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The functional and structural effects of hypothermic storage on ischaemic arterial grafts

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SUMMARY. **The effects of hypothermic ischaemia on blood vessels are unknown. This study aimed to determine the 3 week patency rate and the pathology of 9 experimental groups of hypothermically stored ischaemic arteries and one control group in a rabbit femoral artery model. Ischaemia times were 0 h, 24 h, 1, 2, 4, 6, 8 and 10 weeks (Groups 1–8). Patency was over 80% in all groups after 3 weeks reinsertion. Following reinsertion control grafts maintained normal arterial structure, but cellular degeneration had occurred in all ischaemic grafts and appeared complete after 4 weeks ischaemia. The graft connective tissue framework frequently remained intact. Repair was evident in central graft regions after 2 weeks ischaemia and 3 weeks reinsertion, but occurred only adjacent to the anastomosis in 4–10 week ischaemic arteries. Four week ischaemic arteries (Groups 9 and 10) reinserted for 6 and 12 weeks respectively exhibited near complete repair but patency dropped to 60% in the 12 week group.**

A large number of prosthetic microvascular graft materials have been investigated including polytetrafluoroethylene (PTFE),[1–3] glutaraldehyde tanned vessels,[4–6] bioabsorbable polyurethane grafts,[7,8] and freeze dried vessels.[9–11] None has consistently produced a patency rate to challenge the use of autogenous grafts. However, a readily available microevascular prosthesis would eliminate the harvesting of autogenous grafts, thereby significantly reducing operating time, costs and donor site scarring. This study has investigated the properties of another microvascular prosthesis – hypothermically stored microvascular arteries.

The specific pathological effects of ischaemia on blood vessels are unknown. In replantation surgery it is recognised that amputated parts deteriorate significantly after 6–10 h normothermic ischaemia.[12] However, hypothermic storage during ischaemia (at approximately 4°C) can both increase the chances of successful replantation and lengthen the ischaemia time after which a successful replantation can be achieved.[13]

Although considerable clinical evidence exists to support the effectiveness of using hypothermia as a deterrent to ischaemic necrosis in replantation surgery, there is no documentation of underlying histopathological changes in blood vessels subjected to prolonged cold ischaemia. In addition, the limits of the protective effects of hypothermia for subsequent successful clinical usage are unknown.

Therefore the aims of this study, using the rabbit femoral artery as a model, were to:

(1) determine the functional capabilities, *i.e.* the patency of arterial grafts reinserted into the donor animal after various periods of hypothermic ischaemia, and

(2) ascertain if microscopic structural degeneration occurs in arterial walls subject to varying periods of hypothermic ischaemia, and determine if any repair occurs after reinsertion.

Materials and methods

Experimental model for cold ischaemia

All groups except control Group 1 had 3 operations. For each operation general anaesthesia was induced in the rabbit with intravenous pentobarbital and maintained with nitrous oxide and fluorothane. In the first operation an approximately 3.5–4.0 cm graft was removed from the left femoral artery between, but not including, the epigastric artery and bifurcation of the femoral artery. The rabbit was resuscitated after wound closure. Harvested grafts were wrapped in gauze sponge moistened with saline, placed in a sterile container and stored at 4°C for the duration of a selected ischaemic period. At the end of the ischaemic period, the grafts were brought to body temperature. The donor rabbits were re-anaesthetised for a second operation and the ischaemic grafts gently perfused with heparinised saline before being anastomosed into a 1.8–2.0 cm long defect created in the right femoral artery just distal to the epigastric branch. (After hypothermic storage the graft shrinks to approximately 2.0 cm in length). After anastomosis, the grafts were examined for blood flow and patency. Skin wounds were again closed and the rabbits were resuscitated. In the majority of groups the third operation was performed 3 weeks later (see Exploration).

Experimental groups

One hundred outbred white rabbits were used. Sixty rabbits were allocated into three main groups con-

Table 3-week patency rates of hypothermically stored ischaemic arterial grafts

Period of hypothermic ischaemia	*Group 1 0 h (n = 20)**	*Group 2 24 h (n = 20)*	*Group 3 1 week (n = 20)*	*Group 4 2 weeks (n = 6)*	*Group 5 4 weeks (n = 6)*	*Group 6 6 weeks (n = 6)*	*Group 7 8 weeks (n = 6)*	*Group 8 10 weeks (n = 6)*
Patency rate at 3 weeks	95%	100%	85%	100%	100%	83%	83%	100%

* n = number of grafts in each group.

taining 20 rabbits in each. Group 1 rabbits were used as a control group to represent 0 h ischaemia: in this group the left femoral arterial graft was dissected out and anastomosed immediately to the defect created in the right femoral artery (therefore this was the only group that had 2 operations). In Groups 2 and 3 the left femoral arterial grafts were subjected to 24 h and 1 week cold ischaemia respectively, prior to reinsertion.

After the completion of Groups 1–3, 30 additional rabbits were allocated into 5 smaller groups (Groups 4–8, n = 6). These arterial grafts were maintained at 4°C for ischaemic periods of 2, 4, 6, 8 and 10 weeks, respectively. It was expected that these groups would demonstrate the limiting period of hypothermic storage where subsequent patency fell significantly.

Two additional groups were established to ascertain if longer reinsertion times affect patency rates and pathological appearance. Ten arterial grafts were stored at 4°C (as described above) for 4 weeks. Five grafts (Group 9) were reinserted for 6 weeks, and a further 5 grafts (Group 10) reinserted for 12 weeks prior to patency and pathological assessment.

Exploration

In a third operation (except for Group 1 where exploration was the second operation), patency was assessed by clearing of tissue overlying the graft and the distal recipient artery and observing the colour, diameter and pulsations of the graft and distal artery wall. If patency was in doubt a "milking" patency test was done in the distal recipient artery. Non-patent grafts were removed and immersion fixed in Karnovsky's fixative.[14]

Patent vessels were prepared for perfusion fixation by placing a distally directed catheter in the abdominal aorta. The left iliac vessels were ligated and a small incision placed in the vena cava to provide an outflow for the perfusate. The right iliac and femoral systems were then perfused with heparinised Hanks balanced salt solution to remove any blood. The rabbit was then killed with an overdose of sodium pentobarbitone and the perfusion continued with Karnovsky's fixative for 10 min. The graft was then dissected out together with short segments of proximal and distal recipient arteries and placed in fixative for a minimum of 24 h.

Tissue processing

For light and electron microscopy, segments 2–3 mm long were cut from the mid-section of the graft and from the proximal and distal anastomotic sites. The specimens were processed routinely for Epon/Araldite embedding. Plastic sections 1–2 μm thick were cut longitudinally from the resulting tissue blocks, stained with methylene blue and viewed and photographed under an Olympus BH_2 microscope. Thin sections 80 nm thick were cut from the same blocks, stained with uranyl acetate and lead citrate and viewed and photographed on a Philips CM12 Transmission Electron Microscope.

Statistics

Significant differences in patency rates between groups were determined using Fisher's Exact Text.[15]

Results

Patency

There were no significant differences in patency rates at 3 weeks between groups 1–8. High patency rates were achieved after up to 10 weeks hypothermic ischaemia (see Table).

Group 9 (4 weeks ischaemia, 6 weeks reinsertion) also maintained a high patency (100%), but in Group 10 (4 weeks ischaemia, 12 weeks reinsertion) patency dropped to 60%. Because of the small numbers in each group this was not a statistically significant decrease in patency.

Light and electron microscopy of ischaemic arterial grafts, 3 weeks post reinsertion (patent grafts)

Control grafts (Group 1) exhibited a normal arterial structure 3 weeks later (Fig. 1). Grafts ischaemic for 24 h to 2 weeks and reinserted for 3 weeks (Groups 2–4) contained both relatively normal and damaged areas. Damage increased progressively with ischaemia time and took the form of either tissue debris with scattered dense pyknotic nuclei, or, vacant areas devoid of SMC, but with the basement membrane and thin tract of connective tissue that previously surrounded the SMC persisting (Figs 2, 3). White blood cells were occasionally seen. Endothelium and neointima were absent, but the internal elastic lamina (IEL) was largely intact and occasionally small thrombi were attached to it. There was no evidence of aneurysm formation in any graft.

Areas of regeneration of the media and luminal surface occurred in midgraft and anastomotic regions of Groups 2, 3 and 4 (Fig. 4). Two cell types were represented: (1) synthetic state SMC which contained a few myofilaments confined to a narrow band adjacent to the plasma lemma. The cytoplasm was packed with organelles associated with protein synthesis, *i.e.* granular endoplasmic reticulum, free ribo-

Fig. 1

Fig. 2

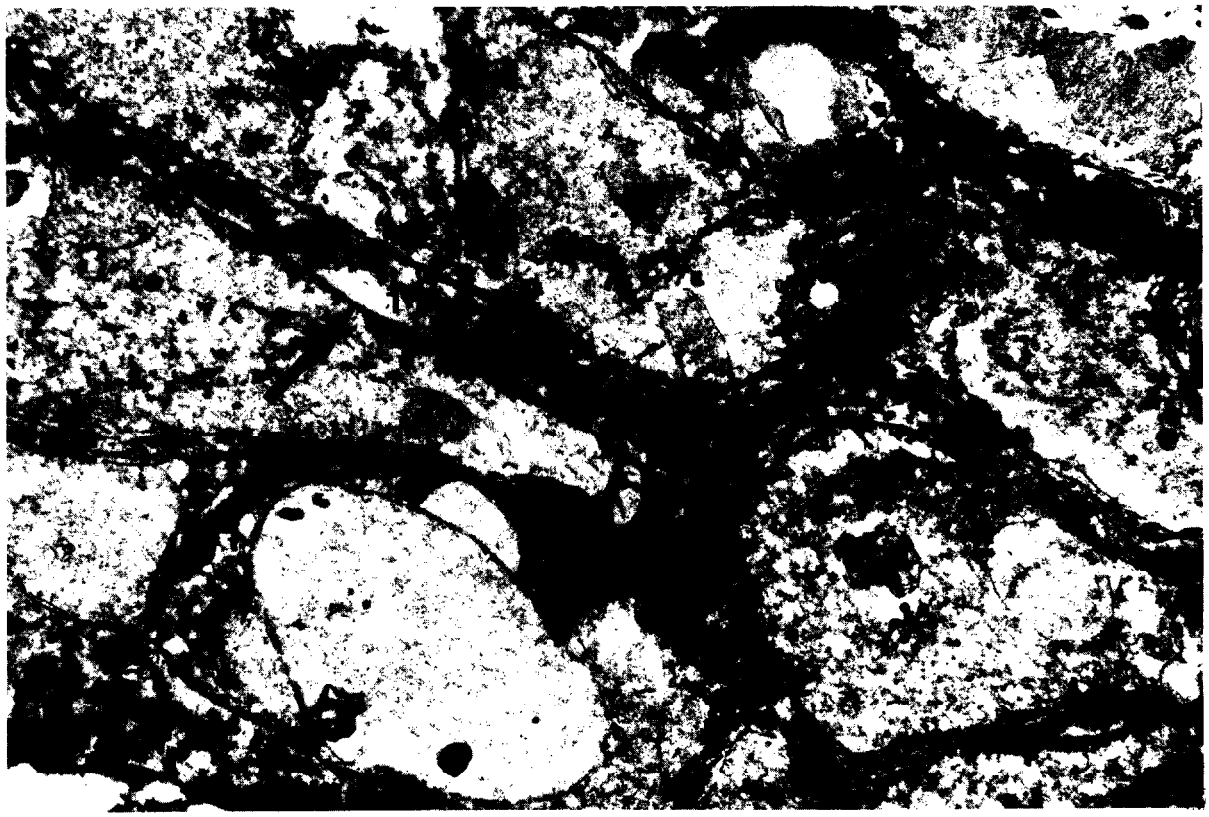

Fig. 3

Figure 1—Light micrograph of control graft (0 h ischaemia) 3 weeks after reinsertion. Arrow: endothelium and thin neointima. 1: media, 2: adventitia. 1 μm thick methylene blue stained, Epon/Araldite section. Scale bar = 10 μm. **Figure 2**—Light micrograph of a degenerated area in a graft ischaemic for 1 week and reinserted for 3 weeks. Most of the cellular components of the graft are dead (no endothelium or SMC) whilst the connective tissue components remain largely intact. Arrow: vacant space which SMC previously occupied in the media. Double arrow: IEL. 1: adventitia. 1 μm thick, methylene blue stained, Epon/Araldite section. Scale bar = 10 μm. **Figure 3**—Transmission electron micrograph of the media of a 2 week ischaemic graft, reinserted for 3 weeks. This area is devoid of SMC. The spaces they previously occupied are evident (*) as too is the basement membrane (arrow) and connective tissue (1) which surrounded the cell. Scale bar = 1 μm.

somes and Golgi apparatus (Fig. 5). Basement membrane around these cells was observed variably. (2) Fibroblasts also had a very active appearance with much granular endoplasmic reticulum, but no myofilaments or basement membrane. Their distinctive nuclei were of a smooth oval shape without chromatin clumping (Fig. 6). Between the cells were tracts of connective tissue. Above the repaired media was an IEL covered by neointima containing longitudinally oriented synthetic state SMC and covered luminally by endothelium.

After 4–10 weeks hypothermic ischaemia and 3 weeks reinsertion the graft wall was very thin and complete cell degeneration had occurred. Occasional fibroblasts and white blood cells were present in the media. The connective tissue framework of the graft,

Fig. 4

Fig. 5

Fig. 6

Figure 4—Light micrograph of an area of regeneration in a graft ischaemic for 2 weeks and reinserted for 3 weeks. The media (1) is quite cellular. Above the media is an IEL (arrow) and neointima (2). 1 μm thick methylene blue stained, Epon/Araldite plastic section. Scale bar = 10 μm. **Figure 5**—Transmission electron micrograph of a synthetic state SMC in a regenerating area (2 weeks ischaemia, 3 weeks reinsertion). A band of myofilaments (1) is evident adjacent to the plasma lemma. The bulk of the cytoplasm is filled with granular endoplasmic reticulum (arrow), Golgi apparatus (2), mitochondria (3) and ribosomes (double arrow). Scale bar = 1 μm. **Figure 6**—Transmission electron micrograph of fibroblast cells (1) in the media of a graft ischaemic for 1 week and reinserted for 3 weeks. The spaces previously occupied by SMC are evident (*). The fibroblast cells contain granular endoplasmic reticulum (arrow), Golgi apparatus (2), ribosomes (double arrow) and mitochondria (3). There are no myofilaments. Scale bar = 1 μm.

i.e. the IEL, and medial connective tissue was largely intact. Thrombus attachment to the IEL was observed.

Within 5 mm of the anastomosis these grafts demonstrated regeneration, having a neointima and thicker media which closely approximated normal arterial thickness and contained fibroblasts and synthetic state SMC.

Light and electron microscopy of 4 week ischaemic grafts reinserted for 6 or 12 weeks

Four week ischaemic grafts reinserted for 6 weeks (Group 9) displayed incomplete repair and patches of degenerated media persisted. At 12 weeks (Group 10) the media was completely replaced by connective

Fig. 7

Figure 7—Light micrograph of a graft ischaemic for 4 weeks and reinserted for 12 weeks. The degenerating media is absent and has been replaced by much connective tissue with synthetic state SMC and fibroblasts (arrow). The IEL remains (double arrow). A neointima (1) covers the luminal surface and some thrombus (2) attaches to its luminal surface. 1 μm thick methylene blue stained, Epon/Araldite section. Scale bar = 10 μm.

tissue, and synthetic state SMC and fibroblasts. A neointima of variable thickness covered the luminal surface throughout (Fig. 7). Thrombi were frequently attached to the neointima, which showed areas of degeneration underlying thrombi.

Nonpatent grafts

Nonpatent grafts were occluded by thrombi of variable organisation. Some thrombosed areas were well organised, being completely replaced by connective tissue and including small blood vessels. In the same grafts less well organised areas showed partial necrosis or stagnant blood. The walls of thrombosed grafts were depleted of SMC and contained numerous blood vessels and some blood cells and connective tissue. Occasionally the graft wall other than the adventitia was entirely absent.

Discussion

The high patency rates of hypothermically stored ischaemic arterial grafts at 3 weeks post insertion indicate that such vessels may be used clinically. It is envisaged that as it is common to harvest extra lengths of blood vessels to create vascular pedicles in replantation and free tissue transfer surgery, these unused segments should be immediately stored at 4°C. If a microvascular anastomosis fails after the initial operation the stored unused lengths of vessel could be used in a second operation to replace an occluded anastomosis. In this situation an artery would be stored for a maximum period of 3 weeks, as microvascular anastomoses are not known to fail after this period. This study has demonstrated that 3 weeks cold storage is well within the functional capabilities of ischaemic arterial grafts.

Hypothermically stored arteries undergo a continuous structural deterioration from 24 h ischaemia which is complete after 4 weeks ischaemia. Thereafter there is no obvious further deterioration; however it appears that blood flow through the 2 cm long conduit can occur despite the fact that there is no endothelium or pseudo-endothelium[16] covering the luminal surface. Platelet and microthrombus attachment to the still largely intact IEL did occur. This suggests that the long term patency of these grafts (*i.e.* at 6 months) may be considerably less than at 3 weeks.

No aneurysms or anastomotic aneurysms were detected in this study. The longest reinsertion period was 12 weeks, most grafts being reinserted for only 3 weeks. It is possible that aneurysms might become apparent only after many months of reinsertion. In clinical microsurgery aneurysm formation is not commonly reported, however most microsurgical procedures are performed on vessels in relatively low blood flow situations, where aneurysms are less likely to occur.

Within 3 weeks reinsertion there was evidence of repair in degenerated grafts. In grafts subjected to 2 weeks ischaemia or less, cells resembling synthetic state SMC and fibroblasts were found in areas of midgraft media, and the luminal surface in these regions was generally covered by neointima. Repair was much slower in grafts ischaemic for 4 or more weeks, which had apparently lost all their cells during

the ischaemic insult. At 3 weeks reinsertion some ingrowth had occurred around the anastomoses; complete repair was not evident until 12 weeks reinsertion.

The repopulation of the media with synthetic state SMC and fibroblasts should not imply that the media was of normal appearance. The synthetic state SMC and fibroblasts were widely scattered, with much connective tissue surrounding individual cells. This is unlike the closely packed, circularly oriented, contractile SMC in normal media.

The origin of cells involved in the repopulation of ischaemic arterial grafts is open to speculation. The cells labelled synthetic state SMC are presumably derived from modulated contractile state SMC[17] which either survived the ischaemic insult in grafts ischaemic for less than 2 weeks, or which migrated into the dead graft from the recipient artery. Evidence of the latter occurred in grafts ischaemic for 4–10 weeks, where regeneration at 3 weeks reinsertion was evident only adjacent to the anastomosis with the recipient artery.

The appearance of fibroblasts within the graft suggests that these cells are also involved in repopulating the dead graft. Their presence in injured media has not been reported previously, however they have been observed in the pores of synthetic PTFE grafts.[3,18] Fibroblasts in the media may originate from surviving cells in the graft adventitia or from connective tissue in the recipient bed.

It is also possible that some or all of the cells designated synthetic state SMC may in fact be myofibroblasts, which occur in granulation tissue and have been described as an intermediate form between SMC and fibroblasts.[19] Morphologically, synthetic state SMC and myofibroblasts appear very similar[19,20] and probably can only be identified by immunofluorescence techniques.[21]

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