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THE IMPACT OF MASTITIS PATHOGENS ON ANTIOXIDANT ENZYME ACTIVITY IN COWS' MILK

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DIMITAR NAKOV[1*], *ACO KUZELOV*[1], *SLAVČA HRISTOV*[2], *VALENTINA VELKOSKA NAKOVA*[3], *BRANISLAV STANKOVIĆ*[2], *JELENA MIOČINOVIĆ*[2]

[1]Goce Delcev University in Stip, Faculty of Agriculture, str. Krste Misirkov 10-A, 2000 Shtip, North Macedonia
[2]University of Belgrade, Faculty of Agriculture, Nemanjina 6, 11080 Belgrade-Zemun, Serbia
[3]Goce Delcev University in Stip, Faculty of Medical Sciences, str. Krste Misirkov 10-A, 2000 Shtip, North Macedonia
**Corresponding author*: dimitar.nakov@ugd.edu.mk

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SUMMARY

The beginning of lactation in dairy cows is a challenging period when cows experience oxidative stress followed by an increased risk of mastitis. This study aimed to establish a correlation between mastitis pathogens and the activity of antioxidant enzymes – superoxide dismutase (SOD) and glutathione peroxidase (GPX) – in milk collected from cows with mastitis compared with their healthy counterparts. During the two-year survey, the udder health status was followed and the changes in SOD and GPX activity in milk were assessed in the period to 42nd day of lactation. The quarter milk samples were screened for detection of abnormal milk secretion (AMS) and intramammary infections (IMI). The spectrophotometric methods were used for detection of antioxidant enzyme activity in milk serum. The prevalence of IMIs from AMS was 43.83% while the isolated mastitis pathogens were grouped as contagious: Streptococcus agalactiae (19.14%) and Staphylococcus aureus (6.17%); or environmental: Enterococcus spp. (8.02%), Pseudomonas aeruginosa (7.41%), Escherichia coli (1.85%), and Aspergillus niger (1.23%). IMI showed statistically significant influence on SOD and GPX activity in milk serum ($p<0.05$). Contagious bacteria lead to increased activity of GPX, while environmental pathogens more drastically increase SOD activity. Providing a balanced diet with adequate antioxidants and managing environmental stressors can help reduce oxidative stress during the transition period and decrease the risk of mastitis in early lactation.

Key words:
glutathione peroxidase, superoxide dismutase, mastitis

Abbreviations:
SOD – superoxide dismutase; GPX – glutathione peroxidase; AMS – abnormal milk secretion; IMI – intramammary infections; ROS – reactive oxygen species; CAT – catalase; P – parity; TDM – test day milk yield; LP_21 – period from beginning of lactation until 21st day in lactation; LP_42 – period from 22nd to 42nd day in lactation; CMT – California Mastitis Test; SCC – somatic cell count; Y_S – year season of calving; GLM – General Linear Model

INTRODUCTION

The most challenging period for management of health, productivity and economic viability of dairy farms is the transition period, spanning from three weeks before to three weeks after parturition (Sordillo, 2005). This period around parturition causes a profound shift in the metabolic processes of dairy cows. During this time, there is a notable decrease in daily dry matter intake (Grummer et al., 2004). Concurrently, the beginning of lactation leads to a surge in energy needs, resulting in a negative energy balance. Consequently, cows need to mobilize body tissues to meet the increased energy demands for milk production, primarily relying on lipids as their energy source (Contreras & Rodriguez, 2011; Wathes et al., 2013). This metabolic intensity brings about alterations in energy metabolism and escalates oxygen consumption. The intensified metabolic processes result in an elevated production of reactive oxygen species (ROS), a group of molecules that can cause cellular damage. In response to this, living organisms typically employ a combination of enzymatic and non-enzymatic antioxidant systems to neutralize ROS, thus safeguarding against oxidative harm (Bionaz et al., 2007; Halliwell & Gutteridge, 2007). Oxidative stress, which refers to an imbalance between oxidants and antioxidants, is commonly quantified through methods that measure oxidants and antioxidants, either directly or indirectly (Celi, 2011). The key antioxidant enzyme systems include superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT), while non-enzymatic antioxidants comprise sulfhydryl (SH) groups of albumin, α-tocopherol, and carotenoids (Havemose et al., 2006). In the transition period, cows are susceptible to oxidative stress, which can potentially lead to periparturient disorders and metabolic ailments (Bernabucci et al., 2002; Bernabucci et al., 2005; Lykkesfeldt & Svendsen, 2007). Therefore, this relatively brief period becomes crucial for health care, significantly impacting dairy producers.

Nevertheless, the existing literature lacks robust evidence linking oxidative stress during the transition period to mastitis occurrence in dairy cows. The most reliable interconnection between oxidative stress and mastitis is through the inflammatory response. When mastitis occurs, the immune system is activated to neutralize bacterial infection. This immune response involves the production of inflammatory molecules and immune cells, which can generate ROS as a natural part of the defense mechanism. However, excessive ROS production can lead to oxidative stress, damaging surrounding tissues and exacerbating the inflammatory process. Additionally, oxidative stress weakens the cows' immune system, making it more susceptible to infections like mastitis. This forms a cycle where mastitis-induced inflammation can contribute to oxidative stress and oxidative stress can impair cows' ability to fight off infections effectively.

Mastitis continues to be a significant health concern in dairy herds, resulting in substantial economic repercussions across the entire milk production chain. The most substantial economic losses in the dairy industry stem from reduced milk production and quality. Depending on the pathway of infection spreading, mastitis pathogens are classified as contagious or environmental (White et al., 2006). The primary contagious udder pathogens commonly found in dairy farms include *Staphylococcus aureus* and *Streptococcus agalactiae*. In addition, there is a diverse array of opportunistic environmental pathogens known to be associated with mastitis. These pathogens include the representatives of family *Enterobacteriaceae* (*Escherichia coli, Klebsiella spp., Enterobacter spp., Serratia spp.*), as well as *Pseudomonas spp., Proteus spp., Corynebacterium pyogenes, Streptococcus uberis,* and *Streptococcus dysgalactiae,* along with other coagulase-negative bacteria within the genus *Staphylococcus* (Taponen & Pyorala, 2009; Holko et al., 2019). Nonetheless, Zadoks et al. (2011) pointed out that this clear-cut division in the transmission of udder pathogens is becoming less distinctive. From an epidemiological perspective, it is increasingly evident that there is no sharp demarcation between contagious and environmental udder pathogens (Piessens et al., 2012).

Hence, the primary objective of this study was to establish a connection between mastitis pathogens and antioxidants enzyme activity (SOD and GPX) in milk samples collected from dairy cows during the period of early lactation.

MATERIAL AND METHODS

A longitudinal survey lasting for two years was carried out to follow udder health status of dairy cows in the period of early lactation, from calving to 42nd day of lactation. Furthermore, the survey aimed to determine udder pathogens and also to evaluate the changes in the activity of antioxidants enzymes SOD and GPX in milk collected from cows with mastitis compared with their healthy counterparts. The population that was followed during the survey comprised 211 black-white dairy cows. The parity of cows (P) had five levels: 1, 2, 3, 4, and 5 or higher. The cows were provided with similar rearing conditions and feeding regimes to avoid additional influence of environmental factors on milk's antioxidant enzyme activity. Cows were milked in the milking parlor twice daily, with the exception of dairy cows in the first month of lactation, which were milked three times daily. The test day milk yield (TDM) of cows in the trial was followed in three milk control points until 42nd day of lactation. The milk samples for

determination of SOD and GPX activities were collected in two physiological periods: the period from the beginning of lactation until 21st day of lactation (lactation period 21 – LP_21) and period from 22nd to 42nd day of lactation (lactation period 42 – LP_42). Daily, before milking and attachment of teat cups, the farmers examined mammary glands for the presence of clinically visible signs of mastitis and abnormalities in milk, such as the appearance of flakes, clots, and blood. The screening of udder quarters for mastitis was performed with California Mastitis Test (CMT) and classified as negative (CMT-) or positive (CMT+). Milk samples were collected for bacteriological examination, under sterile conditions, from each quarter that tested positive to California Mastitis Test (CMT). These samples were analyzed within a 12-hour window following the collection. Identification of bacterial species was carried out in accordance with established microbiological procedures, utilizing reference methodologies aligned with the standards set by the National Mastitis Council (Oliver et al., 2004).

Using the results obtained from screening through clinical examination, CMT, and bacteriological culturing, all cows within the observed population were categorized into three groups: healthy cows, cows exhibiting abnormal milk secretion (AMS), and cows with intramammary infection (IMI). Bulk milk samples were obtained from cows with healthy udder quarters, while milk samples from quarters that exhibited a positive CMT reaction were collected as separate samples. Furthermore, the cows were grouped based on the season of calving (Y_S) to mitigate the potential influence of environmental factors on antioxidant enzyme activity. The seasons of calving were defined according to the season of the year when the cow was calving, as spring (season 1), summer (2), fall (3), and winter (4). Defatting of raw milk samples and abstraction of milk whey was done by centrifugation followed by acidification of skim milk with 1M HCl to adjust pH 4.6 for protein precipitation. After removing the precipitated proteins, the pH value was again adjusted to 7.6 using 1M NaOH. The samples were then stored in sealed plastic tubes at a temperature of -80°C. Prior to analysis, aliquots were thawed. SOD and GPX activity in milk serum was done with spectrophotometric assays expressed as mU/mg protein. SOD activity was measured using kinetic analysis according to Gao et al. (1998), while GPX measuring was used method modified according to Chen et al. (2000). Absorbance measurements were conducted using a spectrophotometer Bio-Rad 680 XR, a microplate reader.

The statistical model Multivariate General Linear Model (GLM) was used to determine the influence of factor variables on changes in SOD and GPX activity in milk. The correlation between SOD and GPX activity in milk was calculated with Pearson's coefficient of correlation.

RESULTS AND DISCUSSION

Table 1 presents the average monthly TDM for cows in early lactation regarding the season of calving. The average TDM was in the interval from 28.15 to 28.79 kg.

Table 1. Average milk yield (kg) ± standard error of mean in 1st, 2nd and 3rd TDM control from the beginning of lactation considering the season of calving

Y_S	n	TDM_1 ($\bar{x} \pm S_{\bar{x}}$)	TDM_2 ($\bar{x} \pm S_{\bar{x}}$)	TDM_3 ($\bar{x} \pm S_{\bar{x}}$)
1_1[a]	24	29.79±1.704	28.82±1.266	28.50±1.018
1_2[a]	26	29.83±0.804	28.92±0.899	27.22±0.935
1_3[a]	18	24.07±1.100	24.90±1.151	23.48±0.756
1_4[a]	22	31.24±0.689	30.07±0.645	28.06±0.726
1[b]	**90**	**28.77±0.645**	**28.17±0.541**	**26.67±0.464**
2_1[a]	35	31.99±1.050	29.48±1.028	26.60±1.150
2_2[a]	20	21.01±1.503	25.02±1.528	23.39±2.071
2_3[a]	39	24.39±1.106	27.39±1.118	28.46±1.001
2_4[a]	27	35.47±1.806	32.95±1.592	33.52±1.392
2[b]	**121**	**28.78±0.847**	**29.14±0.693**	**28.84±0.719**
Total	**211**	**28.76±0.572**	**28.79±0.472**	**28.15±0.485**

Legend: [a]Y_S 1_1 - the first year, season 1; Y_S 1_2 - the first year, season 2; Y_S 1_3 - the first year, season 3; Y_S 1_4 - the first year, season 4; Y_S 2_1 - the second year, season 1; Y_S 2_2 - the second year, season 2; Y_S 2_3 - the second year, season 3; Y_S 2_4 - the second year, season 4; [b]Y_S 1 - the first year of trial; Y_S 2 - the second year of trial

Table 2 presents data for the prevalence of udder quarter disorders in the two physiological periods of early lactation (LP_21 and LP_42) based on the used screening methods.

Table 2. The prevalence of AMS and IMI in dairy cows at the udder quarter level

LP	Total	CMT(-)		CMT(+)		AMS		IMI	
	n	n	%	n	%	n	%	n	%
21	844	765	90.64	79	9.36	45	5.33	34	4.03
42	844	761	90.17	83	9.83	46	5.45	37	4.38

In totally screened mammary gland quarters, the prevalence of AMS was 5.33% and 5.45%, while the prevalence of IMI was 4.03% and 4.38% for the LP_21 and LP_42, respectively. Table 3 shows the results of microbiological examination of milk samples from udder quarters that showed a positive reaction to CMT, and were therefore classified as quarters with persistent AMS.

Table 3. Udder pathogens isolated in milk samples from quarters with positive CMT

	n	%		n	%
CMT(+) quarters	162	100.00			
Microbiologically negative	91	56.17			
Microbiologically positive	71	43.83	***Streptococcus agalactiae***	31	19.14
			Enterococcus Spp.	14	8.02
			Pseudomonas aeruginosa	11	7.41
			Staphylococcus aureus	10	6.17
			Escherichia coli	3	1.85
			Aspergillus niger	2	1.23

Microorganisms causing mastitis were found in 43.83% of udder quarters with persistent AMS. The dominantly isolated microorganisms were grouped as contagious: *Streptococcus agalactiae* (19.14%) and *Staphylococcus aureus* (6.17%) or environmental: *Enterococcus spp.* (8.02%), *Pseudomonas aeruginosa* (7.41%), *Escherichia coli* (1.85%), and *Aspergillus niger* (1.23%).

Effective mastitis diagnosis, followed by implementation of respective control programs, is of crucial importance for sustainable milk production in dairy farms (Reshi et al., 2015). Culture examination is often considered the "gold standard" for identifying infected udder quarters. However, these methods are frequently expensive and time-consuming, which makes them impractical for routine on-farm screening and assessment (Sargeant et al., 2001). In comparison to culturing techniques and somatic cell count (SCC) determination, field screening methods for mastitis diagnosis are convenient and easily applicable, providing more detailed information for preventive measurements. Given that IMIs typically trigger an influx of macrophages into milk, a raised SCC has been extensively utilized as an indicator of mastitis. Consequently, SCC data from quarters, individual cows, and bulk milk are commonly used to monitor mastitis occurrence and milk quality (Berry & Meaney, 2006). While increase in CMT scores often corresponds to heightened SCC values, the extent to which CMT or SCC scores accurately reflect specific pathogen-induced IMIs remains uncertain. Dingwell et al. (2003) underscored limitations in CMT sensitivity and specificity for IMI determination. The primary weakness of scored CMT lies in its limited specificity when identifying udder quarters afflicted with specific mastitis pathogens (Calderon & Rodriguez, 2008). Fairly speaking, CMT is a rapid and cheap test for indirect determination of SCC in milk (Midleton et al., 2004) offering a practical and convenient means of on-farm IMI detection through milk sample testing. The concordance between CMT results and bacteriological findings ranges from 70 to 86%, varying depending on the causative agent (Sanford et al., 2006).

The dynamic changes in SOD and GPX activity in milk at the beginning of lactation, related to udder health status, are shown in Figures 1 and 2.

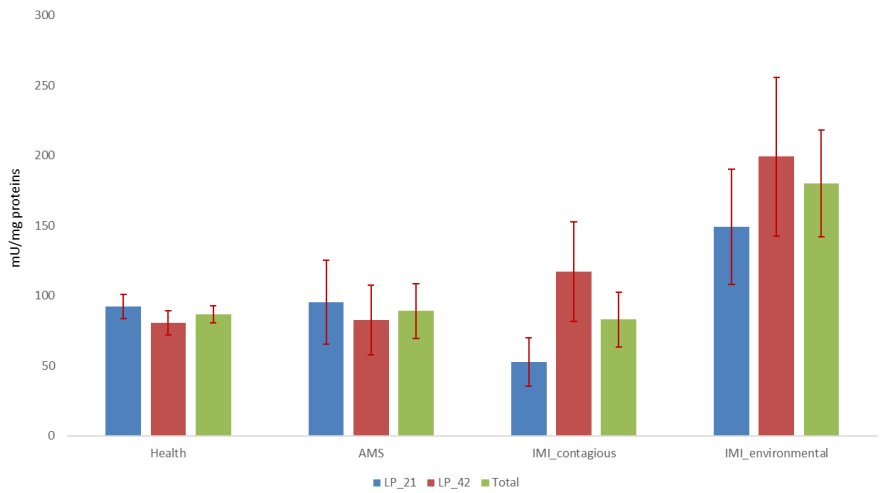

Figure 1. Average activity of SOD (mU/mg proteins) in milk serum related to the health status of cows' mammary gland

Mastitis pathogens have influenced SOD activity in milk, especially in LP_42. The environmental mastitis pathogens increase SOD activity in milk more drastically compared to SOD activity in milk from healthy cows and cows with AMS.

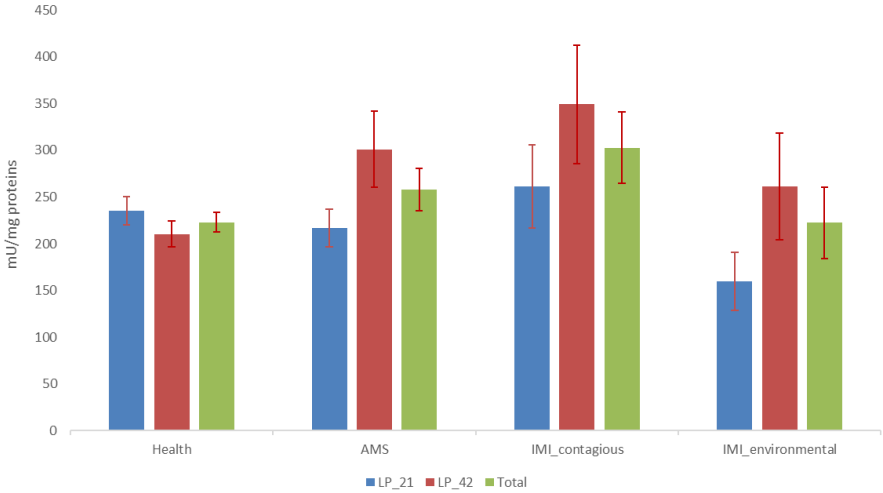

Figure 2. Average activity of GPX (mU/mg proteins) in milk serum related to the health status of cows' mammary gland

As shown in Figure 2, the average GPX activity was higher in milk from cows suffering from IMI caused by contagious mastitis pathogens, especially in LP_42. Both charts indicated that SOD and GPX activity in milk increased when IMI was present.

By reviewing the literature, limited data about SOD and GPX activity in milk were found (Lindmark-Maensson & Akesson, 2000; Filipović et al., 2005; Kasapović et al., 2005). Nevertheless, there is certain disparity in the reported SOD and GPX activity levels in milk across different studies (Stagsted, 2006; Kovačeva et al., 2007).

GLM revealed that Y_S of calving ($p<0.001$) and the presence of mastitis pathogens in milk ($p<0.05$) have a statistically significant influence on SOD and GPX activity in milk (Tab. 4). TDM showed a statistically significant influence on GPX activity in milk ($p<0.05$) but had no statistically significant influence on SOD activity in milk. There was a low ($r = -0.172$), but statistically significant ($p<0.01$) negative correlation between SOD and GPX activity in milk.

Table 4. GLM for the influence of fixed variables on SOD and GPX activity in milk

Fixed variable	df	SOD[a]	GPX[b]
Model[a,b]	17	4.639***	9.986***
P	4	0.441^{NS}	0.112^{NS}
Y_S	7	6.748***	41.781***
LP	1	0.467^{NS}	1.959^{NS}
TDM	1	0.912^{NS}	5.448*
Mastitis pathogen	3	3.224*	2.890*
Error	281		
Total	298		
[a]R^2 = 0.854; [b]R^2 = 0.926			

Legend: ***Significant at level p<0.001; *Significant at level p<0.05; ^{NS}non-significant

Celi et al. (2010) emphasize that SOD is a pivotal player and the first defense line against harmful ROS. GPX activity plays a crucial role in safeguarding animal tissues from oxidative damage by facilitating the reduction of hydrogen and lipid peroxides, and therefore, it is recognized as an indicator of oxidative stress (Tuzun et al., 2002). When parturition begins, there is an uptick in ROS while SOD and GPX values start to decline. In addition, the colostrum is a potential source of ROS due to peroxidation of the omnipresent macromolecules and the role of ROS in unspecific protection against bacteria. As outlined by Shoji et al. (2003), activated phagocytes generate substantial quantities of superoxide as part of the mechanisms employed to neutralize foreign organisms. In this context, the escalated activity of SOD and GPX observed in the period after parturition was a consequence of the elevated synthesis of ROS triggered by the immune response to mastitis pathogens. Protection against ROS is completely achieved through increased GPX activity because SOD actually contributes to dismutation of superoxide to hydrogen peroxide, which in turn serves as a substrate for GPX catalyzing their reduction.

This research has shown that there is a correlation between mastitis pathogens and antioxidative enzymes in dairy cows. When a dairy cow is afflicted with mastitis, whether clinical or subclinical, the immune system responds to the invading pathogens (usually bacteria) by releasing various inflammatory molecules and immune cells. This immune response generates ROS as a part of the body's defense mechanism to eradicate pathogens. While ROS are essential for fighting infections, excessive and uncontrolled production can lead to oxidative stress, causing damage to surrounding tissues and impairing overall cows' well-being. To counterbalance the potentially harmful effects of ROS, cows have antioxidative defense mechanisms in place. Antioxidative enzymes SOD, GPX, and CAT help neutralize ROS and maintain a balanced oxidative state within the body. These enzymes play a crucial role in protecting cells and tissues from oxidative damage. Research indicates that the presence of mastitis pathogens can influence the activity and expression of antioxidative enzymes in the mammary gland (Matei et al., 2011; Darbaz et al., 2019). Different pathogens may elicit varying responses in terms of oxidative stress and antioxidative enzyme activity. The increased activity of antioxidant enzymes in dairy cows that have mastitis in the period of early lactation may indicate that oxidative stress is exacerbated. It is important to note that SOD and GPX are not the only antioxidants that are important for protecting against mastitis. Other antioxidants, such as vitamin E and vitamin C, also play an important role. Andrei et al. (2010) reported SOD activity of 1.044±0.17 U/ml in milk from healthy cows and 1.066±0.15 U/ml in cows afflicted with mastitis. The same authors noted SOD activity in blood from healthy cows at 1688.4±165.48 U/g Hb, compared to 1764.5±110.46 U/g Hb in cows with mastitis. In addition, Berry & Meaney (2006) emphasized the GPX role in protection of mammary gland tissue from the hazardous activity of ROM and alleviating oxidative stress intensity. Andrei et al. (2011) reported heightened GPX activity in milk from cows with AMS compared with their healthy counterparts. They found no significant difference in GPX activity in blood between healthy cows and cows with mastitis. Some studies have shown that cows with mastitis have lower levels of SOD and GPX activity in their milk compared to healthy cows (Stagsted, 2006; Kovačeva et al., 2007). This finding suggests that oxidative stress may play an important role in development and progression of mastitis. However, Sordillo & Aitken (2009) concluded that there is limited knowledge of the relationship between SOD activity and udder health so more research data is needed. In their study related to subclinical mastitis in goats, Darbaz et al. (2019) observed remarkable rises in GPX activity alongside concurrent declines in SOD activity. These alterations exhibited a clear correlation with milk SCC. Other factors, including the age, parity, and the number of offspring, showed no significant association with these antioxidant enzyme activity patterns.

CONCLUSION

Effective management of the transition period critically shapes cows' profitability in the subsequent lactation phase. The farmers managing the transition period of dairy cows are mainly focused on two distinct objectives: optimization of energy intake and disease prevention. However, novel models gain importance in elucidating the genesis of transition-related disorders. Both oxidative stress and mastitis occurring in the most challenging transition period of dairy cows need to be addressed through a holistic approach, emphasizing the interconnectedness and interdependence of feeding, rearing conditions, and immunity. Importantly, the SOD/GPX ratio in milk plays a pivotal role in balancing ROS within milk. The observed increase in SOD and GPX activity in milk is likely to reflect the adaptive responses by cows to combat oxidative stress. The data revealed that environmental mastitis pathogens elevate SOD activity in milk more drastically while increased GPX activity in milk was accompanied by IMI caused by contagious mastitis pathogens. Consequently, an imbalance between the heightened production of ROS and decrease in antioxidant capacity near parturition could amplify oxidative stress and contribute to postpartum disorders in dairy cows. Providing a balanced diet with adequate antioxidants and managing environmental stressors can help reduce oxidative stress during the transition period and decrease the risk of mastitis in early lactation.

Conflict of interest: The authors declare that they have no conflict of interest.

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