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ORIGINAL ARTICLE | BASIC SCIENCE/EXPERIMENTAL

Effects of Cigarette Smoke on Fat Graft Survival in an Experimental Rat Model

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Abstract
Introduction A fat graft is the closest thing to being the ideal soft tissue filler. Although it has many advantages, reliability of late-term survival is a never-ending debate. Although there are observational studies that research the effect of cigarette smoke on fat graft take in clinical setting, there has not been an objective experimental animal study on the affect of smoking on fat graft survival. The aim of our study is to search if smoking has an affect on fat grafts.
Materials and Methods Twenty-two Sprague-Dawley type rats were used. Exposure was maintained via a passive smoke exposure system. Rats were divided into three groups regarding their exposure period. At the end of the study, transferred fat grafts were extracted and weighed with a precision scale, an arterial blood sample was taken for biochemical analysis, and grafts were sent to the pathology laboratory for immunohistochemical assessment.
Results There were meaningful differences between the control group and the other two groups in graft weight loss, serum cotinine, tissue MDA, adipose tissue/fibrosis ratio, stem cell counts, perilipin positive cell density and inflammation density. Furthermore, we detected meaningful correlations between serum cotinine, tissue MDA and graft weight loss.
Conclusion Fat graft takes with the same mechanisms as a wound heals. *So like wound healing, cigarette smoke has a negative affect on fat graft survival*. A fat graft is by its nature an elective procedure so to improve our late-term success, cigarette smoke exposure should be kept to a minimum for increased reliability.
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Keywords Fat grafting · Smoking · Tobacco · Cigarette · Fat graft survival · Fat graft reliability

Introduction

Fat grafting has become the most potent weapon in our arsenal for filling soft tissue defects, body contouring purposes and revitalizing damaged tissues [1, 2]. It is a safe, reproducible, effective and relatively easy procedure. The major problem of fat grafting is most possibly the unreliability of late-term survival (ranging from 20 to 60%); therefore, many studies were carried out to find methods and materials to overcome this issue [3]. Even though the question of 'which conditions can improve survival' is studied extensively, there are relatively few studies looking into conditions that can alter fat graft's survival rates in a negative way.

Smoking, with its all deleterious effects on cell structure and circulation elements, is a commonly pointed out factor for many surgical complications such as fat necrosis, flap ischemia, bad scars, etc. [4]. It decreases the overall chance

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for a successful outcome because it can affect many steps in wound healing immensely [5].

Fat graft taking is, in many ways, similar to wound healing. It follows the same principles and phases. Taking this into consideration, a number of theories have been constructed to explain the life cycle of this newly placed fat graft; such as the 3-zone theory [6]. Assuming a factor that can delay wound healing may affect fat graft taking as well would not be wrong at all. As the most common addiction in society, tobacco is a frequent troublemaker that surgeons have to deal with on a regular basis. Although it is widely accepted and believed that smoking can interfere with fat graft survival, to date it has not been shown quantitatively in an experimental model. Our study objective was to find out whether smoking has an effect on fat graft survival in an experimental passive smoking model using rats as subjects and if it indeed has any effect; to what end.

Materials and Methods

Approval of the Istanbul University Experimental Animal Research Ethics Committee was obtained, and funding from Scientific Research Projects Section had been granted. The study basically consisted of three stages. The first stage was maintaining cigarette's influence on rats to condition them before fat grafting, which took 6 weeks as described in previous studies [7]. The second stage was fat grafting surgery, and the final stage was maturation of the fat graft in its new site, which took 6 weeks as well. All surgical manipulations were performed by the senior author (A.E.).

Smoke Exposure Model and Protocol

The subjects were exposed to cigarette smoke within the smoke exposure model designed by Braga–Gazzalle [8] (Fig. 1). The system built for smoke inhalation consists of a 40 × 33 × 17-cm box divided into two chambers by a perforated metal sheet. The larger chamber was used for the rats (up to 4) and the smaller for the cigarettes, coupled in a metal base with holes. On one wall of the larger chamber, a simple fan (0.14-A computer cooler) was installed. On the wall opposite of the fan, there are a couple of holes for ventilation. This generates a continuous airflow inside the box during the experiment: the smoke is aspirated from the smaller chamber and passes toward the computer fan and leaves there. The volume of the drawn smoke is composed basically of the burning tip (sidestream) as the cigarette filter tip is coupled to the metal base and hermetically isolated from the ambient air. During the experiment, the apparatus is kept closed by a transparent cover to watch the rats. One-week gradual adaptation of the rats to cigarette smoke was maintained, with 1 h exposure per day. The final standardized maximum exposure was reached with eight cigarettes for a period of 2 h per day, for 5 days a week. The experiment was carried out on weekdays not on weekends. All the cigarettes used for the duration of the experiment were the same brand (Camel), which has high levels of tar (10 mg), carbon monoxide (10 mg) and nicotine (0.8 mg).

Fig. 1 The smoke exposure model designed by Braga–Gazzalle

Animals

Twenty-two young, female, Sprague-Dawley type rats weighing between 220 and 240 g were provided from Istanbul University Experimental Animal Research Department for this study. Rats were kept in a temperature controlled sterile environment at 64–79 °F using a 12-h light/12-h dark cycle. Animals were housed in groups of 4 prior to surgery and singly housed postoperatively. The rats were fed standard chow, and water was provided ad libitum. The rats were divided into three groups regarding their period of exposure. Seven rats in group A were exposed to cigarette smoke for a period of 6 weeks before fat graft transfer initially and an another period of 6 weeks after the transfer. They were labeled as the *continuous exposure* group. Seven rats in group B were exposed to cigarette smoke within the same initial 6-week period and with similar conditions as group A, but their exposure was ended after fat graft transfer. They were labeled as the *smoking cessation* group. Eight rats in group C were not exposed to cigarette smoke at all, and they were the control group in this study.

Fat Graft Surgery

At the end of the initial 6-week period, all the subjects were taken to surgery. The left inguinal fat pad was used as the donor site in our study. The surgical procedures were performed in sterile conditions under general anesthesia with intraperitoneal injection of ketamine hydrochloride 100 mg/kg (Ketalar, Pfizer Warner Lambert, New York, NY) and xylazine 10 mg/kg (Rompun 2%, Bayer Türk Kimya Sanayi Ltd. Şti, Istanbul, Turkey). All the subjects were injected with one dose of 0.25 mg cefazolin sodium (Sefazol, Mustafa Nevzat AS, Istanbul, Turkey) for infection prophylaxis.

The rat was fixated to the operation table in supine position, and hair of the left inguinal area was shaved. A 2.5-cm-long incision following the inguinal crease was performed for harvesting the inguinal fat pad (Fig. 2). After dissecting the inguinal fat pad from the femoral pedicle and ligating the nurturing branch, the dissected fat pad was excised én bloc (Fig. 3). After weighing the graft with a precision scale and making sure the graft was approximately 50 mgs, we diced the fat pad into smaller pieces for maximum contact in the recipient area using a No: 15 scalpel. The donor area was sutured with silk sutures after performing careful control of bleeding. With a 3-cm incision passing through skin and panniculus carnosus in the parascapular area, we placed the graft under a 3 × 2 cm surgical pouch (Fig. 4). After fat implantation, the parascapular incision was closed in primary fashion without any tension.

Fat Graft Extraction

After the transfer surgery, the subjects were followed up for an additional period of 6 weeks; it was the necessary time needed for maturation of the newly implanted fat graft. At the end of this second 6-week period, all animals were operated on under general anesthesia. A dorsal skin flap containing all the previous incision sites was elevated on the recipient parascapular area to visualize the fat graft (Fig. 5). Gross analysis, general observation was noted down and photodocumentation of the graft was made at

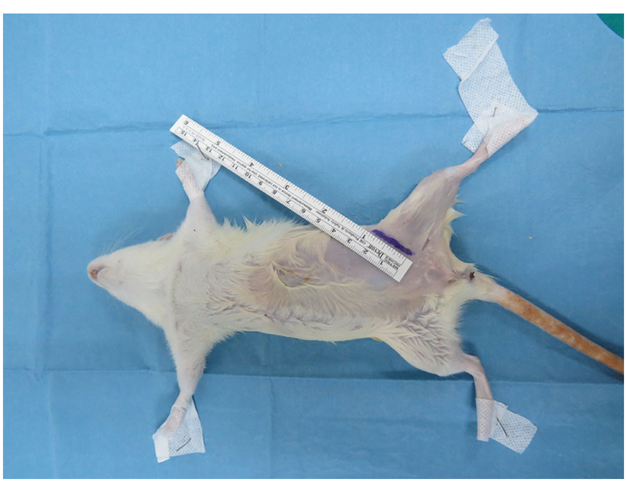


Fig. 2 A simple 2.5-cm incision performed to expose fat pad

Fig. 3 Excised fat pad

this time (Fig. 6). The fat graft was dissected carefully from the surrounding soft tissue/skin block and stored for pathological evaluation after weighing it with the same precision scale. An arterial blood sampling from abdominal aorta was taken for biochemical analysis. After the surgery, deeply anesthetized rats were injected with 0.5 ml of 120 mg/kg pentobarbital barbiturate (Pentothal Sodium, Abbott Turkey, Istanbul, Turkey) for ethical euthanization.

Weight Change Analysis

After the grafts were dissected carefully from under the flaps, they were weighed with a precision scale (Sartorius Quintis, Sartorius, Frankfurt, Germany), before handling them with 10% buffered formalin. The difference in weights between the time of placement and the time of extraction were calculated and recorded.

Histopathological Evaluation

The grafts were cut into two sections (cranial and caudal), and each section was placed in 10% neutral buffered formalin. Sections were prepared in this condition for 48 h, allowing for the tissues to fix. Hematoxylin–eosin staining and immunohistochemical analysis with antibody against perilipin were performed. Following staining, the specimens were evaluated under 40×, 100× and 200× magnifications for the assessment. Percentage of perilipin positive adipocytes and preadipocytes, densities of inflammation in the tissue and ratio of areas undergoing granulation were evaluated.

Scanned photographs from each slide were printed out, and these prints were evaluated by three blinded reviewer pathologists. They were rated from 1 to 5 for adipose tissue content and non-adipose tissue. The higher the adipose tissue content, the higher the rating. Figure 5 shows different examples of grafts with their scores based on the

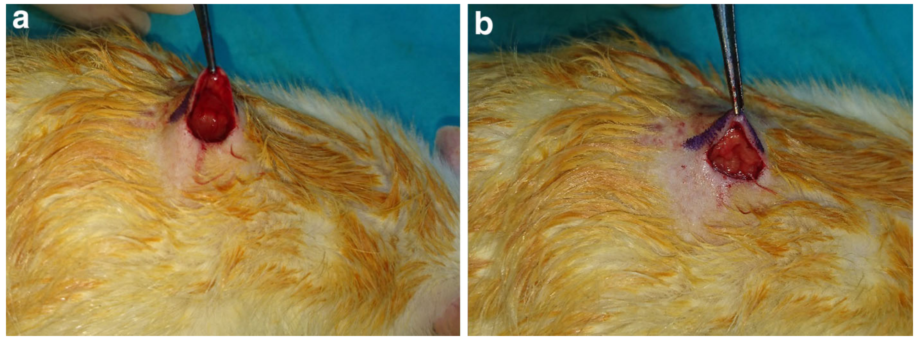


Fig. 4 **a** Surgical pouch under the dorsal skin, **b** surgical pouch filled with fat graft

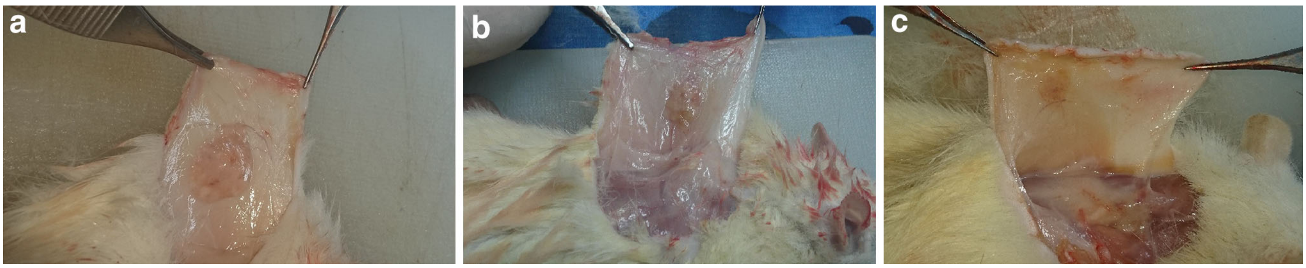


Fig. 5 Mature fat graft under the dorsal skin; **a** sample from control group, **b** sample from smoke cessation group, **c** sample from continuous smoking group

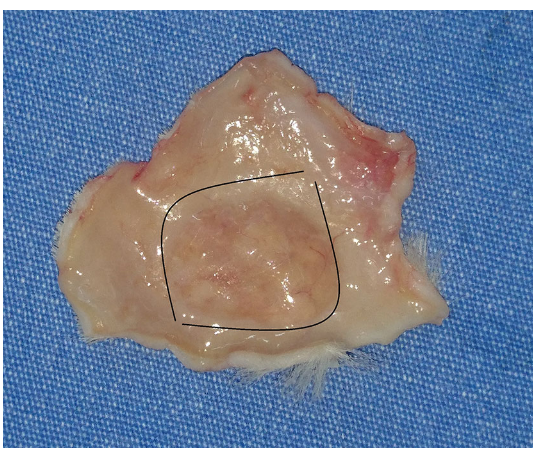


Fig. 6 Excised en bloc fat graft-skin segment (fat graft specimen can be observed inside black outlines)

aforementioned scoring system and the percentage makeup of adipose and non-adipose tissue in each graft. The percentage of perilipin positive adipocytes and preadipocytes was evaluated by the lead pathologist (O.A.) in the study. For assessing inflammation and granulation area density, a self-devised scaling system was used. A score between 0 and 1 was accepted as low density, a score between 1 and 2 as mild density, a score between 2 and 3 as high density and a score between 3 and 4 as very high density.

Biochemical Evaluation

MDA (malondialdehyde) is an end-product of the oxidation cascade, a major by-product of cigarette smoke and a frequently used parameter to assess oxidative stress [9]. Cotinine is an alkaloid found in tobacco leaves and the main metabolite of nicotine [10]. Cotinine, which accumulates in the body as a result of tobacco exposure, crosses the blood–brain barrier and has different pharmacological properties compared with nicotine. Cotinine's longer plasma half-life than nicotine is used for detecting smoking exposure in individuals. In our study, a small sample from graft tissue was taken to assess MDA levels in the fat graft. In addition to that, arterial blood sampling from the abdominal aorta was obtained during the final surgery for assessing serum cotinine levels.

Results

Out of the initial 22 rats, 21 rats completed the 12-week study period. One rat was lost due to bleeding from the femoral pedicle during fat graft harvesting. Representative clinical pictures were taken during both transfer and the final surgery.

Weight Loss Under Continuous Exposure, Smoking Cessation and Non-smoking Conditions

Weight analysis revealed that the mean percentages of weight loss of fat grafts were 80% in group A, 74% in group B and 54% in group C (Fig. 7). Statistically significant differences were found between groups A and C, and between groups B and C. The difference between group A and group B was statistically insignificant (Table 1).

Histological Analysis

The immunohistochemical staining of perilipin showed a significantly higher amount of mature adipocytes and preadipocytes in the control group when compared to the remaining groups. The percentage of mature adipocytes and preadipocytes in the control group were 8% and 5.5%, respectively, whereas in the smoking cessation group they were 2.3% and 1.6%. In the continuous exposure group, percentages were 1.4% and 0.9% (Figs. 8, 9 and 10). The differences between the control group and the remaining two were statistically significant. Even though there were differences between groups A and B, they were found to be statistically insignificant.

Samples at magnification 40× were assessed for adipose to non-adipose tissue ratio in grafts. Gross analysis is performed (Figs. 11, 12).

Using a self-devised grading scale, scores of inflammation and granulation were evaluated (Figs. 13, 14). The areas of granulation and inflammation were seen to get wider as the exposure period to cigarette smoke increased. The differences between group C and the remaining two were statistically meaningful.

Although statistically insignificant, we were able to see a decreasing trend in neovascularization density around fat graft areas as the period of cigarette smoke exposure increases (Fig. 15).

Table 1 The statistical difference between each group in the study. Although significant difference was seen between control group and exposed groups, there was no significant difference between groups B and C

Comparisons A–C	Results Weight loss
Mann–Whitney *U*	2
Wilcoxon W	38
Z	− 3.009
Asymp. sig. (2-tailed)	0.003
Exact sig. [2*(1-tailed sig.)]	0.001
B–C	Weight loss
Mann–Whitney *U*	5
Wilcoxon W	41
Z	− 2.458
Asymp. sig. (2-tailed)	0.014
Exact sig. [2*(1-tailed sig.)]	0.013
A–B	Weight loss
Mann–Whitney *U*	17
Wilcoxon W	38
Z	− 0.573
Asymp. sig. (2-tailed)	0.567
Exact sig. [2*(1-tailed sig.)]	0.628

Biochemical Analysis

Mean serum cotinine levels in the continuous exposure group, the smoking cessation group and the control group were 7.82 ng/ml, 7.22 ng/ml and 4.31 ng/ml, respectively

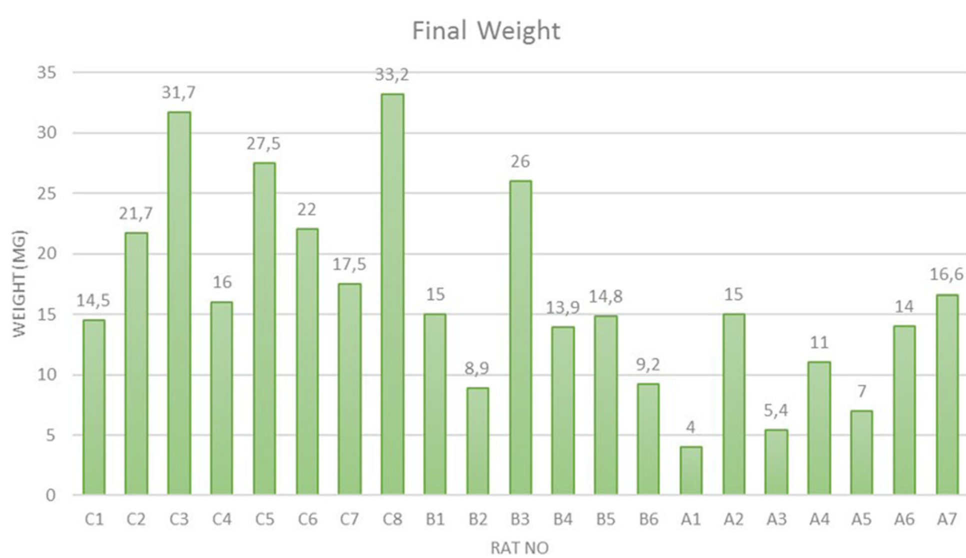


Fig. 7 Final weight of the fat graft specimens; *C* control group, *SC* smoking cessation group, *CS* continuous smoking group

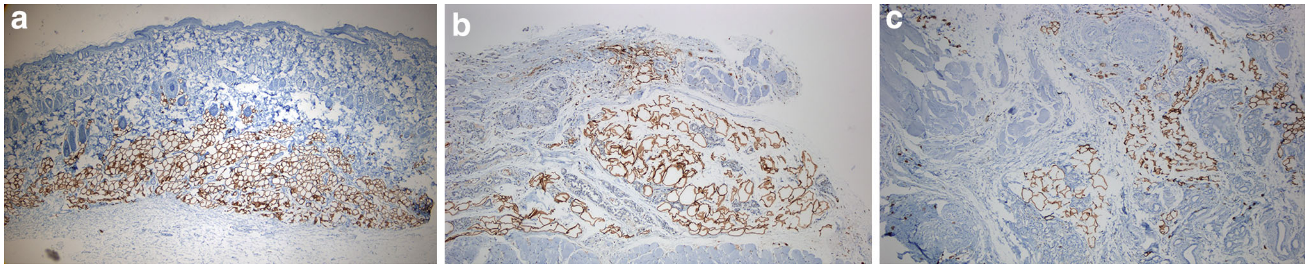
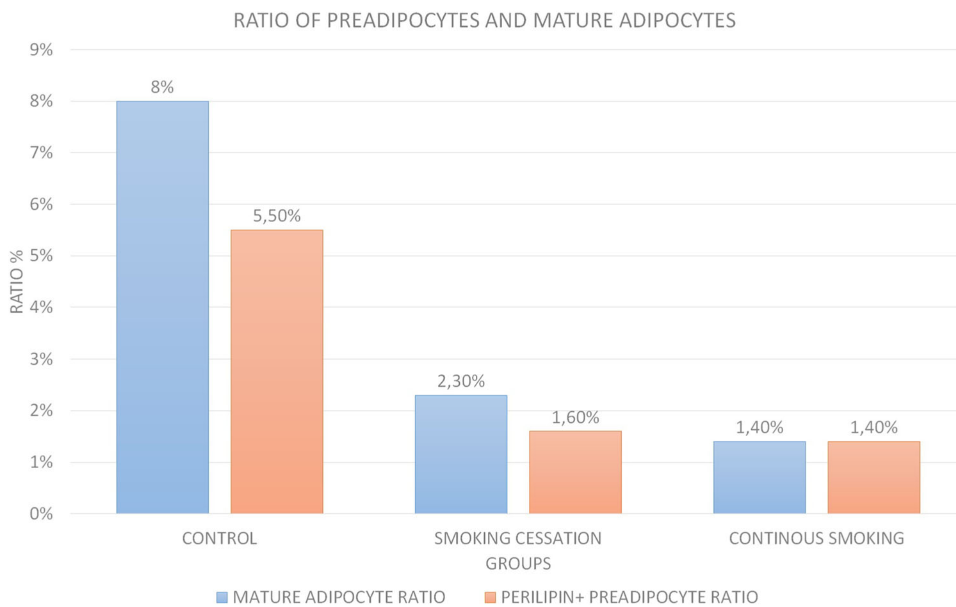


Fig. 8 Perilipin stained preadipocyte and mature adipocyte islands; **a** sample from control group, **b** sample from smoking cessation group, **c** sample from continuous smoking group

Fig. 9 Ratio of mature adipocytes and preadipocytes



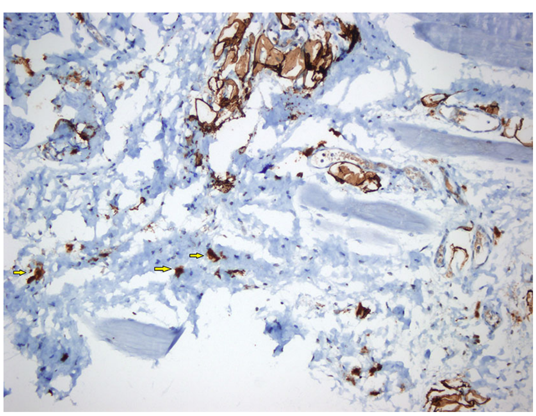


Fig. 10 Perilipin positive preadipocytes marked with arrows

(Fig. 16). All the rats in the smoke-exposed groups had cotinine levels between 5 and 20 ng/ml. These levels represent mid to high second-hand and low active smoking conditions. The differences between the control group and the two other groups were statistically significant. Although there was some difference between groups A and B, it was not deemed statistically significant.

Tissue MDA levels were in correlation with smoking exposure period. The differences between the control group and other two groups were statistically significant, whereas the difference between groups A and B was not.

Weight loss ratios of the grafts were correlated with both tissue MDA levels and serum cotinine levels. Again the differences between the control group and the other two groups were statistically significant, but the difference between A and B was not (Figs. 17, 18).

Discussion

Fat graft popularity has increased over the last two decades [11]. Seemingly everyday, there is a new innovation in surgical technique or a new way for handling the graft. Lately, we are headed into an era of 'micro' and 'nano' fat graft injections, so the focus will be on finding new ways to improve core survival rates.

There are a number of factors effecting fat graft take: ranging from donor site, harvesting technique, the way for handling the graft, placement technique, etc. [12]. Even

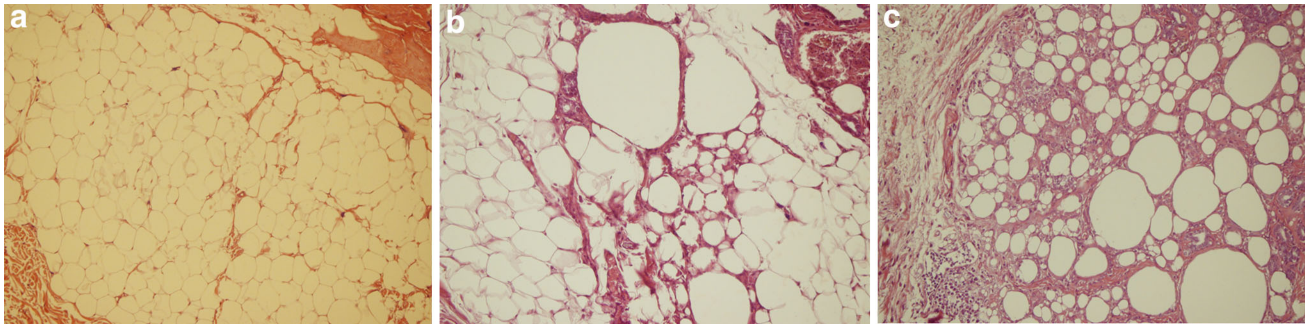


Fig. 11 Light microscope under H&E stain; **a** sample from control group, **b** sample from smoking cessation group, **c** sample from continuous smoking group

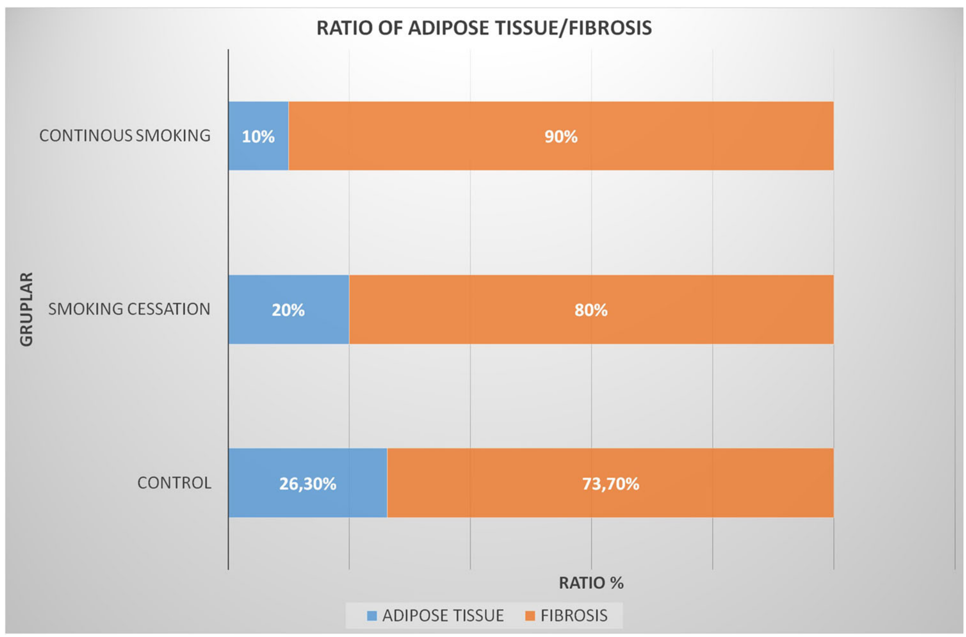


Fig. 12 Ratio of adipose tissue and fibrosis

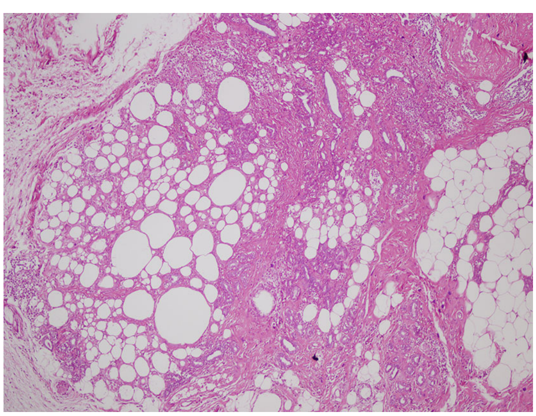


Fig. 13 Extensive inflammation and granulation regions from a sample out of continuous smoking group

with all this accumulated general knowledge, clinical results can still be varied [13]. So it can easily be said that this is a multifactorial process.

The effect of cigarette smoking on postoperative wound complications has been a subject for extensive research [14]. Unsatisfying results for a wide range of operations, from abdominoplasties and breast reductions to facelifts, are attributed to the risk factors stemming from smoking [5]. Because of the nature of this clinical question, the topic does not lend itself well to randomized prospective trials; however, it has been the subject of more than 140 cohort studies (level 2 and 3 evidence) to this date. These studies have been conducted in numerous countries, spanning all surgical sub-specialties and were the subject of a recent large meta-analysis [15]. For the meta-analysis, several end points were separately evaluated. For the end point "necrosis of wound and tissue," which was measured in 19 unique studies comprising 7616 patients, cigarette smoking was found to increase the risk, with an odds ratio of 3.61 [95% confidence interval (CI)]. The end point "healing delay and dehiscence" was evaluated in 18 studies comprising 26,297 patients, with an odds ratio of 2.86 (95%

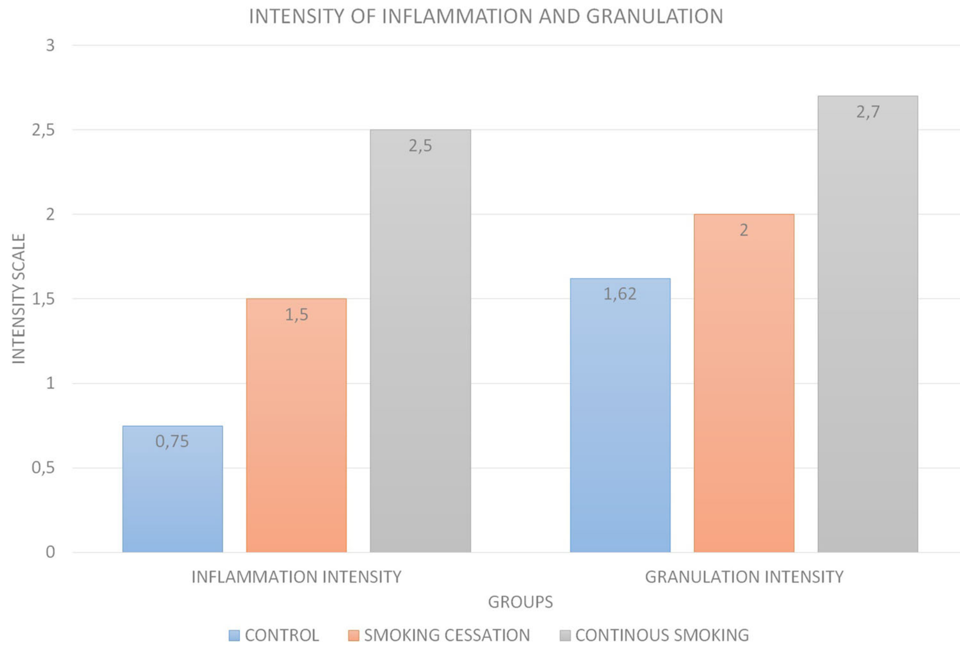


Fig. 14 Intensity of inflammation and granulation

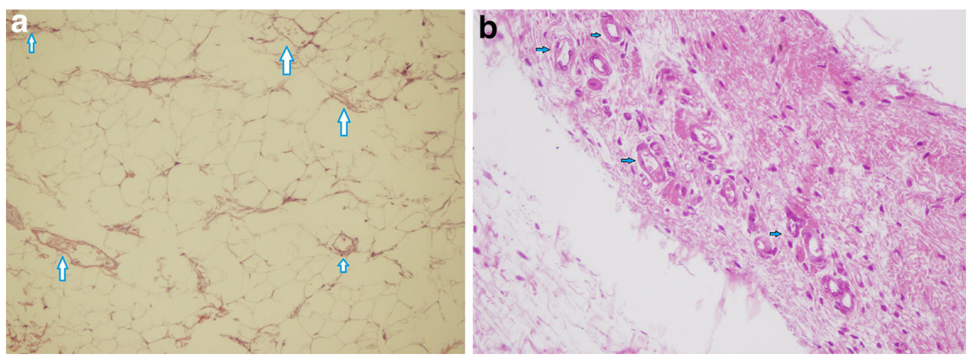


Fig. 15 Neovascularization zones in; **a** control group, **b** continuous smoking group

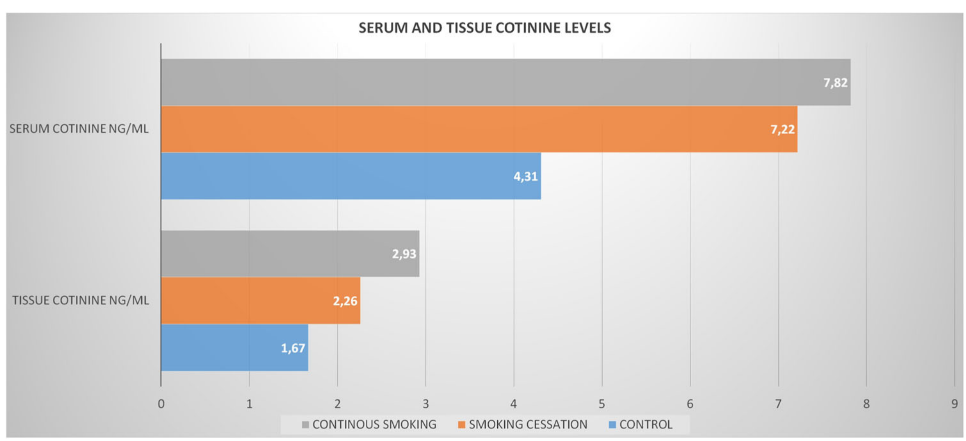


Fig. 16 Tissue and serum cotinine levels

CI). Surgical site infection was an end point in 51 unique studies comprising over 400,000 patients, with an odds ratio of 2.12 (95% CI).

Due to multifactorial effects and still ongoing discussions on fat graft survival, we aimed to demonstrate the deleterious effects of cigarette smoke on fat graft survival in a quantitative manner. In this study, a standardized model of cigarette smoke exposure has been used [8]. To decrease bias in surgical manipulation, a single author (A.E.) performed all the surgeries. The parameters

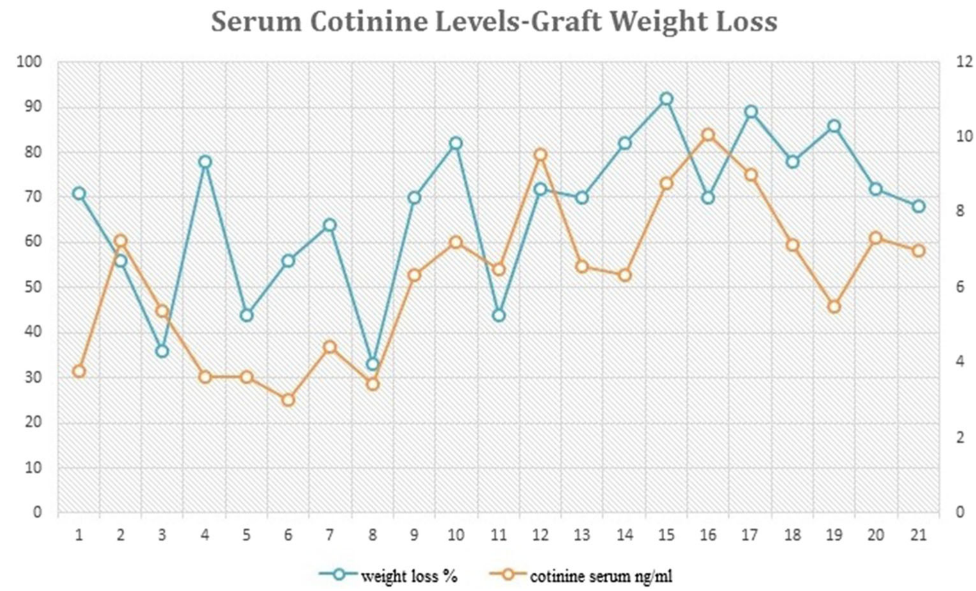


Fig. 17 Correlation between graft weight loss and serum cotinine levels

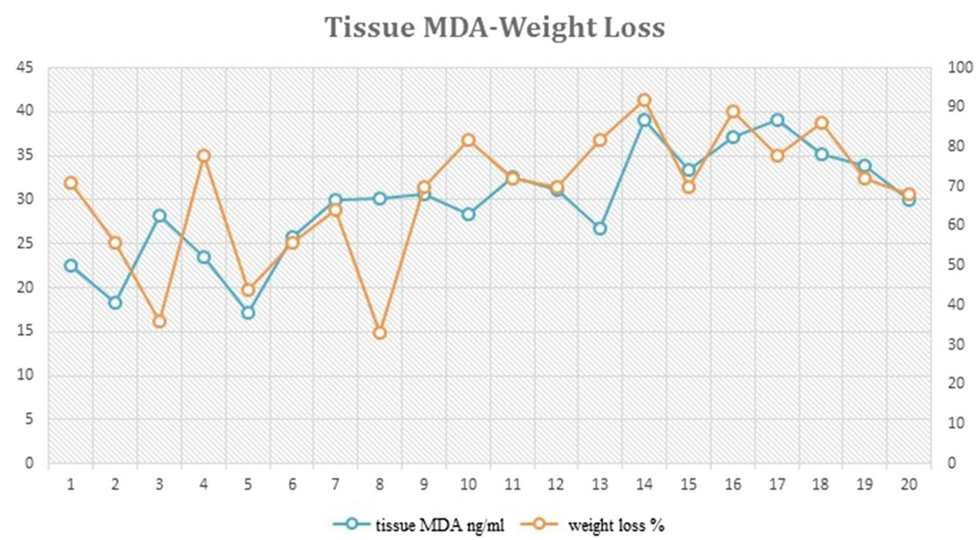


Fig. 18 Correlation between graft weight loss and tissue MDA levels

analyzed were graft weight change, histopathological examinations and biochemical analysis. Histopathological methods were the main focus of this study and were used extensively. The reason for the heavy emphasis on histological parameters was to avoid the subjectivity of radiological and clinical assessment.

The fate of adipocytes after non-vascularized fat grafting has been studied extensively by Yoshimura et al. from the University of Tokyo [16]. The authors have challenged the common belief that transplanted living adipocytes could survive and remain alive for a long period. They created a surgically induced ischemia model by severing blood vessels that supply the inguinal fat pads in mice. Under different degrees of hypoxia, ischemic changes were analyzed by whole-mount staining, immunohistochemistry, flow cytometry and Western blotting. With this animal model, the authors were able to examine the adipocyte survival under hypoxic conditions both in vitro and in vivo with three different degrees of ischemia (mild, intermediate and severe). They found that under severe ischemia, all adipocytes underwent degenerative changes and subsequent tissue remodeling. Adipocytes within the fat pad die easily under ischemic conditions, whereas adipose-derived stem or progenitor cells could survive under ischemic conditions and were activated. These progenitor cells also contributed to adipose tissue repair later on. So in our study, a meaningful decrease in the numbers of ischemia-susceptible mature adipocytes with increasing amount of smoke exposure was to be expected. High levels of tissue MDA and low levels of neovascularization in exposed parties could be leading to ischemia in the area surrounding the fat graft. This phenomenon was consistent with previous works [17]. The unexpected finding was the sharp drop in preadipocyte counts in the continuous exposure and smoking cessation groups. The preadipocytes are known to be resistant to ischemia, and according to many studies, they are the main elements to survive in the graft late term [6]. They are the most important contributors of the final volume. So there should be a factor apart from ischemia for this significant loss under the influence of cigarette smoke. This can be a subject for further study.

Smoking can strongly delay and even interrupt the wound healing phases completely [18]. Not all the areas in a wound heal with the same speed. So it is not abnormal to see areas in different stages of wound healing in a newly placed fat graft tissue. The higher density of granulation tissue and inflammation within the smoke-exposed groups compared to the control group could be explained by these delaying effects of cigarette smoke. Also compared to the control group, we could see that specimens from the exposed groups had wider areas in the earlier stages of wound healing. This also helps to explain how the delaying effect of cigarette smoke can alter the survival of the graft.

Self-reported information about tobacco use is notoriously unreliable, with self-reporting consistently overestimating smoking cessation by 20–30% [10]. The cotinine test is a useful but underused means for confirming cessation compliance before elective surgery. The serum/urine cotinine assay is noninvasive, inexpensive and reliable with a published sensitivity of 98% [10]. Cotinine is a derivative of nicotine with an extended plasma and urine elimination compared to nicotine and has a half-life of 16–20 h. Although serum/urine cotinine is known to be a good indicator for recent period (1–2 weeks) smoke exposure; some studies suggest it can also be used for detecting an exposure up to 8 weeks. In our study, the rats in group B which did not have any contact with cigarette smoke for over 6 weeks, also had serum cotinine levels higher that 5 ng/ml. So the argument above could have a valid point.

Being a significant end-product of oxidation reactions caused by cigarette smoke, tissue MDA is one of the most important indicators of oxidative stress in a tissue. The statistically significant correlation between tissue MDA and graft weight loss can be a supporting point for the idea that oxidative stress can be a direct factor on late-term survival of a fat graft. Moreover, smoking is not the only factor leading to oxidative stress in the body; other causes that produce oxidative stress may also effect the success of fat grafting.

When assessing these findings, there is a valid point that needs emphasis. Even though nicotine is the most famous product of cigarette smoke, it is not the only important chemical inside. Cigarette smoke includes a high number of tobacco-specific and non-specific toxicologically significant chemicals. Besides nicotine, there are over 6000 other products that can be separated: nitrosamines, aldehydes (acrolein, formaldehyde), polycyclic aromatic hydrocarbons (benzopyrene), benzene, hydrogen cyanide to name a few [17, 18]. Although cigarette smoke overall has been proven to have deleterious effects on cell structure and wound healing in many different studies, nicotine as a singular agent has been showed to increase angiogenesis and even improve wound healing in selective cases. Administering nicotine as a separate agent in place of cigarette smoke has the possibility of providing a distinctively different end result regarding fat graft survival. This can be a viable point to investigate in a further study.

Using a rat model, Nishimura et al. [19] reported that fat grafts keep reducing in volume and weight during long-term follow-up. It is difficult to compare the findings for experimental studies and clinical studies. Different studies have used various types of animal models, different methods of transplantation and different methods to estimate the volume and weight reduction of the grafts. Using a rabbit model, Kononas et al. [20] directly weighed the retrieved grafts and reported graft weight reductions after 9 months from 32.7 to 11.4% in the case of suctioned fat and from 42.2 to 19.3% in the case of surgically excised fat. Mikus et al. [21] reported an injected-graft take of only 20% in a canine vocal fold model. Despite specific differences in their findings, these studies reported that only a small part of the fat grafts survived and that fat graft volume was constantly reduced over time. In our study, the weight loss of 54% in the control group was consistent with previously mentioned studies. There were many factors stemming from exposure to cigarette smoke that can lead to resorption of the grafts in our study. Significantly increased weight and volume loss in groups A and B could be attributed to the increasing ischemia, decreasing neovascularization and stalling in wound healing process in both groups. But more extensive analyses are needed for pointing out a specific cause, if there is one.

Plastic surgery is a medical discipline with inherent variability. But we are in the era of *evidence-based medicine*. Smoking has long been claimed as a reason for poor clinical results.

There were a couple of *significant* limitations in our study. Although our exposure model was consistently able to provide meaningful passive exposure, it was not able to provide meaningful active exposure levels.

Rats are commonly used for fat grafting studies, but their external structure is unlike the human body which is a frequently criticized point in experimental flap model studies. The soft tissue connections between skin and muscle planes are not as strong as humans. Again unlike the human body, rats do not possess a natural fat layer under the skin. So the grafts had to be placed in a much less stable surgical plane compared to what is done in a routine fat graft surgery. Although no significant difference is found in survival rates of the grafts regarding their placement in different surgical planes [22], we still think compared to the human model this led to a meaningful difference.

The source of fat grafts in this study was the inguinal fat pad of the rat. It is easy to harvest with few post-op complications, and it is the only high volume fat tissue apart from visceral fat. The main problem is, it contains much more stroma relative to human fat tissue and also a higher density of lymph tissue. So it has different tissue qualities compared to the fat transferred during routine surgery.

In this modern era when performing a fat graft transfer, a suction–injection technique is used, unlike in the past when the fat graft was directly placed into the tissues after excision from the donor site. Our surgical model is a rat-to-rat transfer model and is composed of direct lipectomy followed by direct placement of the fat graft into the created surgical pouch. This model is not the same as what we do in today's fat graft surgery where multiplanar injection into the soft tissue structure is done without undue undermining for a compact and strong placement of the graft.

In conclusion, cigarette smoke had a significant and deleterious effect on late-term fat graft survival in this experimental animal model. When taken into account our previous clinical experiences from many years and results from this study, it is possible to say that the reliability of fat grafting can be questionable in smoking patients compared to non-smokers. Further studies need to be conducted to find the exact mechanisms leading to this final result.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

Ethical Standards Approval of the Istanbul University Experimental Animal Research Ethics Committee had been taken for conducting this experimental study.

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

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