

Immunohistochemical and ultrastructural changes in rat fat tissue related to the local hCG injection

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Abstract. – OBJECTIVES: Recently, it has been observed that weight loss is accelerated by human chorionic gonadotropin (hCG) hormone preparation used for hypothalamic dysfunction in obesity treatment in both sexes. hCG is also used for in vitro fertilization and in treatment of hypogonadotropic hypogonadism.

Our aim was to observe the ultrastructural changes caused by local injections of hCG made for purpose of weight loss and to present them to inform those receiving such therapy.

MATERIALS AND METHODS: In our study, 10 obese female, 10 male obese, 10 non-obese female and 10 non-obese male rats were used. In each group, single dose of subcutaneous hCG injection has been applied to 7 rats for 5 weeks in 5 days of the week, and placebo has been applied to the remaining 3 rats. Following the injection, the tissues were evaluated morphologically, immunohistochemically and ultrastructurally.

RESULTS: Leptin immunoreactivity was similar in all groups. When the adipose tissue samples were examined under electron microscope, they were observed to exhibit normal structure with organelles located around the nuclei and nucleoli, and no distinctive features were found among the groups.

CONCLUSIONS: Administering hCG in addition to diet had no advantage on weight reduction in rats.

Key Words:

hCG, Obesity, Fat tissue, Immunohistochemistry, Ultrastructure.

Introduction

Recently, it has been observed that weight loss is accelerated by human chorionic gonadotropin (hCG) hormone preparation used for hypothalamic

dysfunction in obesity treatment in both sexes¹⁻¹⁹. hCG is used for *in vitro* fertilization and in treatment of hypogonadotropic hypogonadism²⁰. Its mechanism of action is via hypothalamic-pituitary-gonadal axis. It has been shown to have effects on many endocrine organs such as thyroid, suprarenal glands, the ovaries, and the testicles²¹⁻²³. This method has been investigated in many studies on human. Currently, it is widely used in such practices as beauty centers and weight loss resorts because it has been reported to accelerate weight loss and causing regional thinning. Our study was planned because of lack of reports showing effects of widespread use of a hormone preparation affecting whole metabolism for obesity treatment on the tissues at ultrastructural level. Our objective was to observe the ultrastructural changes caused by local injections made for purpose of weight loss therapy.

Materials and Methods

Obtaining and Grouping the Experimental Animals

For the present study, it was planned to use a total of 40 Wistar albino rats. The rats were divided into two groups in such a way that half of them would be male and other half would be female. Then, male and female rats were divided into two sub - groups of obese and non - obese rats. They were used in four groups in such a way that each group would contain ten male and female adult rats. They were obtained in such a way that the female rats of 6 months would be 400 g averagely and male rats of 6 months would be 400 g averagely. For the rats to be used in the study, two sub-groups of female and male rats

from 4 groups were fed *ad libitum* with cafeteria diet for 6 to 8 weeks in order for them to gain weight and then they were continued to be fed standard *ad libitum* for 5 weeks like in the non-obese groups. All rats making the study group were given regular tap water in the laboratory setting under 12:12 hours of light-dark periods.

Administration of the Therapeutic Agents

All of the obese and non-obese groups started to receive hCG and placebo. hCG injection was made subcutaneously once-daily on 5 days every week for 5 weeks. In each group, 7 rats received hCG injections and remaining 3 received placebo. Distant and local fat tissue taken from the rats at the end of previously determined times were morphologically examined under light and electron microscope and expression of estrogen-alpha, estrogen-beta and leptin receptors was assessed immunohistochemically.

Tissue Examination Under Light Microscope

All tissue samples were fixed in 10% neutral formaldehyde for 72 hours. Then, paraffin blocks were prepared. Sections 4 to 5 micron in thickness were taken from the paraffin blocks on the slides with polylysine and they were used in immunohistochemical examination.

Immunohistochemical Method

After the sections in 4 micron thickness taken on the slides with polylysine had been kept in autoclave at 37°C for one night, they were kept in the autoclave for one more hour by elevating the temperature to 57°C in order to facilitate the deparaffinization process. After the slides had been hold in xylene for 15 minutes twice in order to complete the deparaffinization process, they were hold in alcohol series of 100%, 96% and 80% for ten minutes and then cleared of alcohol by holding in distilled water for 5 minutes twice. 3% hydrogen peroxide was prepared with methanole and then applied for 15 minutes to the slides washed with phosphate buffer saline (PBS) and activity of endogenous peroxidase was blocked. Then, Ultra V Block (Lot: K97702D, Splink HRP Broad Spectrum, Golden Bridge International Inc., Life Sciences Division, Mukilteo, WA, USA) was applied for 5 minutes in order to prevent non-specific bindings after the slides were rinsed three times with PBS (pH: 7.4) for 3 minutes. After the blocking stage, the sections were exposed for one night

to primary antibodies to ER- α (sc: 787, Lot: C0209, Santa Cruz Biotechnology, Santa Cruz, CA, USA), ER- β (sc: 8974, Lot: E2809, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Ob-R (sc: 8391, Lot: D1309, Santa Cruz Biotechnology, Santa Cruz, CA, USA) without rinsing. Then, binding to the primary antibody was allowed by applying secondary antibody (Lot: K97702D, Splink HRP Broad Spectrum, Golden Bridge International Inc., Life Sciences Division, Mukilteo, WA, USA) with biotin for 10 minutes after the slides were rinsed three times with PBS for 3 minutes. Then, tissues rinsed with PBS were exposed to streptavidin peroxidase enzyme-complex (Lot: K97702D, Splink HRP Broad Spectrum, Golden Bridge International Inc., Life Sciences Division, Mukilteo, WA, USA) for 10 minutes in order for the tissues to bind biotin. After the slides had been washed with PBS again, visible immune reaction was allowed to appear by using DAB (3,3 aminobenzidine tetrahydrochloride-Plus kit) (Lot: K97717A, Invitrogen, Carlsbad, CA, USA) as chromogen. Harris's hematoxyline was used for background dyeing. The stained slides were subjected to alcohol and xylene series and covered with entellan. The sections were assessed by photographing on Leica Q Vin 3 software in the Leica DM 4000 (Germany) computer-assisted imaging system.

TEM Method

Tissue samples cut into pieces of 1 mm³ were fixed in 2.5% glutaraldehyde (pH: 7.4) in 0.1 M phosphate buffer for 2 hours. The tissues rinsed with buffer three times at the end of fixation process were post-fixed by exposing to 1% osmium tetroxide for 1 hour. The tissues were dehydrated by means of subjecting to serial dilutions of ethanol. Lastly, the tissues exposed to propylene oxide were prepared in blocks with embedding material prepared with Araldite CY212 kit. Semi-thin sections were taken from the blocks polymerized for 48 hours in the autoclave at 56°C and stained with toluidine blue and then examined under light microscope. Fine sections from the marked areas were stained with uranyl acetate-lead citrate and photographed with Carl Zeiss EVO LS 10+ED Transmission Electron Microscope: TEM (Jena, Germany).

Results

In the present study, cells of the fat tissue were distinguished with their normal appearance in the

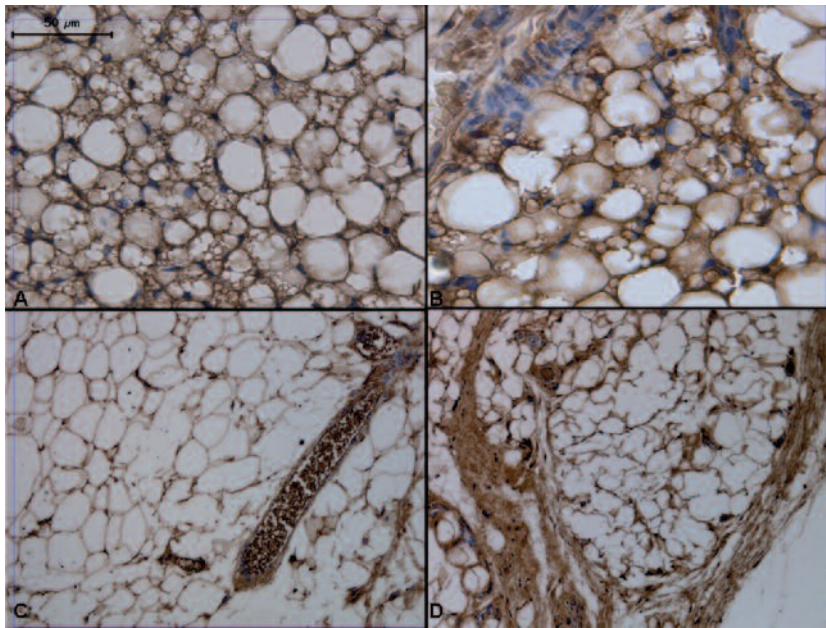


Figure 1. ER- α immunoreactivity of the local fat tissue in the non-obese control group (**A**), non-obese hCG treated group (**B**), obese control group (**C**) and obese hCG treated group (**D**) (Immunoperoxidase-Haematoxylin A, B, C, D X400).

non-obese groups when estrogen receptor (ER)- α immune reaction was assessed in the local fat tissue. Deformation of the fat cells was remarkable in the obese control groups. It was observed that the cells made small groups by coalescing with each other. Reactivity in these groups was remarkable both on the plasma membrane and in the nucleus. Although similar appearance was observed in the obese hCG treated groups, it was remarkable that amount of connective tissue and reactivity in these

groups increased relatively (Figure 1). In the non-obese hCG treated groups, the adipose cells were distinguished with their various sizes in the ER- β staining of local adipose tissue. In the obese groups, however, the plasma membranes were observed to coalesce locally. The coalescing cells were observed to occupy big spaces in whole tissue especially in the obese hCG treated groups. ER- β reactivity was observed to be similar in all groups (Figure 2). In examinations in local adipose tissue, lep-

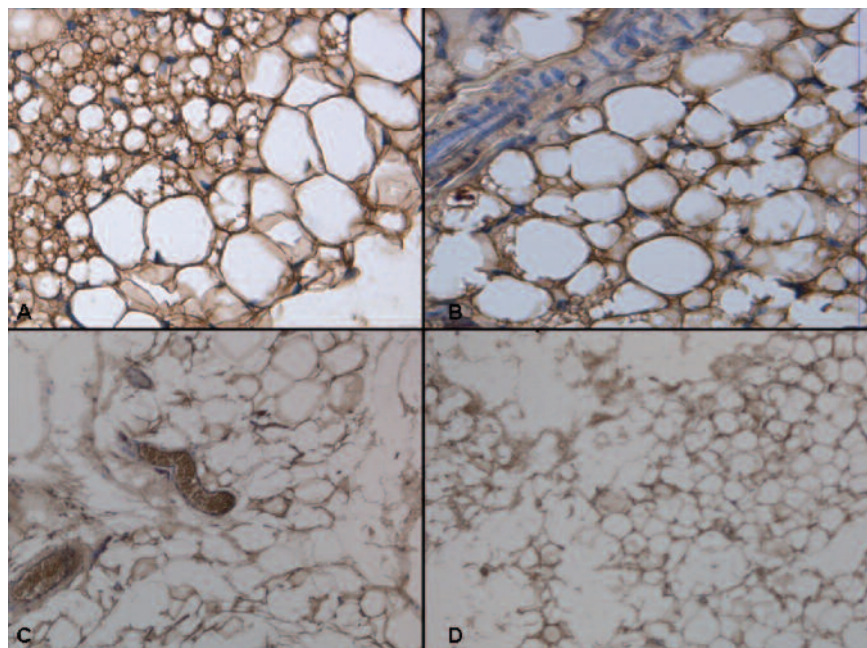


Figure 2. ER- β immunoreactivity of the local fat tissue in the non-obese control group (**A**), non-obese hCG treated group (**B**), obese control group (**C**) and obese hCG treated group (**D**) (Immunoperoxidase-Haematoxylin A, B, C, D X400).

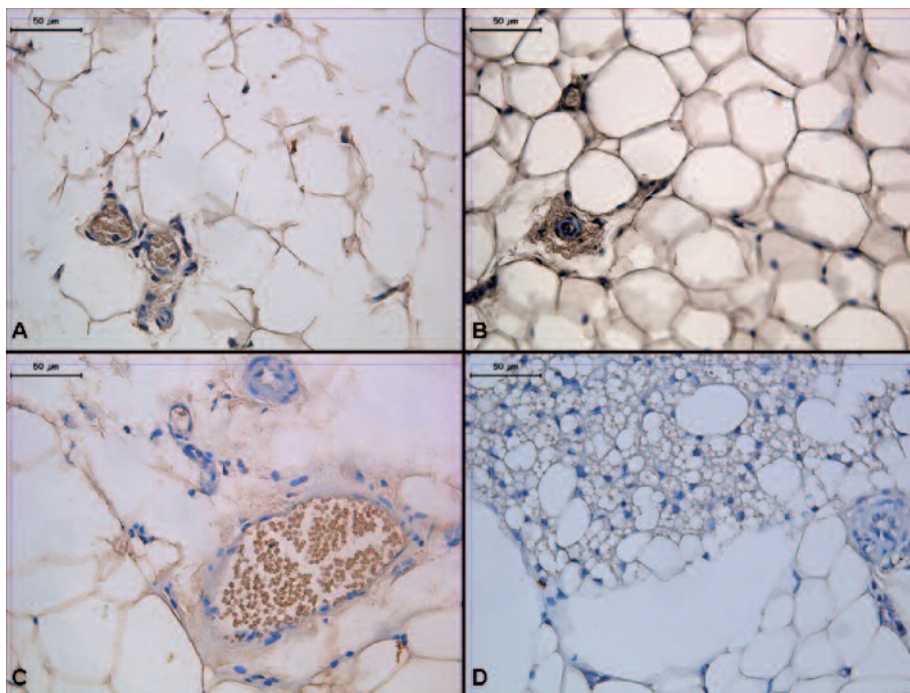


Figure 3. Leptin immunoreactivity of the local fat tissue in the non-obese control group (A), non-obese hCG treated group (B), obese control group (C) and obese hCG treated group (D) (Immunoperoxidase-Haematoxylin A, B, C, D X400).

tin immune-reactivity was observed to exist on the plasma membrane of the adipose cells and connective tissue in the non-obese control groups. Reactivity in the connective tissue was intermediate and widespread in the obese hCG treated groups. However, reactivity of connective tissue in the obese hCG treated groups was decreased compared to the last group (Figure 3). The adipose cells were distin-

guished with their normal shape and appearance when the distant adipose tissue was examined. The connective tissue was normal in structure. ER- α immune reactivity was remarkable on the plasma membranes and in connective tissue and was equal to each other in all groups (Figure 4).

In the ER- β staining of this tissue, the immune reactivity increased in connective tissue in the

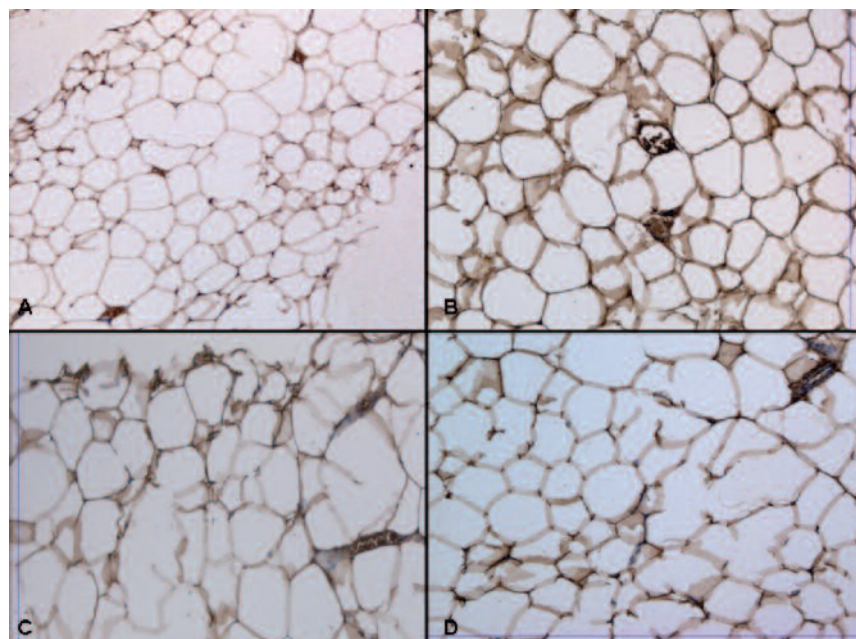


Figure 4. ER- α immunoreactivity of the distant fat tissue in the non-obese control group (A), non-obese hCG treated group (B), obese control group (C) and obese hCG treated group (D) (Immunoperoxidase-Haematoxylin A, B, C, D X400).

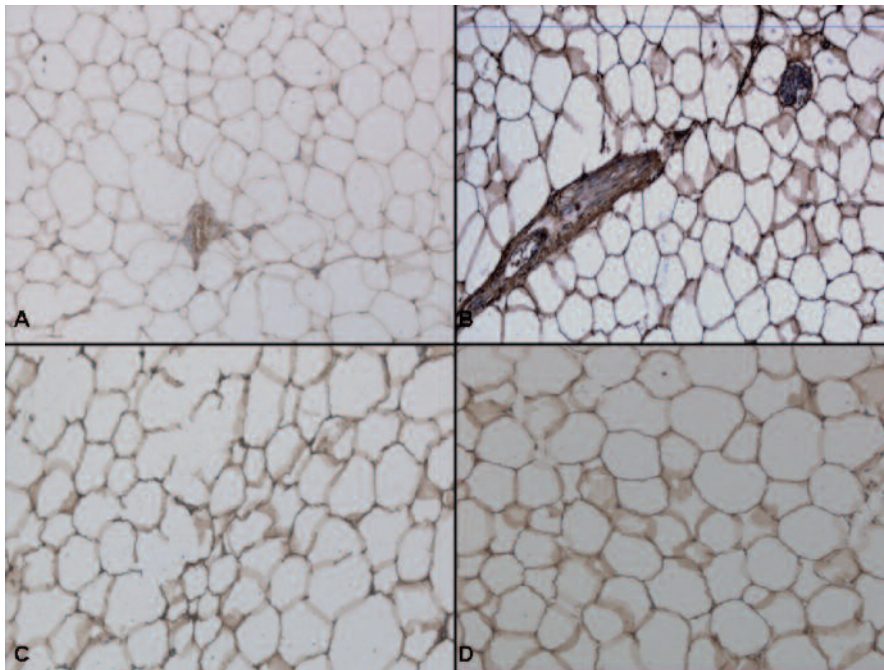


Figure 5. ER- β immunoreactivity of the distant fat tissue in the non-obese control group (**A**), non-obese hCG treated group (**B**), obese control group (**C**) and obese hCG treated group (**D**) (Immunoperoxidase-Haematoxylin A, B, C, D X400).

non-obese hCG treated group in contrast to all other groups. Leptin immune-reactivity in the same tissue was observed to exist on the vessels and on the adipose cells located in the adipose tissue in all groups. Reactivity was similar in all groups (Figure 5). When the adipose tissue samples were examined under electron microscope, they were observed to exhibit normal structure with organelles located around the nuclei and nu-

cleoli, and no distinctive feature was found among four groups (Figure 6).

Discussion

Estrogen and progesterone produced by the ovaries cause total increase of fat in the thoracic and gluteo-femoral regions. These hormones al-

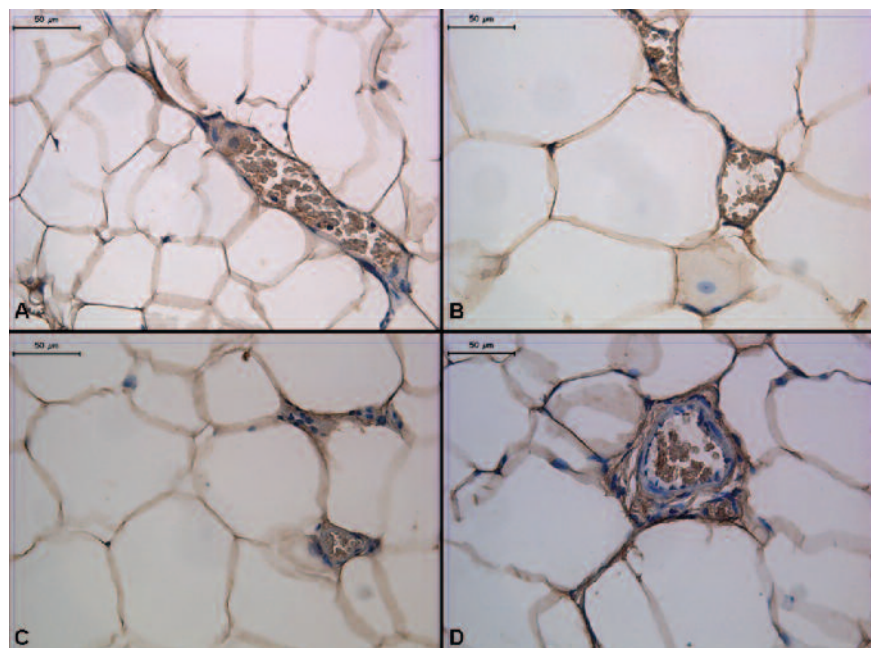


Figure 6. Leptin immunoreactivity of the distant fat tissue in the non-obese control group (**A**), non-obese hCG treated group (**B**), obese control group (**C**) and obese hCG treated group (**D**) (Immunoperoxidase-Haematoxylin A, B, C, D X400).

so have impacts on the ovaries²⁴. The estrogen and progesterone receptors are located on the granulosa cells of the follicle²⁵. In the rat, two subtypes of the estrogen receptor have been defined as estrogen receptor- α and estrogen receptor- β ²⁶. ER- β with high analogy among species has been cloned in mice and humans. Estrogen receptor- α is expressed in Leydig and reproductive cells in the testicles as well as in epithelium of the prostate, vesicular seminalis, breast tissue, ovaries, theca interna cells, and epithelial and stromal cells of the uterus^{27,28}. Furthermore, its mRNA expression has been reported in the epididymis, pituitary, kidneys, suprarenal glands, and in variable extent in the thymus, heart and liver. It has been stated that affinity of estrogen to ER- α is higher than to ER- β and that estrogen increases transcriptional activity of ER- α ²⁹⁻³². Moreover, it has completely opposite transcriptional effects on the ER- α and ER- β activating protein-1 domain. The fact that ER- α and ER- β have similar hormone-binding properties indicates that they respond to the same hormones at comparable levels. It was found in the studies performed at cellular level that ER- β suppressed ER- α when they are transferred to ER-negative cells and reduced sensitivity of the cells to estradiol. It has been observed that obesity may develop in rodents when the ovaries are removed, and similarly fat accumulation increases in human during menopausal period³³⁻³⁵. Peterson observed that the ER- α and ER- β mutant mice developed obesity³⁶. In human adipose tissue, ER- α and ER- β have been demonstrated in the pre-adipocytes and mature adipocytes. Researchers have reported that ER- α and ER- β have similar affinity characteristics and concluded that maintenance of normal body weight is dependent on the balance between them^{36,37}. In the current study, when ER- α immune reactivity in the local adipose tissue was assessed in ER- α and ER- β staining performed in the distant and local adipose tissue, cells of the adipose tissue were distinguished with their normal appearance. It was remarkable that the adipocytes were deformed in the hCG treated obese group. It was observed that the cells made small groups by coalescing with each other. In this group, reactivity was remarkable both on the plasma membranes and on the nuclei. Although similar appearance was observed in the hCG treated obese group, it was remarkable that amount of connective tissue and reactivity in these groups increased relatively. In

ER- β staining on local adipose tissue, plasma membranes were observed to coalesce on some locations in the obese groups, and ER- β reactivity was observed similarly in all groups. ER- α immune reactivity on the distant adipose tissue was remarkable on the plasma membranes and in connective tissue and was equal to each other in all groups. In the ER- β staining of this tissue, the immune reactivity increased in connective tissue in the non-obese group receiving hCG in contrast to all other groups. Therefore, the deformities in adipocytes especially in the obese groups might be a consequence of obesity. Finding coalesced cells due to hCG in the same group suggested that hCG might have dissolved and, thus, reduced content of these cells. Leptin is mainly released by adipocytes in the white adipose tissue and shows its effects on the neurons of arcuate nucleus in the hypothalamus. Many studies have showed that leptin plays important role in regulating body weight in animals and human. The studies revealed that leptin might affect pulsed release of hCG. Plasma level of leptin correlates with body mass index and especially fat rate in the body and its level in the circulation reflects the amount of energy stored in the adipose tissue. As amount of adipose tissue increases, level of leptin increases. Compared to men, it is higher in women. Reason for this difference between two sexes is believed to be that women have more adipose tissue and that testosterone in men inhibits leptin release. hCG hormone, on the other hand, mimics LH released by the pituitary. In the present study, leptin immune reactivity was found on the plasma membranes of the local fat tissue and connective tissue in the non-obese control group not receiving hCG. Reactivity of connective tissue in the hCG treated obese group was intermediate and widespread but increased in the this group compared to the former. Immune reactivity of leptin in distant adipose tissue was observed on the adipocytes and vessels in the connective tissue. Reactivity was similar in all groups. When distant and local adipose tissues were assessed together, the fact that leptin immune reactivity increased in the hCG treated obese group although there was no significant change in the distant adipose tissue suggested that this hormone might have exerted its effects locally. When the samples of adipose tissue were examined under electron microscope, the tissues were found to show normal structure with normal appearing nuclei and nucleoli and organelles located around them

whereas there was no distinctive feature among four groups.

Although the deformities in the adipocytes observed especially in the obese groups in examinations of distant and local adipose tissues were considered to be a consequence of obesity, finding cells with membranes coalesced in this group suggested that hCG administration might have dissolved content of these cells. When distant and local adipose tissues were assessed together, the fact that leptin immune reactivity increased in the hCG treated obese group although there was no significant change in the distant adipose tissue suggested that this hormone might have exerted its effects locally. In ultrastructural assessments, there was no distinctive features among the groups.

Conclusions

The administering hCG in addition to diet had no any advantage on weight reduction in rats.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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