

ENDOTHELIAL CELL CULTURES AS A MODEL FOR ORGAN PRESERVATION STUDIES

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INTRODUCTION

Vascular endothelium represents the primary target for ischemia and reperfusion injury in organ preservation [1]. In comparison to parenchymal tissue, comparatively few studies are available on the tolerance to hypoxia and hypothermia of endothelial cells [2,3]. Cultured endothelial cells offer a promising approach to the substitution of a number of animal experiments in organ preservation studies. In particular, primary cultures of human umbilical vein endothelial cells are a clinically relevant model without constraints for ethical reasons. It goes without saying, exclusively animal models are available for experimental whole organ studies. For instance, the calf is among the widely used animal models in studies of cardiac surgery. For this reason, we compared the viability of human and bovine endothelial cells under preservation conditions.

Successful organ transplantation depends critically on a wide distribution network for donor organs to optimize histocompatibility matching. Preservation time constitutes a limiting factor, particularly for the heart (4 h), lung (4-6 h) and liver (<12 h). Owing to the impracticability of continuous perfusion of the excised organ during transportation, hypoxia constitutes the main cause for damage of the isolated organ [4]. As a remedy, cold storage (4 °C) suppresses metabolic energy demand but comprises a threat to membrane integrity [5]. Before explantation of the donor organ, *in situ* perfusion with preservation solutions is applied to support the tissue energetically and structurally.

The differential applicability of preservation solutions such as Bretschneider solution (HTK), University of Wisconsin solution (UW) and EuroCollins (EC) is well established, although underlying mechanisms are little understood. Clinically, UW and HTK are most commonly used for virtually all transplantable organs, with the exception of the lung (where EC is maintained as a standard [6]). For further optimization, however, it is crucial to elucidate the specific mechanisms of these preservation solutions during preservation and reperfusion. The bioenergetic approach by high-resolution respirometry allows a functional evaluation of preservation in terms of oxidative capacity and membrane integrity.

MATERIALS AND METHODS

Cell culture and preservation experiments: Human endothelial cells were prepared from umbilical cords enzymatically. Bovine endothelial cells were harvested from calf aortae. Both cell types were cultured according to established methods [7,8]. Second to fourth passages of primary cultures were grown under

normoxia (95% air, 5% CO₂) as monolayers and used for preservation and respiration experiments. Organ preservation conditions were simulated by hypoxic cold storage for 4, 24, 48 and 72 h at 4 °C (95% N₂, 5% CO₂). Monolayers maintained under cell culture conditions served as a control. Cold preservation without biochemical support in isotonic salt solution was included as a reference preservation condition. Reperfusion conditions were established by reincubation in normoxic culture medium at 37 °C and 5% CO₂.

Cell viability and morphology: Cell viability was assessed by trypan blue staining; the number of viable cells was counted under the microscope using a grid count. The morphological state was evaluated by means of inversion microscopy, yielding a 3-D image.

High-resolution respirometry: Respiration of endothelial cell suspensions was measured in a two channel titration-injection respirometer (OROBOROS® *Oxygraph*, Paar KG, Graz, Austria) at 37 °C under normoxic reperfusion conditions. The cell density was 1.1 to 1.2·10⁶ cells per ml in the 2 ml measuring chambers which was gently stirred at 350 rpm. See [9] for details of respiratory measurements and preparation of endothelial cells. Oxygen flux, J_{O_2} , was expressed per 10⁶ viable cells.

RESULTS AND DISCUSSION

Human versus bovine endothelial cells

Bovine aortic endothelial cells are frequently used as a cell culture model [7, 10,11]. Therefore, we were interested in a comparison of human and bovine endothelial cells to clarify their relevance for clinical applications. Identical results were obtained with cells from the two sources immediately after cold storage at the two shorter storage times in UW (Fig. 1A). At 72 h cold storage, and particularly after 6 hours of recovery, human endothelial cells preserved in UW appeared to be less cold-storage resistant compared to bovine cells (Fig. 1B).

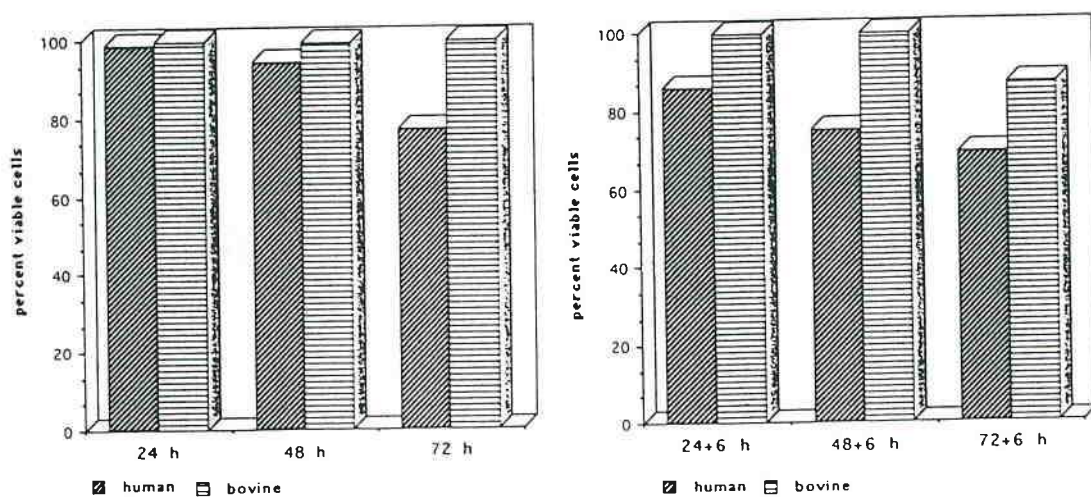


Fig. 1. Cell viability of human and bovine endothelial cells [%] as a function of time of hypoxic cold storage [h] in University of Wisconsin (UW) preservation solution. **A:** Measurements immediately after cold storage; **B:** Measurements after 6 hours of reperfusion conditions following cold storage.

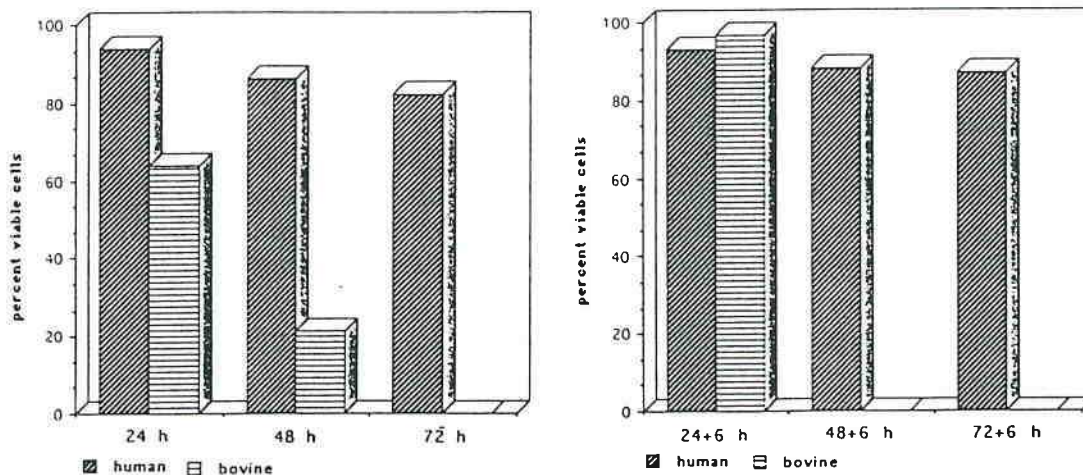


Fig. 2. Cell viability of human and bovine endothelial cells [%] as a function of time of hypoxic cold storage [h] in Bretschneider solution (HTK) preservation solution. **A:** Measurements immediately after cold storage; **B:** Measurements after 6 hours of reperfusion conditions following cold storage.

In contrast to the highly protective effect of HTK on human endothelial cells (see above), this storage solution would not be recommended on the basis of the bovine endothelial cell model: Cell viability dropped to zero after 72 h (Fig. 2A) and after already 48 h when measured at 6 h reperfusion (Fig. 2B). From this significantly different result, we conclude that human endothelial cells are the preferred model in experimental studies with a clinical orientation.

The human endothelial cell culture presents a potential alternative to animal experiments in medical science. The significant difference between human and bovine cells provokes the question as to the clinical relevance of the bovine animal model which is widely used in cardiovascular research.

Viability of human endothelial cells treated with different preservation solutions

In agreement with results reported from animal experiments and cardiomyocytes (for a discussion see [12]), UW solution exerted protective effects on cell viability during hypoxic cold preservation, as compared to EC solution (Fig. 3A). In contrast, however, HTK yields poor protection in the rabbit myocytes [12], but good results were obtained in the human endothelial preservation (Fig. 3A). It is important to note that HTK got a similarly high rating for preservation of human cardiac tissue [13]. Storage in isotonic salt solution and EC solution resulted in significant and increasing loss of viability after 24, 48 and 72 h, without complete recovery after 6 h reperfusion conditions (Fig. 3B). In contrast, cells preserved in HTK and UW were not different from the controls (Fig. 3A), although a trend towards lower viability was significant (at 48 h) with UW when the cells were investigated after 6 h of reperfusion (Fig. 3A). Viability measurements by trypan blue exclusion are not to be taken as evidence of irreversible cell damage, whence partial recovery is possible (compare Fig. 3A and B).

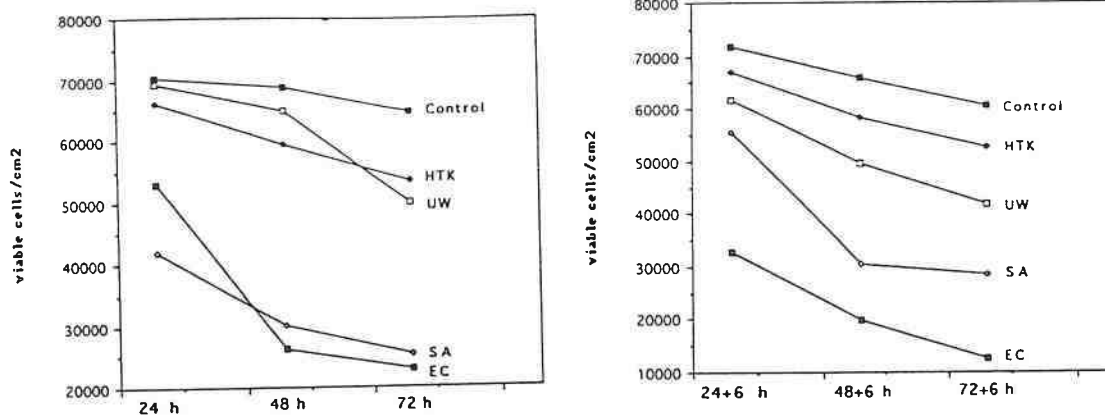
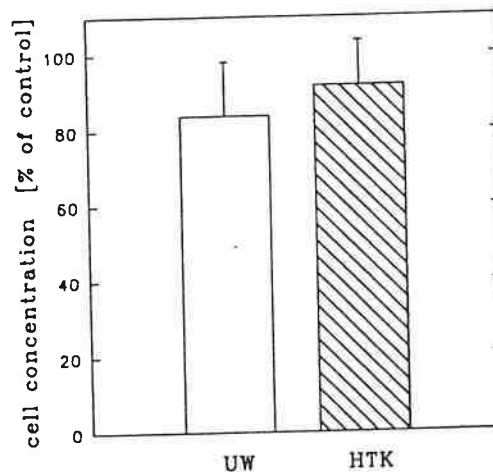


Fig. 3. Cell viability of human endothelial cells [number of viable cells per cm² of culture flask] as a function of time of hypoxic cold storage [h] and preservation solution. CO - control (no cold storage); HTK - Bretschneider solution; UW - University of Wisconsin solution; SA - isotonic salt solution; EC - EuroCollins solution. A: Measurements immediately after cold storage; B: Measurements after 6 hours of reperfusion conditions following cold storage.

The morphological results corroborated the different preservation effects of HTK and UW *versus* EC and isotonic saline solution. Although cell numbers and viability were identical in HTK and UW (Fig. 3), the morphological analysis revealed a different structural integrity of the cells stored for 24 h in HTK and UW, the latter resembling more closely the intact monolayer of the controls.

Whereas cell counts revealed significant differences in the treatments with various storage solutions after 24 to 72 h of hypothermic hypoxia, this test is not sufficiently sensitive to indicate sublethal effects which might occur during shorter preservation periods (Fig. 4).

Fig. 4. Cell counts of human endothelial cells after 4 h cold storage in hypoxic UW and HTK preservation solutions, estimated after 45 min of reperfusion conditions. Cell concentrations in the culture flasks were expressed as % of control culture flasks not subjected to cold storage. Viable cell counts were made after trypsinization. Bars indicate the S.D. ($n=7$). In the controls, viable cell counts were 1.14 ± 0.43 S.D. 10^6 cells·ml⁻¹, after resuspension in culture medium for respirometry. Viabilities of controls, and after storage in UW and HTK were 96.7 ± 1.4 , 93.0 ± 3.1 and 97.0 ± 1.0 %, respectively, where the latter are significantly different.



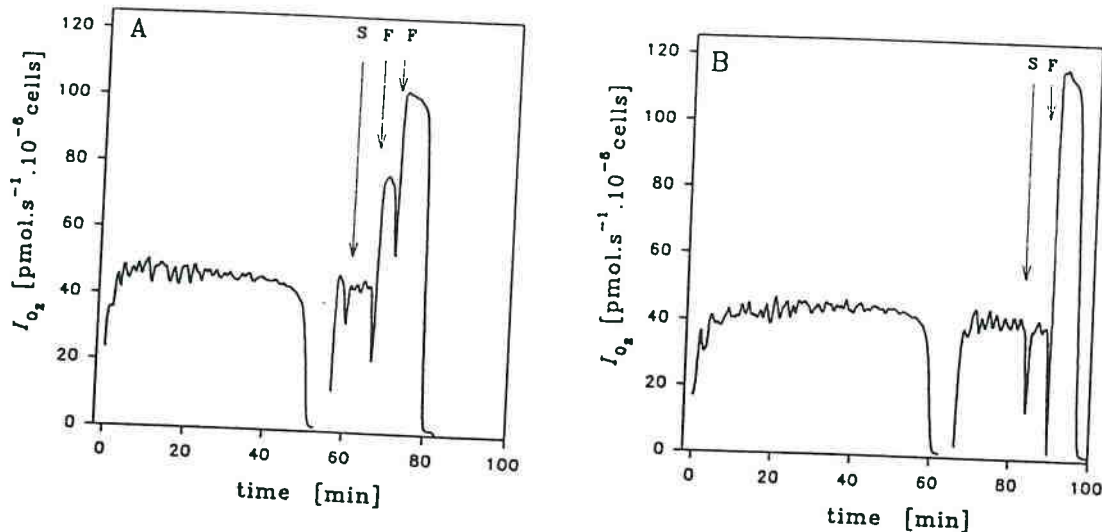


Fig. 5. Oxygen flux, J_{O_2} [$\text{pmol} \cdot \text{s}^{-1} \cdot 10^{-6}$ cells], under reperfusion conditions. **A:** Control without cold-storage; **B:** After hypoxic cold storage for 4 h in UW; **C:** After hypoxic cold storage for 4 h in HTK. Constant rates of respiration were observed in the control up to 50 min until oxygen pressure declined to a critical value. Re-aeration was achieved by opening the stopper for a short time, and an identical oxygen flux was seen in the following period. Addition of succinate (S; 10 mM) had no significant effect on respiration, indicating membrane integrity. The uncoupler FCCP (F; 10 μM final concentration) stimulated oxygen flux >2-fold.

Towards a more sensitive test: High-resolution respirometry

After cold-storage for 4 h in UW, respiration of endothelial cells was only slightly reduced relative to the controls. Respiratory stability in coupled cells and oxidative capacity of uncoupled cells were identical to the controls (see representative experiment shown in Fig. 5A and 5B). In contrast, cold-storage in HTK resulted in a gradual and increasingly significant reduction of oxidative capacity in coupled and uncoupled cells (Fig. 5C).

In conclusion, the functional test by respirometry and the morphological index of structural integrity allow the investigation of preservation conditions at a high level of sensitivity. Sublethal effects of hypoxic cold storage and reperfusion could be differentiated which is a prerequisite for evaluating and optimizing new organ preservation solutions.

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