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Evaluation of Perilipin Expression in Centrifuged Fat Grafts on Different Revolutions Per Minute and Duration Combinations

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Abstract Fat graft applications are one of the most frequently used procedures in reconstructive and aesthetic surgery. Fat graft survival rate is variable and depends on many parameters. Those are the features of recipient area, method of obtaining the fat graft, the procedures applied to the harvest to get pure fat graft and the application techniques. In this study, we aimed to find the optimal revolutions per minute(rpm) and duration to be used in the centrifugation technique, which is one of the most frequently used methods in fat grafting. In our study, we used perilipin protein as a survival marker. Liposuction samples were obtained from 10 Caucasian women individuals; whose age range is between 22 and 29 years without any other reported health problem. Fat grafts from all patients were taken by the same surgical team by the same liposuction procedure. The grafts obtained in our study were divided into 6 different groups, these are A1 group (1 minute–1000 rpm) A2 group (3 minutes–1000 rpm) A3 group (5 minutes–1000 rpm) B1 group (1 minute–3000 rpm) B2 group (3 minutes–3000 rpm) and B3 group (5 minutes–3000 rpm). Perilipin expression measurements were made in the sections taken by immunohistochemistry technique. Three minutes–3000 rpm combination (B2 group) was found to be perilipin stained statistically significantly higher than other combinations. This study seeks to make a notable contribution to the literature by demonstrating the relation between frequently used centrifuge combinations and measuring the expression of perilipin. This protein's role in adipocyte regulation is becoming increasingly apparent.

Level of Evidence III This journal requires that authors assign a level of evidence to each article. For a full description of these Evidence-Based Medicine ratings, please refer to the Table of Contents or the online Instructions to Authors www.springer.com/00266.

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Keywords Fat graft · Centrifuge · Perilipin · Liposuction

Introduction

In recent years, autologous fat grafting has become a common technique for treating volume and contour abnormalities in aesthetic and reconstructive surgery. A study has shown that approximately 80 percent of plastic surgeons use fat grafts in their practice [1]. Fat grafting is used for face contouring, breast augmentation, radiation injury, chest capsule contracture, post-traumatic deformities, congenital anomalies, and burn injuries [2, 3]. Autologous fat grafts have many beneficial features such as not causing a reaction in the immune system, being easily obtained surgically, and being low cost [4].

Studies have shown that variables such as the choice of cannula, the volume of fat graft, and the donor area features have significant effect on fat graft survival [5, 6]. Studies have shown that the volume decreases between 20 and 90% after the first procedure [7]. Autologous fat grafts are considered a more ideal filler than dermal fillers because of the many advantages it provides [8]. Peer's theory of cell survival shows that there is correlation between the number of viable adipocyte cells present during transfer and the volume that demonstrates survival of fat grafts [9].

Perilipin

One of the mechanisms of lipolysis in adipocytes is regulated by phosphorylation of perilipin, a protein that coats lipid droplets. Perilipin is potentially phosphorylated at various sites by adenosine 3',5'-cyclic monophosphate-dependent protein kinase (PKA) [10]. Activation of PKA is the main signaling mechanism by which hormones and neurotransmitters, including epinephrine, stimulate lipolysis in adipocytes. A lipid droplet contains a neutral lipid core surrounded by a single layer of phospholipids and coated with specific proteins [11]. Perilipins are a family of surface proteins that coat lipid droplets and make up 0.25–0.50% of total cell protein [12]. Studies on the biology of lipid droplets and perilipin have increased in recent years. Perilipins act as a physical barrier that protects stored triglycerides from hydrolysis by hormone sensitive lipase (HDL) [13]. Depending on the energy state of the organism, perilipin restricts or provides lipases access to stored triglycerides [14]. White adipose tissue contains unilocular lipid droplets. The mechanisms of induced lipolysis are understood in white adipose tissue where the amount of perilipin 1 is high. Epinephrine and norepinephrine bind to β-adrenergic receptors on the membrane of adipocytes during fasting and exercise. This binding activates adenylyl cyclase and initiates signal sequences that lead to an increase in cyclic adenosine monophosphate(cAMP) levels in the cell [15]. A study has shown that a solution containing epinephrine and lidocaine can increase vulnerability in adipocytes by increasing perilipin phosphorylation. This result reveals that adipocytes may be more prone to lipolysis when exposed to epinephrine and lidocaine [16]. A study in 2018 has show that in lean animals investigated the role of Perilipin 1(Plin 1) in adipose tissue inflammation and insulin sensitivity. According to the obtained data, Plin1 restricts lipolysis and acts as an important regulator against adipose tissue inflammation and insulin resistance. This study reveals that Plin 1 expression can be used as a marker in the treatment of inflammatory metabolic diseases and lipid dysregulation [17].

Centrifuge

One of the frequently used methods to purify the autologous fat graft from this obtained mixed adipose tissue is the centrifugation method. Centrifugation enables the separation of adipocytes from components such as blood cells and tumescent solution obtained with lipoaspirate. Although there are many in vivo and in vitro studies related to centrifugation speed and centrifugation time in the literature, no consensus has yet been reached on a gold standard centrifugation speed and time combination [8, 9, 13, 14]. The centrifuge concentrates adipocytes, separating blood cells, lipids, proteases and other components that may cause their degradation [18–21].

Materials and Methods

In this study, we aimed to find which centrifuge time and duration gives us the most perilipin stained fat grafts. The study at the Istanbul Medipol University Mega Hospital was conducted between March 2022 and September 2022. Approval was obtained from the ethics committee of Medipol University to conduct this study. Informed consent form was obtained from all patients included in the study. All patients who went undergo liposuction surgery were operated by the same surgical team. All operations were performed under general anesthesia. The same liposuction device (VentX infiltration aspiration console, Solta Medical, Bothell WA-USA) was used for obtaining sterile lipoaspirate in all operations. A sterile closed system was used. In order to standardize the cannula in all patients, blunt-tipped straight 4-mm cannulas were used. Liposuction samples were obtained from 10 Caucasian female patients; whose age range is between 22 and 29 years without any other reported health problem. Only female

patients were included in the study because there may be a difference in perilipin expression between genders. In addition, patients with higher or lower than average results in lipid panels were excluded from the study. In addition, patients with a body mass index over 30 were not included in the study. During the operation, it was aimed to prevent time-dependent differences on fat survival while performing centrifugation processes. The tumescent solution used in the operations was 500 mg lidocaine and 0.5 mg adrenaline in 1 liter of 0.9% NaCl solution. Fat samples centrifuged as soon as a sufficient amount of lipoaspirate was obtained for the study. Six different combination groups were formed from adipose tissue samples obtained after centrifugation.

(1) Group A1: Adipose tissue obtained by centrifugation at 1000 rpm for 1 minute
(2) Group A2: Adipose tissue obtained by centrifugation at 1000 rpm for 3 minutes
(3) Group A3: Adipose tissue obtained by centrifugation at 1000 rpm for 5 minutes
(4) Group B1: Adipose tissue obtained by centrifugation at 3000 rpm for 1 minute
(5) Group B2: Adipose tissue obtained by centrifugation at 3000 rpm for 3 minutes
(6) Group B3: Adipose tissue obtained by centrifugation at 3000 rpm for 5 minutes

The obtained tissues were frozen and stored at −80 °C. Eight-micrometer-thick sections were taken from the collected samples with a cryostat (CRYO3 Plus Frozen Device +H90558554D Serial no:58550125-0616, Sakura Finetek, Japan). Sections were taken on adhesive slides and stored at -80 °C again. Transport was carried out on dry ice so that the tissue did not dissolve during the sectioning process. The general tissue morphology in the sections was revealed by Hematoxylin Eosin staining. Perilipin 1 (1:200, rabbit polyclonal; Abcam, UK) immunohistochemical markings were applied to the sections to identify viable adipose cells in tissue samples obtained by different methods.

Hematoxylin Eosin Staining Protocol

After removing the sections from the freezer, they were quickly fixed with methanol chilled at −20 °C, and subsequently taken into distilled water. Following a 10-minute immersion in hematoxylin dye, the sections were placed in tap water. Subsequently, the sections were stained with eosin for 2 minutes and then transferred to distilled water. After passing through an ascending alcohol series, the sections were immersed in xylene for 15 minutes and finally covered with an occlusive medium.

Immunohistochemical Staining Protocol

As soon as the frozen sections were brought to room temperature, they were fixed with methanol cooled at −20 °C for 5 minutes. After being washed three times in PBS, they were immersed in 3% H2O2 for 10 minutes to halt the endogenous peroxidase activity. The sections were then washed three times in PBS and outlined on the slides using a hydrophobic pen. Following this, the sections were incubated in protein block solution for 10 minutes to prevent non-specific antibody binding. After the blocking step, a 1/200 dilution of an anti-perilipin A primary antibody was applied to the sections without washing. The sections were incubated overnight at +4 °C. At the end of the incubation period, the sections were brought to room temperature and washed three times with PBS. Subsequently, the sections were incubated in a biotinylated secondary antibody solution for 10 minutes, followed by three washes with PBS. The sections were then exposed to streptavidin peroxidase solution for 10 minutes, washed three times with PBS, and treated with DAB (Diaminobenzidine) chromogen. Once an adequate reaction was achieved, the sections were taken into distilled water and counterstained with hematoxylin. To complete the process, the sections were dehydrated by passing through a series of ascending alcohols. Sections were kept in xylene for 15 minutes and then covered with closure solution. The sections were viewed using a microscope (Nikon Eclipse, Japan). Tissue sections were immunohistochemically marked with perilipin 1 antibody. Adipocytes exhibiting brown staining on their cell membranes with DAB chromogen were considered viable. The tissue images in the sections were evaluated stereologically using the Cavalier method and the coded counting ruler. The percentage of positive cells within the examined areas was determined.

Statistical Analysis

The percentage of staining of the examined areas in the stereological evaluation obtained using a total of 60 sectional tissue samples consisting of 10 participants, the cavalier method and the dotted counting ruler were calculated for 6 groups according to the time and cycle number differences. Differences between groups were tested by Freidman analysis. Then, with the Durbin–Conover post hoc analysis, pairwise comparisons were made for each group separately to determine which groups the differences originated from. A two-sided p value of 0.05 was considered statistically significant. All data analyses were performed using SPSS version 23.0 software (SPSS Inc., Chicago, IL, USA). Categorical variables were presented as frequency and percentage. Continuous variables

were tested for distribution using the Kolmogorov–Smirnov and Shapiro–Wilk tests. Variables with normal distribution were shown with mean and standard deviation.

Results

A total of 60 samples were obtained from 10 female patients aged 22–29 years with a mean age of 24.2 years. The results obtained as a result of centrifugation and immunohistochemical analysis of the fat grafts obtained from the patients, the average number of cells in the area, the amount of cells stained with perilipin 1 protein, and their percentage in percentage, so that 6 different subgroups are formed for each patient are shared in the table below. In addition, micrographs from 6 different subgroup samples are shown (Table 1).

In the dotted counting ruler, every part of the microscope field is covered with a certain number of equally spaced crosses. Crosses over the adipocytes that show perilipin expression in the image area, that is, that remain viable at the time of fixation, are counted. It is considered a positive value for each field (Figs. 1, 2, 3, 4, 5, 6 and 7).

The ratio of cells displayed in each field to cells expressing perilipin 1 in different combinations for each patient is shown in Table 2.

Comparison of the mean percentage values of the groups (Freidman) (Table 3). Percentage of staining of perilipin at different rpm and time intervals were statistically different between subgroups (x^2 = 27.8, p<0.001).

Comparison of mean p values between groups, pairwise comparisons (Durbin–Conover) (Figs. 8, 9 and 10).

According to the comparison results of the subgroup percentage with the Pairwise (Durbin–Conover) comparison test, it was found statistically significant that the B2 group (3 Minutes–3000 rpm) was more stained with perilipin compared with all other subgroups.

When A1 (1 min–1000 rpm) and A2(3 min–1000 rpm) groups were compared, no statistical difference was found (p 0.068). In the comparison of all subgroups with the A1 group except A2, the A1 group was found to be less stained statistically significant. When the A3 group and B1 were compared, no statistical difference was detected (p=0.312). No significant difference was found in the comparison between B1 and B3 groups (p=0.132).

Discussion

Centrifugation is one of the most commonly used methods to purify the fat graft after obtaining lipoaspirate. Although there are many studies in the literature regarding the optimal centrifugation time and rpm amount that will provide maximum fat graft survival, there is no consensus yet. There is no previous immunohistochemical study with perilipin protein in the literature to reach the optimal centrifuge combination.

In our study, 3 minutes–3000 rpm group was found to be statistically significantly higher in terms of perilipin staining in comparison with all other groups. This combination is still widely used. In the technique described by Coleman, it is recommended to use this combination along with minimizing trauma due to the sensitivity of adipose tissue to trauma, and for this purpose, it is recommended to use hand-assisted suction instead of creating a vacuum effect with high pressure negative pressure. During the application, it was aimed to maximize the contact of the recipient site vascular network by giving a small amount, at low speed and in multiple planes, in order to be atraumatic [21].

In the study conducted by Boschert et al. in 2002 in 4 different groups in 4 different centrifuge power (g=g-force which is a unit meaning relative centrifugal force) (10g, 30g, 50g, 100g) and 4 different centrifugation times (2-4-6-8 minutes), 50g/2 minutes emerged as the best result. This study is valuable both in terms of centrifugal power and time comparison, and it should be evaluated in terms of choosing centrifugal powers close to each other and not covering a wider scale. According to the study by Kurita et al. it is a misconception that the 100g used in the study by Boschert et al. [19] adipocytes. On the contrary, the g force can be further increased according to the excess of the aspirated fat volume. On a patient basis, although adipocyte destruction in patients varies according to the g force, different g forces at the same patient has minor effect on adipocyte destruction.

In the study of Kurita et al. in [21], in which they kept the time constant (3 minutes) both in vitro and in vivo and listed the centrifugal powers (0g, 400g, 800g, 1200g, 3000g, 4200g), 1200g emerged as the best in both in vitro and in vivo groups. This study also supports the Coleman technique. However, this study has a time limit of 3 minutes.

In the comparison made by Pu et al. in [22] in the form of 3000 rpm–5000 rpm and 3 minutes and 10 minutes in vitro, the best result was found to be 3000rpm/3 minutes. Although the same result was found in our technique, this study fully supports Coleman technique and says that it is superior to other techniques.

In the study conducted by Kim et al. in [23] in vitro, the centrifuge time (1 minute–3 minutes–5 minutes) and the centrifugal powers (500 rpm, 1300 rpm, 3000 rpm) were compared to 1300 rpm/5 minutes, which gave the most significant results. However, this study emphasized that adipocyte cell viability depends on multiple factors (patient's fat quality, recipient site, volume delivered, method

Table 1 The ratio of the number of cells visualized to the number of perilipin-expressing cells

Combination groups	The amount of cells in the visualized area	The amount of cells stained with perilipin 1 protein	Ratio of the amount of cells stained with perilipin 1 to the total amount of cells visualized (%)
1000 rpm–1 min	142,8	37,1	25,47
1000 rpm–3 min	148,2	45,6	30,48
1000 rpm–5 min	141,8	41,8	29,33
3000 rpm–1 min	141,3	53,8	37,38
3000 rpm–3 min	134,6	70,3	52,27
3000 rpm–5 min	149,9	62,4	41,3

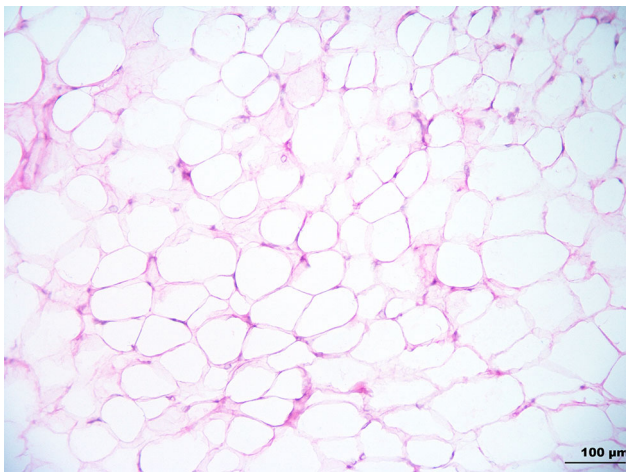

Fig. 1 Hematoxylin–eosin-stained adipose tissue micrograph with 10× zoom

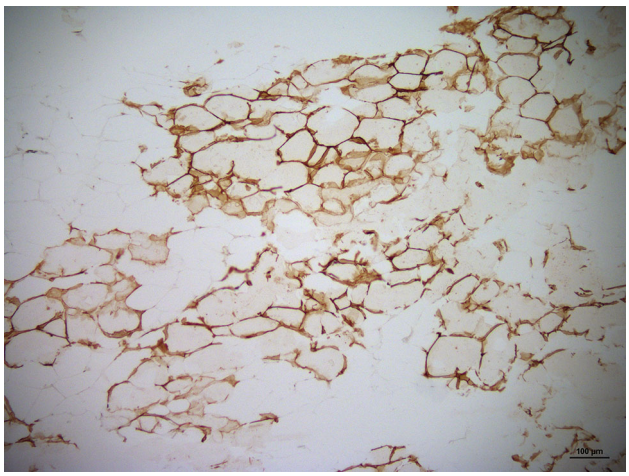

Fig. 3 Perilipin 1 immune reactivity is shown for Group A2 (1000 rpm–3 min) with 10× Zoom

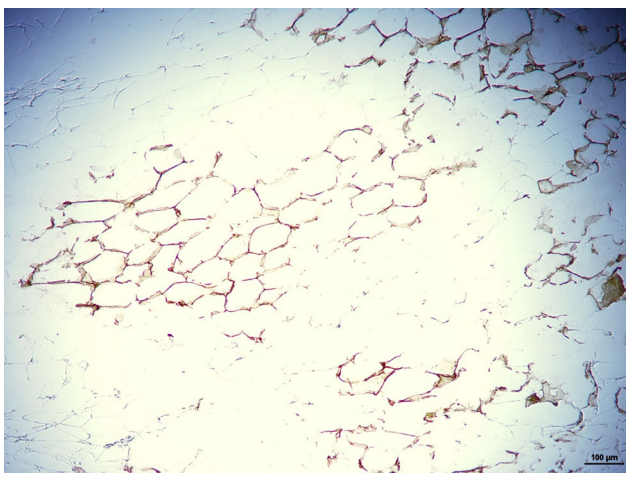

Fig. 2 Perilipin 1 immune reactivity is shown for Group A1 (1000 rpm–1 min) with 10× Zoom

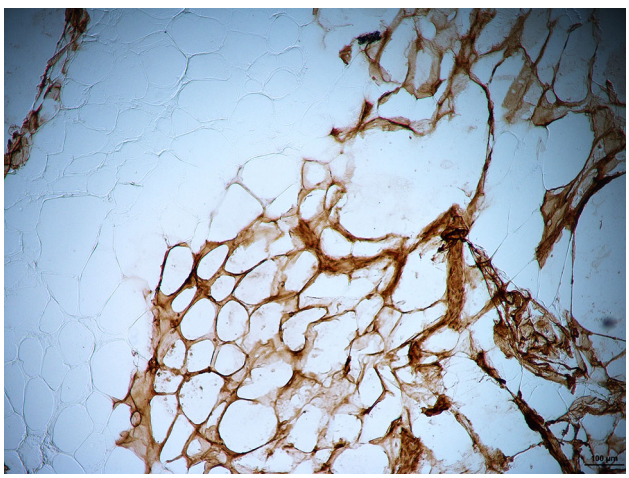

Fig. 4 Perilipin 1 immune reactivity is shown for Group A3 (1000 rpm–5 min) with 10× Zoom

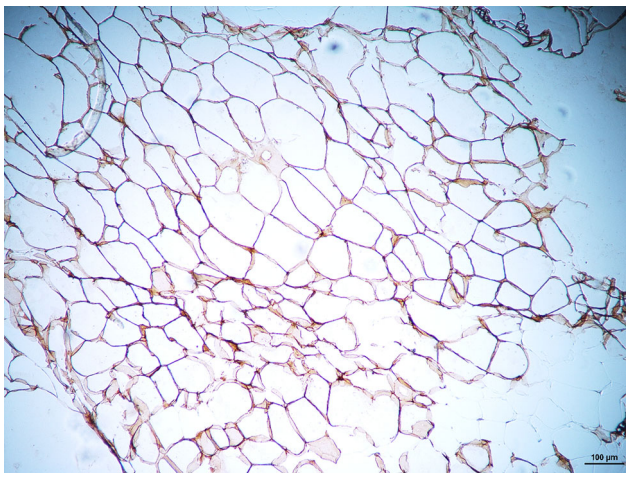

Fig. 5 Perilipin 1 immune reactivity is shown for Group B1 (3000 rpm–1 min) with 10× Zoom

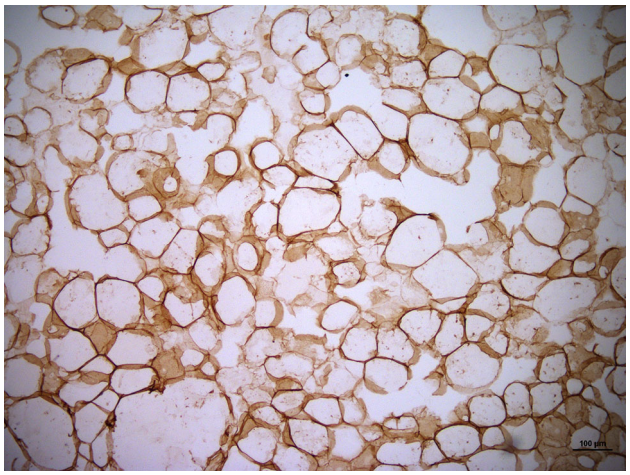

Fig. 6 Perilipin 1 immune reactivity is shown for Group B2 (3000 rpm–3 min) with 10× Zoom

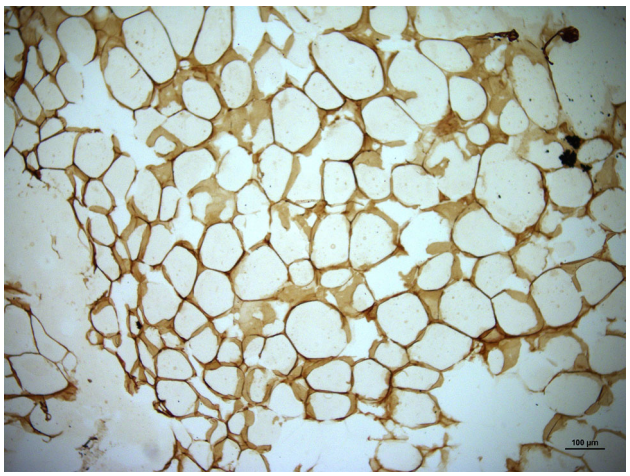

Fig. 7 Perilipin 1 immune reactivity is shown for Group B3 (3000 rpm–5 min) with 10× Zoom

of harvesting, centrifugation, injection method, anesthetic solution used). In addition, this study also tested treatment with different concentrations of epinephrine, which was shown to have minimal effect on adipocyte cell viability.

In the study of Ferraro et al. in [24], both in vitro and in vivo, centrifugation times of the in vitro group (10 minutes, 5 minutes, 3 minutes) and centrifugal powers (500rpm, 1300rpm, 3000rpm) were given. In the in vivo group, the centrifugation times were the same as in the in vitro group, and the centrifugal powers (1300rpm, 3000 rpm and control (sedimentation)) were given, and the best results were obtained at 1300rpm/5 minutes in both in vitro and in vivo groups. Such different results reveal the need for further studies on this subject.

The centrifugal powers (0g, 1000g, 1500g, 3000g, 5000g, 7500g, 10000g, 15000g) were different from each other and the differences in cell damage were examined in the study conducted by Pulsfort et al. [25] and no significant result could be found. From this study we can conclude that centrifugation has minimal impact on adipocyte viability. This contradicts the notion that centrifugation harms fat cells, suggesting it is a safe refinement step.

In the in vitro study of Salinas et al. in [26], the centrifuge time was kept constant as 3 minutes and the centrifugal powers were given as (50g, 1200g, 5000g, 10000g, 23000g). 1200g/3 minutes was found to be the combination of choice. In this study, as in many other studies, the duration was kept constant, and in this study, when compared with other fat collection techniques, the authors stated that the viability of lipoaspirate obtained with the mesh/gauze technique was almost equal to centrifugation in histologic terms and took less time.

In the in vitro study of Son et al. in [27], the centrifugation time was given as 3 minutes. Although the centrifugal powers (control, 1000rpm, 3000rpm, 4000rpm) were different from each other, no difference was found between the groups.

In the in vitro study of Cucchani et al. in [28], the centrifuge time was kept constant at 3 minutes, and there was a difference between centrifugal powers (700rpm, 3000rpm) and the group with 700rpm/3 minutes was found to be significantly higher survival rate.

An in vivo study of Bozkurt et al. in [29], the centrifugation time was given as 4 minutes, and when centrifugal powers (1000rpm, 2000rpm, 3000rpm, 4000rpm, 5000rpm) were compared, 2500rpm/4 minutes was found to be the best. In this article, only hematoxylin and eosin staining was used to investigate cell wall integrity under the microscope.

Centrifugation times (1 second, 1 minute, 3 minutes and 10 minutes) and centrifugal powers (0g, 100 g, 400 g, 500 g, 900 g, 1800 g) were given by Hoareau et al. [30] in their study in vitro and in vivo in 2021. In the in vivo study,

Table 2 The ratio of cells displayed in each field to cells expressing perilipin 1 in different combinations for each patient

Patient no.	A1 (%)	A2 (%)	A3 (%)	B1 (%)	B2 (%)	B3 (%)
1	27.69	20.17	26.79	36.07	47.5	36.18
2	15.08	33.1	31.33	25.41	34.31	33.03
3	17.27	13.42	22.82	22.22	31.88	38.71
4	20.13	30.87	26.32	61.04	44.74	55.56
5	42.58	43.51	21.29	55.97	76.32	57.14
6	31.17	35.76	33.12	31.13	64.81	27.1
7	31.41	26.62	36.54	38.07	59.74	45.64
8	32.26	34.84	38.26	40.6	56.67	44.59
9	21.43	36.94	37.06	29.61	41.94	29.8
10	15.69	29.61	19.87	33.81	64.87	45.39

Table 3 Comparison of the mean percentage values of the groups (Freidman)

Friedman		
χ^2	*df*	*p*
27,8	5	<0.001

centrifugation time (1 minute and 3 minutes) and centrifugal powers (sedimentation, 900 g and 400 g) are given. 400 g/1 minute was found to be the most significant in both in vivo and in vitro studies. Since this is one of the most recent studies, the time that was found to be significantly higher rate of survival was included in our study.

As can be seen, although many studies have been conducted, quite different results have been obtained. In our study, we aimed to change only the centrifuge combination while keeping all other variables that may affect fat graft survival. In our study, we used perilipin protein as a marker of cell survival and found that the 3 min–3000 rpm combination was significantly higher than the other combinations.

Conclusion

Although the centrifugation technique is one of the most common techniques used to purify the fat graft after obtaining lipoaspirate, no consensus has been reached on the time and rpm to be used in centrifugation. In our study, we studied lipoaspirate samples from patients at different centrifugation speeds and times. A total of 6 different combinations were used, including 1000 rpm, 3000 rpm and 1–3–5 minutes. We aimed to obtain an idea about survival of fat grafts by using immunohistochemical techniques and measuring the amount of perilipin surface antigen staining. We found that the 3 minutes–3000 rpm combination was significantly higher stained than the other groups. Perilipin protein has become increasingly popular and the number of studies on its effects on adipocyte physiology has increased in recent years. Due to the limited number of patients included in the study, we think that studies with larger patient groups would be more beneficial. In our opinion, this study will provide important contributions in terms of being unique in the literature by

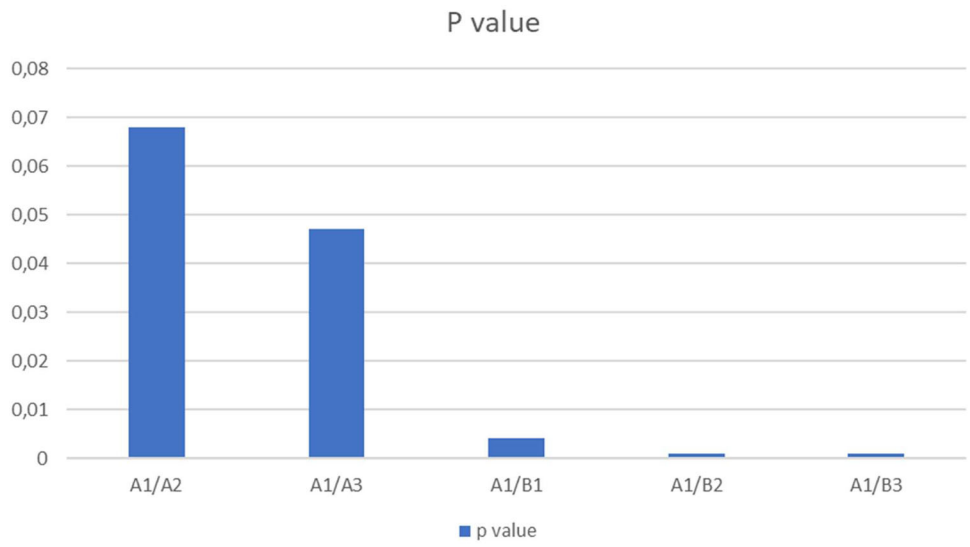

Fig. 8 *P* value comparison of A1 subgroup with others

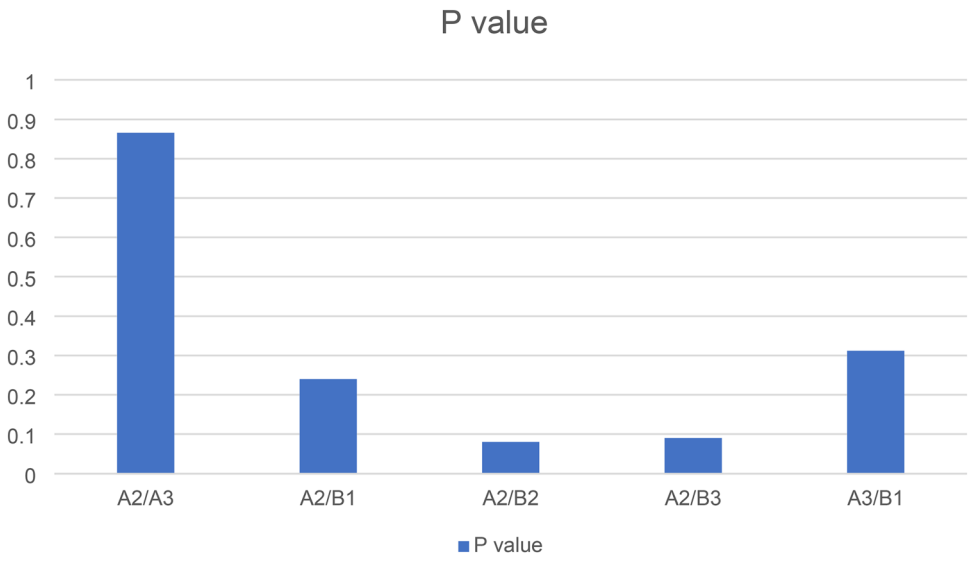

Fig. 9 *P* value comparison of A2 subgroup with others

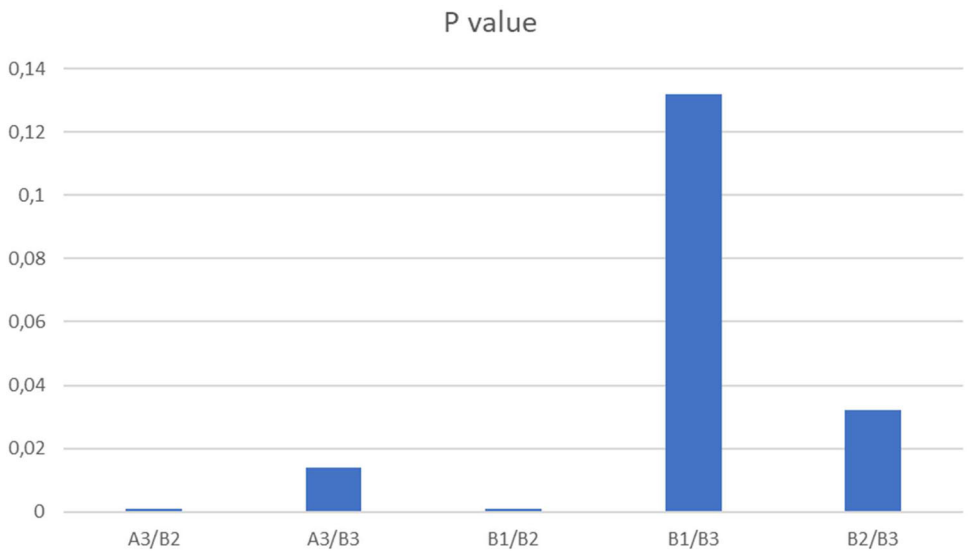

Fig. 10 *P* value comparison of rest of the subgroups

examining the expression of perilipin in most frequently used combinations.

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Declarations

Conflict of interest The authors declare that they have no conflicts of interest to disclose.

Ethical Approval Taken from Istanbul Medipol University Ethical Board on 08.02.2022, E-10840098-772.02-852 numbered. This approval paper is attached.

Informed Consent Informed Consent form is taken from all patients and sample is attached.

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