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Research Article

Investigation of the Relationship Between HMGB1 and Obesity in the Adrenal Gland

Böbreküstü Bezinde HMGB1 ile Obezite İlişkisinin Araştırılması

Muhammed Erdal[1], Berrin Zuhal Altunkaynak[2], Adem Kocaman[3], Isinsu Alkan[4], Emin Oztas[1]

[1]Gulhane Military Hospital, Department of Histology and Embryology, Ankara, Turkey
[2]Okan University, Faculty of Medicine, Department of Histology and Embryology, Istanbul, Turkey
[3]Ondokuz Mayıs University, Faculty of Medicine, Department of Histology and Embryology, Samsun, Turkey
[4]Nevsehir Haci Bektas Veli University, Faculty of Dentistry Department of Basic Science, Nevsehir, Turkey

Abstract

Aim: The interaction between obesity and increased production of pro-inflammatory cytokines results the existence of inflammation HMGB1 secreted from the adrenal gland can play a role in inflammation pathways. The aim of this study is to explain the link between HMGB1 and obesity in the adrenal gland.

Material and Methods: In this study; eighteen female Wistar Albino rats were divided into two groups: untreated control group (n=8) and obese group (n=10). The rats in obese group were fed with high fat diet for ten weeks. Morphometric parameters of adrenal gland were assessed by using stereological techniques. The expression of high mobility group box protein 1 (HMGB1) in adrenal gland was evaluated.

Results: At the end of the analyses; mean volumes of zona fasciculate, zona reticularis, and medulla were significantly increased in obese group. Also, the number of HMGB1 stained cells was significantly increased in the obese group in comparison to control group.

Conclusion: The results suggest that obesity may be one of the reasons of inflammation and hypertrophy in the adrenal gland. HMGB1 may provide a novel perspective into the anti-inflammatory therapeutic strategies in obese patients.

Keywords: Adrenal gland, HMGB1, inflammation, obesity, stereology

Öz

Amaç: Obezite ile artan proinflamatuar sitokin üretimi arasındaki etkileşim sonucu inflamasyonun varlığı adrenal bezden salgılanan HMGB1 inflamasyon yolaklarında rol oynayabilir. Bu çalışmanın amacı, adrenal bezde HMGB1 ile obezite arasındaki bağlantıyı açıklamaktır.

Materyal ve Metot: Bu çalışmada; on sekiz dişi Wistar Albino sıçanı tedavi edilmeyen kontrol grubu (n=8) ve obez grup (n=10) olmak üzere iki gruba ayrıldı. Obez gruptaki ratlar on hafta süreyle yüksek yağlı diyetle beslenirken, control grubu standart diyet ile beslendi. Adrenal bezin morfometrik parametreleri ve mobilite grubu kutu protein 1 (HMGB1) ekspresyonu stereolojik teknikler kullanılarak değerlendirildi.

Bulgular: Analizler sonunda; zona fasikülata, zona retikülaris ve medulla ortalama hacimlerinim obez grupta anlamlı olarak arttığı görüldü. Ayrıca obez grupta HMGB1 ile boyanmış hücre sayısı kontrol grubuna göre önemli ölçüde artmıştı.

Sonuç: Sonuçlar obezitenin adrenal bezdeki inflamasyon ve hipertrofi nedenlerinden biri olabileceğini düşündürmektedir. HMGB1, obez hastalarda anti-inflamatuar terapötik stratejilere yeni bir bakış açısı sağlayabilir.

Anahtar Kelimeler: Adrenal bez, HMGB1, inflamasyon, obezite, stereoloji.

INTRODUCTION

Obesity is a rising prevalent health concern in both developed and developing countries. The excessive accumulation of adipose tissue in the body is defined as obesity by the World Health Organization (1). In particular, increased consumption of saturated fatty acids results in excess visceral adiposity. Obesity is associated with many metabolic and non-metabolic diseases including hyperglycemia (2), type 2 diabetes mellitus (T2DM) (5), cancer (3), hypertension (6), and atherosclerosis (7). These diseases are associated with chronic inflammation therefore, several mechanisms have been proposed to highlight the relationship with adipose tissue (8-10). The

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Corresponding Author: Isinsu Alkan, Nevsehir Haci Bektas Veli University, Faculty of Dentistry Department of Basic Science, Nevsehir, Turkey **E-mail**: isinsuaydin@gmail.com

increased infiltration of immune cells into and around the adipose tissue and elevated production of multiple plasma pro-inflammatory cytokines in the circulation are the most comprehensive approach that shows the relation between obesity and the development of local and systemic chronic low-grade inflammation (4-11).

High mobility group box protein 1 (HMGB1) is defined as a DNA-binding non-histone protein that plays an important role in transcription, repair and replication of DNA (12). The nuclear HMGB1 acts in response to oxidative stress in the cytoplasm (13,14). HMGB1 is released extracellularly from active immune cells and necrotic cells (15). Also, plays an important role to attract immune cells acting as a pro-inflammatory mediator or alarmin (16,17). The increase of H-MGB1 gene expression may be a direct link between inflammation, obesity, and subclinical cardiovascular risk (18,19).

The adrenal steroid level plays a role in obesity and cardiovascular diseases. Elevated adrenal steroid level is associated with obesity (20,21). Interestingly, an increased level of adrenal hormones causes cardiovascular diseases and triggers insulin resistance (22). All this information reveals the necessity of showing the relationship between adrenal glands responsible for adrenal steroid secretion and obesity and investigating the effect of HMGB1 in this relationship. For that reason, the study aimed to investigate the role of HMGB1 in adrenal glands of obese rats.

MATERIAL AND METHOD

Animals

All procedures were conducted according to the experimental protocol approved by the Faculty of Gulhane Military Medicine and authorization of the ethics committee of Gulhane Military Hospital, Turkey (16/37). The study included 18 adults (8 weeks old) female Wistar albino rats (180-200 gr). All animals were kept at constant temperature 22±1°C with a regular 12 hours of light/dark cycle and with free access to food and water. Animals were randomly divided into two groups. (I) control group (n=9): animals were healthy and fed with normal commercial diet for 10 weeks. (II) obese group (n=9): animals were fed with a special diet (42% carbohydrate, 40% lipid and 18% protein) for 10 weeks. The body mass index (BMI) was used to determine obesity. The rats were regularly weighed during the experiment. Weight and height parameters were used to calculate BMI. BMI values greater than 5 kg/m2 were considered as obese. Therefore, two rats were excluded from experiment because of BMI parameters. Animals were anesthetized with a mixture of ketamine (80mg/kg Ketalar i.p.; Eczacıbaşı, Istanbul, Turkey) and xylazine (10mg/kg Rompun i.p.; Bayer, Istanbul, Turkey). Blood samples were taken and rats were perfused with intracardiac 4% formaldehyde. Then, adrenal glands were quickly removed and animals were sacrificed.

Tissue preparation and immunohistochemical procedures

The adrenal glands were fixed in 10% formaldehyde solution (Sigma-Aldrich, St. Louis, MO) for 24 hours. Then, adrenal glands were processed through alcohol (Sigma-Aldrich, St. Louis, MO) and xylene (Merck, Darmstadt, Germany) series and embedded in paraffin blocks. Using a microtome, 5 µm thick sections were taken in the sagittal plane with a sampling rate of 1/6 (RM2125RT; Leica Nussloch, Germany) and stained with Hemotoxylin and Eosin (H&E). For immunohistochemical evaluation, 5 µm sections were stained with anti-HMGB1 rabbit polyclonal antibody (Abcam, Cambridge, United Kingdom) diluted 1/50 (Zymed Laboratory, Cambridge, UK) using the avidin-biotin complex (ABC) method. The process was performed as previously described (23).

Stereological analyses

Cavalieri Principle was used to estimate the total volume (Vt), zona glomerulosa (ZG), zona fasciculate (ZF), zona reticularis (ZR), and medulla of the adrenal gland (23). The sampling interval was 1/6. Choosing the first section was carried out randomly. At least 20 sections were sampled from each adrenal gland. Olympus BX43 microscope was used to analyze sections.

The point counting grid was randomly placed on the screen (d=1 mm) and hitting points on the grid of the interested area were counted (Figure 1). The area among each of four points was called as a unit area and shown with area/point (a/p). The total counted number of points (P) and thickness (t) of the section were multiplied by the unit area to estimate the volume of the adrenal gland (24). The following formula was used for the volume estimations;

$$Vt = t \times \left(\frac{a}{p}\right) \times P$$

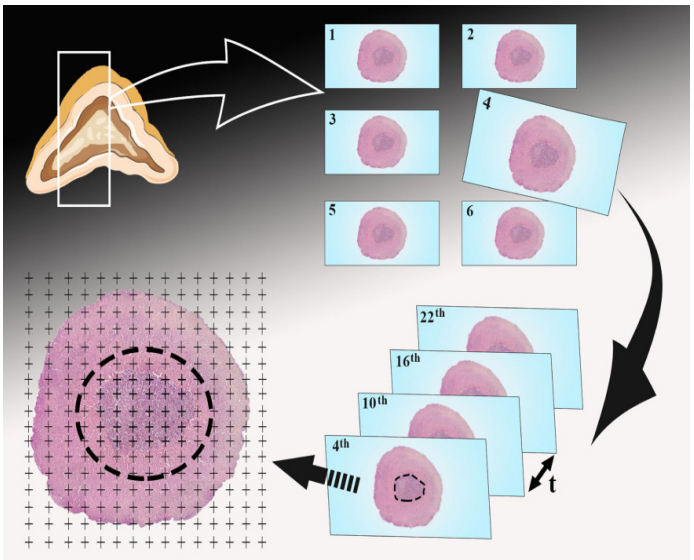

Figure 1. The application of the Cavalieri principle is illustrated on the medulla of adrenal gland

HMGB1 positive cells were counted with physical disector method

Sections were stained with HMGB1 antibody and two consecutive sections were placed upon each glass slide. The first section was called reference and second was called look-up. Sections were photographed at x400 magnification and an unbiased counting frame (2.500 µm2) was placed on the images of reference and the look-up sections on the screen (25). To perform the counting according to the physical disector counting method, the bottom and the left-hand edges of the counting frame were considered as an exclusion line. For that, if the nucleus of the cell touched the left and bottom edges of the frame, it was not counted (red line). All counted nuclei (disector particles) were in the frame or touched the right or upper edges (inclusion lines) of frame (green line). Also, only the nuclei seen in the reference section but not seen in the look-up section were counted. In order to increase the efficiency of the work, the reference and look-up areas of the disector section pairs were also used reversely, as suggested in the literature. Look-up section was used as a reference section while reference section was used as a look-up.

Biochemical analyses

Blood samples (2mL) were collected in the Tubes with EDTA and centrifuged at 10 000g for 3mm at room temperature. After that 1mL plasma was processed according to protocol of the calorimetric glucose, cholesterol, superoxide dismutase and catalase assay kits (Item no: 10009582, 10007640, 707002, 706002 Cayman Chemical Company, Michigan; USA).

Statistical analyses

Leavene test was used to determine whether the data in the groups were parametric. The Independent Samples T-Test was used to compare parametric data of the groups. Mann Whitney U test was used for comparison of nonparametric data. A value of $p<0.01$ and $p<0.001$ were considered statistically highly significant and $p<0.05$ was considered statistically significant. All statistical analyzes were performed with SPSS (Version 15.0 for Windows®, IBM Corp, NY, USA).

RESULTS

Assessment of obesity

Weekly weight gain of subjects was followed. At the end of 10th week, BMI calculations were done to evaluate whether the subjects were obese. The weight increased in obese group than control group. Also, highly significant difference was found between groups in terms of the BMI values (Figure 2.)

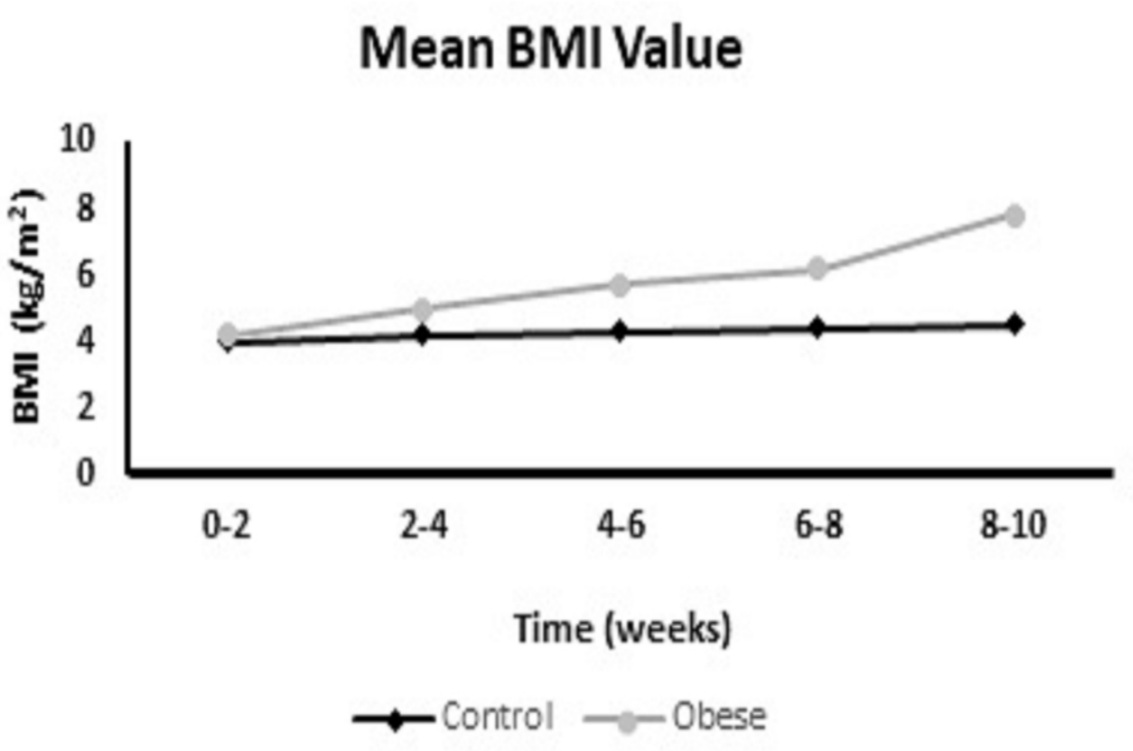

Figure 2. Mean BMI values in control and obese groups. The mean BMI values are increased significantly ($p<0.01$) in the obese group compared to the control group

Stereological evaluation

Stereological results and the mean error coefficient and the coefficient of variation values of the groups showed in Table 1. Stereological results showed that the estimated mean volume of adrenal gland significantly increased in Obese group compared to Control group. In the Obese group, the estimated volume of ZF, ZR and medulla showed highly significant increase ($p<0.001$) whereas the estimated volume of ZG significantly increased in Control group ($p<0.01$) (Figure 3). Both Control and Obese group sections were stained with anti-HMGB1 antibody and sections were evaluated. The number of HMGB1 antibody stained cells was significantly higher in both cortex and medulla of the Obese group compared to Control group ($p<0.001$).

Table 1. The mean error coefficient and coefficient of variation values of the groups

ESTIMATION	Mean CE	Mean CV
Volume of the zona glomerulosa	0.033	0.84
Volume of the zona fasciculata	0.041	1.12
Volume of the zona reticularis	0.037	0.89
Mean number of HMG1 positive cells	0.056	1.4

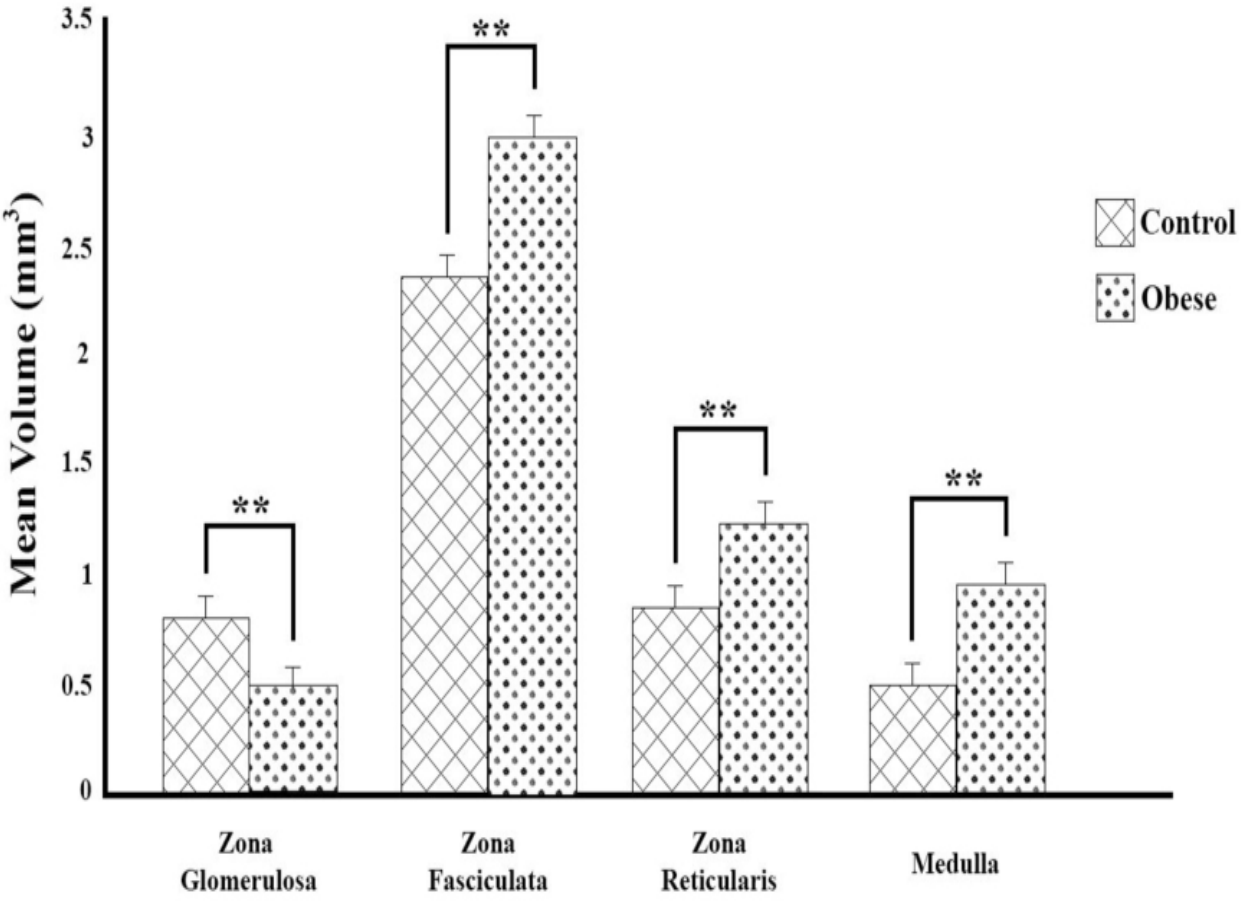

Figure 3. Stereological volume estimations between Control and Obese groups are seen (**; $p<0.01$) (Mann Whitney U test)

Histopathological evaluation

Histological changes were assessed by light microscopic evaluation of tissue sections. In the Control group, the cortex was encircled by a capsule composed of dense irregular connective tissue. Medulla was rich in blood vessels and chromaffin cells. Rarely, healthy appearance of ganglion cells attracted attention. In contrast, the capsule was found thicker in Obese group. Also, there were more and larger fat tissue cells around the capsule of Obese group samples. In Obese group, it was also observed that the ZG layer was thinner than the Control group whereas the ZF layer was thicker than the Control group (Figure 4A, 4B). In the Control group, the ZG layer was found in a normal structure just below the capsule. This layer consisted of parenchymal cells forming concentric or glomerular rings. These small ZG cells were healthy with small dark nuclei containing one or two nucleolus and acidophilic cytoplasm. In contrast to Control group, the selection of cells was difficult and capillaries were dilated in the Obese group.

In the Control group, the ZF was the thickest layer of the cortex. In this layer, sinusoidal capillary vessels that longitudinally located between parallel cell columns were observed. The polygonal cells in this layer had pale acidophilic staining and appeared with vacuoles in some areas. In the ZF of the Obese group, among the steroid content increased cells, new generated cells that had eosinophil cytoplasm and thought to had recently participated on this layer were found. Also, fatty degeneration and necrosis were detected in the ZF of the Obese group (Figure 4C, 4D).

The cells of the ZR in the innermost layer of the cortex were arranged in cords that painted dark acidophiles and anastomosed to each other in the Control group. These cells were contained fewer lipid droplets than ZF cells. Importantly, inflammatory cell infiltration was noted in the ZR layer of the Obese group (Figure 4E, 4F).

The chromaffin cells were arranged as a bundle or cord shaped and sympathetic ganglion cells were dispersed in the connective tissue of the medulla in the Control group. The first notable finding in the medulla of the Obese group was dilated vessel branches compared to the Control group. In the medulla, fibrin depositions were also seen in Obese group. In addition; some of the ganglion cells were healthy, while others were damaged with eosinophilic cytoplasm and shrunken cell borders (Figure 4G, 4H).

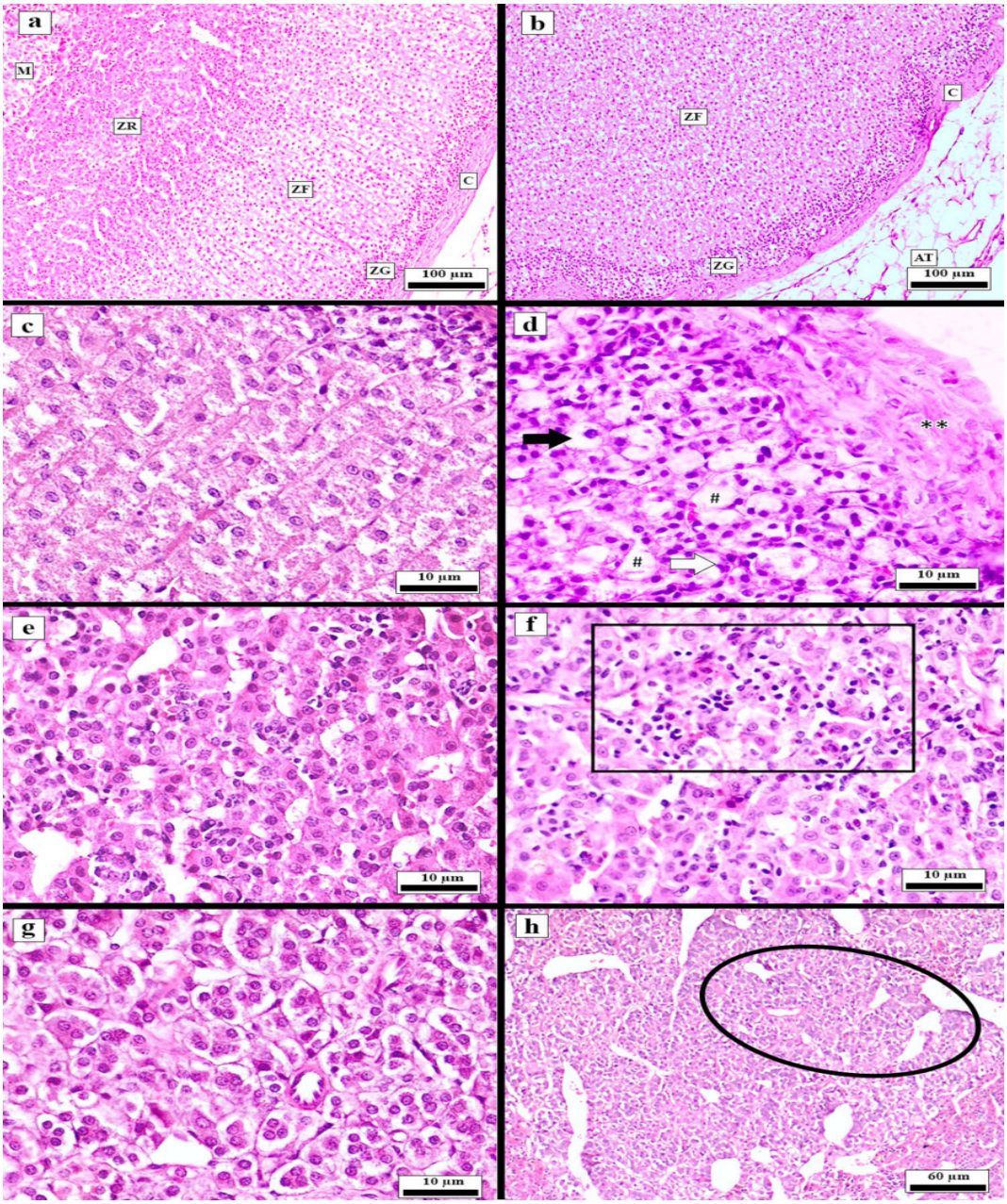

Figure 4. Histopathological evaluation of adrenal glands in both Control and Obese groups are seen. Control group is represented with a, b, c, d. Obese group is represented with e, f, g, h. C; Capsule, ZG; Zona Glomerulosa, ZF; Zona Fasciculata, ZR; Zona Reticularis, M; Medulla, (**); thick capsule, (#); diliated capillaries, black arrow; increased cell volume, white arrow; newborn cells

Immunohistochemical evaluation

The positive stained cells were stereologically evaluated in both groups and a significant difference was found between the groups (p <0.01, Figure 5). According to these results, there was HMGB1 positivity found neither in the cortex layer nor in medullary of the control group.

In the Obese group, strong positive HMGB1 staining was observed in both medullary connective tissue cells and chromaffin cells. Furthermore, a strong HMGB1 positivity was detected in nerve fibers and possible macrophage cells in the medullary of the adrenal gland (Figure 6).

Biochemical results

In this study; glucose and cholesterol levels were calculated. Glucose and cholesterol levels were increased in the obese group compared to the control group (p<0.01, Figure 7).

Catalase and sod levels were calculated for the estimation of oxidative stress levels. These parameters significantly increased in the obese group compared to the control group (p<0.01, Figure 8).

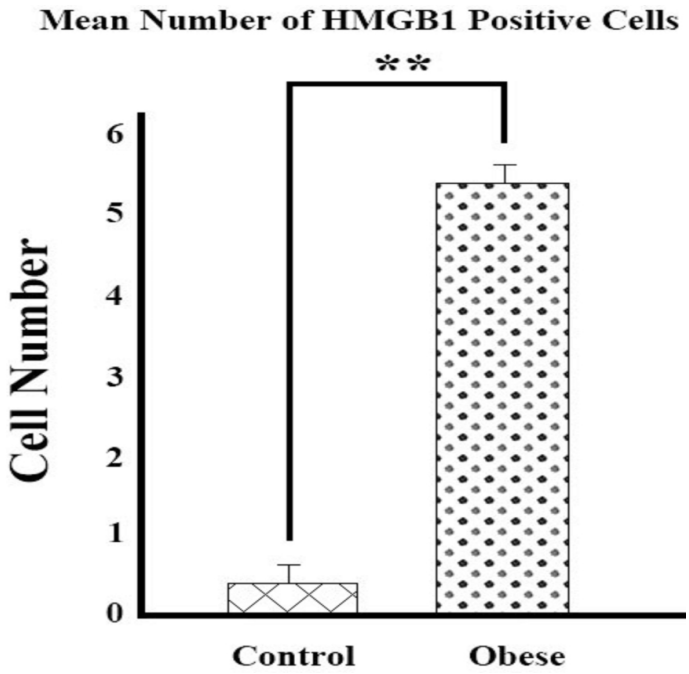

Figure 5. Mean HMGB1 positive cell numbers are seen in both Control and Obese groups. **; p <0.01 (Mann Whitney U test)

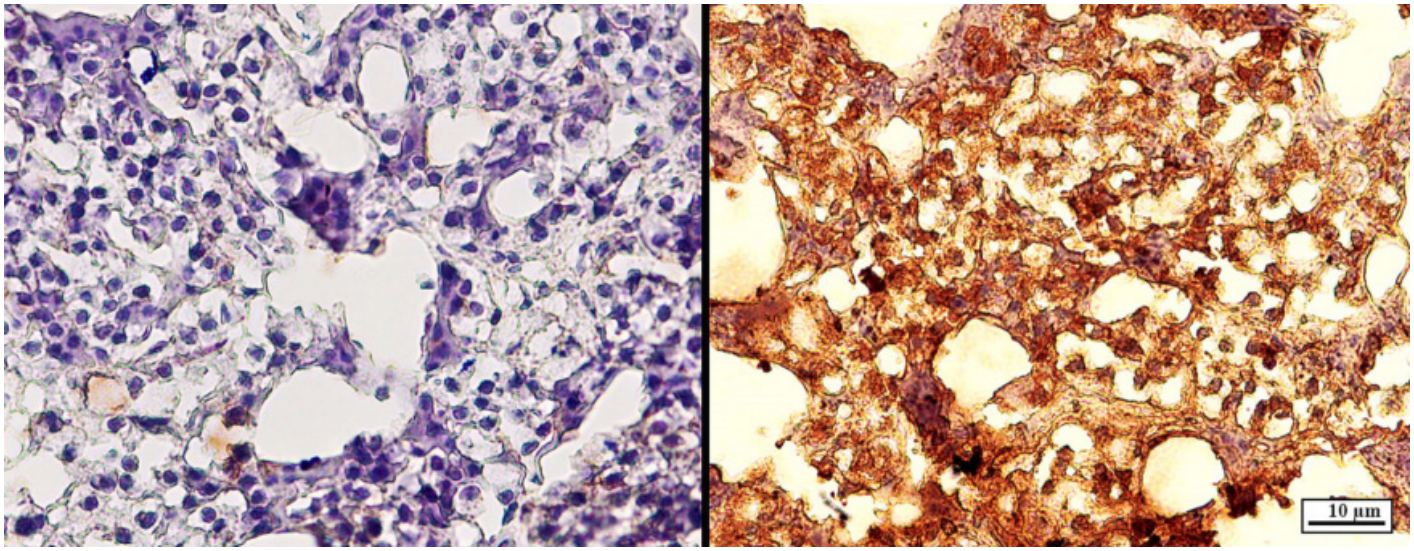

Figure 6. The strong positive HMGB1 staining is observed in the adrenal gland of the Obese group

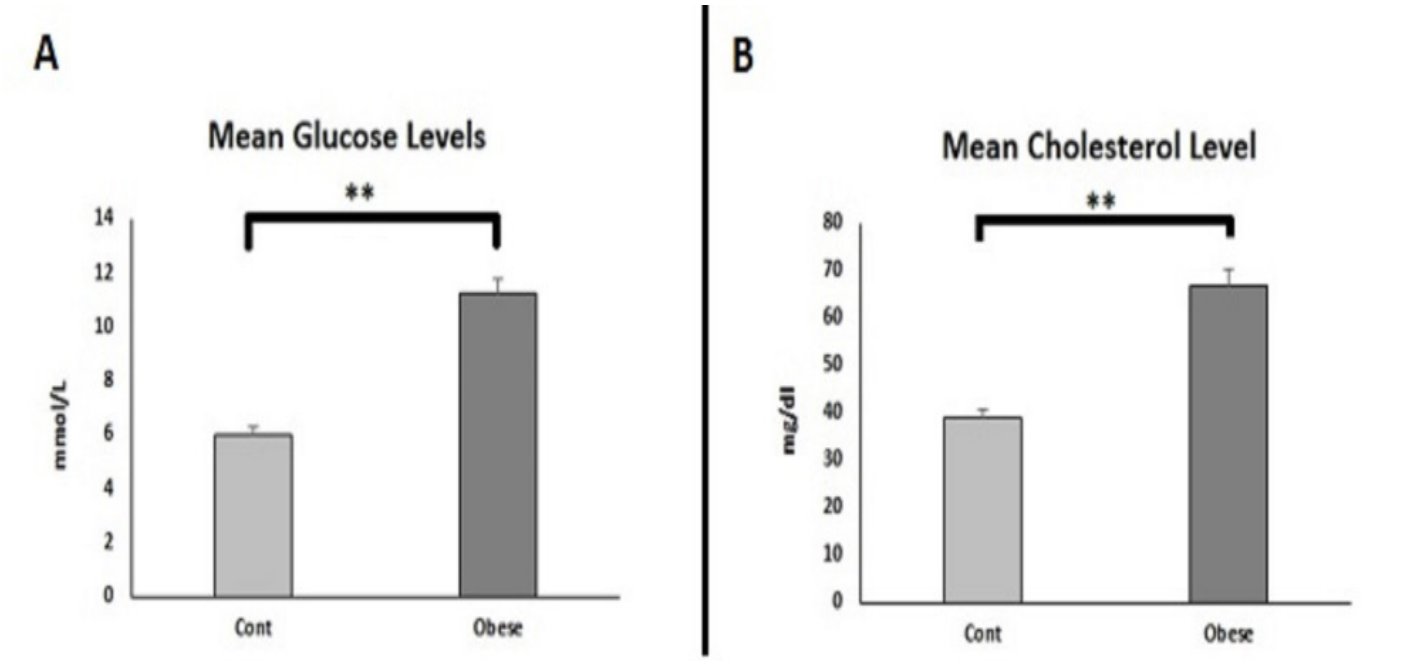

Figure 7. Mean levels of glucose and cholestrerol in all groups are observed (±SD). **Significant differences $p < 0.01$

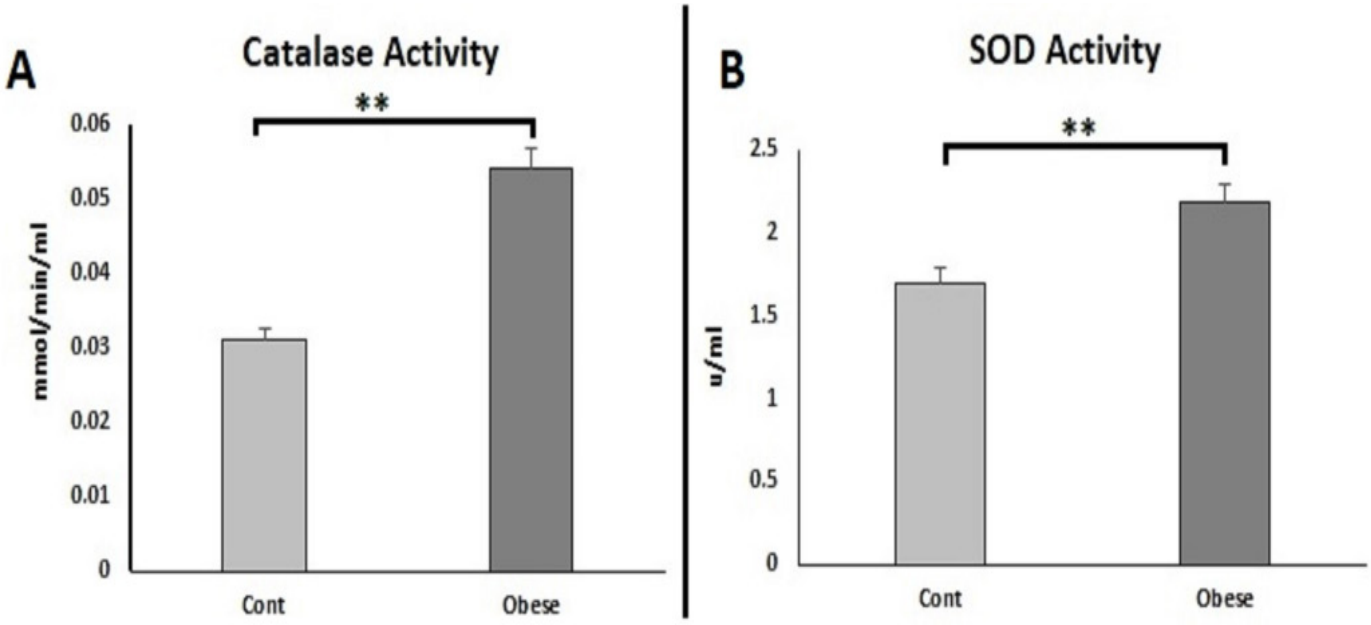

Figure 7. Mean levels of catalase, superoxide dismutase activities in all groups are observed (±SD). **Significant differences $p<0.01$

DISCUSSION

Obesity and overweight are major public health problem worldwide, especially in developing and developed countries (26). The obesity associated adipose inflammation elevates the secretion of pro-inflammatory factors, induces production of reactive oxygen species (ROS) and weakens the antioxidant defence system (27). For that reason, HMGB1 plays a critical role during the inflammation to induce the immune system for the defense (28). In addition, direct relation between development of obesity and BMI and adrenal gland changes has been shown (22).

BMI is used as a standard for classifying weight and is a very useful method to determine the obesity (29). We observed faster weight gain in the diet induced group and that BMI correlates significantly with the body weight only in rats fed with a high fat diet, but not in animals fed a normal diet. For that reason, diet induced group subjects were evaluated as an obese. There were significantly volume differences found in the cortex and medulla of Obese group. Previously, the effects of feeding with high fat diet has been shown as a reason of hyperplasia of the adrenal cortex in response to various environmental stimuli (21,22). Similarly, in the current study, statistically highly

significant increase of estimated mean volumes of ZF, ZR and medulla were found in Obese group. In our results; a significant volume decrease in the ZG of Obese group. In literature, some studies on the relationship between obesity and the adrenal cortex showed that obesity plays a role in changing the ZF and ZR histological structure (22,30). The increase of the ZF cell number caused the change in the ZG cell structure and form the ZF pattern (30). All these result supported by histopathological results. In the obese group there was a thin ZG layer and a thicker ZF layer.

The biochemical study in this research; plasma cholesterol concentration was markedly increased in Obese group. Similar to our results, a positive relationship between plasma cholesterol concentration and lipid peroxidation level was previously shown (31). This study supports the view that the increased oxidative stress may related with elevated cholesterol levels in the adrenal glands of Obese group. In ZF and ZR, cortisol is derived biosynthetically from cholesterol (32). The amount of cholesterol strongly affects the rate of steroidogenesis. In addition, increased cholesterol production and lipoprotein uptake were shown in the adrenal glands of obese animals (22). The stereological analyses of this study support these results because the volumes of the ZF and ZR were increased in the obese group. Also we observed some lipid drop in the cell of the ZF in the histopathological analysis.

Although the relationship between obesity and oxidative stress is clear, it is not clear which is the cause and which is the result (33). It is well known that increased fat tissue is one of the main reasons of the association of elevated oxidative stress with obesity (9). Adipocytes have been described as the source of pro-inflammatory cytokines, and therefore obesity is considered as a chronic inflammatory condition (34). Biochemical markers indicating oxidative stress damage were found very high levels in obese individuals and were directly related to BMI (35). On the other hand some studies shown that oxidative stress, per se, leads to weight gain (33,36). In our study, increased SOD and Catalase enzyme activities were found in obese subject's blood that could be evaluated as a reason of increase in the production of hydrogen peroxide and elevated formation of superoxide radicals.

In particular, HMGB1 from adipocytes plays a dominant role in necrosis. Extracellular HMGB1 activates adipose tissue-resident immune cells, causing active additional HMGB1 secretion from immune cells, and this activation induces adipocyte death (37). Also, the HMGB1 that play a role in inflammation cause to upregulation of cytokines, chemokines, and adhesion molecules, and this regulation is associated with cellular oxidative stress (38,40). In this study, there was a significant increase of the HMGB positive cell in obese groups. Considering that oxidative stress increases in obesity, it is quite possible that HMGB1, which is related to oxidative stress, is increased in the obese group. The HMGB1 positive cells were localized in the all fields of adrenal glands in Obese group while there was a significant density in the ZF. The increased expression of HMGB1 protein might be explained by cholesterol accumulation in immune cells that promotes inflammatory responses (39).

CONCLUSION

Although there are studies in the literature regarding the relationships between each of the parameters in our study separately, no study has been found in which these parameters were studied in chorea in the adrenal gland. It is quite possible that the oxidative stress that develops in the adrenal cortex, especially in the ZF, due to the increased cholesterol and glucose levels in obesity seen in this study, increases the HMGB1 level. However, the relationship between obesity-oxidative stress-HMGB1 in the adrenal gland needs to be examined in detail with pathway analyzes.

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Conflict of Interest: *The authors declare that they have no competing interest.*

Ethical approval: *Faculty of Gulhane Military Medicine and authorization of the ethics committee of Gulhane Military Hospital, Turkey (16/37).*

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