Effects of Recipient Area pH Change on Fat Graft Survival

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Introduction: Predicting the integration and adaptation of adipose tissue grafts in the recipient area remains challenging. Since graft incorporation shares key mechanisms with wound healing, local environmental factors such as pH may influence graft viability. This study aimed to investigate the effects of different pH levels on preadipocyte viability and related cellular responses.

Methods: Adipose tissue obtained from the infraumbilical region of a healthy donor was processed and divided into 13 groups. Isolated preadipocytes were cultured at pH values ranging from 1 to 13. Metabolic activity-based viability was assessed using the MTS assay. Gene expression analyses (IL-6, COX-2, and caspase-3) were performed at selected pH levels (6, 8, 10, and 12) to evaluate inflammatory and apoptotic responses.

Results: Metabolic activity-based viability was significantly higher at mildly acidic to moderately alkaline pH levels (pH: 6–11), with the highest values observed at pH 8 ($P < 0.05$). Extreme pH values (1–5 and 12–13) demonstrated cytotoxic effects. Gene expression analysis revealed the lowest levels of IL-6, COX-2, and caspase-3 at pH 8.

Conclusion: pH levels significantly influence preadipocyte metabolic viability and stress-related cellular responses. Mildly alkaline conditions, particularly around pH 8, may provide a favorable microenvironment during the early phase of fat graft integration. Further in vivo studies are required to validate these findings.

Key Words: Fat graft survival, pH, recipient area

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For the survival of cells and organisms, the regulation of extracellular and intracellular pH is essential. pH is not essential just for maintaining proper ion balance; it is also important for maintaining the optimal function of cellular enzymes and for the optimal binding of hormones and growth factors to cell-surface receptors. Even transient changes in pH value can trigger the production of heat shock proteins, which cause apoptotic cell death and affect cell metabolism.[1]

It has been shown that the adipose tissue graft goes through all phases of wound healing.[2–4] It could be proven that wound healing is affected by wound pH changes, as they can lead to an inhibition of endogenous and therapeutically applied enzymes. Besides, the conformational structure of proteins and their functionality in wound healing are altered.[5] This study was designed to investigate whether the pH value has an impact on adipose tissue graft cellular viability and survival-related processes because it has been proven that wound pH is a potent influential factor for the healing process. For this purpose, fat graft viability, proliferation, differentiation, and apoptosis processes at different pH values were evaluated.[6]

METHODS

Study Design

The current study was performed in the Plastic and Reconstructive Surgery Department of our tertiary care center with approval from the local institutional review board. A liposuction specimen was obtained from a 38-year-old healthy male patient who was admitted to our hospital for abdominal liposuction. Informed consent was obtained from the patient.

Liposuction Procedure and Harvesting Fat Tissue

The patient was operated under general anesthesia; 1 L of Klein solution (1250 mg/L lidocaine; 1.0 mg/L epinephrine; 10 mEq/L sodium bicarbonate) was infiltrated 10 minutes before the liposuction procedure. Aspiration was performed using a 3-mm diameter blunt-tip cannula connected to a 10-mL syringe. Saline solution was used to wash out the lipoaspirate a few times until the blood and tissue residues were removed.

Isolation and Characterization of Cells and Assessment of the Effects of Different pH Values

Adipose tissue graft was distributed evenly into 13 groups, with 30 mL per group. 0.2% phosphate buffer solution (PBS) was filtered through a 22-μL filter, 75 mg of collagenase type 2 enzyme (5 mg/mL) was added to it, and it was filtered through a 22-μL filter again (to prevent contamination). The adipose tissue graft was added to the prepared solution, and it was incubated at 37 °C and 300 rpm and shaken for 2 hours (for homogeneous interaction with the solution). Centrifugation was performed at 400×*g* for 10 minutes to remove floating adipocytes from the stromal-vascular section; the supernatant

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was discarded. Dulbecco modified Eagle medium with culture medium (10% fetal bovine solution) and 1% PSA (10,000 U/mL penicillin, 10,000 μg/mL streptomycin, and 25 μg/mL amphotericin B) (Invitrogen, Carlsbad, CA) 1 g/L was dissolved and seeded on T-75 flasks.

Isolated preadipocytes were incubated in cultures at different pH values between 1 and 13.

Metabolic activity-based viability of preadipocytes was evaluated using the MTS assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]. Preadipocytes in pH 6, 8, 10, and 12 valued culture media were analyzed by the polymerase chain reaction (PCR), and inflammation and apoptosis rates were evaluated.

Cell Passage

The old media of the flasks were discarded after 70% to 80% confluence was achieved in the cells. Media residues were removed by washing the flask surface with 0.2% PBS. A 0.25% trypsin/EDTA solution was added. Cells were removed from the flasks by trypsinizing for 5 minutes at 37 °C. The effect of trypsin was terminated by adding 1:1 complete medium to 0.25% trypsin/EDTA solution. Following centrifugation at 1300 rpm at room temperature, the supernatant was discarded. The required number of cells was replanted, and the passage was completed. The passage was repeated before each subsequent experiment (MTS, flow cytometry, etc.). All experiments were conducted using early-passage preadipocytes (passages 2–4) to minimize alterations in cellular characteristics associated with prolonged in vitro expansion.

Flow Cytometry

Cells in the T-75 flask were removed. PBS at 0.2% was used to wash the cells; a 5-minute centrifugation at 300×*g* was performed, and the supernatant was discarded. The pellet was incubated with 2% paraformaldehyde (PFA) for 10 minutes at room temperature, then washed again with PBS. It was centrifuged at 300×*g* for 5 minutes. The supernatant was discarded. Blocking was performed with 1% bovine serum albumin, and the cells were incubated for 30 minutes at room temperature. The washing procedure (with PBS) was repeated. The pellet was centrifuged for 5 minutes at 300×*g*. The cells were divided into Eppendorf tubes, each with 100 μL. CD73, CD90, CD105, CD44, and CD29 positive marker antibodies and CD14, CD31, CD45, CD34, and CD117 (ABCAM, UK) negative marker antibodies were added to each of them. One Eppendorf tube without antibody was determined as the control group. Spectrophotometric measurements were done with a FACSCalibur flow cytometer.

Cell Viability Assessment Using the MTS Assay

The MTS method (CellTiter 96 Aqueous One Solution, Promega, Madison, WI), which relies on mitochondrial dehydrogenase enzyme activity, was used to evaluate cellular viability. Addition of the MTS to the cell culture medium results in the formation of colored formazan crystals, indicating cellular viability.[7,8] The MTS assay reflects mitochondrial metabolic activity and represents the number of metabolically active viable cells at the time of measurement. It does not directly assess long-term cell survival or proliferative capacity.

Cultivation was performed in 96-well culture dishes with 5000 cells/well for each study and control group. After 24 hours, the cells were detected to acquire morphology in different pH values. HCl and $NaHCO_3$ solutions were titrated into the media of 13 groups at pH values between 1 and 13. Each experimental condition was performed using 3 independent biological replicates, each consisting of 4 technical replicates per condition. Culture media pH was adjusted using sterile 1N hydrochloric acid (HCl) and 1N sodium bicarbonate ($NaHCO_3$) solutions, added dropwise under continuous mixing until the target pH was reached. Final pH values were verified using a calibrated digital pH meter immediately before incubation. The total volume of titrant did not exceed 1% of the culture medium volume in any group, minimizing potential osmolarity changes. As all groups were prepared using the same base medium and only minimal volumes were added, osmolarity-related effects were considered negligible and comparable across groups. The positive control group received 20% dimethyl sulfoxide (DMSO), while the negative control group received only medium. After 24 hours of incubation, the medium was discarded. The MTS solution (10% of the total solution) was added to the mixture of PBS and glucose. The prepared solution was distributed to 96-well culture dishes in 100 μL of all wells. Cells were incubated at 37 °C and 5% CO_2 for 1 hour. Cellular viability of the control and study groups cultured in 13 different pH environments was measured by MTS assay. The color intensity developed after MTS was measured spectrophotometrically with an enzyme-linked immunosorbent assay plate reader at 490 nm absorbance, and the surviving cells were determined.

Cell Culture

From T-75 flasks, cells were removed, counted, and planted in a 6-well tissue culture well with 10^5 cells. After 24 hours, cells were observed to gain a morphology. Preadipocytes proliferate in culture medium and are referred to as fibroblast-like cells because of their morphology.[9] Complete media prepared at pH 6, 8, 10, and 12 were applied to the cells. Cells were incubated for 24 hours at 37 °C in 5% CO_2 medium. Cells were removed and transferred to prelabeled 15-mL Eppendorf tubes and stored at –20 °C for RNA isolation.

RNA Isolation

For 1×10^6 cells, 1 mL Trizol was added to each Eppendorf tube. The samples were inverted for 30 seconds and incubated at room temperature for 5 minutes. The samples were inverted for 10 to 15 seconds after adding 200 μL of chloroform to 1 mL of Trizol, then incubated at room temperature for 3 minutes. Then it was centrifuged at 12,000×*g*. The 400 μL of the upper phase was transferred to a prelabeled Eppendorf tube. Isopropanol was added to an amount equal to that of the supernatant taken and allowed to incubate for 10 minutes at room temperature. Subsequently, 10 minutes of centrifuging was performed at 12,000×*g*, and the supernatant was removed, and 800 μL of 70% ethanol was added to the pellet. After 5 minutes of centrifuging at 12,000×*g*, the supernatant was discarded, and 5 minutes were waited for the alcohol to evaporate. Pellets were dissolved in distilled water, and the data analysis was done on a NanoDrop device.

cDNA Synthesis

The prepared master mix (Supplemental Table 1, Supplemental Digital Content 1, http://links.lww.com/SCS/J720) was added to the prelabeled tubes of 0.2 mL in an amount of 10 μL. The RNA samples to which it was labeled were added to each Eppendorf in an amount of 10 μL (1000 ng/mL). All samples were placed in the MyCycler device, and the DNA was translated to cDNA at the specified temperatures (Supplemental Tables 2, 3, Supplemental Digital Content 2, http://links.lww.com/SCS/J721; Supplemental Digital Content 3, http://links.

lww.com/SCS/J722). Samples were diluted 1/10 with distilled water (without DNase or RNase). cDNAs were stored at –20 ° C.

Real-Time PCR

The real-time PCR aimed to determine the effects of pH on the morphologic and genetic structure of preadipocytes.

In the cDNA samples obtained after RNA isolation, known sequences for these genes were used to evaluate the COX-2, IL-6, and caspase-3 genes. Primer sequences and expected sizes of PCR products are indicated in Supplemental Table 1, Supplemental Digital Content 1, http://links.lww.com/SCS/J720. All primers used for real-time PCR were commercially obtained (OriGene Technologies, Rockville, MD). Primer specificity was verified by melt curve analysis, which demonstrated a single specific amplification peak for each target gene. No evidence of nonspecific amplification or primer-dimer formation was observed. All qPCR assays were performed following standard quantitative PCR guidelines, and amplification reactions showed consistent and reproducible profiles. The master mix was added to a labeled Eppendorf tube in an amount of 8 μL. Two microliters of the cDNA samples were added to each labeled Eppendorf tube. All samples were placed on the Bio-Rad CFX96 real-time system. All genes were normalized to the 18S rRNA gene. Analysis was made at specified temperatures and times, and data were received.

Statistical Evaluation

The data obtained were evaluated biostatistically by one-way ANOVA. Subgroup analyses were performed using the Tukey test. A value of $P \leq 0.05$ was considered significant.

RESULTS

Evaluation of Cell Viability With MTS

Among the groups exposed to different pH values, the control group was considered as 100%, and the highest metabolic activity-based viability was observed at pH 8, with 132%. While the difference between cell viability at pH 7 and 8 is not statistically significant, the difference between pH 8 and 9 is (Supplemental Table 2, Supplemental Digital Content 2, http://links.lww.com/SCS/J721).

It was observed that the cell viability in the groups exposed to pH 6, 8, 9, 10, and 11 increased statistically significantly compared with the negative (medium-only) control group. At pH 1, 2, 3, 4, 5, 12, and 13 values, toxic effect, and decrease in cell viability were observed (Supplemental Table 3, Supplemental Digital Content 3, http://links.lww.com/SCS/J722).

Gene Analysis With Real-Time PCR

Using PCR, apoptotic and inflammatory genes expressed by preadipocytes in different pH environments, and the subsequent expression percentages of genes, were determined. The expression of the IL-6 gene, activated in inflammation, the COX-2 gene, involved in inflammation and apoptosis, and the caspase-3 gene, a marker of apoptosis, was examined. Gene analysis was performed for the cells in pH 6, 8, 10, and 12 media, where cell viability was significantly higher than that of the control group. There are some reasons for choosing these pH values as intermediate values:

pH 6: this value is the second pH value that is significantly higher than the control group.

pH 8: gene analysis was performed for the cells in the pH 8 group, since there was no significant difference between pH 7 and 8 in terms of cellular viability, and the pH 8 group showed higher viability than the pH 9 group. In addition, among all groups, pH 8 also has the highest cell viability.

pH 10: there is a decrease in cellular viability from pH 9 onward. There is no significant difference between pH 9 and 10 in terms of cellular viability. pH 10 was chosen to be an intermediate value.

pH 12: it is the highest pH value at which cell vitality is sufficient for gene analysis. Although the cell viability was decreased compared with the control group, gene analysis could be performed since it contained enough viable cells.

Cytokines

IL-6, COX-2, and caspase-3 gene expressions were found to be highest at pH 12. Expression of these genes at pH 12 was found to be significantly higher than even the positive control group. Overexpression of these genes indicates that inflammatory and apoptotic processes develop in the cell.[10]

IL-6

IL-6, an inflammatory cytokine, has been shown to adversely affect preadipocyte development and differentiation.[11] In another study, the existence of the same effect was proven by showing that preadipocyte differentiation and cell viability were increased by inhibition of IL-6 gene expression.[12] In our study, the pH 12 group was shown to have the highest IL-6 gene expression. Low IL-6 gene expression in the pH 6.8 and 10 values and in the negative control group did not show a significant difference between these 4 groups ($P < 0.05$) (Supplemental Table 4, Supplemental Digital Content 4, http://links.lww.com/SCS/J723).

COX-2

COX-2 is an inflammatory enzyme that increases in bone resorption, mechanical stress, carcinogenesis, and cellular pathologies.[12] In our study, the highest COX-2 expression was at pH 12; low COX-2 gene expression at pH 6, 8, and 10, and in the control group did not show any significant difference between these 4 groups ($P < 0.05$) (Supplemental Table 5, Supplemental Digital Content 5, http://links.lww.com/SCS/J724).

CASPASE-3

Caspase-3, known as the apoptosis gene, has also been shown to indicate cell death in preadipocytes.[10,13] In our study, the highest caspase-3 expression was observed at pH 12; low caspase-3 gene expression at pH 6, 8, and 10, and in the control group did not show a significant difference between these 4 groups ($P < 0.05$) (Supplemental Table 6, Supplemental Digital Content 6, http://links.lww.com/SCS/J725).

DISCUSSION

Stem cells originating from adipose tissue are distributed among mature adipocytes, between the endothelial wall and connective tissue. These cells are considered precursors of both adipocytes and vascular endothelial cells and are responsible for the life cycle of adipose tissue. Although current studies have demonstrated the chondrogenic, neurogenic, and osteogenic potentials of these cells, the term "preadipocyte" is frequently used to describe the cell population obtained after digestion of adipose tissue with collagenase.[14]

There are several reasons for choosing preadipocyte cells for survival evaluation. Preadipocytes are more resistant to trauma than adipocytes. Lipid-filled adipocytes are fragile and can easily be damaged during the liposuction procedure. Mature fat cells can survive if they are fed by inosculation within 4 to

8 days after transplantation. Preadipocytes can live longer without nutrients and consume less oxygen than mature adipocytes.[15,16] Another reason is that while adipocytes cannot increase in number and multiply, preadipocytes have a chance to proliferate and differentiate to increase the number of adipocytes.

It is the most accepted opinion that adipose tissue graft survives by feeding with serum diffusion for the first 48 hours and then regenerates.[2,17,18] The relationship between graft survival and pH has been examined in skin grafts, and it has been observed that the graft shows higher survival at alkaline values.[9,19] The optimal pH value in the recipient bed is between 7.2 and 7.5.[20] When the pH values of the granulation tissue on the wound are examined, the pH value is between 6 and 8. The best pH value of skin grafts has been reported to be 7.4 and above.[19] Sharpe and colleagues reported that 8.55 was the optimal pH value for keratinocyte migration, while fibroblasts and keratinocytes adhered best to the culture medium at pH 8.3.[20,21] Immunization and neovascularization steps are important in adhering to the recipient bed of the skin graft, as in the fat graft.

pH changes affect MMP activity, angiogenesis, macrophage and fibroblast activity, and oxygen diffusion to the wound site.[16] Lengheden and Jansson[22] examined the effects of pH 7.2 to 8.4 values in the human periodontal ligament and embryonic lung cells in vitro and reported that the growth and adhesion properties of these cells decreased significantly below pH 7.8. Zhang et al[23] investigated the effect of different pH values on osteoblasts. When the osteoblasts that were incubated in cell culture at pH 6.4, 6.8, and 7.4 were examined, the highest cell viability and the lowest apoptosis development were detected at pH 7.4. In the same study, it has been shown that a low pH value induces autophagy in osteoblasts. In a recent study, increased osteoblast viability at alkaline pH was demonstrated in vitro, bringing a new perspective to bone regeneration. In this study, it has been shown that cell number and adenosine triphosphate (ATP) content increase at alkaline pH.[24] The positive effects of high pH values on the tissues under the skin barrier correlate with our study results.

MTS measurements showed that extreme pH values (pH 1–5 and 12–13) had a toxic effect on preadipocytes. Cell viability was maintained at weakly acidic (pH 6) and moderately alkaline conditions up to pH 11 (pH 8–11).

The MTS assay used in this study reflects the short-term metabolic activity of viable preadipocytes rather than their long-term survival or proliferative potential. Therefore, the observed differences should be interpreted as relative metabolic viability under different pH conditions. Further studies incorporating long-term survival and proliferation assays are needed to fully elucidate the effects of pH on adipocyte fate.

A 24-hour incubation period was selected to assess early cellular metabolic responses to pH alterations before longer-term adaptive, proliferative, or differentiation-related processes become dominant. This time point was chosen to standardize experimental conditions and to specifically evaluate early stress-related cellular responses.

IL-6, COX-2, and caspase-3 gene expressions examined in cells in pH 6, 8, 10, and 12 groups in our study were found to be the highest at pH 12. The expression of these genes at pH 12 was significantly higher than that of the positive control group (containing the toxic agent DMSO). Overexpression of these genes indicates the development of inflammatory and apoptotic processes in the cell.[10] The genes selected for analysis in this study were chosen to reflect inflammatory and apoptotic responses rather than adipocyte differentiation or endocrine function. IL-6 and COX-2 were included as markers of inflammation-associated cellular stress, whereas caspase-3 was selected as a key effector of apoptosis. Since early inflammatory and apoptotic processes are considered major determinants of fat graft viability during the initial post-transplantation period, these markers were prioritized. Markers related to adipocyte differentiation and metabolic function, such as PPARγ, adiponectin, and leptin, were beyond the primary scope of the present study and should be addressed in future investigations.

When the adipose tissue graft is harvested, the Klein solution is preferably infiltrated into the donor site before the procedure. Jeffrey A. Klein, who first described this solution, stated that it has no standard content and that the amounts/rates of lidocaine and epinephrine in the solution can be adjusted according to the region to be treated. The solution suggested for the abdominal region contains 1250 mg/mL of lidocaine, 1.0 mg/L of epinephrine, and 10 mEq/L of sodium bicarbonate.[25] In our study, the Klein solution was infiltrated into the donor site before the fat graft was harvested. According to the analysis of the effect, there was no significant difference in terms of cell survival between the groups with and without the solution.[26] However, it is not possible to distinguish the effect of bicarbonate since all the components in the solution are examined in a single group. No study has examined the effect of bicarbonate in this solution on graft survival.

Due to the acidic nature of lidocaine, it is aimed to strengthen analgesia by adding sodium bicarbonate to the solution. The pH value of 0.9% NaCl in the solution content is 6.85, and the pH value of 4% sodium bicarbonate is 7.87. The purpose of the prepared solution is to remove the pain during infiltration by obtaining a pH in the range of 7.0 to 7.4.[27] By increasing the sodium bicarbonate ratio of the solution to 8 using the pH values obtained by the Klein solution, we suggest that a mildly alkaline pH environment may support cellular metabolic viability within fat grafts. We argue that the storage of cryopreserved grafts in a pH 8 environment can have a positive effect similar to graft survival. Application of a slightly alkaline solution to the recipient site before injection or incorporation into the graft may have potential implications for graft viability.

In eukaryotic organisms, the energy required for the continuity of life is provided by catabolic reactions that break large molecules (nutrients like glucose, amino acids, and fatty acids) into smaller molecules. Although mitochondria, the powerhouse of the cell, provide the main intracellular energy through oxygen-dependent aerobic cellular respiration, skeletal muscle can undergo anaerobic respiration, also known as fermentation, when energy demand exceeds energy production during strenuous exercise. Fermentation is less efficient than aerobic respiration at using energy from glucose: only 2 ATP are produced per glucose, compared with 36 ATP per glucose produced by aerobic respiration. Lactate (lactic acid) accumulates in the cytoplasm by biochemical feedback mechanisms and eventually stops anaerobic respiration.[28,29]

The viability of the grafts during the imbibition period depends solely on the diffusion of nutrients and oxygen from the receiving site until blood circulation is restored. In other words, the viability of the cells in the graft depends on their tolerance to the hypoxic environment during this critical period.[30,31] Theoretically, the faster the protons released from the lactic acid during the cytoplasmic fermentation process leave the cellular microenvironment, the longer the fermentation con-

tinues to provide vital energy in an oxygen-deprived situation. As a result, the higher pH levels within a limited alkaline range may be associated with improved cellular metabolic viability under hypoxic conditions. While this assumption is true when pH changes are within the weakly alkaline range, very high pH values (increased alkalinity) also compromise cell viability as they will cause denaturation of cells' vital proteins and enzymes.[32,33]

A buffer solution is an aqueous solution consisting of a mixture of a weak acid and its conjugate base (or vice versa). When a small or moderate amount of a strong acid or base is added to a buffer solution, the pH change is very small; therefore, it is used to prevent pH changes in solutions.[34]

According to Belzer and Southard, the first classic concept of tissue acidosis damages cells by destabilizing lysosomal membranes and altering mitochondrial properties. Low pH also inhibits ATP production during glycolysis. The key glycolytic enzyme, phosphofructokinase, is inhibited by increasing hydrogen ion concentrations. For this reason, it was hypothesized that cellular energy metabolism could be stimulated by removing excess hydrogen ions during cold storage. As a result, various preservation solutions with slightly alkaline pH have been tested to achieve low levels of anaerobic glycolysis. This routine fits well with Belzer and Southard concept that the prevention of tissue acidosis is a prerequisite for good organ protection.[35]

It has been shown that the UW solution, which is used in cold storage of the liver, kidney, and pancreas, is a slightly alkaline (pH 7.4) preserving solution, and in studies conducted with this solution, cell viability can be preserved up to 72 hours.[25,36–38] When the pH of the UW solution was changed in cold storage and brought to acidic pH values compared with standard pH values, worse results were obtained (pH 7.4). Since isolated parenchymal cells are not damaged by acidic pH, these results may imply that vascular cells are more sensitive to acidic conditions than parenchymal cells. The results of this study showed us that, unlike isolated cells, cold storage applications actually give better results in an environment where slightly alkaline conditions (pH 7.4 ± 7.8) are provided, rather than protecting the whole organ under acidic conditions.[39,40]

On the basis of the studies mentioned above, we emphasize that a slightly alkaline buffering capacity may be important for graft viability during the imbibition period, rather than the absolute alkaline strength of the solutions that can be used in grafting procedures.

The present study has several limitations. First, it is an in vitro study, and therefore, in vivo experimental studies are required to validate the findings. In addition, all experiments were performed using adipose tissue obtained from a single donor to minimize interindividual variability in cellular responses to pH alterations. However, this may limit the generalizability of the results and should be considered when interpreting the findings. Finally, given the exploratory nature of this study and the lack of prior data on pH-dependent effects on preadipocyte viability, a formal a priori power analysis was not feasible at the study design stage.

To the best of our knowledge, no experimental study has demonstrated the effect of pH on fat tissue graft survival. Although there was no significant difference in inflammation and apoptosis in the other groups except pH 12, considering that the best result was at pH 8 when evaluated in terms of cell viability, we suggest that a mildly alkaline pH environment may support cellular metabolic viability within fat grafts, particularly during the early hypoxic period following transplantation.

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