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ORIGINAL RESEARCH

Comparison of the Effects of Extracorporeal Irradiation and Liquid Nitrogen on Nerve Recovery in a Rat Model

Hüseyin Kaya[a], Dündar Sabah[a], Burçin Keçeci[a], Levent Küçük[a], Oytun Erbaş[b], Fatih Oltulu[c], Gürkan Yiğittürk[d], and Dilek Taskiran[e]

[a]Department of Orthopedics and Traumatology, Faculty of Medicine, Ege University, Izmir, Turkey; [b]Department of Physiology, Faculty of Medicine, Istanbul Bilim University, Istanbul, Turkey; [c]Department of Histology and Embryology, Faculty of Medicine, Ege University, Izmir, Turkey; [d]Department of Histology and Embryology, Faculty of Medicine, Muğla Sıtkı Koçman University, Muğla, Turkey; [e]Department of Physiology, Faculty of Medicine, Ege University, Izmir, Turkey

ABSTRACT

Aim of the study: Biologic reconstruction using tumor-bearing bone autografts devitalized by liquid nitrogen or extracorporeal irradiation (oncological sterilization) is a safe and effective method in musculoskeletal surgery. The purpose of this study was to examine the effects of these two oncological sterilization methods on nerve recovery.

Methods: A total of 48 rats were randomly divided into 3 groups as autograft, irradiation and liquid nitrogen groups. A nerve defect created in the right sciatic nerve was reconstructed with an autograft obtained from the nerve itself. Group I underwent reconstruction with standard nerve autograft. Group II and Group III underwent reconstruction with devitalized nerve autograft treated through extracorporeal irradiation and liquid nitrogen, respectively. The left sciatic nerves of the rats served as control. Electromyography, motor function test and histomorphological analysis were performed to assess the nerve recovery on the 3rd (early stage) and 4th months (late stage).

Results: Electrophysiological assessment revealed better results in irradiation group compared with liquid nitrogen group in terms of myelinization and axonal regeneration. Motor performance of the autograft group was slightly better than the other groups. Histologically, autograft group demonstrated better results compared with other groups. Late-stage assessments revealed high rates of myelinization in the graft segment in liquid nitrogen group and in the segment distal to the graft in irradiation group.

Conclusions: This study has demonstrated that nerve autografts treated by oncological sterilization methods may be used for nerve reconstruction in limb salvage surgery. However, further studies are needed to clarify the applicability of these methods.

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Nerve recovery; limb salvage; extracorporeal irradiation; liquid nitrogen; nerve autograft; bone tumor

Introduction

Amputation used to be the standard surgical treatment method for the musculoskeletal sarcomas of the extremities. Around the 1980s, the developments taking place in diagnostic imaging modalities, adjuvant therapies and surgical techniques increased the accuracy of tumor staging and improved tumor control. In addition, limb salvage surgery has become an alternative to amputation in most cases. Currently, about 70–95% of patients with bone and soft tissue sarcoma are treated with limb salvage surgery, even if the tumor is high grade [1,2].

Wide local excision is the surgical technique preferred for the local control of the malignant bone and soft tissue tumors. However, occasionally, a true wide resection is not possible without sacrificing critical anatomical structures such as bones, major nerves or blood vessels. In limb salvage surgery, bone defects may be reconstructed using intraoperatively treated bone autografts as well as prosthesis and allografts. In order to rid the bone from tumor cells before reimplanting it back to its original place, oncological sterilization methods such as irradiation, liquid nitrogen and autoclaving are used [1,3,4] In addition to being an oncologically safe procedure, this method also has numerous other advantages: there is no risk of allogeneic reactions or transferring infectious diseases, the autograft is a perfect match for the defective area and the surgery is easily applicable [3]. Therefore, in suitable candidates, this method is used in many centers for the reconstruction of bone defects caused by the wide excision of malignant bone and soft tissue tumors [3,5,6]. In cases where major nerve excision is required, the main difficulty in preserving the sensory and motor function of the extremity is the reconstruction of the nerve defect. In such cases, if primary repair is not possible, the standard approach is to repair the defect with an autogenous nerve graft [7–9]. However, finding a suitable nerve to be harvested is difficult. In addition, autogenous nerve grafts may result in significant donor site morbidity, scar and neuroma formation, loss of function and cold intolerance at the distal end of the limb, and may require multiple surgical procedures [9–11]. Such morbidities caused

CONTACT Dilek Taskiran dilek.taskiran@ege.edu.tr Department of Physiology, School of Medicine, Ege University, Izmir, Turkey.

by the use of autografts have directed surgeons toward alternative methods such as using allografts or various conduits. The need for immunosuppression is the most important factor limiting the use of allografts [12]. In addition, while conduit methods yield results similar to those obtained with autografts in the treatment of short peripheral sensory nerve defects, their efficacy in major nerve defects is limited [13–15]. For these reasons, in clinical practice, nerve allografts and conduits have limited application for repairing nerve defects which occur during limb salvage surgery.

In light of these findings, we aimed to examine the effects of these two well-known oncological sterilization methods on the biological healing of the nerve, and to find out whether a nerve defect could be reconstructed successfully with peri-operatively treated devitalized nerve grafts.

Materials and methods

Animals and experimental design

A total of 48 female Sprague-Dawley rats weighing 250–300 g were used for this experimental study. The animals were caged in a controlled environment at 22 °C with 12-hour light/12-hour dark cycles. They were fed by standard pellet diet and tap water ad libitum along the study. The protocol employed in the study was approved by the Institutional Animal Care and Ethical Committee of Ege University. All chemicals were obtained from Sigma–Aldrich Inc. unless otherwise noted.

From each rat, a 10 mm segment of the right sciatic nerve was excised to be used as a nerve graft. The left sciatic nerves of the rats were left in place to be used as the control group. Each animal was randomly assigned to one of three groups:

Group I (autograft): the 10 mm interposition nerve autograft was sutured back to its original place without any intervention.

Group II (radiotherapy): the 10 mm nerve graft was devitalized by a single dose of 50 Gy extracorporeal irradiation before being sutured back to its original place.

Group III (liquid nitrogen): the 10 mm nerve graft was frozen in liquid nitrogen for 20 minutes, thawed at room temperature for 15 minutes and thawed in distilled water for 10 minutes. After the devitalization procedure, the nerve graft was sutured back to its original place.

Surgical procedure

All surgical procedures were performed by a single surgeon using a standard surgical microscope. Anesthesia was induced by intraperitoneal injection of Ketamine–HCl (Alfamine®-i.m.) 50 mg/kg and Xylazine HCl (Rompun®-im) 10 mg/kg. A 10 mm segment of the right sciatic nerve was harvested from the part proximal to the popliteal bifurcation (Figure 1). All nerve grafts were re-implanted orthotopically using three end-to-end epineural sutures (Ethilon® 10-0, Ethicon). Special care was taken to avoid any portion

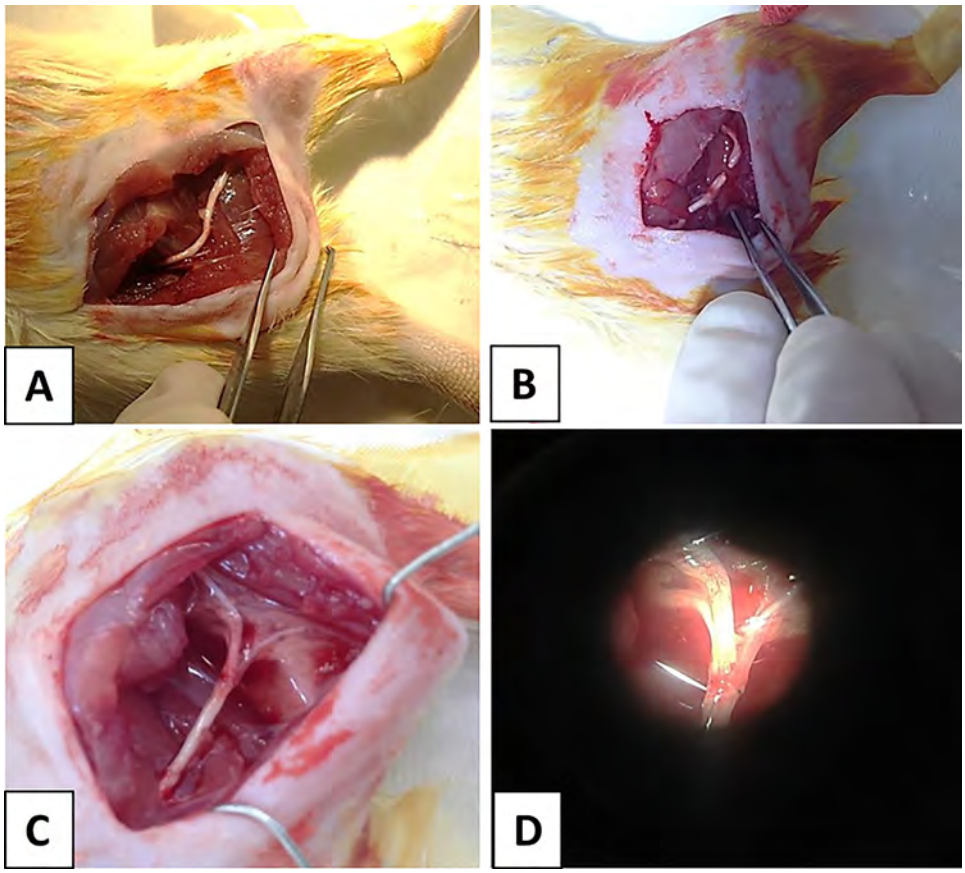

Figure 1. Macroscopic appearance of surgical procedure: (A) right sciatic nerve, (B) a 10 mm long graft is harvested from the nerve, (C) the nerve is orthotopically reimplanted using epineural sutures, (D) anastomosis.

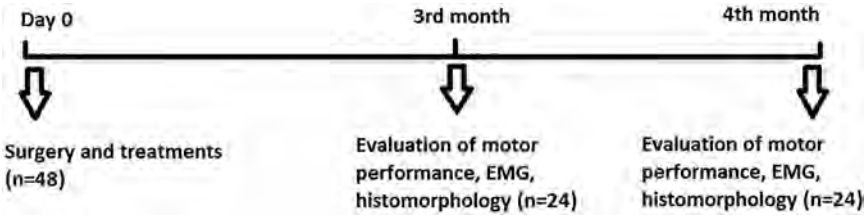

Figure 2. Study design summarizing the timing of surgical procedure and treatments, evaluation of motor function, EMG and histomorphology.

of the nerve. The wounds were closed by 5-0 Vicryl®. All surgical procedures were performed under aseptic conditions. Rats received meperidine (10 mg/kg, s.c.) every 8 h for three times for postoperative pain. Following surgery, all rats were allowed at least 3–4 h recovery period, and then they were placed in their cages. On the 3rd postoperative month, half of each group was sacrificed for early evaluation. The remaining rats were sacrificed on the 4th month for the final investigation. A summary of the study design is presented in Figure 2.

Evaluation of motor function

An inclined plate was used to assess the motor functions of rats as described by Rivlin and Tator previously [16]. The maximum angle in which the animal could maintain its position without falling for 5 seconds was measured three times for each animal and the mean value was recorded and used for analysis. Each trial was performed after a 1-min interval.

Electrophysiological evaluation

Electrophysiological studies are often used for the diagnosis of neuromuscular and neuropathic disorders. The compound muscle action potential (CMAP) recording is a minimally invasive and reliable method which enables to evaluate nerve conduction both in human and animal studies. In peripheral nerve pathologies, the measurement of CMAP amplitudes and latencies provides important information about the axonal loss and demyelination. The distance between the negative and positive peaks in the CMAP curves (p–p distance) obtained by submaximal stimulation is called amplitude. The duration between the stimulus and onset of CMAP is referred as latency (Figure 3). As described in previous studies, while axonal pathologies cause a substantial reduction in the CMAP amplitude, the loss in myelin thickness results in prolonged of distal latency and reduced conduction velocity [17–20].

In the present study, all electrophysiological examinations were performed by the same electromyographer, under general anesthesia (75 mg/kg ketamine and 10 mg/kg xylazine), using an electromyography device (Nicolet Viking IIe electromyography, Nicolet Biomedical, Memphis, TN). Filter setting was 5 Hz to 10 kHz, stimulation time was 0.05 ms and stimulation frequency was 1 Hz. Subcutaneous platinum needle electrodes (Grass; Astro-Med, Inc., West Warwick, RI, USA) were used for stimulation and recording. The sciatic nerve was stimulated supramaximally at the sciatic notch point and CMAPs were recorded from the 2nd and 3rd interdigital muscles [21]. All EMG recordings (distal latency, CMAP duration and amplitude) were evaluated using the Biopac student Lab Pro version 3.6.7 (BIOPAC Systems, Inc.) software. During the EMG recordings, rectal temperature of each rat was monitored by a rectal probe (HP Viridia 24-C; Hewlett–Packard Company, Palo Alto, CA, USA), and carefully maintained at 36–37 °C by using a thermal mattress.

Histopathological evaluation

Immediately after motor function and electrophysiological examinations, rats were sacrificed and histopathological examination was performed. On the 3rd month, half of the rats were sacrificed by anesthetic overdose for early assessment. The remaining rats were sacrificed on the 4th month for late assessment. The right sciatic nerve was dissected and the nerve tissue was removed with the same surgical approach, including both anastomotic patches. The left sciatic nerve was also removed with the same procedure and used as a control group. The sciatic nerve samples were fixed in formalin, embedded in paraffin blocks, sectioned at 5 μm and stained with hematoxylin and eosin staining [22]. All sections were photographed using an Olympus BX51 microscope equipped with an Olympus C-5050 digital camera (Olympus Optical Co., Tokyo, Japan). In the assessment of myelinated nerve fibers and vascular structures in the control and surgical groups (autograft, radiotherapy and liquid nitrogen), digital images of six randomly selected sections were analyzed by two researchers in a double-blinded fashion. The scores of the cross sections were determined by taking the average of the scores of the two researchers for each field separately.

TUNEL (Tdt-mediated d UTP nick end labeling) staining

TUNEL staining was performed to determine apoptotic changes in nerve tissue. TUNEL staining was performed using Apoptag Peroxidase In Situ Apoptosis Detection Kit (Chemicon-Millipore). The staining was performed according to the protocol provided by the manufacturer [23,24]. Ten microscopic fields per section were assessed by a blinded observer at a 40× magnification.

Statistical analysis

All statistical analyses were performed by using the SPSS Version 20.0 program (IBM SPSS Inc., Chicago, IL, USA). Data were expressed as numbers with percentages and

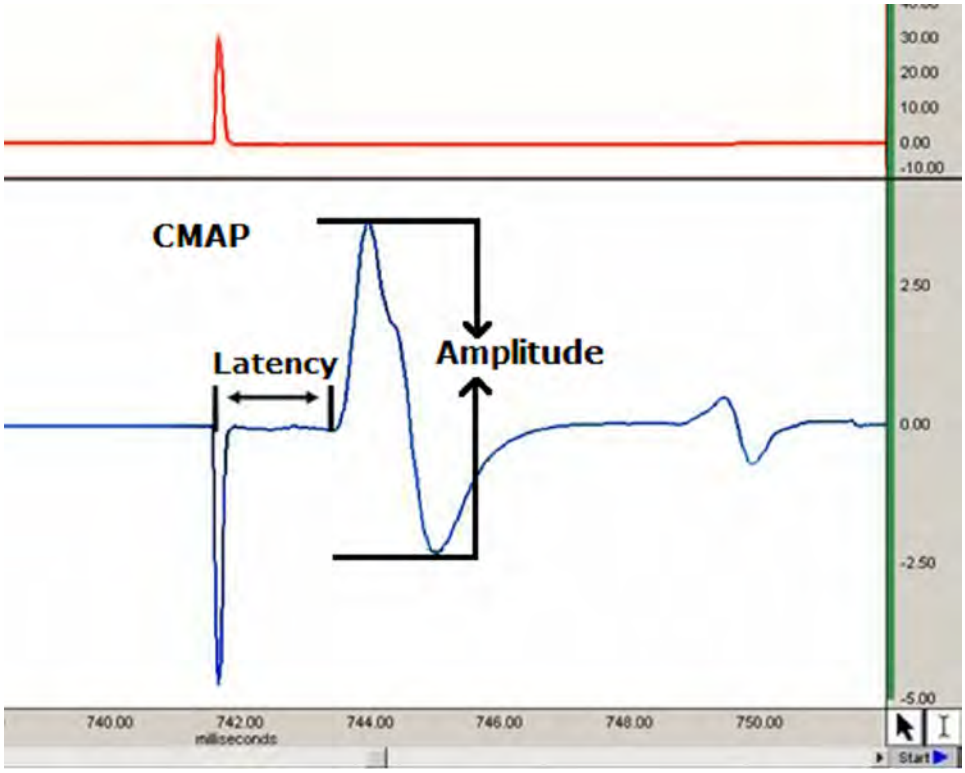

Figure 3. The CMAP and its parameters.

Table 1. Mean climbing angles (degrees) of rats measured with an inclined plate for motor function assessment.

	Pre-operation	Autograft	Radiotherapy	Liquid nitrogen
3rd month	62.6° (±4.3)*	53.0° (±3.7)	50.9° (±3.0)	52.0° (±2.8)
4th month	62.3° (±4.3)*	57.1° (±2.5)	53.1° (±2.3)	53.6° (±3.2)

Data are expressed as mean values ± standard error of the mean (SEM), $n = 8$.
*$p < 0.001$ (different from all other groups).

means with standard deviations. Shapiro–Wilk test was performed to test normality. Groups with non-normal distributions were compared using the Mann–Whitney U test. Factorial ANOVA, one-way ANOVA and *t*-tests were used to compare groups with normal distribution. Tukey-HSD was used for post hoc comparisons. A *p*-value <0.05 was considered statistically significant.

Results

Motor function

Table 1 represents the motor performance of the rats evaluated with inclined plate. The mean pre-operative climbing angles were 62.6° (±4.3) and 62.3° (±4.3) in the groups which were sacrificed on the 3rd and 4th months, respectively. In the 3rd and 4th month evaluations, no significant differences were observed among the three groups (autograft, radiotherapy and liquid nitrogen). However, compared with the 3rd month, motor function was better on the 4th month in all three groups. Pre-operative motor functions of rats were better compared with the 3rd and 4th month results ($p < 0.001$). The motor functions of the autograft group were slightly better than the radiotherapy and liquid nitrogen groups at the 3rd and 4th month, but this difference was not statistically significant.

Electrophysiological assessment

Figure 4 displays an example of EMG recordings obtained from the sciatic nerve of rats. As depicted in Table 2, at the 3rd month, latency was longer in the radiotherapy and liquid nitrogen groups compared with the autograft group ($p < 0.01$) and this result was interpreted as the deterioration of myelinization. On the other hand, myelinization was better in the radiotherapy group compared with the liquid nitrogen group ($p < 0.01$). CMAP amplitude was higher in the autograft group compared with the radiotherapy and liquid nitrogen groups; and higher in the radiotherapy group compared with the liquid nitrogen group ($p < 0.01$), showing that axonal regeneration was better in the radiotherapy group. There was no statistically significant difference between the three groups in terms of myelinization values on the 4th month. Axonal healing was better in the autograft group ($p < 0.05$). No significant difference was found between the radiotherapy and liquid nitrogen groups in terms of axonal healing (Table 3).

Histomorphological assessments

Histological alterations in the nerve sections were assessed with hematoxylin & eosin staining as shown in Figure 5. For histomorphological assessment, the myelinated nerve fibers and vascular structures in the study groups (autograft, radiotherapy and liquid nitrogen) were scored histologically. According to these results, when the graft segment was

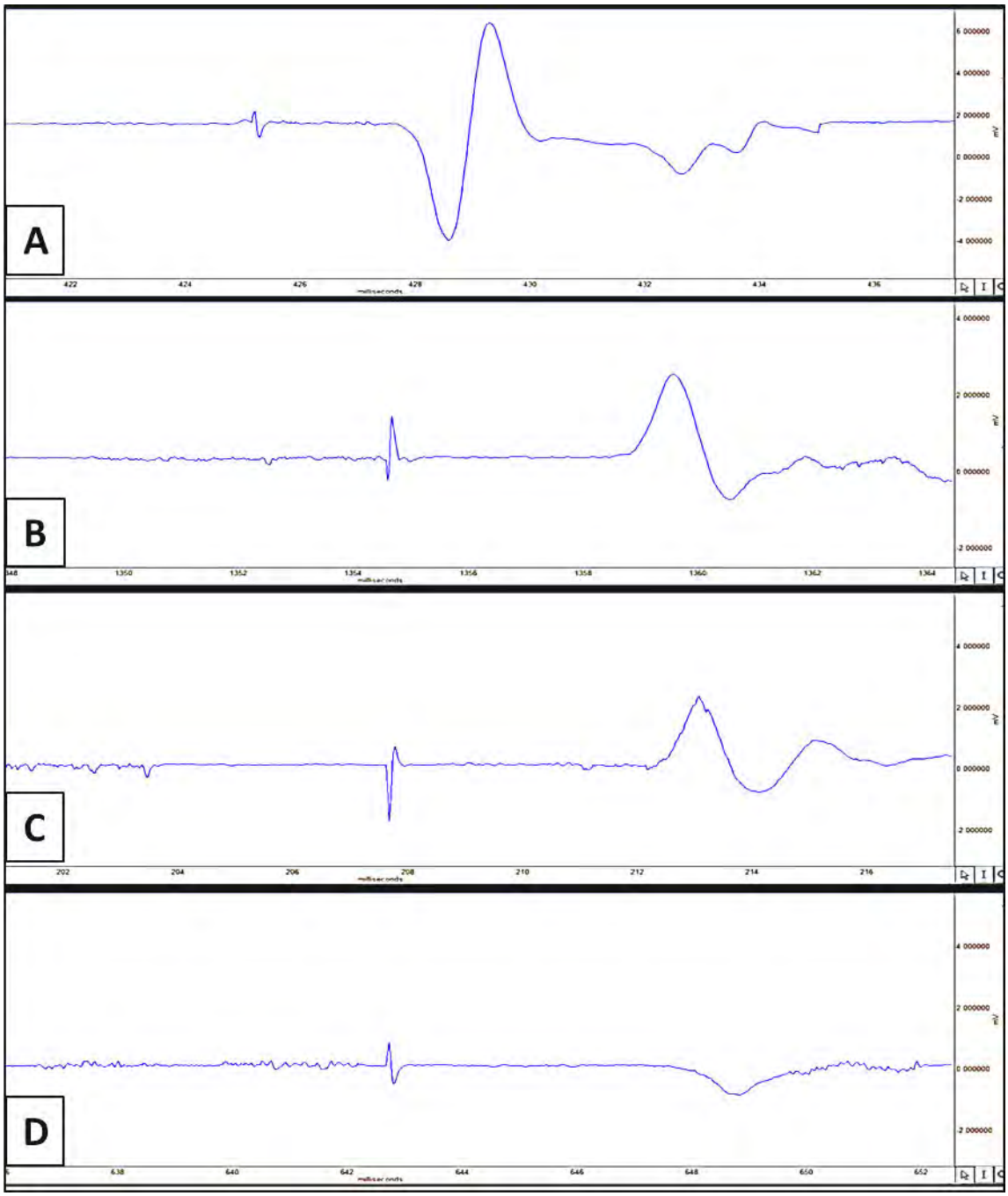

Figure 4. EMG recording samples obtained from (A) control group, (B) autograft group, (C) irradiation group, (D) liquid nitrogen group.

Table 2. Electrophysiological analyses performed on the 3rd month.

	Autograft		Radiotherapy		Liquid nitrogen	
	Right	Left	Right	Left	Right	Left
Latency (ms)	4.3 ± 0.1	2.6 ± 0.1	5.2 ± 0.3*	2.8 ± 0.4	6.7 ± 0.5*#	2.4 ± 0.1
Peak to Peak (mV)	2.6 ± 0.2	13 ± 0.8	1.94 ± 0.3*	11.7 ± 1.7	0.8 ± 0.1*#	15.7 ± 0.9

Data are expressed as mean values ± standard error of the mean (SEM), $n = 8$.
*$p < 0.01$ (different from autograft group).
#$p < 0.01$ (different from radiotherapy group).

assessed, the number of myelinated nerve fibers on the 3rd and 4th months was found to be significantly lower in the three groups compared with the control group. In addition, the number of myelinated nerve fibers in the autograft group was found to be significantly higher than that of the radiotherapy and liquid nitrogen groups on the 3rd month ($p < 0.001$). There was no significant difference between the radiotherapy and liquid nitrogen groups. Similarly, when the myelinated nerve fibers in the nerve graft segment were assessed on the 4th month, it was found to be significantly

Table 3. Electrophysiological analyses performed on the 4th month.

	Autograft		Radiotherapy		Liquid nitrogen	
	Right	Left	Right	Left	Right	Left
Latency (ms)	4.5 ± 0.2	2.9 ± 0.1	4.7 ± 0.2	2.8 ± 0.03	5 ± 0.3	2.8 ± 0.1
Peak to Peak (mV)	4.3 ± 1	13.2 ± 1	2.2 ± 0.4*	15.7 ± 1.1	2.2 ± 0.6*	13.1 ± 0.7

Data are expressed as mean values ± standard error of the mean (SEM), $n = 8$.
*$p < 0.05$ (different from autograft group).

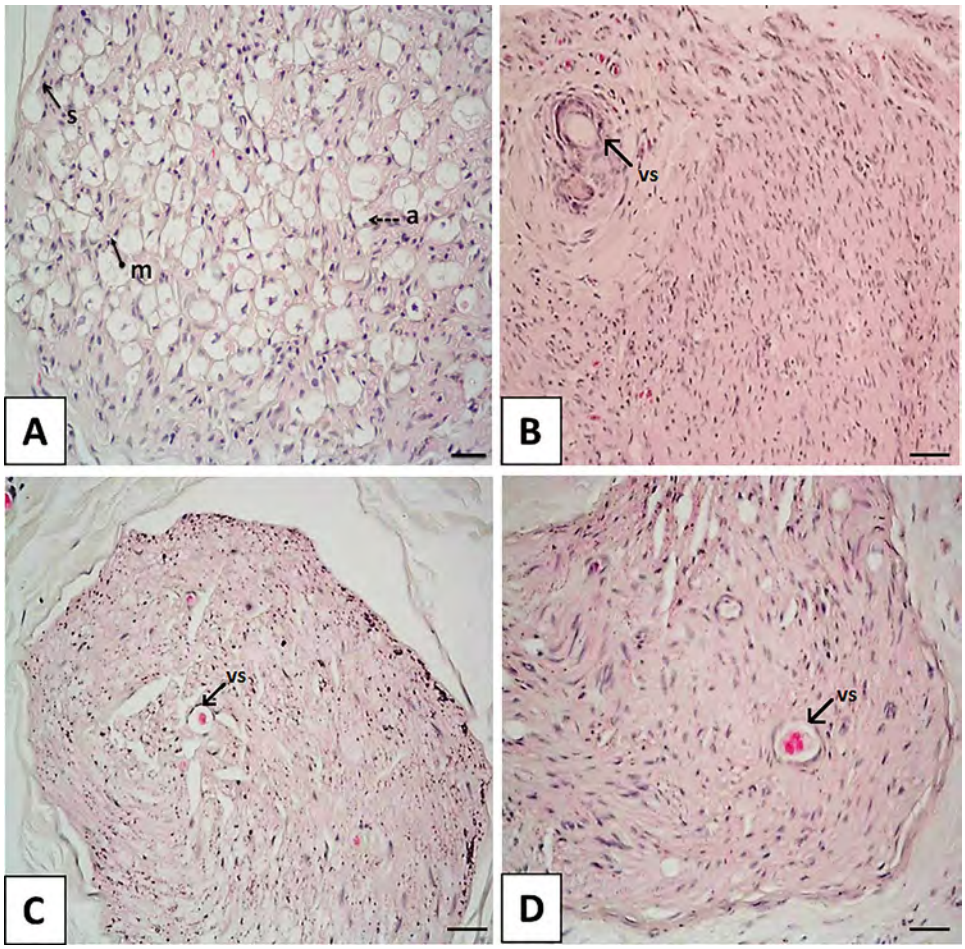

Figure 5. Histomorphological evaluation of graft segment at the 4th month (A) control group, (B) autograft group, (C) irradiation group, (D) liquid nitrogen group. Magnification ×40. Hematoxylin & eosin staining. p: perineurium, a: axon, s: schwann cell, vs: vascular structure.

higher in the autograft group than the radiotherapy and liquid nitrogen groups ($p < 0.001$). In addition, the number of nerve fibers in the liquid nitrogen group was statistically significantly higher than the radiotherapy group ($p < 0.05$) (Figure 6(A)).

In the segment distal to the graft, the number of myelinated nerve fibers on the 3rd month was found to be higher in the autograft group compared with the radiotherapy and liquid nitrogen groups ($p < 0.001$). No significant differences were found between the radiotherapy and liquid nitrogen groups in terms of myelinated nerve fiber counts in the distal segment. At the 4th month evaluation, the autograft group was found to have a significantly higher number of myelinated nerve fibers in the distal segment compared with the radiotherapy and liquid nitrogen groups ($p < 0.001$). Radiotherapy group had significantly higher number of myelinated nerve fibers compared with the liquid nitrogen group ($p < 0.05$). Overall, the number of nerve fibers decreased significantly from the proximal portion of the graft to the distal portion of the graft in all three groups on the 3rd and 4th month evaluations (Figure 6(B)).

In terms of vascular structure assessment of the graft segments, there was no difference between the 3rd and 4th month values of the three groups. The number of vascular structures in the autograft group was significantly higher than the radiotherapy and liquid nitrogen groups ($p < 0.001$). There was no difference between the radiotherapy and liquid nitrogen groups in terms of the number of vascular structures (Figure 7(A)).

When the distal segments were assessed in terms of vascular structure, no differences were observed among the three groups on the 3rd month evaluation. However, on the 4th month evaluation, the number of vascular structures in the radiotherapy group was found to be lower than both the autograft and liquid nitrogen groups ($p < 0.05$) (Figure 7(B)).

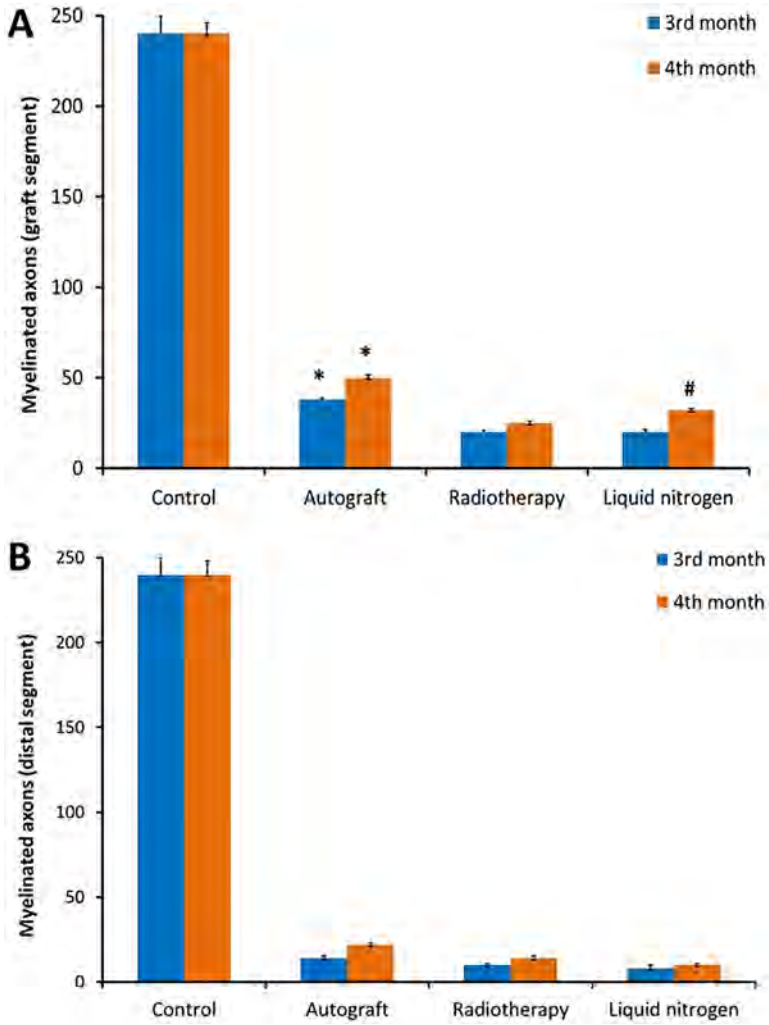

Figure 6. Quantitative evaluation of the myelinated axon counts in the (A) graft segments and (B) distal segment obtained on the 3rd and 4th months. Data are expressed as mean values ± standard error of the mean (SEM), $n = 8$. $^{*}p < 0.001$ (different from other groups), $^{\#}p < 0.05$ (different from irradiation group), $^{\ddagger}p < 0.05$ (different from liquid nitrogen group).

TUNEL (Tdt-mediated d UTP nick end labeling)

There was a significant increase in the number of TUNEL-positive cells in all surgical groups compared with the control group. Specifically, liquid nitrogen group displayed higher apoptotic cells than of the autograft and radiotherapy groups (Figure 8).

Discussion

The main surgical approach to musculoskeletal sarcomas of the extremities is to resect all contaminated tissues (bone, tendon, nerve and vascular structures) along with the tumor. Biological reconstruction using tumor-bearing bone autografts devitalized by liquid nitrogen or extracorporeal irradiation (oncological sterilization) is used as a safe, easy and efficient method in musculoskeletal tumor surgery [3,6]. However, the method described above cannot yet be adapted to repair nerve defects. Therefore, in the present study, we aimed to examine the effects of two oncological sterilization methods, extracorporeal irradiation and liquid nitrogen, on nerve recovery.

There are numerous studies in the literature investigating the effects of radiotherapy and liquid nitrogen on nerve tissues. However, these studies have either focused on nerve allografts or on the effects of surgical-site radiotherapy on nerve recovery [25–28]. To date, there has been no study investigating nerve regeneration in an autograft which was reimplanted following devitalization by extracorporeal single dose radiotherapy. In this respect, our study can be considered as the first study in this field.

It has been previously shown that both radiotherapy and liquid nitrogen have unfavorable effects on nerve regeneration [27–29]. On the other hand, both methods have also been demonstrated to have positive effects in terms of decreasing antigenicity in allografts. Therefore, these methods have been adopted for allograft preparation. By experimenting with different doses of radiation or different liquid nitrogen protocols, researchers have aimed to minimize the unfavorable effects of these methods. For example, Mackinnon et al. have reported that low dose irradiation reduced the antigenicity of the allograft to autograft levels but resulted in a poorer ability to regenerate [26]. In the study performed by Fansa et al., rat sciatic nerve grafts subjected to controlled freezing and cryoprotectant were compared with autografts treated with liquid nitrogen and fresh

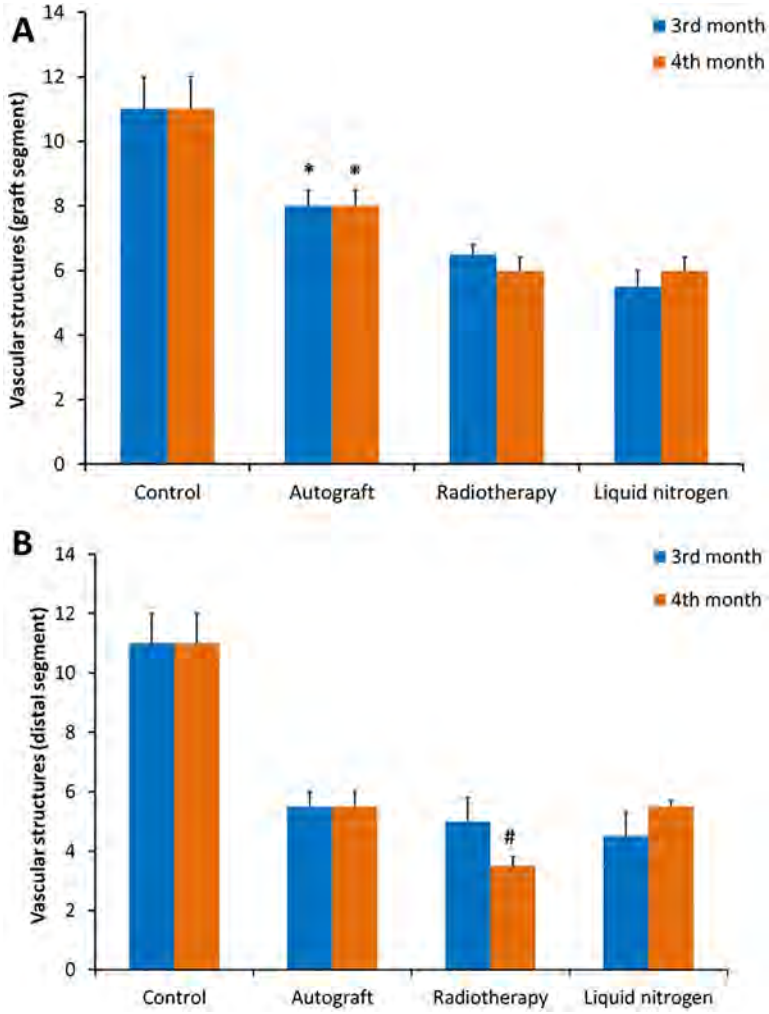

Figure 7. Quantitative evaluation of the vascular structures in the (A) graft segments and (B) distal segments obtained on the 3rd and 4th months. Data are expressed as mean values ± standard error of the mean (SEM), $n = 8$. $^{*}p < 0.001$ (different from other groups), $^{\#}p < 0.05$ (different from liquid nitrogen group).

autografts. They found that axon count and myelinization decreased significantly in the frozen graft groups compared with the fresh autograft group. In addition, grafts which were subjected to controlled freezing yielded better results compared with those treated with liquid nitrogen. The best outcomes were achieved in the fresh autograft group. Impaired regeneration was explained with delayed Wallerian regeneration and slowed revascularization [29].

Previous studies have concluded that the graft should be revascularized in order for the nerve to heal. Some studies have proposed that the main mechanism for graft revascularization is the regeneration of vascular structures within the soft tissue surrounding the nerve [30,31]. In another study, the rat sciatic nerve was observed on the 28th day of graft reimplantation, and it was shown that even though the central part of the graft was hypoperfused, there was revascularization on both ends of the graft, especially on the proximal end [32]. On the other hand, in the study by Mackinnon et al., peripheral revascularization was not deemed important [33]. In another study, Oh et al. have investigated the effects of irradiation on the vascularity of the graft. In this study, compared with the control group, the number of vascular structures was found to decrease significantly in the graft segment of the 86 and 106 Gy irradiation groups, and in the segment distal to the graft in all irradiation groups. The effect of irradiation was found to be dose-dependent [34].

The devitalization methods used in our study were the same as those used for bone tissue treatment. For extracorporeal irradiation, a 50 Gy bolus single dose of radiotherapy (which is considered as the oncologically safe dosage) was administered [5,35] For liquid nitrogen treatment, the method described by Tsuchiya et al. for the devitalization of tumor-bearing bone grafts was used [3]. In our study, compared with the fresh autograft group, revascularization was lower in the early phase in the liquid nitrogen group and in the late phase in the irradiation group. However, revascularization was present both in the proximal and distal ends of the devitalized grafts. These results are in accordance with previous studies indicating that radiotherapy has negative effects on graft revascularization and that freezing impairs revascularization and axonal regeneration in autografts and in allografts subjected to freezing [29,36–38].

In our study, better histological outcomes were observed in the autograft group compared with other groups. However, the radiotherapy and liquid nitrogen groups also showed axonal regeneration to some extent. In the early phase, the outcomes were similar in the radiotherapy and

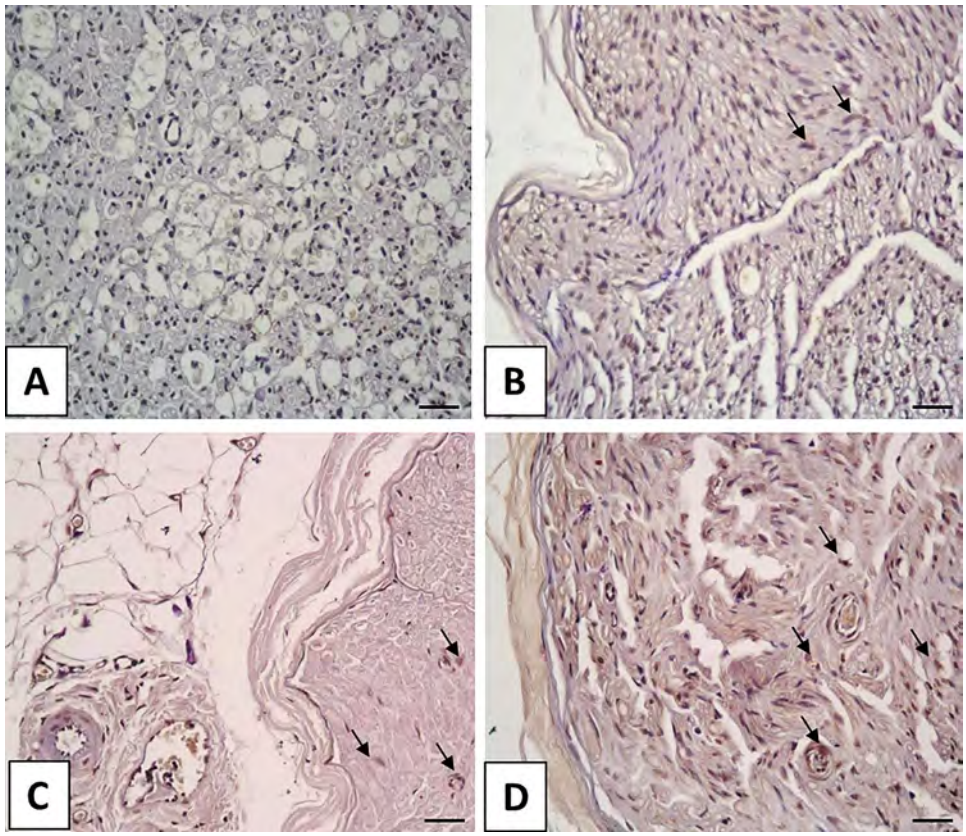

Figure 8. TUNEL immunohistochemistry. Histological sections obtained from the graft segment. Increased apoptotic changes were observed in all surgical groups, but specifically liquid nitrogen group. (A) Control group, (B) autograft group, (C) irradiation group, (D) liquid nitrogen group. Magnification ×40.

liquid nitrogen groups; whereas in the late phase, when the part distal to the graft was evaluated, the radiotherapy group was found to be superior to the liquid nitrogen group. In addition to axonal loss, endoneural fibrosis and fibrotic foci were observed in the graft segment and in the segment distal to the graft in the autograft and radiotherapy groups and in all segments of the liquid nitrogen group. These results show that even though radiotherapy and liquid nitrogen affect axonal regeneration negatively in autografts, they do not cause complete necrosis and fibrosis in the nerve graft. The nerve graft that became oncologically safer after devitalization procedures in our study enabled the axon transfer from proximal of the nerve to the distal in the regeneration process, and did not prevent the regeneration. These findings are in line with the previous studies [27–29].

After the nerve repair, process of nerve regeneration and target organ reinnervation are very complicated. Duration is one of the important factors determining nerve healing. Although various timeframes have been used for evaluating the nerve regeneration in the experimental studies, follow-up periods between 8 and 16 weeks were generally used [38]. In our study, evaluations were made in two different time periods, early (3rd month) and late (4th month). The autograft group was found to be superior in both periods. The radiotherapy group was found to be superior to the nitrogen group in early electrophysiological assessments. However, in late evaluations, no significant difference was found between the two groups. Although better motor function values were detected in the evaluation in the 3rd and 4th month in the autograft group compared with the radiotherapy and liquid nitrogen group, no statistical difference was detected between three groups. This may be due to the need for longer periods for the return of the motor functions. Therefore, studies with a longer follow-up period are required particularly for the evaluation of the motor function.

The limitations of this study may be listed as follows: lack of the electron microscopic assessment, lack of tumor-contaminated nerve tissue and lack of allograft model. Tumor-contaminated neural tissue was not used in the study because the oncological sterilization methods utilized (radiotherapy and liquid nitrogen) are oncologically reliable methods which are currently used in clinical practice. Also, an allograft model was not deemed necessary because previous studies have shown that autografts are superior to allografts. For example, in a longer follow-up experimental study, Strasberg et al. have compared the fresh and cold-preserved autografts and allografts each other at 6 and 10 months. They have found that autografts had significant superiority over allografts, and cold nerve preservation did not enhanced the regeneration [39].

This study demonstrated that nerve regeneration to some degree occurs in autografts treated with radiotherapy or liquid nitrogen. Based on this finding, we propose that nerve autografts treated with oncological sterilization methods can be used for nerve reconstruction in limb salvage surgery. As a result, more comprehensive studies are needed to clarify the applicability and increase the success of these methods.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Authors' contributions

H.K. planned the study, performed all animal experiments and collected the data. H.K. and O.E. wrote the article. O.E. performed motor test and electrophysiological studies. F.O. and G.Y. performed histological evaluations. D.S., B.K. and L.K. helped in general supervision, concept and designing of study. D.T helped in analyzing and interpreting the data and critically revised the manuscript. All authors finally approved the version to be published.

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