitis in dairy farms in the past years, its effects were also overlooked because subclinical mastitis was overlooked (Baştan, 2013).

Undoubtedly, the importance of mastitis, especially subclinical mastitis, increases with the decreasing number of animals and increasing milk yield (Hamann, 2005). In its simplest definition, mastitis means inflammation of the mammary tissue (Bradley, 2002; Hamann, 2005; Blowey and Edmondson, 2010; Baştan, 2013).

Most of the time, mastitis is associated with udder swelling and physical changes in milk, but subclinical mastitis progresses without any change in mammary and milk (Hamann, 2005). In recent years, the effects of subclinical mastitis have been better understood in the light of developing technology and research, and the importance of these mastitis has increased with the loss of milk yield and quality (Baştan, 2013).

In general terms, when balance between oxidant and antioxidant defense system is broken, it is described as oxidative stress (Lykkesfeldt and Svendsen, 2007) and generally observed in different disorders such as respiratory and reproductive system diseases (Lauritzen et al., 2003). Previous studies revealed that decrease in some antioxidant molecules levels during mastitis resulting to an increase in oxidative stress (Komine et al., 2004; Atakisi et al., 2010). Clinical and subclinical mastitis are both associated with decreased antioxidant capacity in milk (Atakisi et al., 2010).

First level of defense against oxidants are antioxidant system. Antioxidants are capable of reducing oxidants reactivity and making them harmless macromolecules (Yang and Li, 2015). Also, they can detect and remove or repair oxidized and damaged molecules. If the oxidative damage exceeds the capacity of repair and removal,

Cite this article as: Elifoğlu TB, Baştan A, Baş B. 2023. Investigation of Some Antioxidant Enzyme Levels in Subclinical Mastitis in Dairy Cows at First 100 Days of Lactation. International Journal of Veterinary and Animal Research, 6(2): 57-62. *DOI: 10.5281/zenodo.8318921*

controlled cell suicide and apoptosis take place finally (Lykkesfeldt and Svendsen, 2007). On the other hand, recent studies have shown that oxidative stress play critical role in apoptosis mechanism. Oxidative stress cause early apoptosis in cell life and, antioxidant system could block early apoptosis (Kannan and Jain, 2000). First defence line against reactive oxygen species are superoxide dismutases (SOD) which are group of metalloenzymes. SOD catalyze the dismutation of superoxide free radical into hydrogen peroxide and molecular oxygen (Younus, 2018). Glutathione peroxidases (GSH-Px) are selenoenzymes which are essential. GSH-Px, SOD and catalase (CAT) form enzymatic antioxidant system which have role of reducing reactive oxygen species (ROS). This enzymatic system also have role of limiting ROS' toxicity (Pei et al., 2023).

The paraoxonase (PON) was firstly discovered as an organophosphate pesticide-hydrolyzing enzyme (Mazur, 1946). Nowadays PON is considered as an antioxidant (Fuhrman, 2012) and acute phase protein (Kulka, 2016). When oxidative stress arises, PON enzyme have role of protective effects agains cellular oxidative damage (Fuhrman, 2012). In addition to this, it is believed that PON have significant role in hydrolyzing hydrogen peroxide and lipid peroxides which helps in protection against oxidative stress as well (Shekhanawar et al., 2013).

Therefore, earlier diagnosis and better understanding about the pathogenesis of subclinical mastitis, and role of oxidative system is important to minimize potential economical losses. Also, it not fully understood how the alterations takes place due to subclinical mastitis in biochemical composition of milk. Consequently, the aim of this presented study was to investigate; the relationship between milk somatic cell count (SCC), total bacterial count (TBC) and serum PON, milk GSH-Px and SOD levels in the first 100 days of lactation, the role of milk SCC, TBC, GSH-Px, SOD and blood serum PON levels in subclinical mastitis.

MATERIALS AND METHODS

Animal material

The presented study was carried out in a commercial dairy farm which had free-stall barn system that has reached certain standards in animal welfare and milk production. Milking have been performed 3 times a day with computerized system and sofware systems are used to follow herd management. Farm is free from herd diseases and had 11.5 tons milk yield per lactation. In addition preventive medicine and vaccination programs are followed as well. According to the farm records, 100 Holstein dairy cows were used in this study which have no history and signs of clinical health problems, gave at least one birth and under the age of five, with similar body conditions (ranging between 2.5 and 3 over 5). All cows were in the first 100 days of lactation, housed in the same conditions and fed by the appropriate nutrition program for the period, and received the same dry period length and treatment. Cows were undergoing 40-45 days of dry period and 20 days close-up period. All cows were received Fatroximin Dry Cow Therapy (Fatro Pharma, Italy). Animals which were in the first 20 days of lactation were not included in the study since SCC results could be effected. Since oxidative stress level and antioxidant mechanisms could be affected by environmental temperatures, the study was carried out in December, January, and February with similar air temperatures to have the same effect of season and environmental temperature on animals.

Study design

After counting milk somatic cells twice with 7-10 days intervals from all the selected animals, two study groups were formed according to the results obtained. Animals with a SCC above $200x10^3$ in both measurements were considered to have subclinical mastitis (Group 1, n=50) and, animals with a SCC below $200x10^3$ were considered healthy (Group 2, n=50). Milk samples were collected from each quarter equally in 50 mL tubes before first milking of the day from the selected animals. On the day of the second milk sample collection, blood samples were collected from the animals which are included in this study after morning milking.

Somatic cell and total bacteria count

Milk samples brought to laboratory within 4 hours after collecting and samples were kept cold in a container for transporting them to the laboratory. Samples were homogenized with a shaker in accordance with the instructions of the IBCm (Bentley Instruments®, USA) device (based on flowcytometry), then samples were prepared with their own solution kits (Bentley Instruments®, USA), first the total bacterial count and then the somatic cell count were performed in accordance with the operating instructions of the device.

Collection of milk and blood sera

The milk samples stored at -80°C were thawed at room temperature. Samples were first centrifuged at -4°C at 21000 rpm (41410 xg) for 15 minutes. After separating the obtained sera into 2 ml tubes, they are centrifuged once again at 26000 rpm (61973 xg) for 15 minutes at -4°C, the separated milk sera are transferred to 1.5 mL Eppendorf tubes and stores at -80°C till biochemical analyzes were performed. The blood samples delivered to the laboratory were centrifuged at 1000 rpm at +4°C for 15 minutes. Collected blood sera were stored at -80°C until the related analysis was performed.

Bacterial culture of milk samples

The samples to be studied were homogenized by vortexing after thawing at 37°C. 50 µL of the prepared samples were taken and inoculated on 7% sheep blood agar, eosin methylene blue agar and MacConkey agar. Petri dishes of the sown samples were incubated at 37°C for 24-48 hours under aerobic conditions. Colonies formed at the end of the incubation period were purified and evaluated macroscopically and microscopically. Each purified colony was stained by Gram staining method, biochemical tests for Gram negative and gram-positive bacteria (Oxidation-fermentation, catalase, oxidase, coagulase, motility examination, urease, H2S, Indole, Citrate, Voges Poskauer and Methyl Red test) according to the results obtained, and bacteria were identified at the species level.

Biochemical Analyses

SOD analysis in milk sera were performed as Sun et al., (1988) described. Analyzes were done with the method based on the inhibition of Nitroblue Tetrazolium reduction of superoxide radicals formed by the xanthine oxidase enzymatic reaction by the SOD enzyme found in the sample. GSH-Px analysis was performed in milk sera with the method developed by Paglia and Valentine, (1967). By

using peroxidase, glutathione is converted to the reduced form GSSG, and the reaction is reversed by a reaction using NADPH+, which can be catalyzed by the glutathione reductase enzyme. PON activity in blood serum was measured by the method described by Armstrong (2008). Paraoxonase activities were determined by measuring the optic densities of p-nitrophenol formed because of enzymatic hydrolysis of paraoxonase using pH: 10.5 buffer.

Statistical Analysis

All obtained variables were checked for normality with Shapiro-Wilk and for homogeneity of variants Levene test was used. Student-t test was used to determine the statistical difference between groups when parametric assumptions were met, and Mann-Whitney U test was used when the assumptions were not met. The strength and direction of the relationship between the obtained parameters were calculated with the Spearman correlation coefficient. All statistical analyzes were performed with a minimum margin of error of 5% with SPSS Statistics (Statistical Package for the Social Sciences) software.

RESULTS

Somatic cell count, which is used as a marker of subclinical mastitis, was higher in subclinical infected cows in the presented study, and the difference was found to be statistically significant ($p<0.001$). Results showed that the GSH-Px was statistically higher in Group 1 than compared with Group 2 ($p<0.001$). The PON levels of the animals in the infected group were found to be lower than those of the healthy animals, the difference was statistically significant ($p<0.001$). It was determined that SOD was higher in Group 1 than Group 2, but the difference was not statistically significant ($p=0.062$) (Table 1). Microbiological analyses were performed from fresh milk of the animals, which were considered as subclinical mastitis. From 50 animals' fresh milk, bacterial growth was observed in only 37 specimens. According to the results of microbiological analyses, while SOD and GSH-Px and PON levels were higher in bacterial growth (+) sub-group in Group 1, no statistically significant difference was found within the group (Table 2).

The cross correlations of milk serum SOD, GSH-Px, SCC, TBC and blood PON values for whole animals are shown in Table 3. Positive correlation of somatic cell number with total bacteria was high ($R=0.766$) and positive correlation with GSH-Px was moderately correlated ($R=0.534$). Similarly, it was observed that the TBC exhibited an increase across all groups, while there was a calculated increase in GSH-Px levels. Moreover, a moderate positive correlation was observed between TBC and GSH-Px. The study findings indicate that Group I demonstrated a significantly higher level of GSH-Px compared to Group 2 (Table 3).

Table 1. Somatic cell and total bacteria count and enzyme levels of subclinical infected (Group 1) and healthy (Group 2).

	Group	*n*	Mean	Std. Err.	Std. Dev.	Median	p
SCC ($x10^3$ scc/ml)	Group 1	50	1674.12	400.08	2829.02	685.00	<0.001
	Group 2	50	36.76	4.17	29.49	24.50	
TBC (cfu/ml)	Group 1	50	845.92	98.44	696.04	654.00	<0.001
	Group 2	50	63.02	8.88	62.78	46.00	
SOD (U/ml)	Group 1	50	0.14	0.01	0.07	0.16	0.062
	Group 2	50	0.13	0.01	0.07	0.12	
GSH-Px (U/ml)	Group 1	50	1.59	0.06	0.44	1.40	<0.001
	Group 2	50	1.33	0.00	0,02	1.33	
PON (U/ml)	Group 1	48	131.74	3.93	27,23	128.83	<0.001
	Group 2	49	152.87	3.51	24,56	152.75	

SCC: Somatic cell count, TBC: Total bacteria count, SOD: Superoxide dismutase, GSH-Px: Glutathione peroxidase, PON: Paraoxonase.

Table 2. Somatic cell and total bacterial count and enzyme activities of milk with and without growth in bacteriological culture in subclinical infected cows (Group 1).

	Bacterial Growth	*n*	Mean	Std. Err.	Std. Dev.	Median	p
SCC ($x10^3$ scc/ml)	Yes	37	1625.84	501.01	3047.55	679.00	0.407
	No	13	1811.54	607.02	2188.63	869.00	
TBC (cfu/ml)	Yes	37	857.46	116.02	705.70	659.00	0.732
	No	13	813.08	192.69	694.76	523.00	
SOD (U/ml)	Yes	37	0.15	0.01	0.07	0.16	0.376
	No	13	0.13	0.01	0.04	0.14	
GSH-Px (U/ml)	Yes	37	1.62	0.08	0.46	1.40	0.509
	No	13	1.52	0.11	0.39	1.37	
PON (U/ml)	Yes	37	129.44	4.61	28.06	126.12	0.288
	No	11	139.47	7.16	23.75	142.12	

SCC: Somatic cell count, TBC: Total bacteria count, SOD: Superoxide dismutase, GSH-Px: Glutathione peroxidase, PON: Paraoxonase.

Table 3. Independent parameters correlations between the groups.

Correlations							
Parameters			**SCC**	**TBC**	**GSH-Px**	**SOD**	**PON**
Spearman's rho	SCC	Correlation coefficient	1.000				
		P					
		N	100				
	TBC	Correlation coefficient	0.766**	1.000			
		P	<0.001				
		N	100	100			
	GSH-Px	Correlation coefficient	0.534**	0.462**	1.000		
		P	<0.001	<0.001			
		N	100	100	100		
	SOD	Correlation coefficient	0.191	0.183	-0.060	1.000	
		P	0.058	0.069	0.556		
		N	100	100	100	100	
	PON	Correlation coefficient	-0.297**	-0.293**	-0.334**	-0.150	1.000
		P	0.003	0.004	0.001	0.142	
		N	97	97	97	97	97

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

SCC: Somatic cell count, TBC: Total bacteria count, SOD: Superoxide dismutase, GSH-Px: Glutathione peroxidase, PON: Paraoxonase.

DISCUSSION AND CONCLUSION

In the presented study, somatic cell counts in two milk samples taken 7-10 days apart from four mammary lobes were used to define the groups. Animals with higher than $200x10^3$ cells/ml in both measurements were considered to have subclinical mastitis, while animals with less than $200x10^3$ cells/mL in both measurements were considered healthy as in the researches (Schukken et al., 2003; Lievaart et al., 2007; Baştan, 2013). In the study, the difference between the groups according to the number of somatic cells was found to be statistically very significant. The difference in SCC between groups thought to be important for intergroup comparisons.

According to the researchers, the milk TBC of cows with subclinical mastitis is higher than healthy ones (Baştan, 2013; Küplülü and Vural, 2016). The results of the TBC of the study are consistent with the literature. The TBC in milk of cows with subclinical mastitis was higher than that of healthy cows, and the difference was statistically significant ($p<0.001$).

It was reported that there is a positive correlation between SCC and TBC (Colakoglu et al., 2017). Olechnowicz and Jaśkowski, (2012) reported that an increase in the total number of bacteria causes an increase in the number of somatic cells. The presented study has similar results with other studies. The variation in the total bacterial count between healthy and infected cows was statistically highly significant ($p<0.001$), while the positive correlation between the somatic cell count and the total bacterial count was statistically highly correlated ($R=0.766$).

In the presented study, GSH-Px (U/mL) in the milk of subclinical infected cows was found to be higher than that of the healthy group, the difference was statistically significant ($p<0.001$). A significant positive correlation ($R=0.534$ and 0.462) of GSH-Px was found with TBC and SCC. In a study investigating tank milk SCC and GSH-Px activity, researchers reported a positive correlation between SCC and GSH-Px activity. They reported that the increased GSH-Px activity as the number of SCC increased may be related to increased free radicals and polyunsaturated fatty acids in milk (Hamed et al. 2008). The results in the presented study are similar. It was thought that there may be more than one reason for the increase in GSH-Px in milk with subclinical mastitis. It is thought that the GSH-Px found in milk comes to the udder through the circulation and passes into the milk. Studies show that GSH-Px and SOD values in the blood of cows with mastitis decrease (Atroshi et al., 1986; Erskine et al., 1987; Ghasemian et al., 2011). Rehman et al., (2017) reported that blood serum GSH-Px level was lower in cows with subclinical mastitis than in healthy cows. In the light of this literature information, it was thought that one of the reasons for the increase in the milk GSH-Px enzyme level might be the increased transition from blood to milk, but since blood serum GSH-Px levels were not investigated in the study, no definite conclusion could be reached.

It has been reported that enzyme activity increases because of casein hydrolysis in subclinical mastitis (Andrei et al., 2010). Some mastitis agents can increase GSH-Px levels during adaptation to oxidative stress. Cells synthesize superoxide to take advantage of its antibacterial activity during phagocytosis. It has been reported that the bacteria responsible for the infection synthesize enzymes such as SOD, GSH-Px and catalase in order to prevent this mechanism (Matei et al., 2011). It has been reported that some bacteria also synthesize glutathione peroxidase (Andrei et al., 2010; Matei et al., 2011). It was thought that one of the reasons for the increase in milk GSH-Px level may be due to the hydrolysis of casein and/or the defense mechanism of the pathogen that causes mastitis.

Higher milk SOD activity in infected cows was considered statistically significant ($p=0.062$). Yang et al., (2011) reported that SOD activity in milk with subclinical mastitis was lower, but the difference was not statistically significant. Andrei et al., (2010), on the other hand, reported in their study that SOD was higher in subclinical infected milk, but this level was not related to the number of somatic cells, and they could not find a correlation. The presented study has similar results with the literature, milk SOD was found to be higher in subclinical infected cows, but with limited statistical significance, and no correlation with SCC was determined. It has been reported that milk SOD is only in the serum phase of milk, there is no enzyme activity in the fat fraction, and it is the same according to the electrophoretic properties of the bacterial and somatic cell-derived SOD enzyme (Rizzo et al., 2012). Holbrook

and Hicks, (1978) could not determine whether the milk SOD activity originated from bacteria or somatic cells. Since the milk SOD in the analyzes reflects the total activity originating from bacteria and somatic cells, it was thought that no correlation could be determined with SCC. In the light of this literature information, although the difference between the groups in SOD activity was of limited significance, it was concluded that the change in milk SOD activity in subclinical mastitis was uncertain.

The lack of relationship between milk SOD activity and SCC in the study suggested that PMNs were associated with low superoxide anion release. Oxidative stress, which is formed due to high somatic cell number, bypasses the first part of the antioxidant defense system, and the formed free radicals do not need to interact with SOD (Dimri et al., 2013). It has been reported in an in vitro study that PMNs activated by *E. coli* damage the mammary epithelium with oxygen radicals, and that SOD cannot prevent the cytotoxic effects of these radicals (Boulanger et al., 2002).

According to the results of biochemical analyzes and statistics, PON activity decreased in subclinical mastitis and this decrease was evaluated as statistically significant. These results are consistent with the current literature (Turk et al., 2012; Kovačić et al., 2019; Nedić et al., 2019). Nedić et al., (2019) reported that serum PON activity decreased due to infection in subclinical mastitis in their study, and the presented study contains similar results with these literatures. At the same time, a negative correlation was determined between PON activity and the number of somatic cells (R=-0.297) and the total number of bacteria (R=-0.293). These correlations suggested that PON activity could be evaluated as a negative acute phase protein in subclinical mastitis and that PON activity could be used as a marker showing udder inflammation and in monitoring udder health. In addition, a negative correlation (R=-0.334) was found between serum PON activity and milk GSH-Px activity. It was concluded that the antioxidant system of the mammary gland is systemically affected in subclinical mastitis and that PON activity may be a marker in the evaluation of the oxidative state of the mammary gland.

In cows with subclinical mastitis, serum PON was lower and milk GSH-Px was higher than in healthy cows, the change in milk SOD and GSH-Px are an indication that the antioxidant system of the mammary gland is affected, and oxidative stress is formed in subclinical mastitis. It was concluded that there is a negative correlation between PON activity and milk SCC, TBC and GSH-Px due to oxidative stress in the udder. Since the antioxidant system is affected, especially in subclinical mastitis in the first 100 days of lactation, approaches to support immunity will reduce the incidence of subclinical mastitis in this period.

In conclusion, more detailed studies on PON should be done to better understand the pathogenesis of subclinical mastitis and mammary gland immunity. Also, to better understand the pathogenesis of subclinical mastitis in the first 100 days of lactation, further investigation of oxidative stress and antioxidant mechanisms of the mammary gland should be done.

Acknowledgement. This article was produced from the first author's PhD dissertation.

Conflict of Interest

The authors declared that there is no conflict of interest.

Authorship contributions

Design: T.B.E., A.B., Data Collection or Processing: T.B.E., Analysis or Interpretation: T.B.E., B.B., Literature Search: T.B.E., A.B., Writing: T.B.E., A.B., B.B.

Financial Support

This study was supported by The Office of Scientific Research Projects at Ankara University (Turkey) with grant number; 19l0239003.

Ethical Approval

This study was conducted pursuant to the 02/01/2019 dated and 2019-1-4 numbered approval decision of the Local Ethics Board for Animal Experiments of Ankara University.

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