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ORIGINAL ARTICLE BASIC SCIENCE/EXPERIMENTAL

Effects of Insulin, Metoprolol and Deferoxamine on Fat Graft Survival

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Abstract

Background The main problem faced with fat grafting is unpredictable resorption rates. Many substances have been reported to increase the survival of fat grafts. The aim of this study was to compare the effects of insulin, metoprolol and deferoxamine on fat graft survival.

Methods Inguinal fat pads of male Sprague–Dawley rats were harvested and split into four parts as grafts. The grafts were placed in subcutaneous pockets in four quadrants on the back area of the rats. The insulin and metoprolol group fat grafts were incubated in regular insulin and metoprolol solutions, until they were placed. Deferoxamine and control group fat grafts were placed without incubation. After surgery, the control group fat grafts were injected with 10 doses of NaCl solution once every 3 days, and the deferoxamine group fat grafts were injected with 10 doses of deferoxamine solution once every 3 days. After a graft maturation period of 3 months, the grafts were harvested for weight measurements and histological and immunohistochemical evaluation.

Results According to the rate of perilipin staining, the metoprolol group had 30% more mature viable adipocytes than the control and insulin group fat grafts ($p < 0.05$ and $p < 0.01$, respectively). CD31 activation rates were significantly higher in the deferoxamine and insulin group than in the metoprolol group ($p < 0.05$). CD34 staining rates did not differ between any groups ($p > 0.05$).

Conclusions In this experimental study, we have shown that there was no significantly increased fat graft survival rate seen in any drug treatment group. Low survival rates of stem cells demonstrated that the adipogenesis period ended at 3 months. Treatment of fat grafts with the selective β_1-blocker metoprolol resulted in good quality better graft take with more viable mature adipocytes. However, better viability of adipocytes did not result in increased weight of the fat graft. Studies aiming to compare the effects on fat graft survival of beta-blockers with long or short durations of action, different potencies and different receptor selectivity may be designed in the future. In addition, further studies may be performed, in which immunohistochemical markers used to assess inflammation and fibrosis are added to the study after the completion of the fat graft maturation period at the end of the first year to test the permanence of the results.

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Keywords Fat graft · Survival · Metoprolol · Deferoxamine · Insulin

Introduction

Fat grafting is a widely used procedure in plastic surgery for both reconstructive and aesthetic purposes [1–3]. The main problem faced with fat grafting is unpredictable resorption rates, which create unpredictable long-term results. Many substances have been reported to increase the survival of fat grafts, including insulin, growth factors, oestradiol, beta-blockers and deferoxamine [4–10]. In 2000, Yuksel et al. found that the effect of insulin in increasing fat graft survival was similar to that of insulin-like growth factor 1 (IGF-1) due to receptor similarity [4]. In 2001, Ayhan et al., in a comparison of the effects of insulin and metoprolol, a selective β_1-blocker that has lipolysis-inhibiting effects on adipocyte cell membranes via cyclic AMP, reported that metoprolol had a greater effect on fat graft survival [5]. Deferoxamine has been studied because of its indirect contribution to angiogenesis via chelation of the iron ion cofactor of prolyl hydroxylase, a degrading enzyme of hypoxia-inducing factor 1α (HIF-1α) and was reported to have positive results, with increased levels of HIF and vascular endothelial growth factor [6]. In 2016, Temiz et al. found that deferoxamine, acting indirectly through HIF-1α, led to hypertrophy of fat grafts [7]. The aim of the present study was to evaluate the effects of insulin, metoprolol and deferoxamine on fat graft survival.

Materials and Methods

Animal Model

Ethical approval was obtained from the Experimental Animals Local Ethics Committee of Aziz Sancar Experimental Medical Research Institute of Istanbul University, and the experimental protocols for animal studies were adhered to throughout the study. All applicable institutional and/or national guidelines for the care and use of animals were followed. Twenty-two male Sprague–Dawley rats aged 8 to 9 weeks weighing $\sim$ 300 $\pm$ 10 g were used. The rats were kept in individual cages maintained at standard room temperature and were fed ad libitum. The back of each rat was separated into four quadrants (Fig. 1). Inguinal fat pads of the rats were used as donor sites, and surgically created dorsal subcutaneous pockets as recipient sites. The study comprised three types of experimental fat graft groups: the right cranial insulin group, which were incubated in regular insulin solution; the right caudal metoprolol group, which were incubated in metoprolol solution; and the left caudal deferoxamine group, in which deferoxamine solution was injected periodically after surgery. Injection, instead of incubation method for deferoxamine, was used. Because the methods that we chose for this study were used in previous similar articles [5–7] and we tried to mimic the same methods in order to compare the results. The control group was the left cranial serum group, in which isotonic serum solution was injected periodically after surgery. In addition, during killing of the rats, one adipose tissue biopsy sample from each animal, called the self-fat tissue sample which was not used as a graft, was harvested to compare with the study group fat grafts histologically.

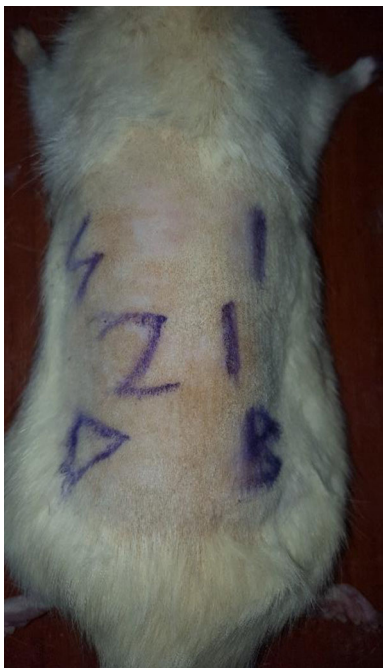

Fig. 1 Determination of the groups [21: rat number (1–22), S: serum/control group, I: insulin group, D: deferoxamine group, B: beta-blocker (metoprolol) group quadrants for placement of fat grafts]

Surgical Procedure

After induction of anaesthesia with 30 to 35 mg/kg of ketamine HCl (Ketalar 500 mg flakon; Pfizer İlaçları Ltd. Şti., Istanbul, Turkey) and 10 mg/kg of xylazine (Rompun 2% injectable; Bayer Healthcare LLC, Kansas, USA) solution intraperitoneally, under appropriate local antisepsis conditions, the dorsal and inguinal areas of the rats were shaved. The inguinal fat pads of the rats were split into four nearly equal parts after being harvested as grafts. The obtained fat grafts were quickly weighed on a precision scale (Costech I2000 Superior Mini Digital Platform Scale;

Costech Tanzania Commission for Science and Technology, Dar es Salaam, Tanzania) before they were subjected to any treatment and without allowing them to dry. The insulin group fat grafts were incubated for 5 min inside an incubator under appropriate conditions equal to the body temperature of male Sprague–Dawley rats in 5 mL of regular insulin solution (NovoRapid FlexPen 3 mL 100 IU/mL; Novo Nordisk Sağlık Ürünleri Tic. Ltd. Şti., Istanbul, Turkey) until they were placed in the rats' backs. [5] The metoprolol group fat grafts were incubated for 5 min in 5 mL of metoprolol solution (Mepolex 5 mg/5 mL IV ampul; Keymen İlaç San. ve Tic. Ltd. Şti., Istanbul, Turkey) inside an incubator under the same conditions as the insulin group fat grafts until they were placed in the rats' backs [5]. Four 1 × 1 cm subcutaneous pockets were dissected between the dermis and the panniculus carnosus, one in each quadrant, on the dorsal area of each rat. Each fat graft was placed on its predetermined pocket after incubation, except for the deferoxamine and control group fat grafts, which were placed without incubation. After surgery, the control group fat grafts were injected with 10 doses of 0.2 mL 0.9% NaCl solution once every 3 days, and the deferoxamine group fat grafts were injected with 10 doses of 0.2 mL 0.9% NaCl solution prepared with 300 mg deferoxamine once every 3 days [7].

After a fat graft maturation period of 3 months, the rats were euthanised by a high dose of 150 mg/kg pentobarbital injected intraperitoneally. Five rats died while undergoing the first surgical procedure. The grafts were harvested together with their transition zones through longitudinal incisions made over the midline of the dorsal area (Fig. 2), for weighing with a precision scale and histological and immunohistochemical evaluation. The euthanised rats were removed in accordance with the rules of the Istanbul University Aziz Sancar Experimental Medical Research Institute Animal Sciences Experimental Animals Laboratory.

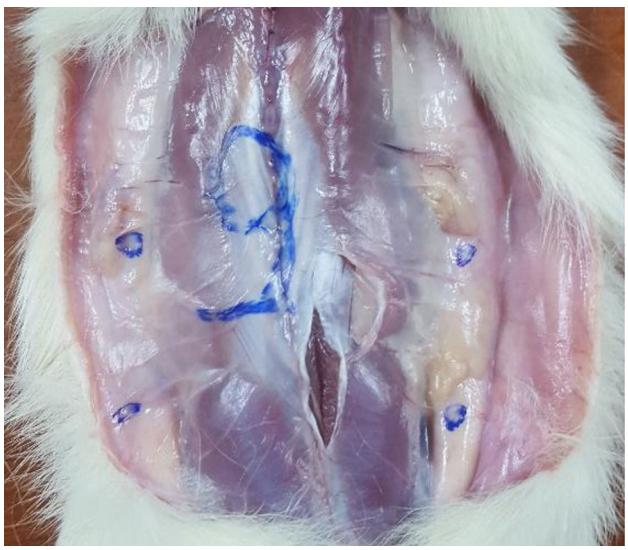

Fig. 2 Fat grafts after maturation period. Note the new blood vessels growing in areas perpendicular to the fat grafts

Histological and Immunohistochemical Analysis

All materials were stained and evaluated histopathologically and immunohistochemically in the Pathology Department of Istanbul University Cerrahpaşa Medical Faculty. Immunohistochemical staining procedures were performed using a compatible kit with an automated immunohistochemical staining device (Ventana Benchmark XT, Ventana Medical Systems, Tucson, AZ, USA). The grafts were divided into two parts longitudinally, and 4-μm serial sections were made from paraffin blocks. The first section was stained with haematoxylin and eosin to evaluate the rate of fat necrosis. The second section was stained with perilipin immunofluorescent antibody [1:100 D1D8 (Cell Signaling, Leiden, the Netherlands)] to evaluate the rate of viable adipocytes. Perilipin expression was focal and weak in the areas where immature cells were observed. Perilipin staining was considered positive only in strongly stained areas and was expressed as a viable adipocyte percentage. The third section was stained with CD34 immunofluorescent antibody [1:400 QBEnd/10 (Cell Marque, Rocklin, CA, USA)] to evaluate the rate of stem cell survival. The fourth section was stained with CD31 immunofluorescent antibody [1:100 EP78 (Cell Marque, Rocklin, CA, USA)] to evaluate the rate of angiogenesis. The presence of CD31 or CD34 was scored as 0 = none, 1 = light, 2 = moderate, 3 = strong. Light microscopical evaluation was carried out at 40× magnification in 5 × 5 mm areas, with the values taken to include the entire cross-sectional area.

Statistical Analysis

Statistical evaluation was performed with IBM SPSS Statistics software, version 21.0 (IBM Corp., Armonk, NY, USA, 2012). Mean values and standard deviations of all data were calculated. All results are expressed as mean ± standard deviation. The distribution of the variables among the groups was determined by the Kolmogorov–Smirnov test. For numerical data, the *t* test was used for dependent groups and delta was calculated for the percentage change. In comparisons between two groups, the Mann–Whitney *U* test was used for nonparametric data, the McNemar test for intermittent data and the Kruskal–Wallis test for nonparametric numerical data. The Mann–Whitney *U* test was used for post hoc analysis, and statistical significance was determined by Bonferroni

correction. A p value < 0.05 was considered to indicate statistical significance.

Results

Weight maintenance was calculated as a percentage by dividing the final fat graft weight at the end of the study by the pregrafting weight. Although graft weights significantly decreased between pre- and post-study in all groups ($p < 0.05$), there were no statistically significant differences between the four groups in percentage weight loss ($p > 0.05$) (Table 1).

There were also no statistically significant differences between the four groups in percentage fat necrosis ($p > 0.05$).

There was a statistically significant difference between the four groups of fat grafts in the percentage of perilipin staining ($p < 0.05$). In the post hoc analysis, the metoprolol group fat grafts had a significantly higher percentage of perilipin staining than the control group fat grafts and a significantly higher percentage of perilipin staining than the insulin group fat grafts ($p < 0.05$ and $p < 0.01$, respectively) (Fig. 3).

The insulin group fat grafts were more strongly stained with CD31 than the self-fat tissue group ($p < 0.01$); this was the only difference in CD31 staining rate between the self-fat tissue group and the study groups. The CD31 staining rate in the control group fat grafts was not significantly higher than that in the other three study group fat grafts ($p > 0.50$). There was a significant difference in CD31 staining rate between the deferoxamine and the metoprolol group fat grafts and a more significant difference in CD31 staining rate between the insulin and the metoprolol group fat grafts ($p < 0.05$ and $p < 0.005$, respectively); that is, the CD 31 staining rate was strong in the deferoxamine group fat grafts and stronger in the insulin group fat grafts compared with the metoprolol group fat grafts. However, there was no statistically significant difference in CD31 staining rate between the deferoxamine and the insulin group fat grafts ($p > 0.05$) (Fig. 4). There were no statistically significant differences in CD34 staining rates between any groups, including the control group ($p > 0.05$) (Fig. 5).

Table 1 % Change in weight

	Preop weight (g) Mean ± SD	Postop weight (g) Mean ± SD	p	X^2
Control	0.64 ± 0.16	0.45 ± 0.22	0.589	1.92
Deferoxamine	0.63 ± 0.16	0.36 ± 0.15		
Insulin	0.63 ± 0.18	0.39 ± 0.10		
Metoprolol	0.63 ± 0.16	0.42 ± 0.15		

Kruskal–Wallis test. SD, standard deviation

Discussion

Fat grafting has been used for both aesthetic and reconstructive purposes [1–3, 11]. Many studies of fat grafting have reported controversial results [11, 12]. In 1989, Fournier initiated a new era with a technique called 'lipofilling', based on the injection of fats obtained by liposuction [13], but the results were regarded as unpredictable [2, 14–16]. In 1997, Coleman identified a popular technique called 'lipostructure' [17]. Thus, fat grafting has gained popularity again [1, 14, 18].

Increasing the quality of skin and softening scar tissue, filling a soft-tissue defect and increasing the projection of body parts for aesthetic purposes are several uses of fat grafting [1, 2, 14]. Although fat grafting has major advantages compared with implant materials, unpredictable resorption rates may lead to disappointing results [2, 19]. This has led to the use of many materials to improve the results [4–10].

Genetic, physiological and metabolic variables are determinants of fat graft survival [20–22]. In most experimental studies, each animal has received one type of fat graft study group. In our study, each rat's dorsal area was divided into four quadrants, and all experimental group fat grafts were examined on each animal (Fig. 1). Thus, the effect of genetic and epigenetic conditions on the outcome was minimised. In addition, the study groups were more homogeneous and reliable, and the number of experimental animals was reduced. Because of the known effect of the oestrogen cycle on fat metabolism [9, 23–26], only male rats were used in our study.

Insulin has high lipogenic activity due to the similarity of its receptors to those of IGF-1 [27] and its induction of acetyl-CoA carboxylase [28]. In 2000, Yuksel et al. found that the effect of insulin on fat graft survival was similar to that of IGF-1 [4]. Adipose tissue has a neuroregulatory mechanism that leads to lipolysis due to β-adrenergic receptors [29]. Therefore, the effect of selective β_1-receptor blocking agents such as metoprolol on adipose tissue metabolism will favour lipogenesis. In 2001, Ayhan et al. compared the effects of metoprolol and insulin on fat graft survival. They reported that the survival rate of the metoprolol group increased with the volume of the fat graft [5]. The most interesting result we have found in our study was the decrease in the weight of fat grafts in all groups at the end of 3 months. In fact, the weight or volume of fat grafts is reduced in experimental models [5–7] and in clinical practice. However, in our study the decrease in the weight

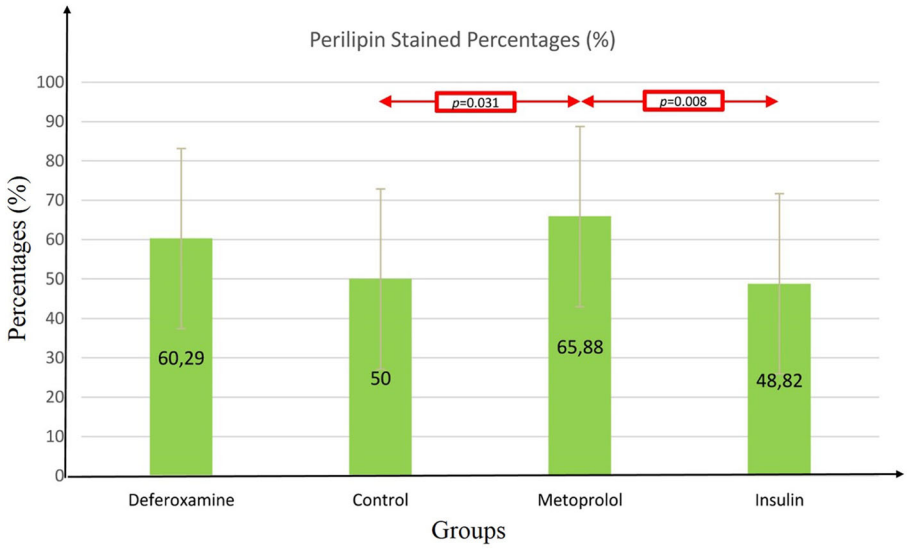

Fig. 3 Percentage of perilipin staining for the four groups

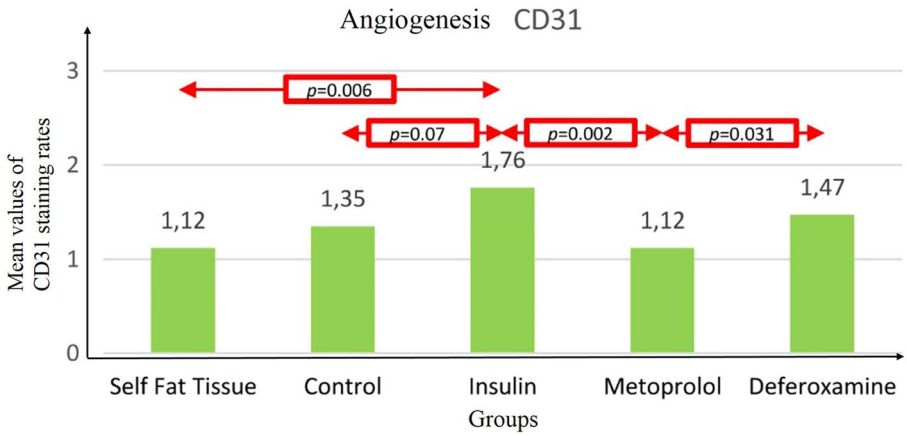

Fig. 4 Mean values of CD31 staining rates

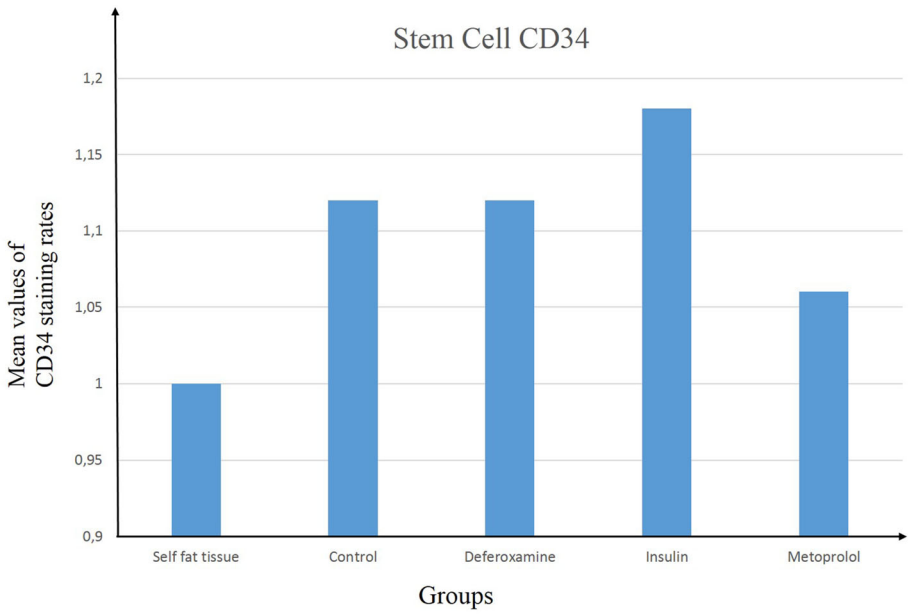

Fig. 5 Mean values of CD34 staining rates

of fat grafts was statistically non-significant. On the other hand, Yüksel et al. [4] found the absolute and real increase in the weight or volume of fat grafts at the end of 3 months. They used subcutaneous injection of fat grafts mixed with insulin-containing polymerised microspheres in order to release drugs for a long period. However, they calculated the fat graft weights as the sum of fat graft weight and weight of microspheres all together after the experiment. It was hard to understand whether the increase in weight of specimens was due to the increase only in the weight of the fat graft or due to the increase in the weight of both the mixture of fat graft and the microspheres, but they did not clarify. In our study, we found that the insulin, deferoxamine and metoprolol groups did not differ significantly from the control group.

In our study, the viable adipocyte rate was more than 30% higher for the metoprolol group than for the control and insulin groups (Fig. 3). In the study of Ayhan et al., they found that mature viable adipocytes were statistically predominant in fat grafts treated with metoprolol. In addition, they found less amounts of mature viable adipocytes with higher amounts of fibrosis, multinuclear giant cells and fat necrosis in fat grafts treated with insulin [5]. In the study of Yuksel et al., the effects of insulin on fat graft survival were similar to those found by Ayhan et al. [4]. However, Yuksel et al. studied subcutaneous injection of fat grafts mixed with insulin-containing polymerised microspheres [4]. In both studies, a smaller number of animals for each study group were examined with a different methodology than in our study, which may explain the results we found are different from their study.

Deferoxamine is an iron-chelating agent used clinically in the treatment of iron poisoning. Prolyl hydroxylase is a degrading enzyme of HIF-1α that uses the iron ion as a cofactor [6]. Recent studies have found that deferoxamine increases fat graft survival [6, 7]. In contrast to recent findings, our results showed that deferoxamine did not increase the weight of fat grafts. However, in our study, the deferoxamine group was found to have a non-significant increase in viable adipocyte rate compared with the control group ($p > 0.05$). According to the study of Temiz et al., deferoxamine significantly ($p < 0.002$) increased viable adipocyte rates histologically [7]. In comparison with the studies of Yuksel, Ayhan and Temiz, our study examined a larger number of animals for each group, all experimental groups were examined on each rat and evaluations were performed with immunohistochemical stains such as perilipin, CD31 and CD34, which are reported to be more specific than haematoxylin and eosin staining [30–33].

Eto et al. reported three zones from the periphery to the centre of the fat graft: an outer 'surviving' zone, an intermediate 'regenerating' zone and a central 'necrotic' zone [34]. According to their study, survival of the adipose-derived stem cells (ADSCs) in the regenerating zone determines the overall final fat graft volume and this period is called adipogenesis [30]. Yoshimura et al. showed that the rate of newly formed viable small adipocytes in the regenerating zone returns to baseline levels by 12 weeks of postgrafting. Thus, they stated that adipogenesis occurred until the end of the third month [33]. After that period, the partly necrotised adipose tissue does not lose its volume, because dead adipocytes stay nonabsorbed as lipid droplets for a long time [31, 34]. Therefore, fat grafts were harvested at the end of the third month in our study. In addition to viable adipocyte rates, we cross-checked the end of the adipogenesis period between the study groups and the self-fat tissue group according to stem cell survival rates. The low stem cell survival rates that we found in all groups at the end of 3 months (Fig. 5) confirm the same length of the adipogenesis period that Yoshimura et al. found in their study.

Low stem cell survival rates and low mature viable adipocytes with high angiogenesis, which were found in the insulin group (Fig. 4), may be explained by contamination with haematopoietic cells, the presence of inflammation and/or increase in fibrotic activity [30, 35–39]. Low angiogenesis but high levels of mature viable adipocytes found in the metoprolol group may suggest that the effect of mechanism for fat graft survival may be due to adipogenesis regardless of angiogenesis. In contrast to metoprolol, the increase in mature viable adipocytes and angiogenesis found in deferoxamine suggests that it is due to angiogenesis-dependent adipogenesis. Further studies are needed, which should include immunohistochemical markers of inflammation, fibrosis and stem cell rates within a 3-month period, to explain the higher angiogenesis rates seen in the insulin and deferoxamine groups.

Conclusions

In this experimental study, we have shown that there was no significantly increased fat graft survival rate seen in any drug treatment group. Treatment of fat grafts with the selective β_1-blocker metoprolol resulted in better graft take. However, better viability of adipocytes did not result in increased weight of the fat graft. Low survival rates of stem cells demonstrated that the adipogenesis period ended at 3 months. Studies aiming to compare the effects on fat graft survival of beta-blockers with long or short durations of action, different potencies and different receptor selectivities may be designed in the future. In addition, further studies may be performed in which immunohistochemical markers are used to assess inflammation and fibrosis at the end of the first year to test the permanence of the results.

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Compliance with Ethical Standards

Conflict of interest All authors report no conflicts of interest. No competing financial interests exist.

Ethical Approval Ethical approval was obtained from the Experimental Animals Local Ethics Committee of Aziz Sancar Experimental Medical Research Institute of Istanbul University, and the experimental protocols for animal studies were adhered to throughout the study. All applicable institutional and/or national guidelines for the care and use of animals were followed.

Informed Consent For this type of study, informed consent is not required.

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