

The Effect of Trophic Factor Supplementation on Cold Ischemia-Induced Early Apoptotic Changes

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We have previously shown that trophic factor supplementation (TFS) of University of Wisconsin (UW) solution enhanced kidney viability after cold storage. Here, we use an in vitro model to study the effect of TFS on early apoptotic changes after cold ischemic storage. Mitochondrial membrane potential was determined by fluorescence intensity in primary canine kidney tubule cells, Madin-Darby canine kidney cells, and human umbilical vein endothelial cells. In addition, caspase 3 enzyme activity assay and immunofluorescence staining were performed to evaluate apoptosis. There was a 15% increase in mitochondrial membrane potential in human umbilical vein endothelial cells stored in trophic factor supplemented University of Wisconsin solution after four-hour rewarming ($P < 0.05$). TFS suppressed caspase 3 enzyme activity and activation in human umbilical vein endothelial cells. We confirmed that the presence of TFS in UW solution has a beneficial effect by protecting mitochondrial function and reducing early apoptotic changes in vascular endothelial cells.

Keywords: Cold storage, Trophic factor supplementation, Apoptosis.

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Transplantation therapy for chronic renal failure remains significantly limited by donor kidney shortages (1). Delayed graft function, seen in 20% to 30% of transplanted cadaver kidneys, is a major risk factor for sequelae that affect long-term graft survival, patient management, and costs of transplantation (2). Cold ischemic (CI) injury during organ preservation plays an important role in not only inducing delayed graft function and allograft nephropathy, but also promoting apoptosis in certain cell types, including vascular and glomerular endothelial cells (3–6).

Tubular injury has long been a hallmark of CI injury. Particularly, the proximal convoluted tubule and the thick ascending limb of the loop of Henle are prone to injury induced by cold ischemia. Vascular endothelial cells are also a target of preservation injury. Recent work suggests that vascular injury, which affects blood flow in the outer medullary and juxtamedullary region, is a significant factor in tubular damage in ischemic kidneys (7).

Apoptosis is a form of programmed cell death morphologically characterized by cell shrinkage and condensation of both nuclear chromatin and cytoplasm (8, 9). On a molecular basis, one of the distinct signaling pathways is the mitochondrial pathway. It is known that various apoptotic stimuli such as oxidative stress, cold shock, and hypoxia can result in apoptotic cell death by the collapse of mitochondrial membrane potential (MMP), disruption of the outer mitochondrial

membrane, and release of apoptogenic molecules from mitochondria such as cytochrome c.

Previously, we have shown that trophic factor supplementation (TFS) of University of Wisconsin (UW) solution (TFS-UW) with a mixture of nerve growth factor β , substance P, insulin-like growth factor I, and batenecin (also known as bovine neutrophil peptide-1) reduced CI injury during cold storage of kidneys (10). Therefore, we hypothesize that TFS may have a beneficial effect on apoptosis induced by CI injury. The aim of the present study was to investigate whether TFS inhibits early apoptotic events induced by CI storage. In addition, we examined multiple cell lines to determine whether such effects may be cell type-specific.

Primary canine kidney tubule cells, Madin-Darby canine kidney cells, and human umbilical vein endothelial cells (HUVECs) were grown in RK-1 medium, Dulbecco minimum essential media, and EGM-2 media (Clonetics), respectively. All three cell lines were cultured at 37°C in a humidified atmosphere containing 5% CO₂. Cells were grown to approximately 70% confluence. After washing with phosphate-buffered saline, cells were stored at 4°C under nitrogen for four days in UW solution or TFS-UW containing nerve growth factor β (20 $\mu\text{g/L}$), substance P (2.5 $\mu\text{g/L}$), insulin-like growth factor I (10 $\mu\text{g/L}$), and bovine neutrophil peptide-1 (1 $\mu\text{g/L}$). Cells were then rewarmed in their proper cell medium for one, four, six, and 24 hours.

The membrane potential-dependent stain MitoTracker Red CMXRos (Molecular Probes) was used to assess the mitochondrial membrane potential in cells. Cells were incubated in each appropriate media containing a 0.1 μM final concentration of MitoTracker Red CMXRos dissolved in dimethyl sulfoxide for 30 minutes in a 37°C, 5% CO₂ gas incubator. Cells were washed in phosphate-buffered saline and qualitative analysis was carried out by capturing digital images of five fields per well. To determine whether the observed increase in staining was significant, spectrofluorometric analysis was used to quantitatively measure changes in fluorescence. Quantitative analysis was carried out using a fluorescence plate reader (Synergy HT; Bio-tek Instruments Inc.).

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Caspase 3 activity was determined by a CaspACE Assay System (Promega). In brief, an equal amount of cells were washed and lysed with cell lysis buffer containing 1% Triton X-100, 50 mM-HCl (pH 7.4), 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. Lysates were incubated on ice for 15 minutes and centrifuged at 15,000 g for 20 minutes at 4°C. The supernatant fractions were collected and incubated with the caspase 3 substrate, acetyl- Asp-Glu-Val-Asp *p*-nitroanilide (Ac-DEVD *p*NA), at 37°C for four hours. Caspase 3 activity was assessed by measuring the absorbance at a wavelength of 405 nm with a fluorescence plate reader (Synergy HT; Bio-tek Instruments Inc.).

HUVECs were grown and stored at 4°C under nitrogen

for four days in UW solution or TFS-UW as described earlier. After incubation in a humidified atmosphere containing 5% CO₂ at 37°C for four hours, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 5% bovine serum albumin, and stained with a 1:100 dilution of rabbit anticleaved caspase-3 antibody (Promega) at 4°C overnight. Primary antibodies were detected using a 1:200 dilution of fluorescein isothiocyanate-conjugated donkey anti-rabbit Cy2 (Jackson Immuno Research Laboratories, Inc.) at room temperature for one hour. Nuclei were stained with 0.8% DAPI (Roche Diagnostics, Indianapolis, IN) in phosphate-buffered saline for 10 minutes at room temperature. The number of caspase 3-positive cells was counted in

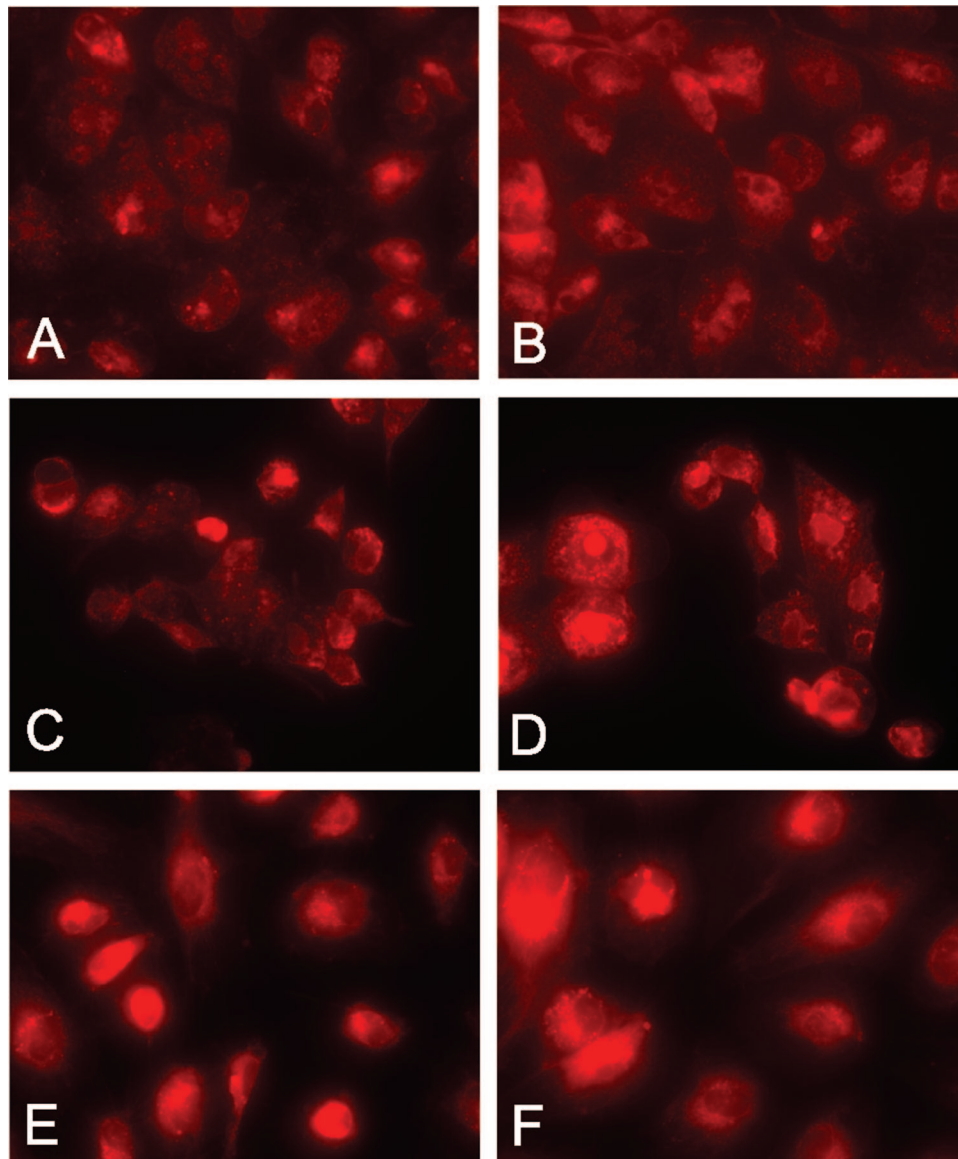


FIGURE 1. Mitochondrial staining with MitoTracker Red in (A, B) canine kidney tubule cells, (C, D) Madin-Darby canine kidney cells, and (E, F) human umbilical vein endothelial cells. As a result of six-hour (A–D) rewarming, red mitochondria staining of trophic factor-treated cells (B, D) was slightly higher than cells stored in University of Wisconsin solution for (A) kidney tubule and (C) Madin-Darby canine kidney cells. The mitochondrial membrane potential of human umbilical vein endothelial cells was also higher when the cell was incubated in trophic factor supplemented University of Wisconsin solution at 4°C for four days and then rewarmed in cell culture medium at 37°C for 24 hours (F) than when the cell was stored in unmodified University of Wisconsin solution for four-day cold ischemic storage followed by 24-hour rewarming (E) ($\times 400$).

five randomly chosen fields at a magnification of 400 \times , and the mean and standard deviation was then calculated.

All statistical analyses were performed using the Student *t* test. $P < 0.05$ was considered statistically significant.

The mitochondria of cells stored under CI conditions for 12 hours (positive control) and four days showed a remarkable decrease in MMP compared with control groups for all three cell lines. As the rewarming time increased, MMP collapse was gradually recovered. When cells were treated with trophic factors, red mitochondrial staining of the TFS-UW group was slightly higher than that of the UW group for all cell lines. This cytoplasmic staining pattern was similar in all rewarmed UW and TFS-UW groups (Fig. 1).

We found a significant 15% increase after four-hour rewarming in fluorescence intensity in HUVECs in the presence of TFS ($P < 0.05$). However, there was no significant difference in fluorescence intensity in primary canine kidney tubule and Madin-Darby canine kidney cells. These results indicate that TFS protects MMP collapse most preferentially in HUVECs and that TFS may have a beneficial effect on apoptosis induced by CI injury (Fig. 2).

Loss of MMP has been shown to activate caspase 3 and thus promote apoptosis. Therefore, we were interested in determining whether TFS had an inhibitory effect on caspase 3 activity in cells after CI storage. We found that CI injury led to a significant increase in caspase 3 activity compared with cells stored in their own appropriate medium. The high caspase 3 activity seen with storage in UW solution of HUVECs was slightly decreased by addition of trophic factors to the UW solution. The level of caspase 3 activity was 154, 528, and 456 pmol min⁻¹mg⁻¹ in the control, UW, and TFS-UW groups, respectively (Fig. 3A).

To determine whether the caspase 3 enzyme activity correlated with activated caspase 3, we used immunocytochemistry to probe for the activated form of caspase 3. As shown in Figure 3B, cells stored in UW solution stained positive for the active form of caspase 3. The number of caspase 3-positive cells were 0.60 ± 0.89 , 4.00 ± 1.00 , and 1.80 ± 0.84 in control, UW, and TFS-UW groups, respectively. The number of caspase 3 active cells was significantly decreased by TFS ($P < 0.05$). In addition, these cells also showed nuclear condensation and fragmentation, morphologic changes characteristic of apoptosis. These results are in agreement with the MitoTracker Red staining results suggesting that TFS decreases early apoptotic events in vascular endothelial cells.

To determine the effect of TFS on early apoptotic change induced by CI injury in primary canine kidney tubule cells, Madin-Darby canine kidney cells, and HUVECs, we used CMXRosamine dye as a measure of mitochondrial function. The fluorescence intensity of cells remarkably decreased after cold hypoxic storage suggesting that cold ischemia induces damage that results in a loss of MMP. Consistent with our results, it was reported that a reduction in mitochondrial size has been associated with a marked decrease in MMP (11) and that endothelial cells also exhibited decreased MMP when exposed to cold hypoxia (12). Interestingly, in the present study, the collapsed MMP after CI injury showed a tendency to increase by TFS when compared with that of cells stored in UW solution in all three types of cells studied here, further suggesting that TFS may have an antiapoptotic effect during cold hypoxic storage.

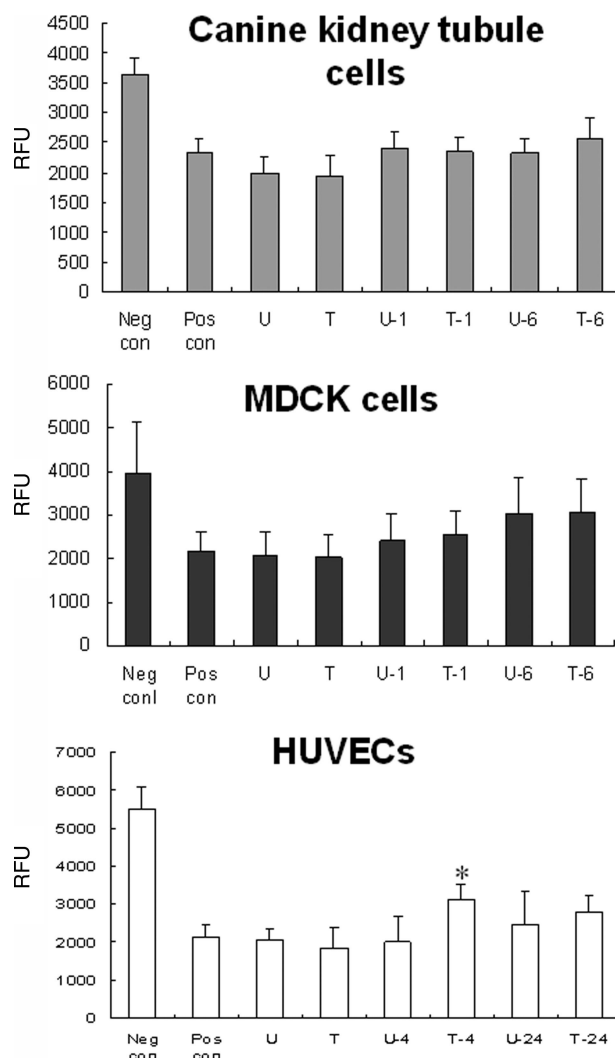


FIGURE 2. Effect of trophic factor supplementation on mitochondrial fluorescence intensity. The intensity was decreased after cold ischemic storage for 12 hours (positive control) and four days in all cell lines. As the cells were rewarmed for one hour or more, mitochondrial fluorescence intensity gradually increased. There was a significant increase after four-hour rewarming in human umbilical vein endothelial cells in the presence of trophic factor supplementation (TFS) ($P < 0.05$ compared with cells stored in University of Wisconsin [UW] solution and rewarmed for four hours). Neg con (), negative control, untreated normal cells; Pos con (), positive control, cells stored in UW solution for 12 hours; U, cells stored in UW solution for four days; T, cells stored in TFS-UW solution for four days; U-1, cells stored in UW solution for four days followed by one-hour rewarming; T-1, cells stored in TFS-UW solution for four days followed by one-hour rewarming; U-4, cells stored in UW solution for four days followed by four-hour rewarming; T-4, cells stored in TFS-UW solution for four days followed by four-hour rewarming; U-6, cells stored in UW solution for four days followed by six-hour rewarming; T-6, cells stored in TFS-UW solution for four days followed by six-hour rewarming; U-24, cells stored in UW solution for four days followed by 24-hour rewarming; T-24, cells stored in TFS-UW solution for four days followed by 24-hour rewarming. RFU, relative fluorescence units.

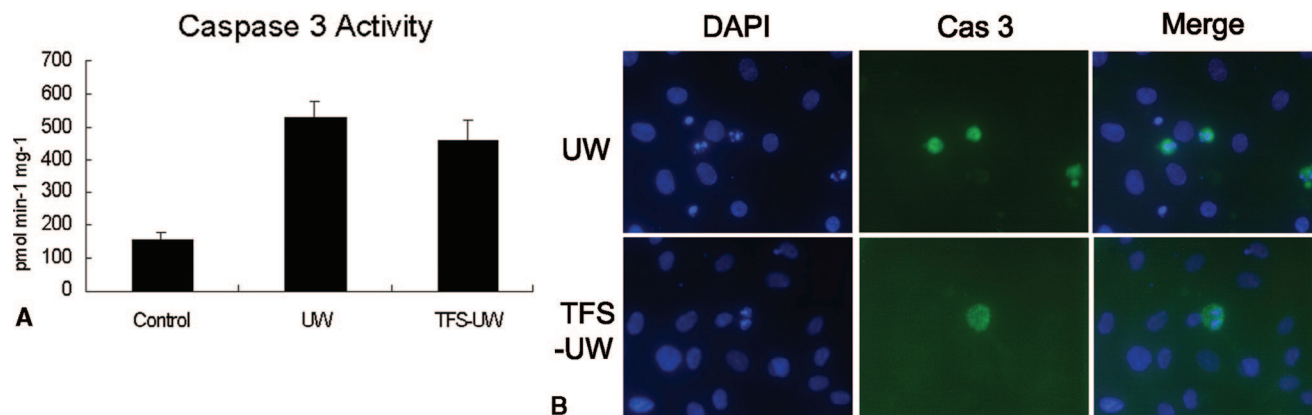


FIGURE 3. The effect of trophic factor supplementation on (A) caspase 3 enzyme activity and (B) immunofluorescence staining in human umbilical vein endothelial cells. The increased caspase 3 activity seen in cells stored in University of Wisconsin solution was slightly decreased by treatment with trophic factors in University of Wisconsin solution (A). Nuclear condensation and fragmentation and caspase 3 expression were observed in the same cells after four days cold ischemic storage in University of Wisconsin solution (Upper pictures). Caspase 3-positive cells were decreased by supplementation with trophic factors (lower pictures) (B).

When the MMP is collapsed, the release of cytochrome c, a mitochondrial apoptotic protein, leads to caspase activation and apoptotic cell death. Among the caspase family of proteins, caspase 3 is the critical effector caspase in the apoptotic pathway (13–15) and is activated by a mitochondria-dependent pathway (16–20). Based on these reports, we could suppose that TFS, having a protective effect on the MMP, would inhibit the activation of caspase 3 after CI injury and rewarming. In the current study, results from the caspase 3 enzyme assay and immunofluorescence staining strongly suggested that the activation of caspase 3 was inhibited by TFS in cold ischemic vascular endothelial cells.

Our results show that TFS protects against MMP collapse and that activation of caspase 3 was inhibited during early apoptosis in vascular endothelial cells. Collectively, the protective effects of TFS on mitochondrial-dependent apoptosis may be one mechanism whereby TFS is able to improve kidney preservation for transplantation.

REFERENCES

- Ojo AO, Wolfe RA, Leichtman AB, Dickinson DM, Port FK, Young EW. A practical approach to evaluate the potential donor pool and trends in cadaveric kidney donation. *Transplantation* 1999; 67: 548.
- Matas AJ, Gillingham KJ, Humar A, Dunn DL, Sutherland DE, Najarian JS. Immunologic and nonimmunologic factors: different risks for cadaver and living donor transplantation. *Transplantation* 2000; 69: 54.
- Roberts JR, Rowe PA, Demaine AG. Activation of NF-kappaB and MAP kinase cascades by hypothermic stress in endothelial cells. *Cryobiology* 2002; 44: 161.
- Kruman II, Gukovskaya AS, Petrunkova VV, Beletsky IP, Trepakova ES. Apoptosis of murine BW 5147 thymoma cells induced by cold shock. *J Cell Physiol* 1992; 153: 112.
- Rauen U, Polzar B, Stephan H, Mannherz HG, de Groot H. Cold-induced apoptosis in cultured hepatocytes and liver endothelial cells: mediation by reactive oxygen species. *FASEB J* 1999; 13: 155.
- Tanaka T, Miyata T, Inagi R, et al. Hypoxia-induced apoptosis in cultured glomerular endothelial cells: involvement of mitochondrial pathways. *Kidney Int* 2003; 64: 2020.
- Fuller TF, Sattler B, Binder L, Vetterlein F, Ringe B, Lorf T. Reduction of severe ischemia/reperfusion injury in rat kidney grafts by a soluble P-selectin glycoprotein ligand. *Transplantation* 2001; 72: 216.
- Shankland SJ, Wolf G. Cell cycle regulatory proteins in renal disease: role in hypertrophy, proliferation, and apoptosis. *Am J Physiol Renal Physiol* 2000; 278: 515.
- Savill J, Mooney A, Hughes J. Apoptosis and renal scarring. *Kidney Int Suppl* 1996; 54: 14.
- McAnulty JF, Reid TW, Waller KR, Murphy CJ. Successful six-day kidney preservation using trophic factor supplemented media and simple cold storage. *Am J Transplant* 2002; 2: 712.
- Tiano L, Ballarini P, Santoni G, Wozniak M, Falcioni G. Morphological and functional changes of mitochondria from density separated trout erythrocytes. *Biochim Biophys Acta* 2000; 1457: 118.
- Amberger A, Weiss H, Haller T, et al. A subpopulation of mitochondria prevents cytosolic calcium overload in endothelial cells after cold ischemia/reperfusion. *Transplantation* 2001; 71: 1821.
- Khwaja A, Tatton L. Caspase-mediated proteolysis and activation of protein kinase C-delta plays a central role in neutrophil apoptosis. *Blood* 1999; 94: 291.
- Pongracz J, Webb P, Wang K, Deacon E, Lunn OJ, Lord JM. Spontaneous neutrophil apoptosis involves caspase 3-mediated activation of protein kinase C-delta. *J Biol Chem* 1999; 274: 37329.
- Daigle I, Simon HU. Critical role for caspases 3 and 8 in neutrophil but not eosinophil apoptosis. *Int Arch Allergy Immunol* 2001; 126: 147.
- Watson RW, O'Neill A, Brannigan AE, et al. Regulation of Fas antibody induced neutrophil apoptosis is both caspase and mitochondrial dependent. *FEBS Lett* 1999; 453: 67.
- Maianaki NA, Mul FP, van Buul JD, Roos D, Kuijpers TW. Granulocyte colony-stimulating factor inhibits the mitochondria-dependent activation of caspase-3 in neutrophils. *Blood* 2002; 99: 672.
- Murphy BM, O'Neill AJ, Adrain C, Watson RW, Martin SJ. The apoptosome pathway to caspase activation in primary human neutrophils exhibits dramatically reduced requirements for cytochrome C. *J Exp Med* 2003; 197: 625.
- Altmann F, Conus S, Cavalli A, Folkers G, Simon HU. Calpain-1 regulates Bax and subsequent Smac-dependent caspase-3 activation in neutrophil apoptosis. *J Biol Chem* 2004; 279: 5947.
- Maianaki NA, Geissler J, Srinivasula SM, Alnemri ES, Roos D, Kuijpers TW. Functional characterization of mitochondria in neutrophils: a role restricted to apoptosis. *Cell Death Differ* 2004; 11: 143.