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IGF-1 and GH alterations in lambs with intestinal inflammation

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Abstract: Insulin-like growth factor-1 (IGF-1) acts on the development of internal organs such as the small intestine and muscle in all animal species. Similar to IGF-1, GH is also essential for growth and is an effective hormone on intestinal development during neonatal period. The aim of this study was to investigate the effects of diarrhea on IGF-1 and GH hormones in lambs with intestinal inflammation up to 63 days of age. The study material consisted of 15 healthy and 15 diarrheic lambs. Blood and fecal samples were collected on the first day of life and on the 7th, 14th, 21st, 35th, 49th and 63rd postnatal days consecutively. Diarrhea was observed on 6 animals on the 7th day and 9 animals on 14th day visitation. IGF-1 showed statistically significant differences ($P < 0.01$) between diarrheic and healthy animals on all measurement days. A significant association was found between the cut-off values determined after ROC analysis of the 7th day (Sens: 93.33%, Spec: 66.67%, $P = 0.004$) and 14th day (Sens: 73.33%, Spec: 80%, $P = 0.0002$) values of IGF-1 and diarrhea. Considering 14th day of IGF-1, a logistic regression analysis was performed, the risk of diarrhea is OR = 7.00 times higher if the concentration of the parameter is above the cut-off value. ROC analysis also showed significant cut-off values for GH on 14th day. In terms of sensitivity, the highest value was IGF-1's 7th day value, therefore it can be preferred for the determination of intestinal inflammation in cases with diarrhea. As a general evaluation, it is seen that the highest performance was given by the 7th day IGF-1 values according to the Youden's J index. In conclusion, elevations in IGF-1 and GH concentrations may be associated with intestinal inflammation. The inducing effect of the inflammatory response on IGF-I and GH may strengthen the relationship between the two parameters.

Key words: Lamb, diarrhea, cryptosporidium, IGF-1, growth hormone

1. Introduction

Insulin-like growth factor-1 (IGF-1) is a hormone that is mainly secreted by the liver and works along with the presence of growth hormone (GH) and acts on the development of internal organs such as the small intestine and muscle in all animal species [1]. Serum IGF-1 and GH concentrations are affected by genetic and nongenetic factors. These factors include sex, breed, birth weight, feed ration, nutritional status, litter size, weaning age and internal parasite load [1–4]. IGF-1 is highly expressed in the intestinal epithelial cells during the fetal and neonatal period and has been shown to stimulate intestinal development [5,6]. In cases with gastroenteritis, IGF-1 supports the intestinal barrier and helps to prevent the harmful effects of luminal pathogenic bacteria and their toxins [7,8].

Similar to IGF-1, GH is also essential for growth and body development in general [7]. In addition to its growth-related effects, GH is also an effective hormone on intestinal development during the neonatal period [9]. Intestinal epithelial cells with high turnover capacity are directly induced by GH [10,11]. Similar to IGF-1, it is GH is also associated with infective diarrhea. It has been reported that GH concentrations may be elevated just before the detection of infective factors causing intestinal inflammation and after recovery as well [7].

The aim of this study was to investigate the effects of diarrhea on IGF-1 and GH hormones in lambs with intestinal inflammation up to 63 days of age.

2. Materials and method

2.1. Study population and grouping

The study material consisted of 14 female and 16 male lambs in a total of 30 lambs of Kıvırcık breed. The lambs used in the study were fed exclusively with milk from their dams without separation from the time of birth. Visitations were conducted on the first day after a lamb was born and after on the 7th, 14th, 21st, 35th, 49th and

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63rd postnatal days consecutively. In any time of the visits, lambs that showed gastrointestinal symptoms were taken into the study group and lambs that remained healthy formed the control group. Lambs that formed the study group showed exclusively gastrointestinal symptoms. The gastrointestinal status was scored as mild, moderate and severe in help with the universal diarrhea scaling system called Vesikari Score [12]. Lambs were marked and given a unique number right after birth to be distinguishable throughout the study and their weight measurements were performed in all visitations. Their sex and litter size were also recorded. Their dams had been fully vaccinated and given routine antiparasitic medicine. Routine clinical examinations (body temperature measurement, heart rate and respiration count measurement) have also been conducted.

2.2. Sample collection

Blood samples were collected from vena jugularis and taken into 7 mL EDTA and 7 mL serum vacutainer tubes. First blood samples were collected approximately 12 h after the lambs were born and fed on colostrum on the first day of life and on the 7th, 14th, 21st, 35th, 49th and 63rd postnatal days consecutively. Stool samples were also obtained on the stated days while observing the animal or through rectal manipulation into single-use 10 mL fecal sample tubes. Blood and fecal samples were brought to Bursa Uludağ University Veterinary Faculty Central Laboratory immediately. Blood tubes were centrifuged at 5000 rpm for 15 min, plasma and serum samples were separated into commercially available cryotubes and stored at –80 °C.

2.3. Laboratory analysis

Sheep specific commercial ELISA kits were used to measure GH and IGF-1 concentrations (Shanghai Sunred Biological Technology Co., Ltd, Shanghai, China; Sheep GH Elisa kit Catalog no: 201-07-0075 and Sheep IGF-1 Elisa kit Catalog no: 201-07-0005). Serum samples were measured by double-antibody sandwich ELISA technique and Epoch microplate reader (BioTek Instruments, Winooski, VT, USA) was used for the results. The highest standard concentration range of GH to be measured in serum was 12 ng/mL; the lowest concentration was 0.75 ng/mL (12, 6, 3, 1.5, 0.75 ng/mL). In the measurement of IGF-1 values, the highest standard concentration was 600 ng/mL and the lowest was 37.5 ng/mL (600, 300, 150, 75, 37.5 ng/mL). No dilution was required in serum samples when performing the ELISA kit procedure. As a result of GH measurement, the R^2 value of the standard graph (% accuracy of the study) was 0.997 (99%); The R^2 value of IGF-1 standard graph was found to be 1.00 (100%).

Commercial rapid ELISA test kits (Antigen rapid BoviD-5 Ag Test Kit; BioNote Inc., Hwaseong, South Korea) were used for the etiological analysis of fecal samples *(Cryptosporidium parvum, rotavirus, coronavirus, E.coli K99, Giardia spp.).* These tests are qualitatively based on immunochromatographic measurements and their sensitivity and specificity are featured by the manufacturer as 98% and 99%, respectively. Parasitological examinations of fecal samples to confirm *Cryptosporidium parvum* presence were performed in addition to rapid diagnostic kits. For this purpose, a modified Ziehl–Neelsen staining technique was used.

2.4. Statistical analysis

Normality of the data was tested with the Shapiro–Wilk test. One way repeated measurement ANOVA test for nonnormally distributed data (Friedman's method) were used in both groups to show possible differences among each measurement days. In case of normal distribution, data were analyzed by t-test for independent groups. For the data that are nonnormally distributed, Mann–Whitney U tests were used. Descriptive statistic outcomes were given as mean ± SE for parametric test and median (min-max) for nonparametric test. Error bar graphics have been generated for normally distributed data to compare healthy and diarrheic animals for IGF-1 and GH while boxplot graphics were used for nonnormally data. Relationships between variables were examined by Pearson correlation and Spearman rank correlation coefficients. Cut-off values of IGF-1 and GH variables for determining of diarrhea positivity were analyzed by ROC analysis. The cut-off values were determined according to the Youden's J index in those with statistically significant area under curve (AUC) values in direct with the study of Fluss et al. [13]. Odds ratio (OR) was calculated according to the significant cut-off values with a chi-square test for risk amount of diarrhea positivity. Statistical analyzes were performed with IBM SPSS v22 (IBM Corp., Armonk, NY, USA) and MedCalc V19.1.3 (MedCalc Software Ltd., Ostend, Belgium) programs.

3. Results

Study material consisted of 30 lambs. Four of the lambs included in the study group (n = 15) were twin births and 7 were single births whereas 3 of the lambs in the control group (n = 15) were twinborn and 9 of the lambs were single born. Clinical examination parameters for both groups are shown in Table 1. Diarrhea was observed on 6 animals on the 7th day and 9 animals on the 14th day visitation. *Cryptosporidium parvum* was detected in two of the six lambs with diarrhea on 7th day visit, while no agents were found in four of them. Six diarrheic lambs on the 7th day were treated with halofuginone lactate for 7 days and diarrhea was resolved on the 4th day of the treatment. On the 14th day visit, *Cryptosporidium parvum* was also detected in all 9 animals showing signs of diarrhea. Same treatment was given to the lambs and course of diarrhea

was resolved in 3 to 5 days. In the treatment period, there were regular contacts with the veterinarian working in the farm for updates. Besides, stool samples were stained with Ziehl-Neelsen technique and presence of *Cryptosporidium* spp. oocysts were confirmed.

Statistical analysis which was conducted on the 7th and 14th day was applied in two statistical models in this study. In first model, 6 animals with diarrhea on 7th day and 9 animals with diarrhea on 14th day were compared with 15 healthy animals of those same days (Table 2). There were no statistically significant differences between the repeated measurement days in terms of IGF-1 among each group. Friedman's method for repeated measurements was performed and outcome showed no statistical differences for control (P = 0.762) and study group (P = 0.269).

There were statistically significant differences between study and control animals on all measurement days in the first statistical model (Table 2). IGF-1 concentrations

Table 1. Mean (± SE) clinical parameters of study (n = 15) and control (n = 15) groups. T (˚C): body temperature, R (min): respiratory count, P (min): heart rate.

	Healthy lambs			Diarrheic lambs		
	T (˚C)	R (min)	P (min)	T (˚C)	R (min)	P (min)
1st day	38.60 ± 0.10	44.80 ± 1.64	100.46 ± 3.10	39.58 ± 0.13	68.93 ± 1.67	98.26 ± 3.28
7th day	38.78 ± 0.12	46.66 ± 1.73	83.86 ± 1.37	40.43 ± 0.21	60.40 ± 5.17	110.93 ± 5.30
14th day	38.56 ± 0.12	37.73 ± 1.10	82.93 ± 2.73	40.90 ± 0.19	68.0 ± 1.77	86.80 ± 2.23
21st day	38.74 ± 0.09	49.60 ± 0.99	82.40 ± 1.87	39.11 ± 0.29	56.66 ± 1.73	86.53 ± 1.95
35th day	38.76 ± 0.08	42.00 ± 1.97	80.26 ± 1.35	38.08 ± 0.22	47.66 ± 1.99	90.40 ± 3.14
49th day	37.63 ± 0.14	40.46 ± 1.29	86.00 ± 2.78	38.51 ± 0.25	54.33 ± 1.64	91.60 ± 2.12
63rd day	38.48 ± 0.13	39.33 ± 0.96	80.93 ± 1.11	38.04 ± 0.25	48.06 ± 1.39	87.33 ± 1.75

Table 2. Mean (± SE) or median (min-max) values of body weight (BW), IGF-1 and GH concentrations of study (1st and 2nd statistical model) and control groups and statistical significancies between them. *(C: control group) (Both statistical model are comparison with the control groups.)

	1st day	7th day	14th day	21st day	35th day	49th day	63rd day
BW (kg)–1st		5.8 (4.7–8.25)	7.17 (5.16–10.20)				
BW (kg)–2nd	3.40 ± 0.21	5.9 (4.9–8.45)	7.15 (5.15–10.30)	8.15 (6.5–11.5)	11.3 (6.25–16.3)	12.99 ± 1.07	13.64 ± 1.36
BW (kg)–C	4.28 ± 0.24	6.0 (3.25–8.15)	7.35 (4.1–10.85)	8.4 (4.75–13.1)	10.8 (7.65–16.1)	15.56 ± 0.85	20.05 ± 1.10
P values 1st		0.379	0.840				
P values 2nd	0.006	0.399	0.820	0.902	0.291	0.01	< 0.001
IGF-1 (ng/mL)–1st		260.22 ± 20.40[a]	269.88 ± 17.25[a]				
IGF-1 (ng/mL)–2nd	219.29 ± 15.42	256.690 ± 19.63[a]	253.68 ± 15.63[a]	236.71± 13.63	233.13 ± 11.96	257.47 ± 27.40	246.63 ± 14.81
IGF-1 (ng/mL)–C	175.51 ± 14.96	184.60 ± 17.47[b]	185.44 ± 11.74[b]	185.44 ± 11.74	176.57 ± 9.14	171.31 ± 9.17	180.47 ± 8.45
P values 1st		0.012	0.003				
P values 2nd	0.028	0.011	0.007	0.008	0.004	0.004	< 0.001
GH (ng/mL)–1st		3.39 ± 0.13[a]					
GH (ng/mL)–2nd	2.50 (1.14–4.34)	2.52 (1.05–5.61)	3.02 ± 0.27[a]	2.69 ± 0.17	2.70 ± 0.17	2.43 (0.87–7.95)	2.64 ± 0.21
GH (ng/mL)–C	2.22 (0.41–4.38)	2.44 (0.77–3.72)	1.87 ± 0.29[b]	1.98 ± 0.23	1.92 ± 0.25	1.31 (0.84–3.38)	1.79 ± 0.21
P values 1st			0.004				
P values 2nd	0.328	0.175	0.008	0.02	0.018	0.431	0.009

[a,b]: Define statistical signifigacnce among columns for the same parameter between diarrheic and control groups for the first statistical model.
1st statistical model defines only 7th and 14th comparison for IGF-1 and BW, and 14th day comparison for GH only.
2nd statistical model defines comparisons for all of the measurement days.

on day 7 (n = 6) and day 14 (n = 9) which was the time diarrhea was first observed was found to be 260.22 ± 20.40 ng/mL and 269.88 ± 17.25 ng/mL, respectively. IGF-1 values for healthy group were measured as 184.60 ± 17.47 and 185.44 ± 11.74 ng/mL, respectively on same days (P < 0.01) (Table 2). Cut-off values for IGF-1 were >246.085 ng/mL (Sens: 93.33%, Spec: 66.67%, P = 0.0043) and >187.421 ng/mL (Sens: 73.33%, Spec: 80%, P = 0.0002) on 7th (Figure 1) and 14th (Figure 2) day, respectively which was attributed to initial term of observed diarrhea of lambs**.** Besides, as a result of the ROC analysis on the 7th day of IGF-1, the risk of diarrhea is OR = 7.00 times higher if the concentration of the parameter is above cut-off value (CI 95%: 2.27–95.75, P = 0.01). The risk of diarrhea is OR = 5.50 times higher for the 14th day (CI 95%: 2.56–95.75, P = 0.02). ROC analysis also showed significant cut-off values for GH on the 14th day with the diarrheic animals involved (Cut-off: > 2.569 ng/mL, Sens: 73.33%, Spec: 100%, P = 0.0001, AUC: 0.853, Youden's J index: 0.7333) (Figure 3, Table 3). Comparison of healthy and diarrheic groups in case of IGF-1 has also been shown in error bar graphics in Figure 4 for each sampling days.

Repeated measurements of GH levels for the first statistical model of study group and healthy group are shown in Table 2. Statistical differences were not observed (P = 0.420). Comparison of control and study groups in case of GH have also been shown in error bar graphics for normally distributed data while boxplot graphics were used to express nonnormally distributed data in Figure 5 for each sampling days.

In the present study there were positive correlations between IGF-I and GH on 1st (R = 0.48, P < 0.05), 7th (R = 0.52, P < 0.05), 14th (R = 0.63, P < 0.01), 49th (R = 0.80, P < 0.01) and 63rd (R = 0.71, P < 0.01) days in diarrheic group. In addition, there were positive correlations between IGF-I and GH on 7th (R = 0.60, P < 0.01) and 35th (R = 0.52, P < 0.01) days in healthy lambs' group. Negative correlations between body weight and IGF-1 was found on 35th (R = –0.66, P < 0.01) , 49th (R = –0.67, P < 0.01), and 63rd (R = –0.63, P < 0.01) days in diarrheic group.

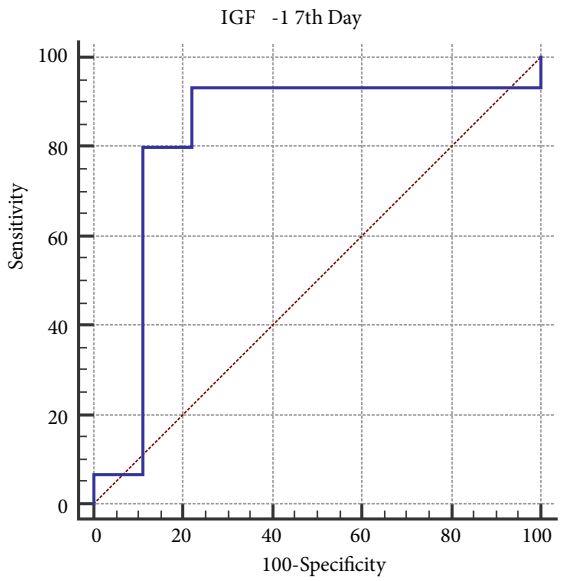

Figure 1. ROC graphic in terms of cut-off values of IGF-1 on 7th day for first model (n = 6).

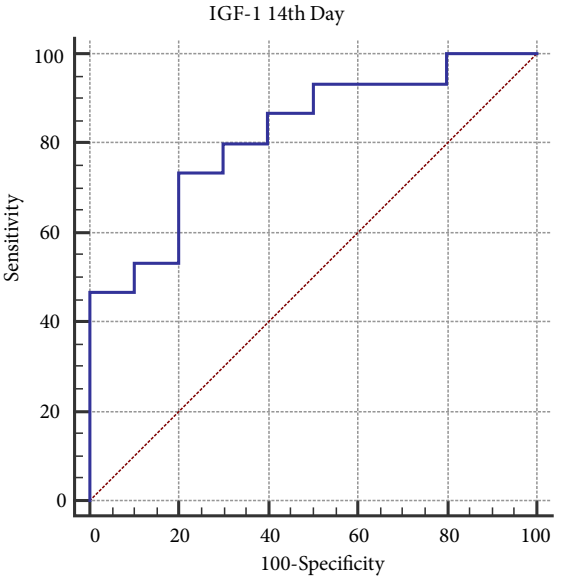

Figure 2. ROC graphic in terms of cut-off values of IGF-1 on 14th day for first model (n = 9).

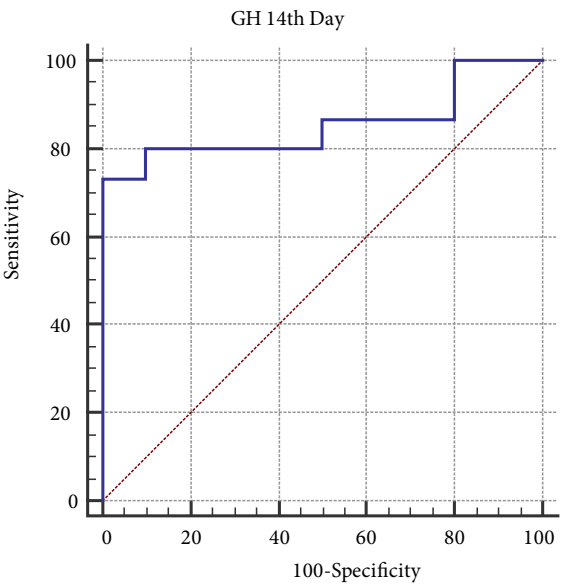

Figure 3. ROC graphic in terms of cut-off values of GH on 14th day for first model (n = 14).

Table 3. Cut-off, sensitivity, specifity, AUC, Youden's J index and P values of ROCN analysis of IGF-1 and GH on 7th and 14th days for both statistical model.

	Cut-off value	Sensitivity (%)	Specificity (%)	Youden's J	AUC	Standart error	95% CI	P values
IGF-1–7th day 1st model	>246.085	93.33	66.67	0.7111	0.822	0.113	0.613 to 0.947	0.0043
IGF-1–14th day 1st model	>187.421	73.33	80.00	0.5333	0.820	0.0854	0.616 to 0.943	0.0002
GH–14th day 1st model	>2.569	73.33	100.00	0.7333	0.853	0.0810	0.655 to 0.962	0.0001
IGF-1–7th day 2nd model	>209.794	80	80	0.6000	0.778	0.0952	0.589 to 0.908	0.003
IGF-1–14th day 2nd model	>187.421	93.33	73.33	0.6667	0.782	0.0936	0.594 to 0.911	0.002
GH–14th day 2nd model	>2.569	86.67	73.33	0.6000	0.764	0.0964	0.575 to 0.899	0.006

In the second statistical model of the analysis, lambs that had diarrhea in any time of the visit (which were 7th and 14th days) were put in the study group to form an equal number of animals for both of the groups throughout the study and rest of the visitations days were also analyzed statistically (21st, 35th, 49th, and 63rd days). Repeated measurement analysis of IGF-1 were also showed no statistical differences similar to the first model ($P = 269$). There were statistically significant differences between study and control animals on all measurement days in the second model (Table 2). IGF-1 concentrations were measured as 256.690 ± 19.63 ng/mL for study group on 7th day ($n = 15$) and 253.68 ± 13.63 ng/mL for healthy group ($n = 15$) on 14th day, respectively. Similar to the first model, cut-off values for IGF-1 when all 15 animals compared were >209.794 ng/mL (Sens: 80%, Spec: 80%, $P = 0.003$) and >220.129 ng/mL (Sens: 93.33%, Spec: 73.33%, $P = 0.002$) on 7th (Figure 1) and 14th (Figure 2) day, respectively. Repeated measurements of GH levels of the study group are shown in Table 2. Statistical differences were not observed ($P = 0.480$).

In the second statistical model, there were positive correlations between IGF-I and GH on 1st ($R = 0.48$, $P < 0.05$), 7th ($R = 0.57$, $P < 0.05$), 14th ($R = 0.61$, $P < 0.01$), 49th ($R = 0.80$, $P < 0.01$) and 63rd ($R = 0.71$, $P < 0.01$) days in diarrheic group. In addition, there were positive correlations between IGF-I and GH on 7th ($R = 0.60$, $P < 0.01$) and 35th ($R = 0.58$, $P < 0.01$) days in healthy lambs' group. Negative correlations between body weight and IGF-1 were found on 35th ($R = -0.66$, $P < 0.01$) , 49th ($R = -0.67$, $P < 0.01$) and 63rd ($R = -0.63$, $P < 0.01$) days in diarrheic group.

4. Discussion

The fluctuating course of plasma IGF-1 in neonatal ruminants may be associated with a significant effect of feeding period e.g. weaning [1]. A study by Medrano and Bradford [3] has shown that IGF-1 plasma concentrations are high in neonatal lambs and tend to decrease over time. However, the hormone measurements in that researchers study were performed with an interval of 1 month until the age of 4 months, and this situation cannot fully reveal the possible fluctuating course of IGF-1 in neonatal period. In the present study, there were no statistically significant changes in IGF-I levels among each sampling days for second statistical model. However, the initial IGF-I levels were measured lower compared to the 7th and 14th sampling days in healthy and diarrheic group for the first and second statistical models. Also, in our study, hormone measurements were conducted up to only 63 days of age when the lambs were feeding milk from their dams. In that fact, weaning age can be a particular factor in IGF-1 and GH concentration variations because of the high IGF-1 and GH composition in milk [14].

The increase in serum IGF-1 in the recovery of malnourished children with persistent diarrhea suggests that this increase may be correlated with the recovery status [15]. A study in infants notes that infants with lower birth weight compared to their peers had higher IGF-1 serum concentrations [16]. Babies with lower birthweight also have higher levels of GH which stimulates more IGF-1 expression and increases growth response in order to cope up with the weight gain delay [16]. This situation is parallel to our study, lambs with lower birth weight are more prone to have diarrhea in the 7th and 14th days and express IGF-1 in higher concentrations. Bhutta et al. [17] have shown that severe infections affect both IGF-1 production and induction of a related protease for IGF binding protein 3 in plasma. On the other hand, some studies suggest that malabsorption, maldigestion and endotoxemia may cause a decrease in IGF-1 levels [18–20]. In our study, there was no evidence of endotoxemia but moderate intestinal inflammation associated with *Cryptosporidium parvum* infection may have developed in lambs. Therefore, an increase in IGF-I levels may be associated with moderate inflammation and a nonsevere maldigestion. Differences in IGF-I levels at the time of birth between the diarrheic group and the control group may be related to the

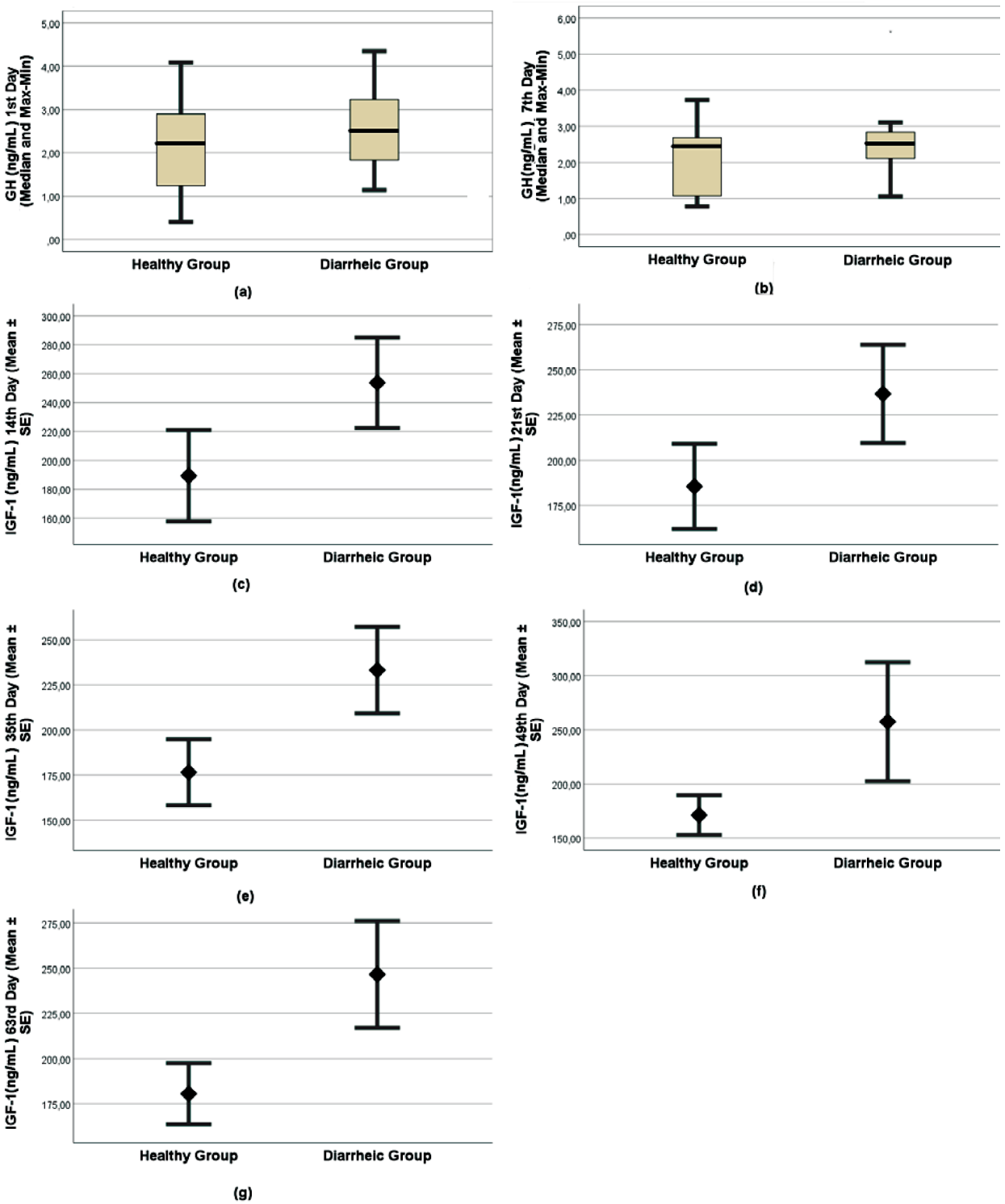

Figure 4. Error bar graphics of IGF-1 concentrations of healthy and diarrheic lambs in days 1(a), 7(b), 14(c), 21(d), 35(e), 49(f) and 63(g).

bodyweight differences between the groups. Although the difference between live weights disappeared on the 7th and 14th days, it is seen that the difference between the diarrheic and healthy animals in terms of IGF-1 levels continues (Table 2). In addition, positive correlations were obtained between IGF-1 and GH for both groups

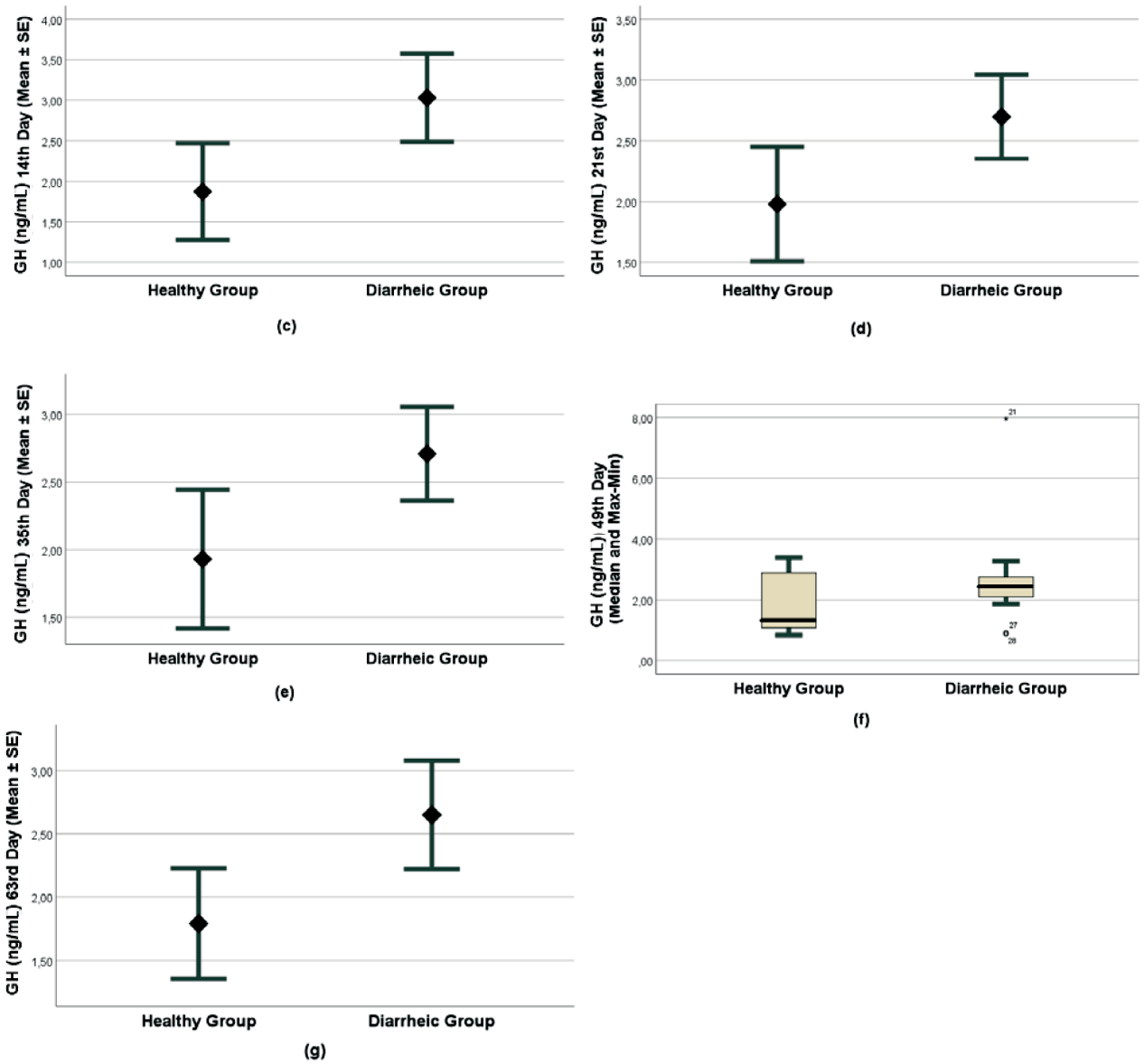

Figure 5. Error bar graphics and boxplot graphics of GH concentrations of healthy and diarrheic lambs in days 1(a), 7(b), 14(c), 21(d), 35(e), 49(f) and 63(g).

on certain days, but the most striking correlation link was found on the 7th and 14th days for the diarrheic group. It is thought that elevations in both hormones may be logical on the 7th and 14th days, which were the times that diarrhea was first observed. All of this can be interpreted that significantly high GH and IGF-1 concentrations may have had a role in intestinal regeneration and improved recovery status for these lambs.

In this study, cut-off limits, sensitivity and specificity values of the IGF-I and GH levels were evaluated in diarrheic and healthy lambs using the ROC analysis. [13]. In first statistical model, ROC analysis of IGF-1's revealed (7th day: OR: 7.00, Sens-Spec: 93.33%, 73.33%, P = 0.004, 14th day: OR: 5.5, Sens-Spec: 73.33%, 80%, P = 0.0002, Table 3) high sensitivitiy for both of them, respectively. Thus the cutoff value of IGF-1 on the 7^{tth}day (OR: 7.00) for first statistical model can be preferred for the determination of intestinal inflammation in cases with diarrhea. In second statistical model, ROC analysis (7th day: OR: 16.00, Sens-Spec: 80%, 80%, P = 0.003, 14th day: ROC (OR: 5.5, Sens-Spec: 93.33%, 73.33%, P = 0.003) showed that sensitivity and odds ratio of 7th day was more powerful in order to detect diarrheic cases. As a general evaluation, it is seen that the highest performance was given by the 7th day IGF-1 values according to the Youden's J index in both of the statistical models (Table 3).

ROC analysis for GH also showed a high sensitivity and specificity for the 14th day with a cut-off value of >2.569 ng/mL (Sens: 73.33, Spec: 100, AUC: 0.853, P = 0.0001). In parallel with the statistical differences found on the 14th day for GH hormone, ROC analysis result may also be a good marker in predicting concentration changes in intestinal inflammatory conditions.

GH and IGF-1 levels can increase following acute phase reaction secondary to infection which arise from different causes in sheep [18]. Moore et al. [18] found that IGF-1 concentrations tended to gradually increase over 6 days throughout experimentally induced yeast infection. IGF-I levels were observed to remain higher up to 8 days after the infection resolved compared to the control group. There is a similar pattern with IGF-1 in our study. IGF-1 levels are higher compared to healthy lambs especially when diarrhea was resolved. In addition, the elevation of GH in calves with diarrhea was reported by Brückmann et al. [7]. In the same study it was reported that the high GH levels return to concentrations of those healthy calves only in measurements on the 21st day. The above studies suggested that IGF-I and GH can be elevated for a period of time after diarrhea. Our findings are corraleted with those results mentioned above but also many variable can be effective on the surge of GH and IGF-I which include feeding, weaning, body weight etc. [1–4].

In conclusion, elevations in IGF-1 and GH concentrations may be associated with intestinal inflammation. The inducing effect of the inflammatory response on IGF-I and GH may strengthen the relationship between the two parameters. So, further studies are needed to evaluate factors modulating the IGF-I and GH.

Acknowledgment/conflict of interest
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