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Serum Zonulin Levels and Fecal Scoring as Probable Early Predictor of Intestinal Inflammation Among Calves with Diarrhea: Cohort Study

İshalli Buzağılarda Bağırsak İnflamasyonunun Olası Erken Göstergesi Olarak Serum Zonulin Seviyeleri ve Dışkı Skorlaması: Kohort Çalışması

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ABSTRACT Objective: Zonulin, probably very well known protein, modulate the integrity of intercellular connections within the gut. The objective of this present field study was to validate the relationship among fecal scores and circulating zonulin levels as probable early indicator of intesinal inflammation among calves with diarrhea. **Material and Methods:** Upon arrival at a milk-fed veal facility, 34 calves were scored for fecal consistency on a scale of 0 to 3. Calves with a fecal score of 2 (loose feces)-3 (watery feces) were classified as diarrheic, with score of 0 and 1 were denoted as apparently healthy calves. Commercially available specific Bovine Zonulin ELISA test kits were used. The present study was approved by the local ethic committee of Aydın Adnan Menderes University-HADYEK and ethical guidelines were stricly followed up with written owner consent was available for all calves enrolled. **Results:** The mean zonulin levels (ng/mL) diarrheic calves with score 2 and score 3 were detected as 41.04±4.5 and 61.27±4.80, respectively presenting statistically significant alterations in contrast to healthy calves 20.35±2.93 ($p<0.05$). **Conclusion:** It should not be unwise to draw take home messages that intestinal inflammation and thus circulating zonulin levels should in correlation with fecal scoring be interpreted on field conditions for prognosis and available treatment practices.

Keywords: Intestinal inflamation; fecal score; zonulin

ÖZET Amaç: Muhtemelen çok iyi bilinen bir protein olan zonulin, bağırsaktaki hücreler arası bağlantıların bütünlüğünü modüle eder. Bu mevcut saha çalışmasının amacı, ishalli buzağılarda bağırsak inflamasyonunun olası erken göstergesi olarak dışkı skorları ve dolaşımdaki zonulin seviyeleri arasındaki ilişkiyi doğrulamaktı. **Gereç ve Yöntemler:** Sütçü yönde besi işletmesine varıldığında, 34 buzağı dışkı kıvamı için 0 ila 3 arasında bir ölçekte puanlandı. Dışkı puanı 2 (gevşek dışkı)-3 (sulu dışkı) olan buzağılar ishalli olarak sınıflandırılırken, 0 ve 1 görünüşte sağlıklı buzağılar olarak gösterildi. Sığır spesifik ticari Zonulin ELISA test kitleri kullanıldı. Bu çalışma, Aydın Adnan Menderes Üniversitesi yerel etik komitesi HADYEK tarafından onaylanarak, etik kurallar sıkıca takip edilirken çalışmaya dâhil edilen her bir buzağı için yazılı onam formu elde edildi. **Bulgular:** Ortalama zonulin seviyeleri (ng/mL) ishalli buzağılar arasında skor 2 ve skor 3 olanlar açısından sırasıyla 41,04±4,5 ve 61,27±4,80 olarak saptandı ve sağlıklı buzağıların 20,35±2,93 aksine istatistiksel olarak anlamlı değişiklikler gösterdi ($p<0,05$). **Sonuç:** Bağırsak inflamasyonu ve dolayısıyla dolaşımdaki zonulin seviyelerinin; prognoz ve mevcut sağaltım uygulamaları için saha koşullarında fekal skorlama ile korelasyon içinde yorumlanmasının mümkün olacağını öne sürmek yerinde olabilir.

Anahtar Kelimeler: İntestinal inflamasyon; fekal skor; zonulin

Arousing interest was gained significant modifications as significance of ordinary gut barrier function is enhanced in ruminants via understanding multistorey vulnerability to microbial antigens impending from pre-gastric fermentation compartments.[1] Nowadays due to increasing evidence of proof the efficacy of intestinal health on animal production and health status have better understood.[2] Multifactorial alterations disrupting available management applications resuting with animal's intestinal health switching from health to disease conditions [i.e. altered barrier function with a consequence of leaky gut]. Selected factors included weaning, heat stress and diarrhea.[3-10] To the present

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author knowledge no previous study attempted to evaluate relationship among leaky gut, intestinal inflammation, fecal scoring and circulating zonulin levels, which prompted to perform this study at this purpose.

Zonulin, a very well recognized analogue of *Vibrio cholerae* zonula occludens toxin, has been elucidated as gut permeability biomarker. Though it has been denoted as significant regulator of epithelial tight junctions, several disease conditions in calves and cattle dedicated expressed zonulin levels.[6-8,10] Hence zonulin are mainly expressed within the small intestine, elevated concentration of zonulin levels could support this notion, in which another purpose of this study evolved serum zonulin levels and fecal scoring as probable early predictor of intestinal inflammation among calves with diarrhea.

MATERIAL AND METHODS

DEMOGRAPHIC FIELD DATA

This prospective field study was performed (along by the help of a veterinary surgeon, whom withdrawn blood samples) through a milk-fed veal facility in Aydın Municipality, Türkiye. All enrolled calves were participated at this study with written owner consent. The present study was approved by the local ethic committee of Aydın Adnan Menderes University-HADYEK on October 27, 2021, with no: 64583101/2021/146. Throughout this cohort study ethical guidelines were strictly followed up, and written owner consent was available for all calves enrolled. Guide for the Care and Use of Laboratory Animals (www.nap.edu/catalog/5140.html) were deemed available, were taken into consideration throughout all work. There were no pain or discomfort reported or detected in any animal, as this study did not involved any drug administration.

SAMPLING AND FIELD RESEARCH

In a total of 34 calves enrolled, 1.5 mL blood was withdrawn (via field veterinary surgeon) from *Vena jugularis* into anticoagulated tubes. There afterwards obtained samples were entirely sent to laboratory work. Commercially available Bovine Zonulin ELISA test kits (MyBiosource ELISA kits, USA) were purchased (Turkish side distributor RDA Group, İstanbul). Performed and used methodology was similar to prior articles by the present author.[6,7] Fecal scoring system adopted from Graham et al[11] were shown above in Table 1.

TABLE 1: Fecal scoring chart.[11]

Fecal score	
0	Normal consistency
1	Semiformed or pasty
2	Loose feces
3	Watery feces

STATISTICAL ANALYSIS

Zonulin levels obtained from healthy and diarrheic calves in different fecal scores were tabulated as mean and standard error of mean. Statistical analyses were performed via Kruskal-Wallis ANOVA with multiple comparison tests. Statistical analyses were performed with Graphpad Prism Software (GraphPad Software, Boston, USA) and p value were set as <0.05.

RESULTS

All tabulated data along with statistical values were given at Table 2 and Figure 1 with boxplot analytes. Additional relevant data belonging to scores and enrolled cases were shown above (Figure 2, Figure 3).

DISCUSSION

In a prior study serum zonulin levels were analyzed in diarrhea-predominant irritable bowel syndrome and constipation-predominant irritable bowel syndrome. Matchin groups were evolved as comparison of healthy controls and celiac disease cases. Interestingly permeability increase was clinically relevant as zonulin levels showed

TABLE 2: Mean zonulin levels among diarrheic and healthy calves enrolled at this study.

Groups	Zonulin (ng/mL) $\bar{X}\pm SE$
Healthy calves	20.35 ± 2.93^{a}
Diarrheic calves (Score 2)	41.04 ± 4.5^{b}
Diarrheic calves (Score 3)	61.27 ± 4.80^{b}

^{a,b}: Different letters in column is statistically significant.

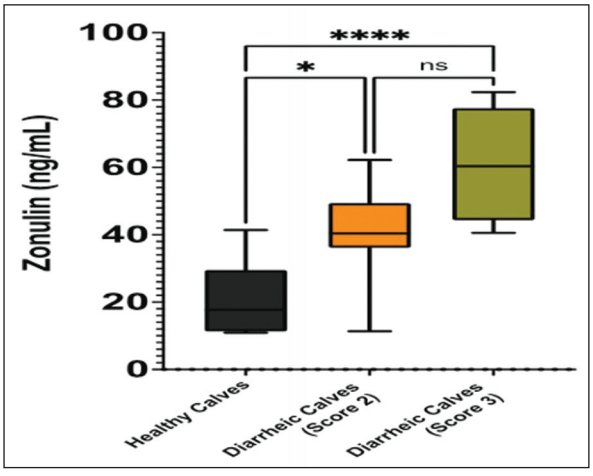

FIGURE 1: Boxplot analytes of serum zonulin levels and fecal scoring among calves enrolled at this study.

FIGURE 2: Scores denoting as 1, 1 and 3 in healthy 2 and other one diarrheic calf.

FIGURE 3: Healthy (on the left side) and diarrheic 2 other calves (on the middle and right side).

correlated with stool frequency in patients with irritable bowel syndrome with diarrhea.[12] Zonulin has been elucidated as regulator of intestinal permeability in which increased circulating zonulin throughly been detected in several gastrointestinal/non-gastrointestinal issues.[13-15] Zonulin changes small intestinal intercellular tight junction integrity through influencing epidermal growth factor receptor via proteinase-activated receptor 2.[13] Furthermore zonulin is capable of elevating colonic permeability as a repond against existing enteric bacteria.[16] In the present study mean zonulin levels (ng/mL) among diarrheic calves with score 2 and score 3 were expressed as 41.04±4.5 and 61.27±4.80, respectively, denoting significant elevation in comparison to healthy calves 20.35±2.93 ($p<0.05$) (Table 1, Figure 1, Figure 2).

A novel research with a subjected experimental indomethacin injection protocole aimed at analyzing for replication of leaky gut in calves, attained interesting results. Repeated indomethacin injections over a 2 days period exhibited leaky-gut-like symptoms mainly occuring at distal jejunum, ileum, and colon along with elevated gut permeability.[2] In the present study with the prior experience and field studies of the presenting author all diarrheic calves were enrolled, were free of any previous medication (Figure 3).[6-8,10] However at the end of the study, responsible veterinary surgeons were informed about relevant data, for their self treatment decision and applications.

In a prior research, also performed by the present author, the specificity of zonulin as a noninvasive selected biomarker of gut barrier functioning for detecting and differentiating calves suffering from diarrhea was analyzed. Besides supportive aim composed determining the existence of leaky gut. Through purchased commercially available Bovine Zonulin ELISA test kits, eleven diarrheic and other relevant healthy calves zonulin concentrations (ng/mL) expressed as (±SEM), presented marked differences ($p<0.001$) as 57.97±4.250 vs. 26.43±3.528, respectively.[10] Apart from the latter mentioned research, in this field study the relationship between fecal scoring and circulating zonulin levels were subjected to interpretation. Calves with scores 2 and 3 were deemed diarrheic, were shown to have elevated zonulin levels, probably indicating intestinal permability alterations and intestinal inflammation.[6-8,10,17-22]

CONCLUSION

Limited studies have shown increased intestinal permeability in several disorders among calves. Validating serum biomarkers for altered intestinal permeability in intestinal inflammation among calves with diarrhea will facilitate research and pathophysiology-based therapy.

Source of Finance

During this study, no financial or spiritual support was received neither from any pharmaceutical company that has a direct connection with the research subject, nor from a company that provides or produces medical instruments and materials which may negatively affect the evaluation process of this study.

Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

This study is entirely author's own work and no other author contribution.

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