Original article

Viability of nerve grafts preserved in different storage medium: ultrastructural features

Scabora, JE.[1], Carone, AL.[2], Figueiredo, JF.[3], Esquisatto, MAM.[1]* and Langone, F.[4]

[1]Centro Universitário Hermínio Ometto – UNIARARAS,
Av. Dr. Maximiliano Baruto, 500 Jd. Universitário, CEP 13607-339, Araras, SP, Brasil

[2]Departamento de Neurocirurgia, Faculdade de Medicina,
Universidade do Vale do Sapucaí – UNIVAS, Pouso Alegre, MG, Brazil

[3]Núcleo de Medicina e Cirurgia Experimental, Faculdade de Ciências Médicas,
Universidade Estadual de Campinas – UNICAMP, São Paulo, Brazil

[4]Departamento de Fisiologia e Biofísica, Instituto de Biologia,
Universidade Estadual de Campinas – UNICAMP, São Paulo, Brazil

*E-mail: marcelosquisatto@uniararas.br

Abstract

We investigated the ultrastructural organization of transplanted autologous grafts after storage in two different solutions. Male Wistar rats were divided into groups to obtain normal tibial nerves, freshly transplanted nerves, and nerves stored in Wisconsin/Belzer or Collins solution for 24 or 72 hours at 4 °C and transplanted (W1, W3, C1, C3). After storage or transplantation, the specimens were processed for ultrastructural analysis. All grafts showed alterations in collagen fiber organization in the endoneurial space compared to normal nerves. These fibers were more loosely organized among nerve fibers, a finding that was significantly more marked in group C3 compared to groups W1 and W3. Important alterations were also observed in the myelin sheath structure of grafts stored in the two media. These changes were characterized by separation of the lipid lamellae, clearly visible in larger diameter nerve fibers. These findings were more marked and frequent in the C1 and C3 groups compared to the W1 and W3 groups. Ultrastructural analysis showed better preservation of Schwann cells and other elements that support axonal regeneration for grafts stored in Wisconsin/Belzer solution. These results support ongoing studies for the formulation of storage solutions that permit the creation of nerve banks for heterologous transplantation.

Keywords: peripheral nerve, regeneration, graft, ultrastructure.

1 Introduction

Autologous nerve grafts are currently the best choice for the reconstruction of peripheral nerve injuries. The use of these nerve grafts in combination with tension-free suture is the most widely employed method in surgical routine (EVANS, BRANDT, KATZ et al., 2002) However, the availability of donor nerves is limited in these cases, and sensitivity loss is observed in the donor regions (De MEDINACELLI and SEABER, 1989) In an attempt to overcome this problem, various techniques have been tested to replace autologous grafts. Among these technologies are synthetic or biological conduits, arterial and venous grafts, and acellular muscle grafts which are used to ligate the nerve stumps (CHEN, SEABER, URBANIAK et al., 1994; ATCHABAHIAN, MACKINNON and HUNTER, 1999; FANSA, LASSNER, KOOK et al., 2000; SUZUKI, SUZUKI, TANIHARA et al., 2000; KARACAOGLU, YUKSEL, PEKER et al., 2001; KREKOSKI, NEUBAUER, ZUO et al., 2001; EVANS, BRANDT, KATZ et al., 2002). However, these alternatives are inefficient in promoting an adequate environment for functional repair of peripheral nerves.

An alternative method is to use the allograft. However, one disadvantage of this type of transplant is the fact that it can cause rejection which is only controlled by the use of immunosuppressive agents. Nevertheless, allografts are considered to be the another choice for use in humans. Thus, many efforts are made to reduce the immunogenicity of these grafts and, at the same time, to preserve the viability of Schwann cells.

The principle of graft preservation is based on hypothermia which reduces the metabolic rate and activity of enzymes and prevents the accumulation of toxic products resulting from anaerobic metabolism. The deleterious effects of ischemia such as metabolic acidosis and edema are also minimized by low temperatures. In the case of peripheral nerves, the pre-condition for regeneration basically consists of the preservation of the basement membrane as an axonal conduit and the viability of Schwann cells. In this case, hypothermia should be combined with the use of substances that preserve these elements and, consequently, increase the storage period of grafts and maintain their viability (HARE, EVANS and MACKINNON, 1993; EVANS, MACKINNON, BEST et al., 1995; EVANS, MACKINNON, LEVI et al., 1998; FANSA, KEILHOFF, PLOGMEIER et al., 1999; EVANS, BRANDT, KATZ et al., 2002).

In this respect, an important step has been made with the development of solutions that, combined with hypothermia, preserve the viability of grafts after adequate storage periods and, in the case of allografts, can eliminate or significantly minimize the antigenicity of these grafts. In fact, investigations regarding the action of preservation media that permit the creation of nerve graft banks have been intensified over the last years (LEVI, EVANS, MACKINNON et al., 1994; EVANS, MACKINNON, BEST et al., 1995; EVANS, MACKINNON, LEVI et al., 1998; ATCHABAHIAN, MACKINNON, HUNTER, 1999). However, little is still known about the effects of these media on the nerve elements structure that can modify the regenerative capability during different times of stock.

In the present study, we investigated the ultrastructural characteristics of the nerve components in the grafts after 50 days of autologous transplantation and pretreatment with two types of storage solutions for two different periods of time.

2 Material and methods

Male Wistar rats weighing 300 to 350 g obtained from the Animal House of UNICAMP were used. Before each surgical procedure, the animals were anesthetized with sodium pentobarbital (50 $mg.kg^{-1}$ body weight; Hypnol, Laboratórios Cristália). All experimental procedures were approved by the Ethics Committee on Animal Experimentation of the Institute of Biology, UNICAMP.

Wisconsin/Belzer solution was purchased from Du Pont Pharma (Amsterdam, The Netherlands) and had the following composition: 25 mM KH_2PO_4, 5 mM $MgSO_4$, 100 mM potassium lactobionate, 5 mM adenosine, 3 mM total glutathione, 30 mM raffinose, 1 mM allopurinol, and 50 $g.L^{-1}$ hydroxyethyl starch, pH 7.4 (osmolarity: 320 $mOsmol.L^{-1}$). The activity of Wisconsin/Belzer solution is attributed to its components. Allopurinol inhibits intracellular xanthine oxidase and protects the cell against free oxygen radicals produced by ischemia of the organ or tissue during preservation. Similarly, potassium lactobionate also protects against free oxygen radicals produced by the cells. In addition to these component supplies the cell with potassium and raffinose, support an osmotic action and reducing tissue edema. Another component of Wisconsin/Belzer solution that guarantees cell viability is glutathione. This substance participates in many intracellular reactions, including those involved in the metabolism of hydrogen peroxide and lipoperoxides.

Collins solution was purchased from B. Braun S/A (Rio de Janeiro, Brazil) and had the following composition: 15 mM KCl, 10 mM $NaHCO_3$, 28 mM $MgSO_4$, 15 mM KH_2PO_4, 42 mM K_2HPO_4, and 139 mM glucose, pH 7.0 (osmolarity: 317 $mOsmol.L^{-1}$).

The ionic components are the same founded in Wisconsin/Belzer solution. Among these components, magnesium ion acts as an inhibitor of Na+-K+ ATPase and protects the cell membrane of organs stored for transplantation. The presence of such ion in these solutions may have effectively contributed to preservation and viability of cells in the transplanted organs.

For analysis of the ultrastructural characteristics of the nerve grafts after preservation, a group of 12 animals had their left and right tibial nerves dissected and a 15 mm segment was removed and stored in Wisconsin/Belzer or Collins solution for 24 or 72 hours at 4 °C (groups W1, W3, C1 and C3, respectively).

For observation of the grafts after preservation and autologous transplantation, a group of 12 animals had their left tibial nerve removed and stored in Wisconsin/Belzer or Collins solution for 24 or 72 hours (groups W1-T, W3-T, C1-T and C3-T, respectively). After these periods, the nerves were transplanted into the right side of the same animals. After 50 days, a segment of the nerve containing the graft and portions of its ends was removed from the animal and treated as described below.

Two other groups of animals were used as control. In three animals, a 15 mm segment of the left tibial nerve was removed and immediately transplanted into the right side (NF group). Fifty days after surgery, the grafts were harvested, processed and analyzed as described below. Three animals of the same age had their right and left tibial nerves removed and immediately treated as described below (NN group – normal).

The segments obtained after storage and transplantation were fixed in Karnovsky solution, post-fixed with 2% osmium tetroxide and processed for embedding in Araldite F resin (CY-205, Uegama). For ultrastructural analysis, ultrathin sections (0.1 μm) were cut with an LKB ultramicrotome (Bromma 8800), counterstained with uranyl acetate and lead citrate (REYNOLDS, 1963), and observed under an electron microscope (LEO 906, Jeol).

3 Results

Grafts stored in the two solutions (C1, C3, W1 and W3) presented similar ultrastructural characteristics, which became more marked after 72 hours of storage, with all grafts showing alterations in collagen fiber organization in the endoneurial space when compared to normal nerves (NN group). These fibers were found to be more loosely organized among nerve fibers, with this alteration being significantly more marked in the C3 group than in the W1 and W3 groups. On the other hand, no signs of collagen fiber disintegration were observed in any of the specimens stored in Collins or Wisconsin solution for 24 or 72 hours (Figure 1 a-e).

Important alterations were also observed in the myelin sheath structure of grafts stored in the two media. These changes were characterized by the separation of the lipid lamellae, a finding clearly visible in larger diameter nerve fibers. These findings were more marked and frequent in groups C1 and C3 than in groups W1 and W3.

The alterations in the myelin sheath were accompanied by changes in axonal structure. These axons were found to be retracted and exhibited fragmentation of their cytoskeleton demonstrated by the agglutination of neurofilaments which appeared to form isolated bundles when observed in cross-sections. These axonal changes were the more intense, the greater the derangement of the myelin sheath lamellae. On the other hand, no structural changes were observed for amyelinated axons in any of the groups studied. Even larger-caliber axons had a morphology similar to that of axons of normal nerves. Interestingly, no structural alterations were observed in the basal lamina around myelinated and

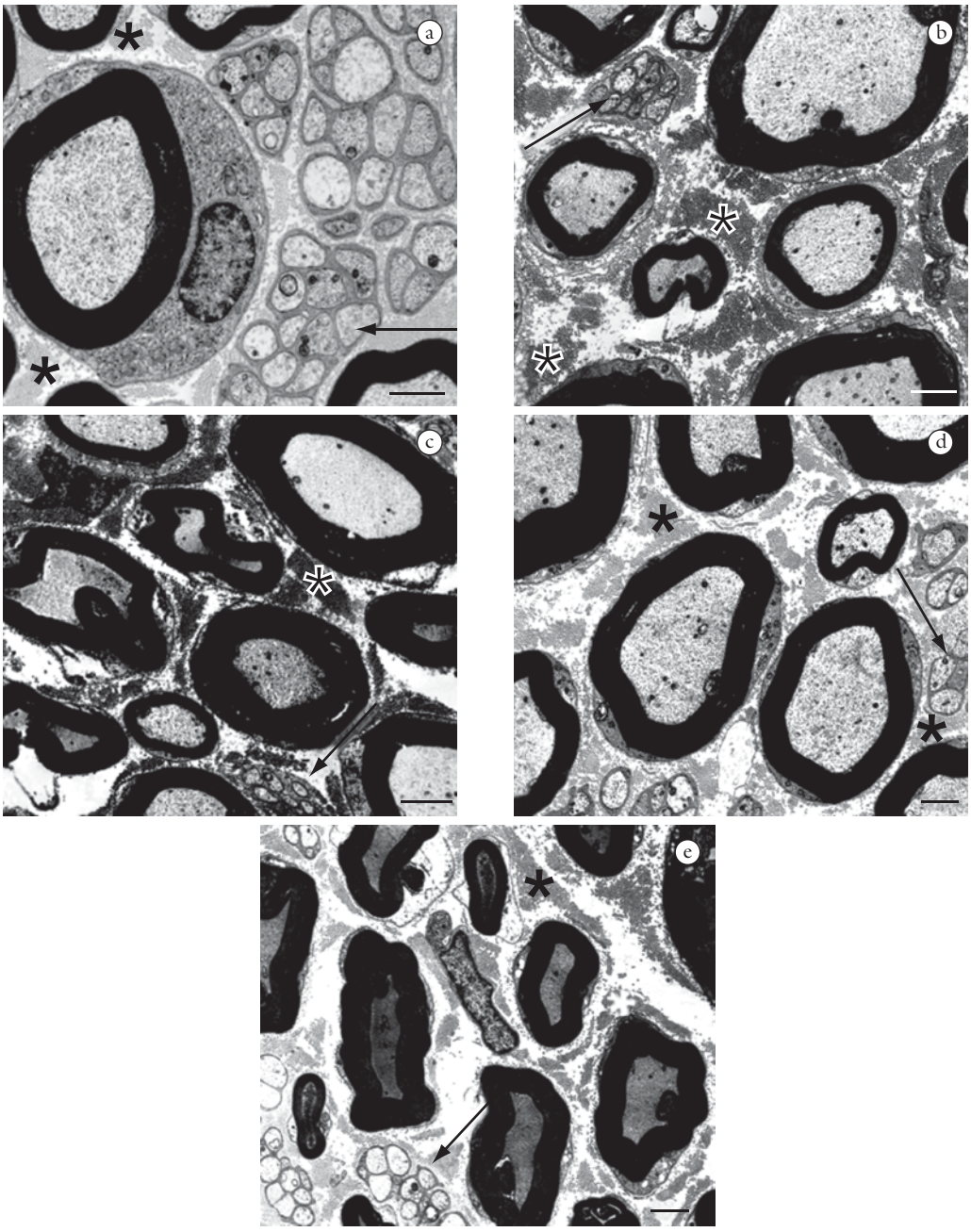

Figure 1. a) Electron photomicrographs of cross-sections cut along the major axis of normal nerves; b) nerve grafts stored in Collins solution for 24 hours; c) 72 hours; d) in Wisconsin/Belzer solution for 24 hours; and e) 72 hours. Note the similar ultrastructural alterations of grafts stored in the two solutions which were more marked after 72 hours of storage, with all grafts showing changes in collagen fiber organization in the endoneurial space when compared to normal nerves. These fibers were more loosely organized among nerve fibers, with this finding being significantly more marked in the C3 group compared to the W1 and W3 groups. There were no signs of collagen fiber disintegration in any of the preserved specimens. Bar = 1 μm. (*) = collagen fibrils. Arrow = myelinated axons.

amyelinated fibers for either treatment or period of storage, i.e., there were no signs of interruption of the basal lamina even around myelinated fibers whose lamellae were highly deranged. On the other hand, the structural integrity of Schwann cells was preserved in grafts stored for 24 hours in the two media. However, cells exhibiting ultrastructural alterations such as cytoplasmic vacuolization and edema were observed after 72 hours of storage. This phenomenon was more frequent in the C3 group than in the W3 group (Figure 2 a-f).

In the groups of grafts stored for 72 hours, the epineurial and perineurial connective tissue membranes showed no changes in their organization when compared to normal nerves (data not shown), suggesting that the association

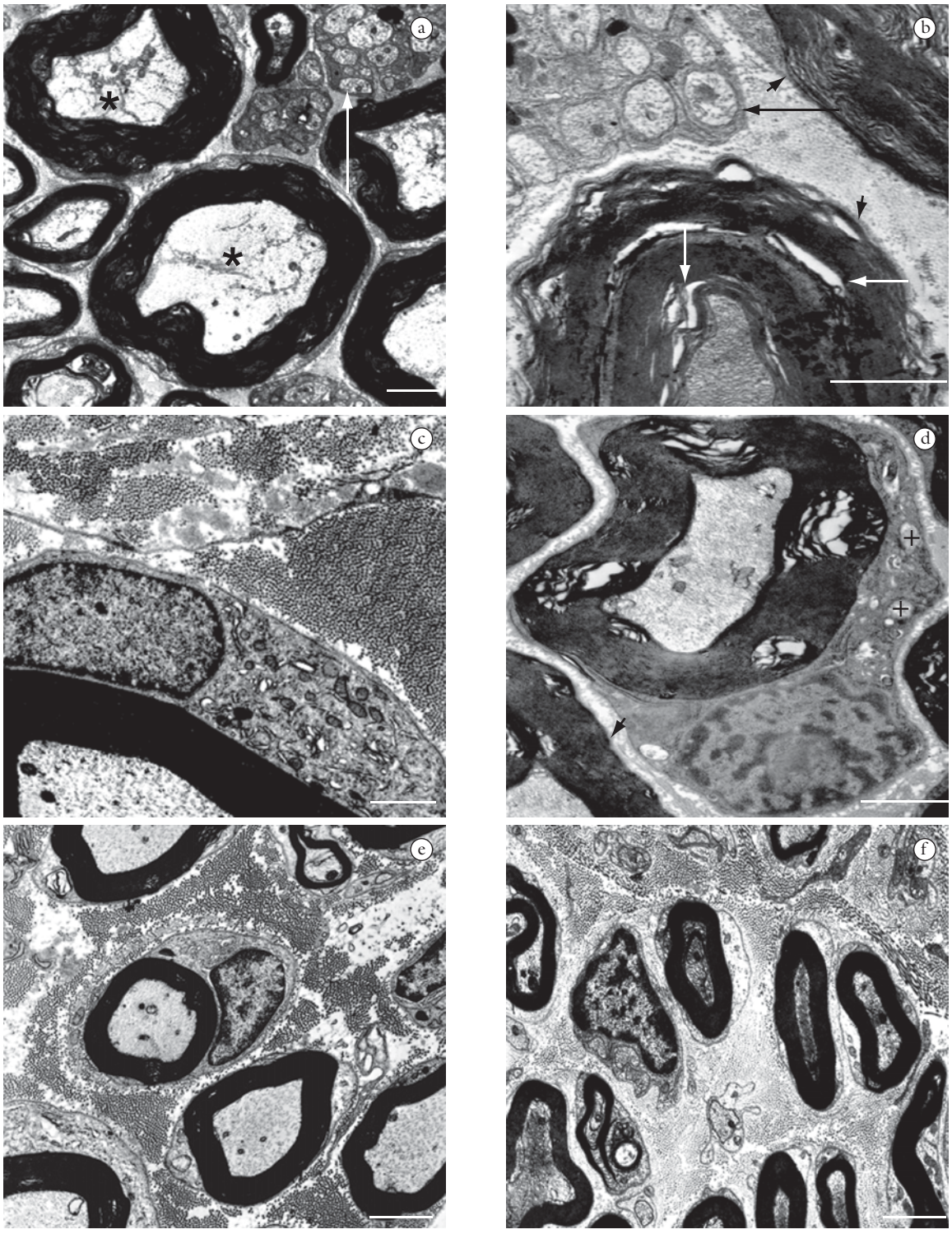

Figure 2. a, b) Electron photomicrographs of cross-sections cut along the major axis of nerves stored in Collins solution for 24 hours; c, d) 72 hours; e) in Wisconsin/Belzer solution for 24 hours; and f) 72 hours. Note the important alterations in the myelin sheath structure of grafts stored in the two media characterized by separation of the lipid lamellae, clearly visible in larger diameter nerve fibers (arrow). These findings were more marked and frequent in groups C1 and C3 compared to groups W1 and W3. Changes in the myelin sheath were accompanied by alterations in axonal structure. These axons were retracted and exhibited fragmentation of their cytoskeleton demonstrated by agglutination of neurofilaments (*) which appeared to form isolated bundles. Note the lack of changes in the structure of amyelinated axons in all groups studied (thin arrow). Observe also the lack of structural alterations in the basal lamina (arrowhead) around myelinated and amyelinated fibers for the two treatments and storage periods, and the structural integrity of Schwann cells after 24 hours of storage in the two media. On the other hand, note the presence of cells exhibiting ultrastructural alterations such as cytoplasmic vacuolization and edema (+) after 72 hours of storage. Bar = 1 μm.

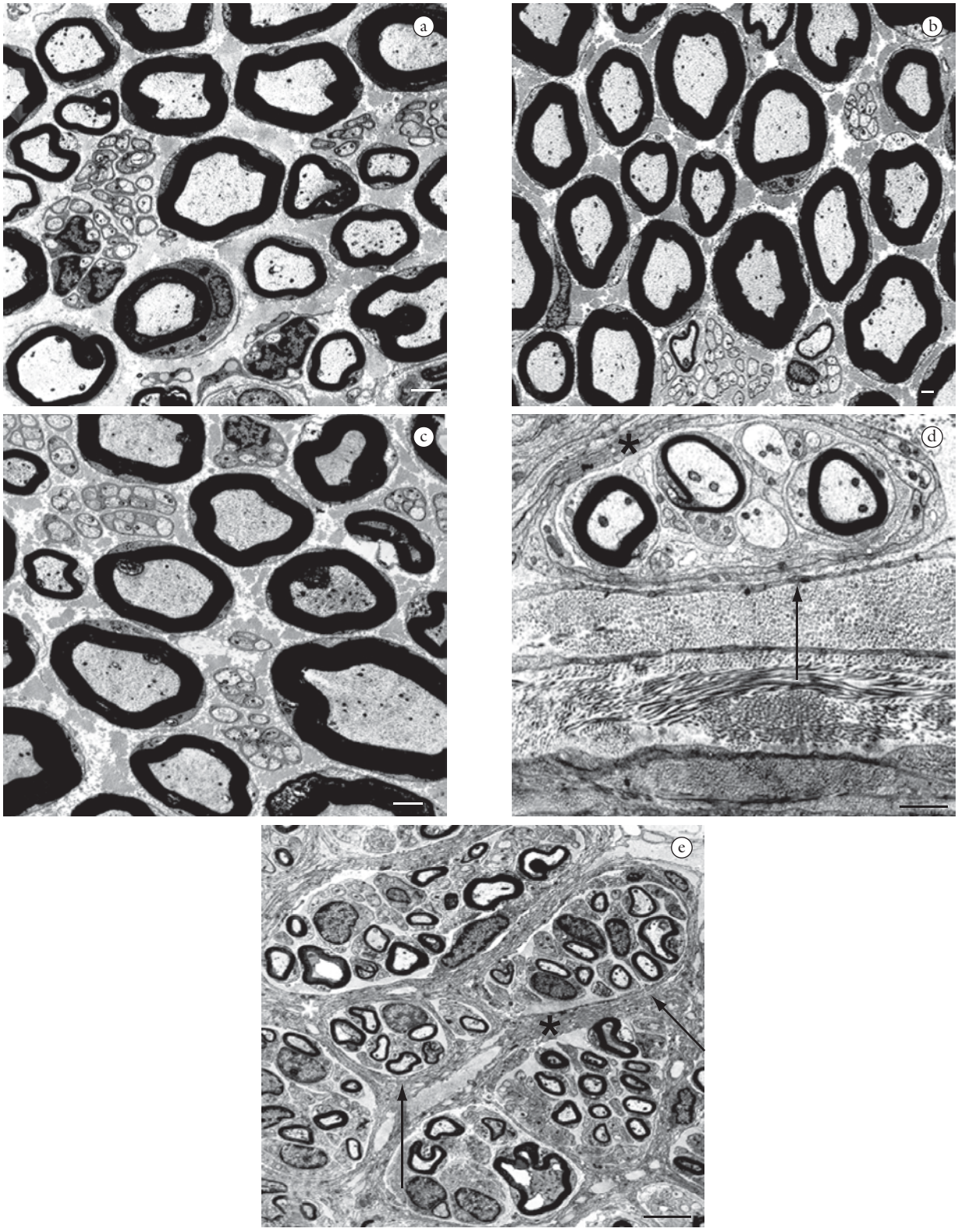

Figure 3. a) Electron photomicrographs of cross-sections cut along the major axis of freshly transplanted nerves; b) nerves transplanted after storage in Wisconsin/Belzer solution for 24 hours; c) 72 hours; d) in Collins solution for 24 hours; and e) 72 hours. Observe the apparently larger number of myelinated fibers and a similar tissue organization pattern in groups W1-T and W3-T compared to the NF group. Note also the presence of numerous minifascicles (arrow) in the C1-T and C3-T groups. At the same time the number of collagen fibrils (*) in the endoneurium was increased in these groups. Bar = 1 μm.

between these cells was preserved during the period of storage.

Fifty days after transplantation (Figure 3 a-e), ultrastructural analysis showed an apparently larger number of myelinated fibers and a similar tissue organization pattern in the W1-T and W3-T groups compared to the NF group. However, the density of myelinated fibers seemed to be smaller than that observed for the NN group. In addition, minifascicles were observed in all groups but were more frequent in the C1-T and C3-T groups. At the same time, an increase in the number of collagen fibers in the endoneurium was noted in these groups. The ultrastructural organization of the epineurium and perineurium was similar in grafts preserved in the storage solutions and fresh nerve grafts (NF group, data not shown).

4 Conclusion

In the present study, we evaluated the ultrastructural aspects of regeneration of peripheral nerve grafts after pretreatment with Collins and Wisconsin/Belzer solution for 24 and 72 hours under conditions of ischemia and hypothermia. After the storage period, Schwann cell morphology, connective sheaths organization and axonal regeneration were evaluated 50 days after transplantation and compared to a control (NF) group and to normal nerves (NN group).

The present results agree with the our previous findings (CARONE, SCABORA, BARROS et al., 2007). We showed that preservation of rat tibial nerve segments in Wisconsin/Belzer and Collins solution at 4 °C for 24 or 72 hours preserves the viability of transplanted nerves. However, the nerve grafts preserved in Wisconsin/Belzer solution were more efficient in the axonal regeneration.

The efficacy of nerve graft regeneration is related to the structural and functional preservation of the extracellular matrix molecules and Schwann cells, and the quality of the microenvironment that interacts with these cells (CHEN, HSIEH, TSAI et al., 2000). Schwann cells are the main factor responsible for the maintenance of an adequate trophic environment for axonal regeneration (TRUMBLE and PARVIN, 1994). The preservation of these cells depends on the integrity of their basement membrane components and of the cell receptors that interact with this membrane (TERENGHI, 1995; EVANS, MACKINNON, LEVI et al., 1998).

Observation of the ultrastructural characteristics of grafts 50 days after transplantation revealed the presence of an expressive number of collagen fibers in the endoneurium of C1-T and C3-T grafts, whereas in the W1-T and W3-T groups this number was similar to that observed for the NF group. These findings, together with the fact that minifascicles were more abundant and smaller in C1-T and C3-T grafts compared to groups W1-T and W3-T, led us to infer that the number of functionally preserved Schwann cells was larger in the latter groups. In addition, fibroblasts and perineurial cells predominated in C1-T and C3-T grafts. Our results agree with those reported by Strasberg and co-works (1996) who observed little collagen fiber deposition in connective tissue sheaths of nerve allografts stored in Wisconsin solution for 7 days before transplantation in a sheep model. Furthermore, our data are also supported by the findings of Carone and co-works (2007) who employed the same experimental model as that used in the present study. We found, in that study, a significantly larger number of regenerated myelinated axons 50 days after transplantation in grafts stored in Wisconsin/Belzer solution compared to those preserved in Collins solution.

In this respect, our data suggest that Collins solution is unable to ensure the viability of Schwann cell populations to permit adequate regeneration of nerve stumps close to the preserved graft. Another side, Wisconsin/Belzer solution contributes to the functional preservation of these cells, thus improving axonal regeneration. The efficiency of Wisconsin/Belzer solution, in this case, is probably associated with its composition. Its components have a strong antioxidants effect and can protect Schwann and others cells, present in the peripheral nerve, against the action of free radicals produced during organ ischemia and reduce tissue edema (TOLEDO-PEREYRA, 1977; KOYAMA, 1985; SOUTHARD, MARSH, McANULTY et al., 1987; JANIESON, LINDELL, SOUTHARD et al., 1989; AMETANI, SOUTHARD, BELZER, 1990; KERR-CONTE, BOUDJEMA, SOUTHARD et al., 1991; SUMITOMO, DOHI, URISHIRAHA et al., 1992a; SUMITOMO, LINDELL, SOUTHARD et al., 1992b; VREUGDENHIL, EVANS, BELZER et al., 1992; EVANS, MACKINNON, BEST et al., 1995; STRASBERG, MACKINNON, GENDEN et al., 1996; FIGUEIREDO, 1997; EVANS, MACKINNON, LEVI et al., 1998; HADLOCK, SUNDBACK, HUNTER et al., 2001). Several investigators have demonstrated that ischemia produced by the procedures of cold organ storage depletes cellular antioxidants, a fact that renders the cells more susceptible to the action of free oxygen radicals (AMETANI, SOUTHARD and BELZER, 1990; KERR-CONTE, BOUDJEMA, SOUTHARD et al., 1991; VREUGDENHIL, EVANS, BELZER et al., 1992). We believe that the components of Wisconsin/Belzer solution, associated to cold storage, must increases the survival of the Schwann cell population during the preservation period and, consequently, contributed to a more favorable outcome of nerve regeneration.

We thus confirm, using ultrastructural investigation, the best of Schwann cells preservation seems to be the basic principle of preservation of peripheral nerve grafts. In this respect, storage of nerve segments in Collins solution for the periods proposed here results in significant damage to the Schwann cell population and Wisconsin/Belzer solution can therefore be considered to be a more efficient storage medium for the preservation of nerve grafts, as reported by several investigators (EVANS, MACKINNON, BEST et al., 1995; ATCHABAHIAN, GENDER, MACKINNON et al., 1998; EVANS, MACKINNON, LEVI et al., 1998; EVANS, BRANDT, KATZ et al., 2002; CARONE, SCABORA, BARROS et al., 2007).

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