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(54) Title: MEANS AND METHODS OF PRESERVATION OF GRAFTS DURING TRANSPORT

(57) Abstract: This invention provides a composition in the preservation of a medical graft during transport from the donor to the recipient. Also, methods of preserving medical grafts during transport are provided by the present invention.



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MEANS AND METHODS OF PRESERVATION OF GRAFTS DURING TRANSPORT

This invention provides a composition in the preservation of a medical graft during transport from the donor to the recipient. Also, methods of preserving medical grafts during transport are provided by the present invention.

A progressive increase in the prevalence of lifestyle diseases such as diabetes mellitus type 2 and essential arterial hypertension provides for the basis for multiple diseases. They often involve vascular injury and have systemic impact on the entire organism leading to structural and functional damage of various organs and tissues. The rising demand for graft transplantation is the result. For example, kidney transplantation is the only curative approach for chronic renal failure (Port et al., JAMA, 270 (1993), 1339-1343; Schnuelle et al., J Am Soc Nephrol, 9 (1998), 2135-2141).

After successful transplantation, loss of function due to immunological rejection of the transplanted organ exhibits one of the most critical complications. Over the past years, the optimization of immunosuppressive protocols considerably reduced the incidence of acute rejection after e.g. kidney transplantation. However, very little progress has been achieved regarding improvement of long-term transplant function and survival, respectively (Hariharan et al., N Engl J Med, 342 (1988-1996), 605-612). Chronic allograft dysfunction still represents the major cause for graft loss and the underlying multi-factorial immunological as well as non-immunological processes remain poorly understood.

Prolonged ischemia does not only lead to early tissue damage with reduced graft function, but also inhibits deteriorating long-term effects on transplanted kidneys by increasing their immunogenicity and predisposition to fibrosis (Basile, Curr Opin Nephrol, 13 (2004), 1-7). This is also true for any other kind of graft, e.g. lung

(Whitson, J Thorac Cardiovasc Surg., 131 (2006), 73-80.), heart (Faggian, Transplant Proc., 36 (2004), 617-9) and liver transplants (Adam, Lancet, 340 (1992), 1373-6). The impact of ischemia-reperfusion injury on organ donation was convincingly demonstrated by improved results for living donor transplants (Terasaki et al., N Engl J Med, 333 (1995), 333-336). Importantly, ischemia-reperfusion injury is already initiated in the brain-death donor and persists during cold preservation until a considerable period of time after transplantation. In fact, there is growing evidence for chronic deleterious effects of acute ischemia (Opelz et al., Transplantation, 83 (2007), 247-253).

HMG-CoA-reductase inhibitors (statins) are usually known to lower cholesterol levels in patients with or at risk of cardiovascular disease. This is mediated by inhibiting the HMG-CoA reductase, which is the rate-limiting enzyme of the mevalonate pathway of cholesterol synthesis. Inhibition of this enzyme in turn stimulates low-density lipoprotein (LDL) receptors, resulting in an increased clearance of LDL from the bloodstream and a decrease in blood cholesterol levels. Also, statins such as pravastatin are reported to lessen the severity of renal ischemia-reperfusion injury, probably by mediating the inhibition of the mevalonate-isoprenoid pathway independent of its lipid lowering action (Sharyo et al., Kidney Int, 74 (2008), 577-584). Also, statins are used for reduction of brain injury during cerebral ischemia by increasing blood flow (Endres et al., Proc Natl Acad Sci U S A, 95 (1998), 8880-8885). Another effect of statins is to reduce the area and myocardial no-reflow after ischemia and reperfusion. This effect is due to its activation of mitochondrial K(ATP) channels. Also, statins were found to be already active during ischemia by preventing apoptosis (Nydegger et al., Transplant Immun, 9 (2002), 215-225).

However, an unresolved issue is the provision of means and methods suitable to prevent a decrease of graft function caused during transport of the graft in order to avoid acute rejection and graft loss.

The present invention provides a composition comprising one or more statins for use in the preservation of a medical graft. Also, a method of preserving a medical graft comprising the placing and the transport of a graft in a container filled with a composition comprising one or more statins is provided in the present invention.

In one embodiment, the composition is fluid, preferably aqueous. Also, a method of preserving a medical graft is provided in the present invention. In accordance with the present invention, the preservation of the medical graft may be between the removal of the graft from the donor and the implantation of the graft into the recipient.

In accordance with the present invention, the method of preserving a medical graft may comprise the placing of the graft into a suitable container, and the transport of the graft in said container to the recipient, wherein said container is filled with a composition comprising one or more statins before, during and/or after the placing of the graft into said container. In accordance with the present invention, the composition is for use in the preservation of a medical graft. The transport of the graft in the container may further be spatially and timely immediate.

In accordance with the present invention, the container the graft is placed into may be cooled to a temperature lower than the ambient temperature while containing the graft. In one embodiment, the container is cooled to about 4° C. The container may also be closable. The container may be filled with the herein described composition comprising statins before, during and/or after the graft has been placed into the container.

In accordance with the present invention, the donor and/or the recipient of the graft may be mammal. In one embodiment, at least the recipient is human. The donor may be alive or deceased at the time the graft is removed. The graft may be an autograft, an allograft or a xenograft. In one embodiment, the graft is an allograft. The graft may be kidney, heart, lung, liver, pancreas, intestine, hand, foot, arm, leg, digit, toe, cornea, skin, penis, Islet of Langerhans, bone, free flaps, or parts thereof. In a preferred embodiment, the graft is a kidney. The graft may be placed into the container at any time before, during or after the container has been filled with the composition comprising statins. In one embodiment, the graft is placed into the container immediately after the removal from its donor. The graft may be maintained in the container containing the composition comprising one or more statins according to the present invention during the entire transport or be removed in between. In one

embodiment, the graft is maintained in the container during the entire transport. The graft may also be perfused with the statin containing composition of the present invention during the transport.

In accordance with the present invention, the composition may comprise hydrophobic statins or hydrophilic statins. In one embodiment, one or more statins are hydrophobic. In another embodiment, all statins are hydrophobic. In context with the present invention, the hydrophobic statins may require activation (i.e. statins which are prodrugs). The composition may comprise hydrophobic statins with a final concentration of 0,1-100 μM . In one embodiment, the final concentration of hydrophobic statins in the composition is 0,1-20 μM , preferably 1,25-15 μM . In a most preferred embodiment, the final concentration of hydrophobic statins in the composition is 8-12 μM . The composition may also comprise hydrophilic statins with a final concentration of 10-400 μM . In one embodiment, the final concentration of hydrophilic statins in the composition is 10-200 μM , preferably 50-150 μM . In a most preferred embodiment, the final concentration of hydrophilic statins in the composition is 75-100 μM . The hydrophobic statins may be solved in chloroform at 610 mg/ml, in DMSO at 540 mg/ml, in methanol at 200 mg/ml, in ethanol at 160 mg/ml, in 0,1 M HCl at 60 $\mu\text{g/ml}$, in PEG-400 at 70 mg/ml, in 0,1 M NaOH at 70 mg/ml or in water at 30 $\mu\text{g/ml}$. In one embodiment, the hydrophobic statins are solved in ethanol. Hydrophobic prodrug statins may be activated according to the instructions provided by the manufacturer. In one embodiment, 4 mg the hydrophobic prodrug statins are solved in 100 μl ethanol and activated by 0,1 N NaOH, followed by incubation at 50° C for 2 hours at a pH set to 7,0 by HCl. The final stock solution may then be adjusted to 4 mg/ml hydrophobic prodrug statins. Hydrophobic prodrug statins may be activated before, during or after adding the statins to the composition. In accordance with the present invention, the hydrophobic prodrug statins may be activated before they are added to the composition.

In accordance with the present invention, the composition may further comprise sodium, potassium, magnesium and a buffer reagent. In one embodiment, the buffer reagent is an anionic compound, such as lactobionate or phosphate. The final concentration of sodium in the composition may be 10-160 μM or, preferably, 15-100

μM . In one embodiment, the final concentration of sodium in the composition is 15-30 μM . The final concentration of potassium in the composition may be 2-150 μM or, preferably, 100-130 μM . In one embodiment, the final concentration of potassium in the composition is 110-125 μM . The final concentration of magnesium in the composition may be 2-30 μM or, preferably, 3-20 μM . In one embodiment, the final concentration of magnesium in the composition is 4-13 μM . The final concentration of lactobionate in the composition may be 10-150 μM or, preferably, 25-110 μM . In one embodiment, the final concentration of lactobionate in the composition is 80-105 μM . The final concentration of phosphate in the composition may be 10-80 μM or, preferably, 20-60 μM . In one embodiment, the final concentration of phosphate in the composition is 30-35 μM . The sodium, potassium, magnesium and/or the buffer reagent may each be added to the composition comprising statins before, during or after the composition is filled into the container. In one embodiment of the present invention, the sodium, potassium, magnesium and/or the buffer reagent are added to the composition comprising statins before the composition is filled into the container. The herein described composition comprising statins may be fluid, preferably aqueous, before, during and/or after adding the composition to the container. The composition may be lyophilized, vacuum-dried or spray-dried before adding the composition to the container.

In accordance with the present invention, the composition comprising statins may also be added to existing preservation solutions approved for organ transplantation known in the art. Such solutions may be commercially obtained and comprise, e.g., Eurocollins solution, Viaspan UW Solution, Custodiol HTK solution, Celsior solution, or the like.

Brief description of the Figures

Figure 1: Comparison of kidney function between pretreated and postoperatively treated murine kidney following ischemia reperfusion injury:
Simvastatin pretreatment effectively preserved renal function after ischemia-reperfusion injury. In a murine model of ischemia-reperfusion injury, the pretreatment group received simvastatin 4 hours prior to

induction of ischemia, whereas postoperative treatment was initiated immediately after reperfusion. Mice were treated with simvastatin 10 mg/kg i.p. The decrease of renal function following renal ischemia-reperfusion injury is effectively decelerated by simvastatin pretreatment. Renal excretion capacity on day 2 only declined to 39.8% in the pretreatment group versus 22.7% and 24.8% in the postoperatively treated and control group, respectively ($p < 0.05$).

Figure 2: Histological pictures of renal tissue treated and non-treated with pravastatin after cold ischemia:

Tissue sections show murine renal cortex subjected to 24 hours cold ischemia (4° C). Systemic perfusion of murine kidneys with respective solutions via Vena cava inferior in vivo with 5 ml ice-cold solution (4° C); optical control of optimal perfusion until organs appear pale/decolorized. During ischemia, kidneys have been stored in ice-cold solutions (4° C): 0,9% NaCl (**A**), 0,9% NaCl + 5 mg/l pravastatin (**B**), PBS (**C**), PBS + 5 mg/l pravastatin (**D**); original magnification was x200. Fixation of kidneys O/N in 4% formaldehyde, coating with paraffin, colorizing of sections with hemalaun/eosin. Renal corteces treated with solution comprising pravastatin (**B** + **D**) exhibited significantly improved vitality according to the well-preserved nuclei compared to tissue not treated with pravastatin (**A** + **C**). Also, tissue of **B** + **D** shows less decrease of cell height and morphologically less structural changes than tissue of **A** + **C**.

Figure 3: Histological sections of renal parenchyma treated for 24 h with ice-cold solution (4 °C) comprising Eurocollins alone (**A**, left), Eurocollins + simvastatin 20 μ M (**B**, left), and Eurocollins + pravastatin 200 μ M (**C**, left), respectively, were compared with their transplanted counterparts that were additionally exposed to 15 min reperfusion (**A**, **B**, **C**, right). Compared to kidneys treated with Eurocollins alone (**A**), statin-treated kidneys exhibited significantly improved vitality according to the well-preserved nuclei (**B**+**C**).

Moreover, less decrease of cell height and less structural changes were found. Kidneys treated with the pravastatin-comprising preservation solution showed granular degeneration within the cytoplasm (C). In contrast, Eurocollins + simvastatin prevented cellular degeneration and improved morphological appearance of tubular cells (B). Compared to kidneys subjected to cold ischemia alone (A, B, C, left), transplanted kidneys showed increased tissue damage (A, B, C, right). Pre-existing ischemic damage was further aggravated by reperfusion injury.

Hereinafter, the present invention is specifically illustrated with reference to Examples, but it is not to be construed as being limited thereto.

Examples

Example 1

Renal ischemia-reperfusion injury model

Studies were performed in a murine model of ischemia-reperfusion injury. Mice were anesthetized by intraperitoneal injection with a combination of ketamine (150 mg/kg) and xylazine (15 mg/kg) and placed on a heated surgical pad to keep constant body temperature. The right kidney was exposed through median abdominal incision, and mice were subjected to ischemia by clamping the renal pedicle with a non-traumatic microaneurysm clamp (Braun, Melsungen, Germany) which was removed after 45 min. The incision was closed with a 5-0 suture and surgical staples.

Determination of renal function by ^{99m}Tc -MAG3 imaging (Technescan MAG3, Covidien, Neustadt/ Donau). After hydration with sterile saline and induction of anesthesia with a combination of ketamine (150 mg/kg) and xylazine (15 mg/kg), mice underwent whole-body scintigraphy in a gamma camera (Philips – former Picker – Prism 3000 XP, Cleveland, USA) using dynamic imaging protocols with ^{99m}Tc -MAG3, respectively. Intravenous injection of a standardized dose of $\sim 3.7 \times 10^7$ Bq per mouse and dynamic acquisition were simultaneously started. 1 frame per 5

sec was collected with a total scan time amounting to 10 min. To determine baseline renal function, ^{99m}Tc -MAG3 imaging was carried out 4 days before ischemia-reperfusion surgery. Postoperative renal scans were performed on day 2.

Image analysis.

Image files were analyzed using Hermes kidney analysis software V4.1 (Hermes Medical Solution, Stockholm, Sweden) by standard manual region of interest (ROI) analyses of the whole body, both kidneys including their background regions as well as the site of injection. Data were exported to Microsoft Excel to assess renal function represented as percentage injected dose (%ID). %ID was obtained by division of the background corrected kidney ROI by the injection site corrected whole body ROI. In addition to renal function curves (%ID), the peak %ID minus %ID at 10min serving as a marker of tubular excretion capacity were determined. The pretreatment group received simvastatin 4 hours prior to induction of ischemia, whereas postoperative treatment was initiated immediately after reperfusion. Mice were treated with simvastatin 10 mg/kg i.p. The decrease of renal function following renal ischemia-reperfusion injury is effectively decelerated by simvastatin pretreatment. Renal excretion capacity on day 2 only declined to 39.8% in the pretreatment group versus 22.7% and 24.8% in the postoperatively treated and control group, respectively ($p < 0.05$).

Example 2

Effect of statins on ischemia

This study was to elucidate the exact time point of action of statins in transplantation-related ischemia-reperfusion injury. Therefore, the effect of statins in ischemia alone (without reperfusion) was examined:

Mice were anesthetized by intraperitoneal injection with a combination of ketamine (150 mg/kg) and xylazine (15 mg/kg) and placed on a heated surgical pad to keep constant body temperature. Median abdominal incision was performed. Systemic perfusion of murine kidneys with respective solutions via Vena cava inferior *in vivo*

with 5 ml ice-cold solution (4° C) using a 30-gauge needle; optical control of optimal perfusion until organs appear pale/decolorized. Then, kidneys were carefully removed and were stored in ice-cold solutions (4° C; cf. Figure 2): 0,9% NaCl (**2A**), 0,9% NaCl + 5 mg/l pravastatin (**2B**), PBS (**2C**), PBS + 5 mg/l pravastatin (**2D**); original magnification was x200. Fixation of kidneys O/N in 4% formaldehyde, coating with paraffin, colorizing of sections with hemalaun/eosin. Renal corteces treated with solution comprising pravastatin (B + D) exhibited significantly improved vitality according to the well-preserved nuclei compared to tissue not treated with pravastatin (A + C). Also, tissue of B + D shows less decrease of cell height and morphologically less structural changes than tissue of A + C.

Example 3

Experimental design

Kidney transplantation

Anesthesia

Mice were anesthetized by intraperitoneal injection with a combination of ketamine (150 mg/kg) and xylazine (15 mg/kg) and placed on a heated surgical pad to keep constant body temperature.

Donor preparation

The left kidney was exposed through median abdominal incision. The abdominal vessels were dissected and ligatures prepared in the following order:

- supra-renal aorta (3-0 ligature)
- renal vein as closer as possible to the cavae (6-0 ligature)
- infra-renal aorta (3-0 ligature to fix the canulla)
- infra-renal aorta+cavae en bloc (3-0 ligature)

The ureter was dissected preserving its surrounding fat and divided close to the bladder. After knotting the infra-renal aorta+cava en bloc the infra-renal aorta was clamped. Then, a cannula was inserted and fixed in the infra-renal aorta. Then, the vascular clamp was removed for Systemic perfusion of murine kidneys via infra-renal cavae in vivo with 5 ml of the respective ice-cold preservation solution (4 °C) using a

30-gauge needle. Optimal perfusion was determined by optical control until organs appeared pale/decolorized. Then, kidneys were carefully removed and were stored in the following ice-cold solutions (4 °C) for 24 h:

1. Eurocollins (composition: NaHCO_3 10 mM, KCL 15 mM, K_2HPO_4 42.5 mM, KH_2PO_4 15.1 mM, Glucose 195 mM; Osm: 320-330 mOsm/l)
2. Eurocollins + simvastatin (activated) 20 μM
3. Eurocollins + pravastatin 200 μM .

Transplantation

The left kidney was exposed through median abdominal incision. The ureter was carefully dissected and divided close to the kidney in order to preserve its whole length. Then, nephrectomy of the left kidney was performed. The abdominal vessels were dissected and clamped in the following order:

1. cava caudal
2. cava cranial
3. aorta cranial
4. aorta caudal

Vascular anastomosis of artery and vein were performed. Once the vascular anastomosis were completed, the clamps were removed in the following sequence to allow the restoration of natural blood flow in the kidney:

1. aorta cranial
2. vena cranial
3. vena caudal
4. aorta caudal

Histological analysis

Kidneys were carefully removed. Fixation of kidneys O/N in 4% formaldehyde was performed as well as coating with paraffin, colorizing of sections with hemalaun/eosin.

Results

Histological sections of renal parenchyma treated for 24 h with ice-cold solution (4 °C) comprising Eurocollins alone (Figure 3A left), Eurocollins + simvastatin 20 μ M (Figure 3B left), and Eurocollins + pravastatin 200 μ M (Figure 3C left), respectively, were compared with their transplanted counterparts that were additionally exposed to 15 min reperfusion (Figures 3A to 3C right).

Compared to kidneys treated with Eurocollins alone, statin-treated kidneys exhibited significantly improved vitality according to the well-preserved nuclei. Moreover, less decrease of cell height and less structural changes were found. Kidneys treated with the pravastatin-comprising preservation solution showed granular degeneration within the cytoplasm. In contrast, Eurocollins + simvastatin prevented cellular degeneration and improved morphological appearance of tubular cells.

Compared to kidneys subjected to cold ischemia alone, transplanted kidneys showed increased tissue damage. Pre-existing ischemic damage was further aggravated by reperfusion injury.

In conclusion, the data obtained from the murine kidney transplantation model support pre-existing data from the renal ischemia-reperfusion injury model and in vitro experiments in that statins significantly prevent tissue damage during both warm (renal ischemia-reperfusion injury model) and cold ischemia (transplantation model) and thus decrease reperfusion injury.

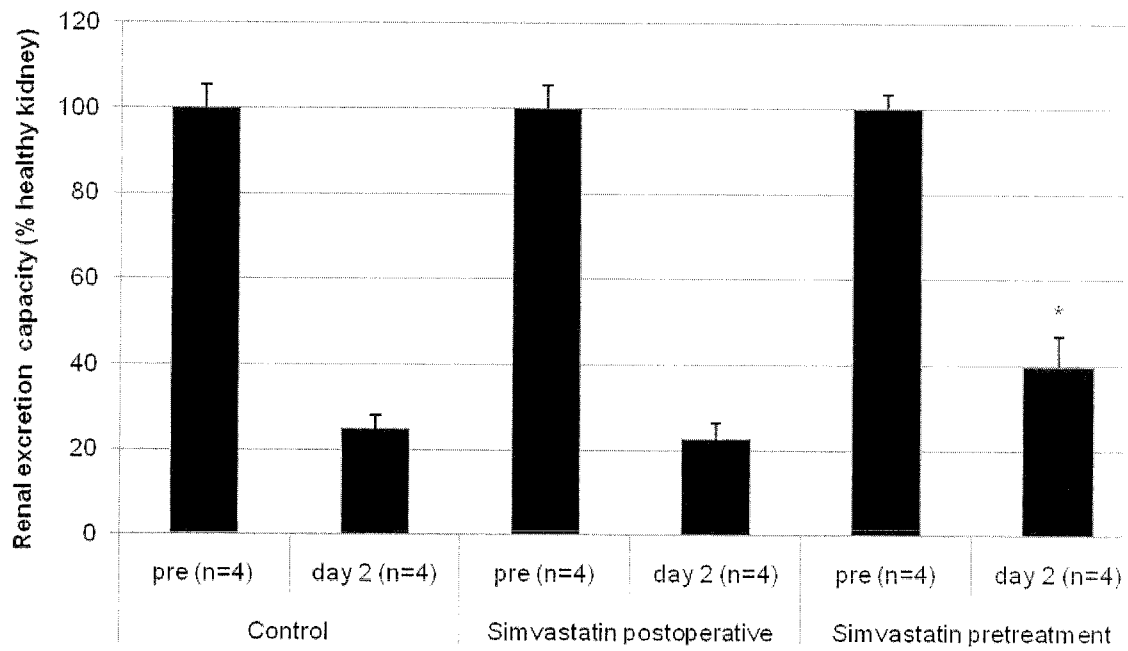
Claims

1. A composition comprising one or more statins for use in the preservation of a medical graft.
2. Method of preserving a medical graft comprising the following steps:
(a) placing the graft into a suitable container; and
(b) transporting the graft in the container of (a) to the recipient,
wherein the container of (a) is filled with the composition according to claim 1 before, during, and/or after step (a).
3. The method according to claim 2, wherein the transport of step (b) is spatially and timely immediate.
4. The composition according to claim 1 or the method according to claim 2 or 3, wherein the donor and the recipient of the graft is mammal.
5. The composition or the method according to claim 4, wherein at least the recipient is human.
6. The composition according to any of claims 1, 4 or 5 or the method according to any one of claims 2 to 5, wherein the statins are hydrophobic or hydrophilic.
7. The composition according to any one of claims 1 or 4 to 6 or the method according to any one of claims 2 to 6, wherein one or more statins are hydrophobic.
8. The composition according to any one of claims 1 or 4 to 7 or the method according to any one of claims 2 to 7, wherein the final concentration of the hydrophilic statins is 10-200 μM and the final concentration of the hydrophobic statins is 0,1-20 μM .

9. The composition according to any one of claims 1 or 4 to 8 or the method according to any one of claims 2 to 8, which further comprises sodium, potassium, magnesium and a buffer reagent.
10. The composition or the method according to claim 9, wherein the buffer reagent is anionic.
11. The composition or the method according to claim 10, wherein the anionic buffer reagent is lactobionate or phosphate.
12. The composition or the method according to any one of claims 9 to 11, wherein the final concentration of sodium is 10-160 μM , the final concentration of potassium is 2-150 μM , and the final concentration of magnesium is 4-30 μM .
13. The composition or the method according to claim 11 or 12, wherein the final concentration of lactobionate is 20-120 μM and the final concentration of phosphate is 10-60 μM .
14. The composition according to any one of claims 1 or 4 to 13 or the method according to any one of claims 2 to 13, wherein the graft is an autograft, allograft, isograft or xenograft.
15. The composition according to any one of claims 1 or 4 to 14 or the method according to any one of claims 2 to 14, wherein the graft is kidney, heart, lung, liver, pancreas, intestine, hand, foot, arm, leg, digit, toe, cornea, skin, penis, Islet of Langerhans, bone, free flaps, or parts thereof.
16. The method according to any one of claims 2 to 15, wherein the container is cooled to a temperature lower than the ambient temperature while containing the graft.

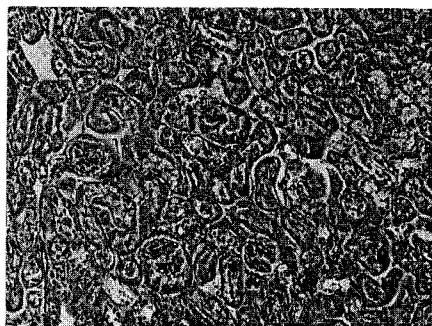
17. The method according to any one of claims 1 to 16, wherein the container is closable.
18. Use of one or more statins for the preparation of a composition according to any one of claims 1 or 4 to 15 for the preservation of a medical graft between removal of the graft from the donor and implantation of the graft into the recipient.

Figure 1



p < 0.05 vs. day 2 simvastatin postoperative

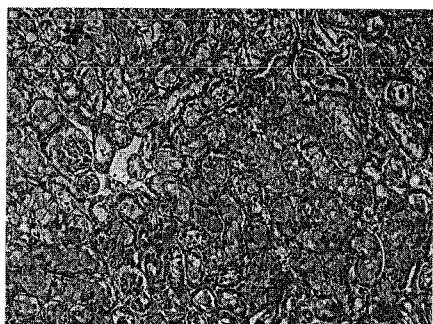
Figure 2



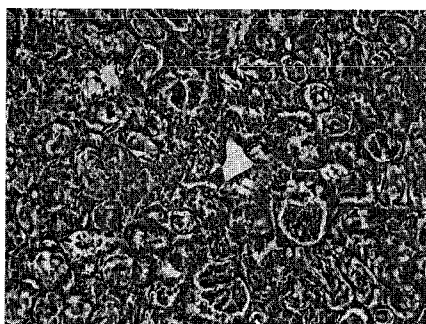
A



B



C



D

Figure 3

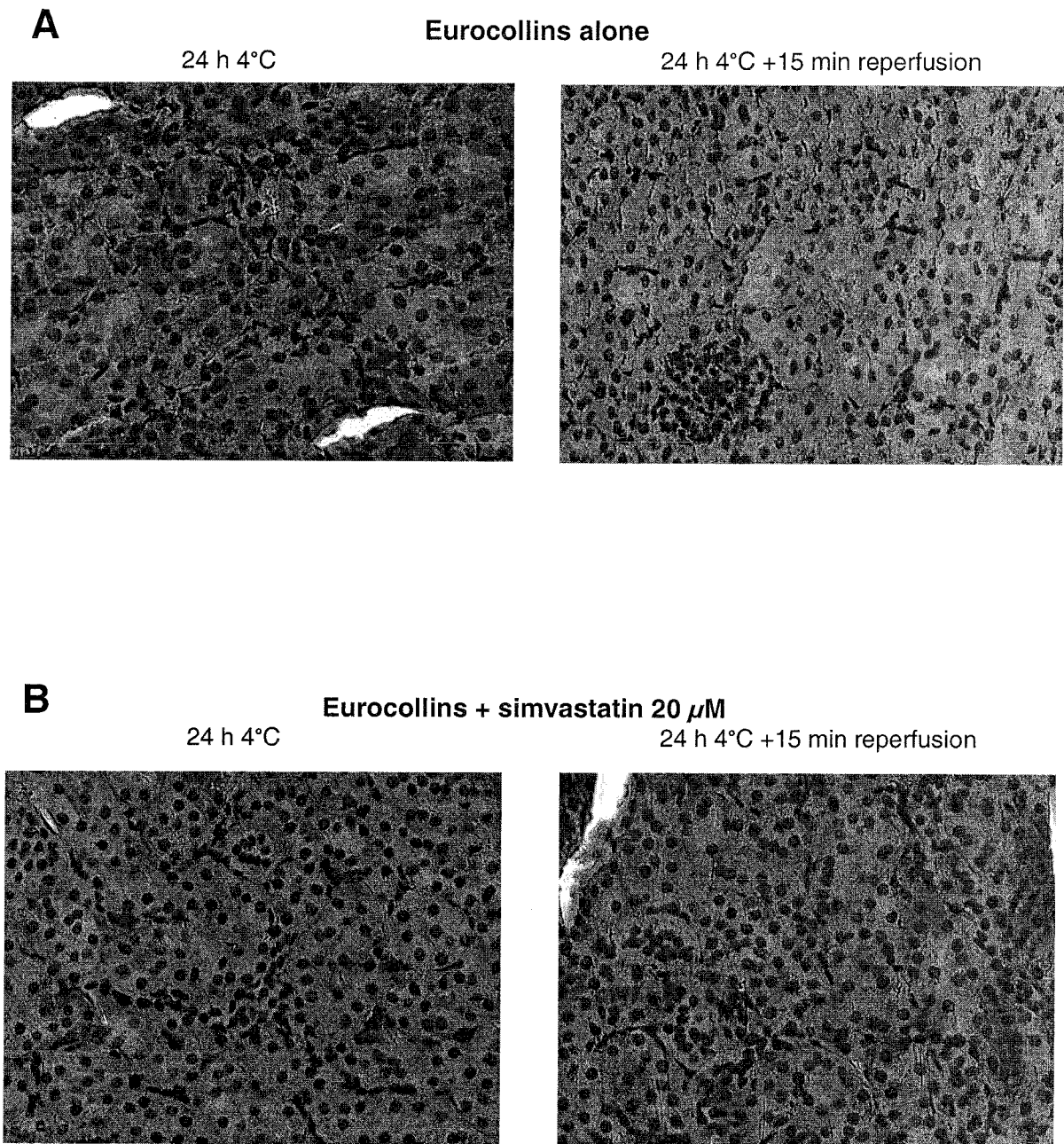


Figure 3

