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RESEARCH

Effect of lactoferrin in rats fed with a high-fat diet and with a full-thickness skin defect model

Yüksek yağlı diyet ile beslenen ve tam kalınlıkta deri defekti modeli oluşturulan sıçanlarda laktoferrinin etkisi

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Abstract

Purpose: Lactoferrin is a member of the milk protein family with a broad spectrum of bioactivities and has therapeutic effects against many microbes, viruses, and other pathogens. This study aims to show the healing effect of lactoferrin against obesity-related wound healing.
Materials and Methods: In this study, 48 male rats were grouped as the Control (C) (n =8), Full-thickness skin defect model (FT) (n =8), Bovine Lactoferrin (bLf) (200 mg/kg)(n= 8), High Fat Diet (HFD) + Bovine Lactoferrin (bLf) (n = 8), High-Fat Diet (HFD)+Full-thickness skin defect model (FT) (n=8), and HFD+Full-thickness skin defect model (FT) + Bovine Lactoferrin bLf (n=8). High-Fat Diet + bLf and High-Fat Diet + Full-thickness skin defect + bLf group rats were given a high-fat diet and Bovine Lactoferrin orally. IL-6 and VEGF biochemical parameters were examined in serum.
Results: When the serum IL-6 protein amounts of the groups were examined, it was the highest in the HFD+FT+bLf group on the 21st day, and the lowest in the FT group, when the serum VEGF protein amounts, which were the other biochemical parameters, were evaluated, these amounts were found to be the highest in the HFD+FT+bLf group and the lowest in the FT group on the 21st day.
Conclusion: Lactoferrin has been shown to accelerate wound healing, and the fact that lactoferrin is readily available and abundant in milk makes it an exciting treatment option for wound healing and inflammation in the future.
Keywords: Wound healing, lactoferrin, diet, high-fat, rats.

Öz

Amaç: Laktoferrin, geniş bir biyoaktivite yelpazesine sahip süt proteini ailesinin bir üyesidir ve birçok mikrop, virüs ve diğer patojenlere karşı tedavi edici etkilere sahiptir. Bu çalışmanın amacı laktoferinin, obeziteye bağlı yara iyileşmesine karşı iyileştirici etkisi göstermesi amaçlanmaktadır.
Gereç ve Yöntem: Bu amaçla 48 adet erkek sıçan Kontrol (C) (n=8), Tam kalınlıkta deri defekti modeli (FT) (n=8), Sığır Laktoferrin (bLf) (n= 8), Yüksek Yağlı Diyet olarak gruplandırıldı. (HFD) + Sığır Laktoferrin (bLf) (n = 8)(200 mg/kg), Yüksek Yağlı Diyet (HFD)+Tam kalınlıkta cilt defekti modeli (FT) (n=8) ve HFD+Tam kalınlıkta cilt defekti modeli (FT) + Sığır Laktoferrin bLf (n=8). Yüksek Yağlı Diyet + bLf ve Yüksek Yağlı Diyet + Tam Kalınlık cilt defekti + bLf grubundaki sıçanlara yüksek yağlı diyet ve sığır laktoferrin oral olarak verildi.IL-6 ve VEGF biyokimyasal parametrelerine serumda bakılmıştır.
Bulgular: Grupların serum IL-6 protein miktarları incelendiğinde 21. günde en yüksek HFD+FT+bLf grubunda, diğer grup olan serum VEGF protein miktarları ise FT grubunda en düşüktü. Biyokimyasal parametreler değerlendirildiğinde bu miktarların 21. günde en yüksek HFD+FT+bLf grubunda, en düşük ise FT grubunda olduğu görüldü.
Sonuç: Laktoferrinin yara iyileşmesini hızlandırdığı gösterilmiştir ve laktoferrinin kolaylıkla bulunabilmesi ve sütte bol miktarda bulunması, onu gelecekte yara iyileşmesi ve iltihaplanma için heyecan verici bir tedavi seçeneği haline getirmektedir.
Anahtar kelimeler: Yara iyileşmesi, laktoferin, yüksek yağlı diyet, sıçan.

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INTRODUCTION

Obesity is a complex and multifactorial disease. About one-third of the world's population today is overweight or obese[1]. Obesity increases the risk of mortality in ischemic heart and cardiovascular[2]. Obesity triggers a process that leads to many diseases, the first of which is the slowing of the wound-healing process[3]. Previous studies reported that cytokines and growth factors accelerate wound healing in many animal models[4].

Lactoferrin (Lf) is mostly found in colostrum and milk. However, it is also found in mucous secretions such as saliva, vaginal discharges, gastrointestinal secretions, tears, nose and sperm, bile, and bronchial discharges. After casein, Lf is the second most abundant protein in milk[5]. Lactoferrin is an important component in the first line of defense of the host with its ability to respond to different physiological and environmental changes[6]. Lactoferrin has multiple biological properties such as antiparasitic, antifungal, antibacterial, antiviral, antitumor activity, regulation of iron absorption, and immunomodulatory effects during inflammation and infection[7,8]. Lactoferrin is a glycoprotein used therapeutically against a wide variety of viruses, including the Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV-2), which is closely related to the novel Severe Acute Respiratory Syndrome Coronavirus-2 [9]. Many alternative treatment agents were developed to cure diseases that occur with conditions such as obesity and overweight, and one of them is Lactoferrin, which has become widespread in recent days.

With this study, it was aimed to determine whether Lactoferrin, which has increased interest as an adjuvant and natural treatment today, would affect the inflammatory state in rats with a high-fat diet and a full-thickness skin defect model, and to reveal the comparative effects of healing in full-fat rats. In this study, we focused on the effects of Bovine Lactoferrin administration on macroscopic imaging and changes in biochemical parameters (VEGF, IL-6) in rats.

MATERIALS AND METHODS

The study was conducted in the laboratories of Gaziantep University, Faculty of Medicine, Department of Physiology and Biochemistry, and Gaziantep University Experimental Animals Research Center (GAUNDAM) and Application Center and were housed in separate standard cages (7 to 7.5 inches) under standard pellet and water ad libitum and standard light and dark cycle conditions (20-22 °C; 60-70% humidity; 12 h light/dark cycle). A total of 48 Sprague Dawley (SD), male rats weighing 250-300 grams were randomly selected and grouped to form experimental groups in the study. All stages of the study were performed after the approval with the number 2022/56 from Gaziantep University Animal Experiments Local Ethics Committee. All experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals National Research Council (US) Committee Update of the Guide for the Care and Use of Laboratory Animals Citation 2011).

Determination of live weights

Before the experimental study was initiated, the body weights of the experimental animals were determined. The body weight measurements of the rats in the experimental study group were determined twice a week on the same days and hours.

Experimental groups

A total of 48 Sprague Dawley (SD) male rats were divided into 6 different groups. There were 8 male SD rats in each group.

1st group: Control group (C) was fed *ad libitum* with standard feed and drinking water throughout the study period (21 days).

2nd group: Bovine Lactoferrin group (bLf) was the group that was fed with Bovine Lactoferrin, and it was mixed with standard food for 21 days and given orally at a dose of 200 mg/kg/day[10].

3rd group: Full-Thickness skin defect (FT) After shaving the back hair of the rats under anesthesia with a razor blade, taking care not to damage the skin, 1 circular full-thickness skin defect was induced in the dorsal part with a biopsy punch diameter. The rats with a full-thickness skin defect model were divided into groups and housed one by one.

4th group: High Fat Diet (HFD) + Bovine Lactoferrin (bLf) group was fed with 200 mg/kg bovine Lactoferrin mixed with the feed in addition to the fatty diet and was given orally during the study period (21 days).

5th group: High-Fat Diet (HFD) + Full Thickness Skin Defect (FT) group was fed with a high-fat diet

and a skin defect model was created biopsy punch in the dorsal part of 1 circular full-thickness during the study period (21 days).

6th group (High-Fat Diet (HFD) + Full Thickness Skin Defect Model (FT) + Bovine Lactoferrin group (bLf) was fed with a high-fat diet and 200 mg/kg Lactoferrin added to the feed, and a circular full-thickness skin defect model was created with punch in the dorsal parts of the rats.

Full layer skin defect model

The back hairs of the rats were cleaned carefully under anesthesia without damaging the back tissues. Then, 1 circular full-thickness skin defect was created in the dorsal part with a 12 mm (1.2 cm) mm diameter biopsy punch.

Applied diet model

The standard diet and high-fat diet were prepared according to the specially prepared AIN-93 diet at the rates specified in the previous literature data. The fat ratio of the standard diet was 12% of the energy and 42% of the high-fat diet [11].

Imaging and measuring the wound surface area macroscopically

To view the rats that had full-thickness skin defects and were divided into groups, the camera height was fixed, the angle was vertical, and the light level and the camera were the same. The rats with full-thickness skin defects were imaged macroscopically to compare the wound area diameters on the first day (day 1) and the last day (day 21).

Biochemical measurements

IL-6 and TNFα levels were measured using the sandwich Enzyme Linked Immunosorbent Assay (ELISA) method. Tissue injuries are known to cause stimulation of pro-inflammatory cytokines such as VEGF and IL-6, and the release of such inflammatory cytokines can induce stimulation of vascular endothelium. IL-6 and VEGF biomarkers were examined in the sera of the rats that were included in all groups that were created in the experimental design of the present study.

Statistical analysis

Statistical analysis was performed by using the Statistical Package for Social Science (SPSS) Version 22.0 (SPSS Inc., Chicago, USA) package program. Descriptive statistics for numerical variables were expressed as group mean ± Standard Deviation (SD) (n: number of animals used in the experiment). The Shapiro-Wilk Test was used for normality analysis. The difference between the means of the biochemical parameters of VEGF and IL-6 in more than two independent groups was evaluated by using the One-Way ANOVA. The difference between the groups was evaluated by using Post-Hoc Tukey. The results were evaluated by using the p value at the 95% Confidence Interval and the results were considered statistically significant when the p value was <0.05. As a result of the power analysis made to ensure that the sample selected in the study represents the universe; It was calculated that if the number of animals to be used in the experiment was at least 8 in each group, with a medium effect size ($f = 0.25$), 80% power could be achieved with 95% confidence (Type 1 error margin $\alpha = 0.05$).

RESULTS

In the experimental study, the rats were weighed on the 1st, 4th, 7th, 10th, 13th, 17th, and 21st days at the same hours (Figure 1). When the distribution of the weight changes of the groups was evaluated according to the groups and days (Figure 1), in the weight change of the C group, the weights of the 1st day (266.1+27.07) compared to the 4th day (241.01+24.78) and 13th day (250.46+19.88) decreased, and the difference (263.95+38.41) on the 21st day was almost equal, but there was no statistically significant difference. When the weight changes in the FT group were examined, it was seen that there was no statistically significant difference between the days, as well as a noticeable increase or decrease. In the Bovine Lactoferrin administered group (bLf), there was no change in the body weight change from day 1 (246.8+28.1) to the 4th, 7th, and 10th days, it was observed that the live weight gain continued to increase on the other days after the 13th day (250.86+20.32). There was no statistically significant difference in the live weight gain or decrease in the bLf group. Live weights of the high-fat diet+full-thickness skin defect group increased starting from the 1st day (254.99+27.63), 4th day (259.84+26.31), 7th day (269.05+27.74), 10th day (269.70+29.07), 13th day (274.95+27.40), 17th day (276.76+25.36), 21st day (275.04+27.07).

Table 1. Weight changes of experimental groups (ANOVA)

Days	**Experimental Groups**	**Mean**	**95% CI**	**SE**	**df**	**F**	**P-Value**
1	Control	266.100	241.159-291.141	10.134	5	1.424	0.239
	FTSD	244.436	212.099-276-772	13.215			
	bLf	246.800	246.800-223.293	9.941			
	HFD+FTSD	254.992	225.991-283.993	11.282			
	HFD+bLf	223.231	184.688-261.774	16.300			
	HFD+FTSD+bLF	243.321	222.031-264.612	8.701			
4	Control	241.014	218.090-263.938	9.369	5	0.465	0.800
	FTSD	244.943	215.267-274.618	12.128			
	bLf	248.306	227.683-268.930	8.722			
	HFD+FTSD	259.842	259.815-232.228	10.742			
	HFD+bLf	235.500	193.808-277.192	17.632			
	HFD+FTSD+bLF	238.636	212.432-264.839	10.709			
7	Control	255.859	259.000-286.716	12.611	5	0.504	0.771
	FTSD	245.593	214.832-276.254	12.571			
	bLf	246.763	227.262-266.263	8.247			
	HFD+FTSD	269.058	239.940-298.177	11.327			
	HFD+bLf	242.206	197.777-286.636	18.789			
	HFD+FTSD+bLF	248.536	221.545-275.527	11.031			
10	Control	258.764	235.691-281.838	9.430	5	0.484	0.786
	FTSD	246.243	215.728-276.758	12.471			
	bLf	246.169	226.126-266.211	8.476			
	HFD+FTSD	269.708	239.194-300.223	11.871			
	HFD+bLf	247.081	202.534-291.628	18.839			
	HFD+FTSD+bLF	251.814	223.368-280.260	11.625			
13	Control	250.464	232.071-268.857	7.517	5	0.708	0.621
	FTSD	243.361	211.957-274.766	12.834			
	bLf	250.862	233.866-267.859	7.188			
	HFD+FTSD	274.950	246.191-303.709	11.188			
	HFD+bLf	250.315	207.571-293.059	18.076			
	HFD+FTSD+bLF	257.221	229.975-284.468	11.135			
17	Control	258.267	239.510-277.027	7.666	5	0.741	0.598
	FTSD	246.203	213.302-279.104	13.446			
	bLf	255.900	237.788-274.012	7.660			
	HFD+FTSD	276.767	250.150-303.383	10.354			
	HFD+bLf	251.675	214.647-288.702	15.658			
	HFD+FTSD+bLF	262.250	234.636-289.864	11.285			
21	Control	263.953	247.258-280.647	6.823	5	0.764	0.582
	FTSD	248.456	212.982-283.983	14.520			
	bLf	259.008	244.156-273.862	6.281			
	HFD+FTSD	275.045	246.628-303.462	11.055			
	HFD+bLf	250.121	215.170-285.072	14.781			
	HFD+FTSD+bLF	266.843	293.732-293.953	11.079			

$P < 0.05$ is significant.; CI: confidence interval, SE: standart error, df; degrees of freedom; FTSD: Full-thickness skin defect model, bLF:Bovine Lactoferrin; HFD: High Fat Diet).

Although the weight gains were positive, it was found that there was no statistically significant difference. Weight gain of HFD+bLf group increased on day 1 (223.23+ 46.10) and continued on 4th, 7th, 13th, 10th, 17th and 21st days (251.72+41.80). Despite the continuous increase, it was observed that there was no statistically significant difference. The live weight gains in the HFD+bLf group increased on the 1st day (223.23 + 46.10) and on the 4th, 7th, 10th, 13th, 17th, and 21st days (251.72+41.80). Despite the continuous increase, there was no statistically significant difference (Table 1).

Although there was a decrease in the weight change of the HFD+bLf+FT group on the 4th day (238.63+ 28.3), it was seen that the weight change was positive on the other days. The wound area was evaluated statistically (Table 2).

On the first day of this experimental study, the full-thickness skin defect model was created in 3 groups (FT, HFD+FT, HFD + FT + bLf) with punch. It was found that there was a statistically significant difference between the HFD + FT group and the HFD + FT + bLf group on the 21st day, and the wound surface area was less in the group given HFD + FT + bLf (Table 2). The wound area diameter and scar tissue of the FT+HFD+bLf group were even smaller than the other FT and FT+HFD groups. When the multiple comparison analysis results were evaluated between the 1st and 21st day, no statistically significant differences were detected between the 1st-day groups, but a statistically significant difference ($P < 0.05$) was found between the HFD+FT group and the HFD+FT+bLf group on the 21st day. The wound surface area was seen to be even smaller in the HFD+FT+ bLf group (Figure 2).

Table 2. Wound area analysis between groups at day 1 and day 21 (ANOVA).

Days	Experimental Groups	Mean	% 95 CI	SE	df	F	P-Value
Day 1	FTSD	0.447	0.434±0.459	0.005			
	HFD+FTSD	0.445	0.433±0.457	0.005	2	0.125	0.883
	HFD+FTSD+bLf	0.443	0.432±0.455	0.005			
Day 21	FTSD	0.156	-0.027±0.340	0.075			
	HFD+FTSD	0.153	0.140±0.166	0.005	2	3.611	0.049*
	HFD+FTSD+bLf	0.006	-0.000±0.012	0.002			

*P < 0.05 is significant. CI; confidence interval, SE; standard error, df; degrees of freedom; FTSD: Full-thickness skin defect model, bLF:Bovine Lactoferrin; HFD: High Fat Diet).

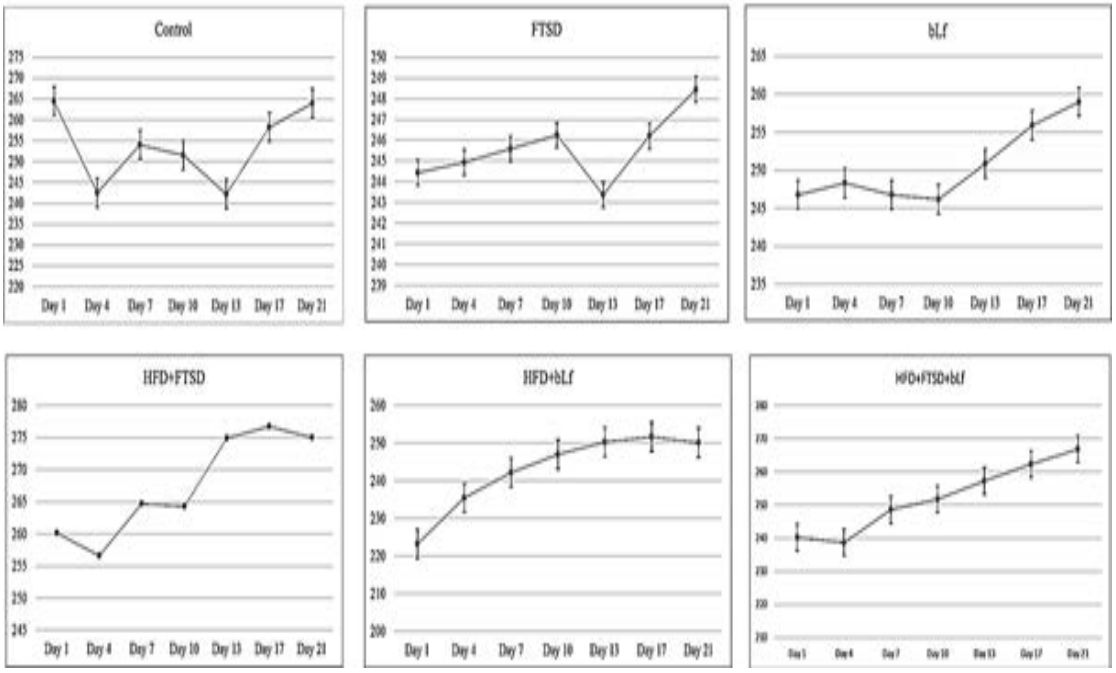

Figure 1. Weight (gram) changes graph of the groups depending on the day.

FTSD: Full-thickness skin defect model, bLF:Bovine Lactoferrin; HFD: High Fat Diet.

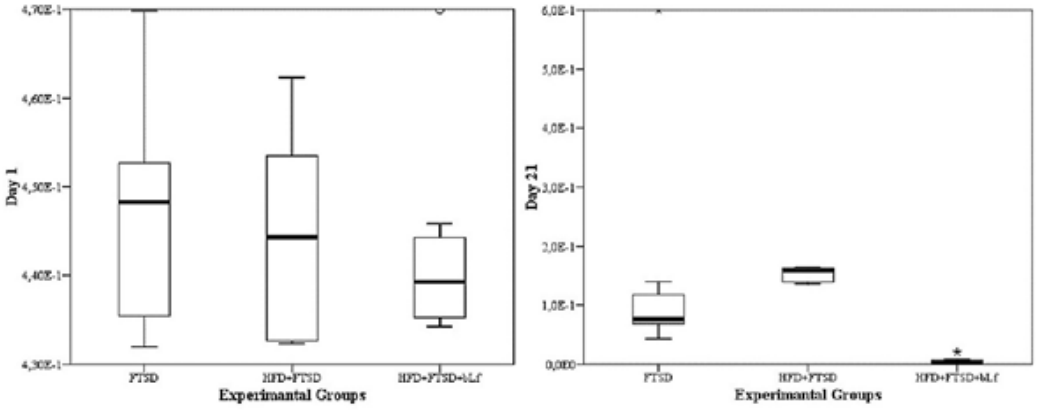

Figure 2. Box-plot plot showing the differences in the wound areas according to the groups on the 1st and 21st days.

Normality analyzes were performed by Shapiro-Wilk and differences between groups by ANOVA. Post hoc analyzes were performed with the Tukey test. The graph was expressed as 95% median. Asterisks indicate statistically significant ($P < 0.05$).

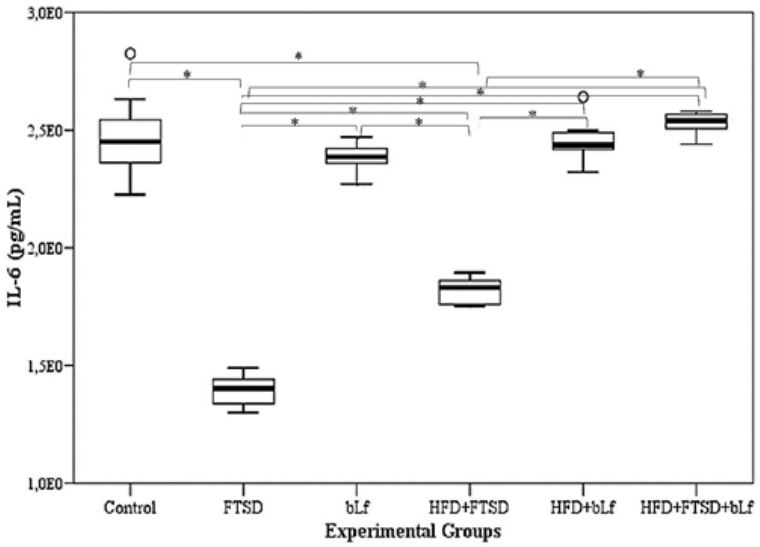

Figure 3. Box-plot plot showing IL-6 results in groups.

Normality analyzes were performed by Shapiro-Wilk and differences between groups by ANOVA. Post hoc analyzes were performed with the Tukey test. The graph was expressed as 95% median. Asterisks indicate statistically significant related groups ($P < 0.05$) and circles indicate excessive values; FTSD: Full-thickness skin defect model, bLF:Bovine Lactoferrin; HFD: High Fat Diet.

When the serum IL-6 protein amount of the groups was evaluated, it was found to be highest in the HFD+FT+bLf group and lowest in the FT group on the 21st day (Figure 3). When the serum VEGF protein amounts of the groups were examined, it was highest in the HFD+FT+bLf group and the lowest in the FT group on the 21st day (Figure 4). Considering the logarithmic mean value of the groups on the 21st day, the group with the lowest mean value of IL-6 compared to the C group was the

FT group. According to the group mean values on day 21, the group with the highest average of IL-6 levels was HFD+FT+bLf. Considering the group mean values for VEGF on the 21st day, the group with the lowest mean compared to the C group was the FT group. According to the group mean values on the 21st day, the group with the highest mean value of IL-6 was HFD+FT+bLf.

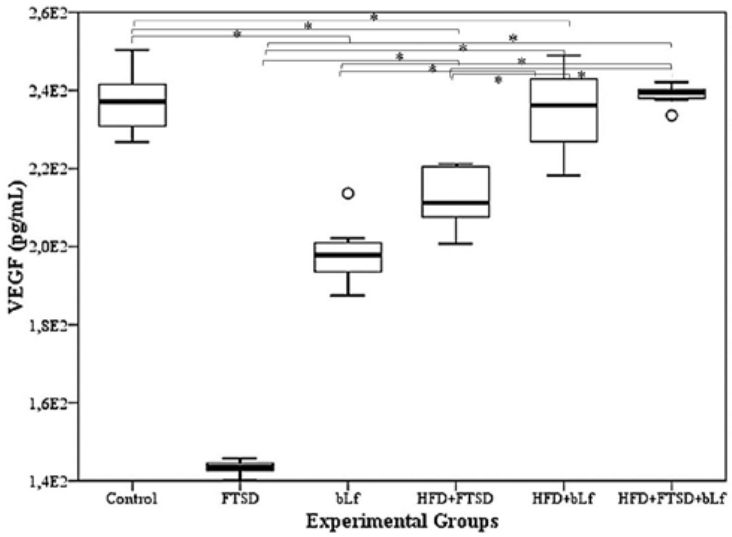

Figure 4. Box-plot plot showing VEGF results in groups.

Normality analyzes were performed by Shapiro-Wilk and differences between groups by ANOVA. Post hoc analyzes were performed with the Tukey test. The graph was expressed as 95% median. Asterisks indicate statistically significant related groups ($P < 0.05$) and circles indicate excessive values; FTSD: Full-thickness skin defect model, bLF:Bovine Lactoferrin; HFD: High Fat Diet.

DISCUSSION

In the present study, the effect of Bovine Lactoferrin on wound healing was measured with macroscopic imaging in rats fed with a high-fat diet and a full-thickness skin defect model was created, and the effect of biochemical parameters was investigated. An important effect of lactoferrin on the development of adaptive immune responses is that it directly activates the first-line defense activity[12].

Animal experiments were used to examine the effects of VEGF expression in the bLF-treated full-thickness skin defect group and it was found that oral administration of bLf can reduce VEGF overexpression concerning vasculogenesis and angiogenesis in full-thickness skin defect. When the findings of the study were evaluated, it was found that bLf had negative effects, especially on the level of VEGF. Cytokines act in synergy and have an overlapping activity that can occur at their receptors or along intracellular signal transduction pathways[13,14]. In this study, the administration of Bovine Lactoferrin to rats on pellets (200 mg/kg body weight) was extensively shown to be an important negative regulator of IL-6 in both in vitro and animal models of Lf, as well as in clinical trials. Andrich De et al. conducted a study in which high-fat diets and diet-related obesity were examined, metabolic homeostasis and oxidative stress and inflammation levels in many organs, including skeletal muscle, and found an increase of up to 4.5-fold in proinflammatory Interleukin 6, which is pro-inflammatory in the high-fat diet groups when compared to the C group[15]. Elmenam et al. In their study, they investigated sterile inflammations in people with pregnancies between 28 and 34 weeks and showed that maternal serum IL-6 levels were significantly higher in the study group than in the controls[16] . Similar results were obtained in the present study with the above data. In this regard, it was concluded in the study that feeding with a High-Fat Diet affected increasing IL-6 levels. When the IL-

6 levels of bLf and the C group were compared, it was seen that the IL-6 levels of the bLf group were lower, and according to these data, it was concluded that bLf lowers the serum IL-6 level, albeit at insignificant levels. Orhan C. et al. evaluated the protective potential of Mesozeaxantin against retinal oxidative damage and retinal growth and transcription factors in rats fed with a High-Fat Diet (HFD). In their study conducted on rats, they also observed that VEGF levels increased in groups given a High-Fat Diet[17]. Choi et al. conducted a study to evaluate HFD treatments for lymphedema in mice. They also evaluated VEGF protein expression in tissue samples. They found that VEGF protein levels were significantly higher in HFD-fed mice than in operated mice fed a standard chow diet[18]. Similar results were obtained in this study. It was concluded that it increased VEGF levels in groups fed with a High-Fat Diet. When the VEGF levels of bLf were examined, it was seen that it was lower than the Control group and HFD+FT+bLf group, and it was concluded that bLf had an inhibitory effect on VEGF levels. Imaging was performed under anesthesia on the 1st and 21st days to view the wound areas macroscopically in rats that had a full-thickness skin defect model. The reduction rates of the wound areas obtained on the 1st day and the 21st day were compared. In the study, the group fed with bLf and a high-fat diet (FT+HFD+bLf) had the smallest wound area when measured on the last day (21st day) compared to the other full-thickness skin defect model groups (FT, HFD+FT). It is possible to argue that Lactoferrin causes more closure of the wound area and the disappearance of the scar trace when compared to other groups, and that bLf promotes epithelial cell migration.

In another study, Saaristo et al. reported that growth factors and cytokines increase VEGF amounts during the wound healing process. In the present study, findings supporting this idea were obtained. It was determined that the wound areas were smaller in the groups given Bovine Lactoferrin. Similar results were obtained in this study[19]. In their study, Shams et al. aimed to evaluate the status of VEGF-overexpressing fibroblast cells to evaluate the angiogenesis function in wound healing. They found that VEGF-expressing fibroblast cells are responsible for increasing the number of blood vessels and accelerating angiogenesis during the wound healing process[20]. The effect of oral administration of 200 mg/kg bL for 21 days on the development of obesity was investigated and it was found that 200 mg/kg bLF supplementation reduced body weight gain and fat index, although there were no statistically significant differences in weight gain. It was also found that weight gain was higher in the groups fed with HFD compared to the other groups. In this study, no changes were detected between the fatty diet group and HFD+bLf, indicating that the anti-obesity effect of bLf is probably not related. However, it was also found that this was the opposite and the weight gain of the groups given Lactoferrin increased less than the groups fed a high-fat diet.

As a result, it shows that bLf accelerates wound healing and better nutrition of tissues by increasing immune responses and angiogenesis factors. Especially in the groups whose diets were supplemented with bLf, wound healing was faster and macroscopically, the wound areas were reduced in size. Lf, exerts positive effects on cell cycle progression and cell migration towards normal cells and immune cells. Lactoferrin, and specifically its bovine milk-derived form Bovine Lactoferrin, is emerging as a potential strong candidate for alternative treatment in many types of inflammation and wound healing.

HFD diets have various application periods. Accordingly, it presents different pathologies and processes. Diet durations and the effects of long-term treatment are limitations of this study. Short and long-term monitoring of the diet application according to age range and weeks will allow to monitor the changes of the parameters at the beginning and end of the diet and on a weekly basis, and thus will enable planning with the right timing as a treatment target. The potential of these parameters as markers can be effectively evaluated with the correct weeks and treatment durations.

Author Contributions: Concept/Design: ASB, GSY; Data acquisition: ASB, MO, HU; Data analysis and interpretation: ASB, GSY, MO, HU; Drafting manuscript: ASB; Critical revision of manuscript: ASB; Final approval and accountability: GSY, ASB, MO, HU; Technical or material support: GSY; Supervision: ASB; Securing funding (if available): n/a.
Ethical Approval: Gaziantep University Animal Experiments Local Ethics Committee Presidency Research Application Approval Ethical approval was obtained with the decision dated 10.11.2022 and numbered 2022/56.
Peer-review: Externally peer-reviewed.
Conflict of Interest: No potential conflict of interest relevant to this article was reported..
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