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M. A. Konerding
University of Mainz, konerdin@mzdmza.zdv.uni-mainz.de

M. Knocks
University of Essen

H. -R. Zerkowski
University of Halle

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IMPACT OF THE INCUBATION MEDIUM ON THE ENDOTHELIUM OF AUTOLOGOUS VEIN GRAFTS: DAMAGE SCORING BY SCANNING ELECTRON MICROSCOPY

M.A. Konerding*, M. Knocks[1] and H.-R. Zerkowski[2]

Institute of Anatomy, Department of Macroscopic Anatomy, University of Mainz, Germany
[1]Department of Thoracic and Cardiovascular Surgery, University of Essen, Germany
[2]Clinic for Thoracic and Cardiovascular Surgery, University of Halle, Germany

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Abstract

The aim of this study was to examine the influence of different incubation media on the morphology of the endothelium of great saphenous vein grafts and establish a suitable scoring system for the evaluation of damage caused by these media. Fifty specimens of saphenous veins from ten patients during elective aorto-coronary bypass surgery were used. Ten specimens served as controls; the others were assigned to test groups and exposed to heparinized whole blood, Bretschneider's HTK, human albumin or Ringer's solution. Specimens exposed to heparinized blood showed only slight morphological alterations, whereas the other three mediums caused severe damage. Thus, heparinized blood seems to be most suitable as a rinsing and incubation medium.

A widely accepted scoring system for the quantification of endothelial damage caused by the incubation media did not adequately reflect the morphological alterations in the cytoskeleton and membrane topology. The proposed scoring system, which is based on endothelial cell separation, endothelial cell loss, amount of deposits, endothelial cell surface homogeneity, in addition to the frequency of spikes and blebs, seems to be suitable for characterizing differences in endothelial morphology.

Key Words: Endothelium, coronary artery bypass grafting, organ preservation, cardioplegic solutions, Bretschneider's HTK, heparinized whole blood, Ringer's solution, scanning electron microscopy.

*Address for correspondence:
M.A. Konerding
Anatomisches Institut,
Makroskopischer Bereich,
Johannes Gutenberg-Universität Mainz,
D-55099 Mainz, Germany
Telephone number: (49) 6131-392549
FAX number: (49) 6131-394710
E.mail: konerdin@mzdmza.zdv.uni-mainz.de

Introduction

Aorto-coronary bypass surgery is well established as a therapeutic modality in cases of coronary vascular disease. However, the occlusion rate of the bypasses still remains as high as 10-20% within the first year; thereafter, an occlusion rate of about 2% per year is common (Grondin, 1984). Such an unsatisfactory outcome is mainly attributable to three factors: progress of coronary heart disease, the run-off into the coronary vascular bed, and the biological properties of the grafted vessel (Lawrie, 1990; Lüscher, 1991). However, a part of the injuries to the grafted tissue occur during surgical harvesting, preparation and storage (Cartier *et al.*, 1991; Lüscher, 1991; Sottiurai *et al.*, 1983).

Ischemia and reperfusion (Forman *et al.*, 1989), distension with uncontrolled pressure (Bonchek, 1980; Hasse *et al.*, 1981), and stretching (Hofer *et al.*, 1981) are the most likely causes of structural damage to the vascular wall and, in particular, to the endothelium. Rinsing of the vein graft, which is necessary in order to check possible leakages, as well as storage in cardioplegic or other solutions, also contribute to endothelial damage (for detailed reviews of the literature, see Angelini *et al.*, 1989; Chiavarelli *et al.*, 1992; Lawrie, 1990; Mattila *et al.*, 1987; Sottiurai *et al.*, 1983; Wagner, 1990). Several authors have attempted to assess the extent of endothelial damage after different treatments of the grafts (Hofer *et al.*, 1981; Sottiurai *et al.*, 1983; Wagner, 1990). However, damage scores such as those of Gundry *et al.* (1980) do not provide adequate predictive parameters.

Against this background, the aim of our study was to investigate the extent of endothelial damage of vein segments after standardized storage in different media, and to determine the most suitable and least damaging medium for rinsing and storage, as well as to establish a modified endothelial damage score.

Materials and Methods

Fifty specimens of great saphenous veins, from ten

male patients aged 42 to 79 years (mean 58.4 ± 1.2 years) during elective aorto-coronary bypass grafting, were used for this study. All operations were performed by the same two surgeons under standardized conditions: the veins were harvested atraumatically after exposure ("no touch technique"), without any further surgical manipulation, before cardioplegia. All side branches were ligated with silk (4-0), and blood flow was interrupted within seconds before excision. Immediately after removal, each vein was cut into two segments: one to be used for bypass surgery and the other for our experiments.

The vein segments were each cut into five pieces with an average length of 3 mm. Four of these specimens were randomly assigned to the test groups and one to the control group. The test groups were exposed (without rinsing out of blood) to heparinized whole blood (group II; 5000 IU/l), Bretschneider's HTK (group III; pH 7.02-7.20, 310 mosmol/l; Köhler Chemie, Alsbach, Germany), 3.5% watery human albumin solution (group IV; 150 mmol natrium, 1.5 mmol potassium, pH 7.00, 245 mosmol/l; Immuno, Heidelburg, Germany) or Ringer's solution (group V, pH 7.40, 309 mosmol/l; Fresenius, Bad Homburg, Germany) for 1 hour at room temperature (mean temperature 21.1 ± 0.5°C). Bretschneider's HTK (group III) is a widely used solution for cardioplegia and multiple organ protection with comparatively low potassium content (9 mmol; sodium chloride 15 mmol, magnesium chloride 4 mmol, histidine 198 mmol, tryptophan 2 mmol, mannitol 30 mmol, calcium chloride 0.015 mmol). Time and temperature chosen should reflect the situation in cardiovascular surgery. The controls (group I) were fixed immediately. After incubation, the specimens were fixed in a cacodylate-buffered solution of glutaraldehyde and paraformaldehyde (2% glutaraldehyde, 2% paraformaldehyde; 860 mOsmol, pH 7.40) at 4°C for 4 hours. After rinsing in cacodylate-buffered saline (pH 7.40, 4°C), the specimens were dehydrated in an ascending alcohol series and critical-point dried without postfixation. Amyl acetate was used as the intermedium. In order to expose the intimal surface, the vein rings were cut into two pieces. The specimens were mounted on stubs with carbon cement (Leit-C, Neubauer, Germany) and sputtered with gold in an argon atmosphere.

All examinations were carried out, after double blind randomization, using a Stereoscan 180 scanning electron microscope (Cambridge, UK) at an acceleration voltage of 20 keV. In all specimens, 10 or more areas with approximately 0.25 mm^2 were viewed. As a rule, at least three images with a primary magnification of x300, x800, and x1500, respectively, as well as detailed images were recorded for each specimen. Based on these images, the following morphological parameters were examined both qualitatively and semi-quantitatively in order to establish a damage score, as suggested by Gundry *et al.* (1980): (**1**) Extent of coverage of the intima with endothelial cells; (**2**) separation of endothelial cells; (**3**) loss of endothelial cells; (**4**) exposed bundles of collagen fibers; and (**5**) intimal fractures. These parameters were assessed qualitatively and semi-quantitatively on a scale of 0 to 4 on the basis of the extent of involvement of the specimen, as follows: 0, no damage; 1, less than 10%; 2, 10 to 25%; 3, 25 to 50%; and 4, more than 50%, with respect to the luminal surface area. Intimal and medial edema, as well as exposed basement membrane, proposed as parameters by Gundry *et al.* (1980) were not graded, since in our opinion, these features cannot be appropriately assessed by the method used.

In order to reflect possible pathological changes in the membrane topology of single endothelial cells, five additional parameters were scored, as follows: (**1**) Amount of deposits; (**2**) degree of homogeneity of the endothelial cells surface; (**3**) frequency of spikes; and (**4**) frequency of blebs. These additional parameters were also assessed on a scale of 0 to 4.

All specimens were scored double-blindly by two independent investigators. The deviation of the investigators was less than five per cent. After decoding, the average of both scorers was utilized. Since the group scores represent semi-quantitative data, no statistical analysis was carried out. Prior to decoding, two investigators attempted independently to identify those specimens that belonged to the same groups.

Results

Group specificity

The specimens showed evidence of different morphological and pathological changes and could be assigned to groups before decoding on the basis of their appearance. All control specimens were recognized as such (10 out of 10). In the case of the experimental groups, 40 to 70% of the specimens were assigned correctly.

Morphological findings and general features (Fig. 1)

In all specimens, there were variable numbers of longitudinally orientated folds and clefts, visible both macroscopically and microscopically, as a result of vessel spasm, fixation, and drying-induced shrinkage. However, these features did not interfere with the examination of the endothelium since the majority of the endothelial surface could always be inspected.

Controls (Fig. 2): All controls had flat, continuous endothelium without any indication of cell loss. The endothelial cells were mainly orientated along the longi-

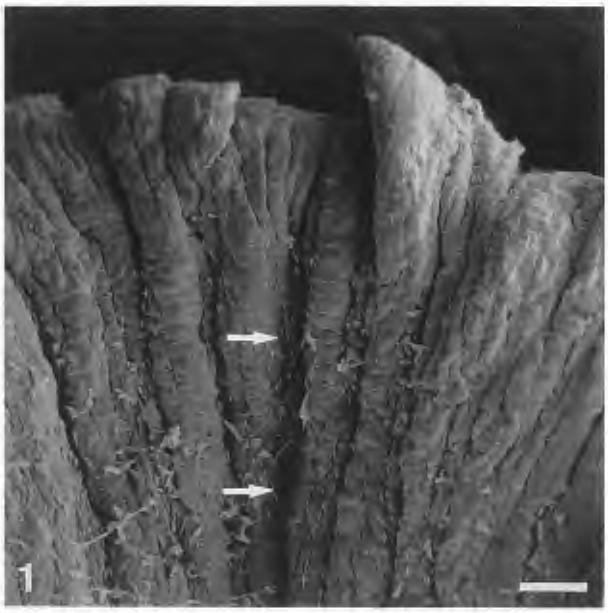

Figure 1. General appearance of a dried segment of an autologous vein graft, which was cut longitudinally before dehydration, drying and mounting. Note the longitudinally arranged folds (arrows) caused by shrinkage and media spasm, which obscure a part of the intimal surface. Bar = 100 μm.

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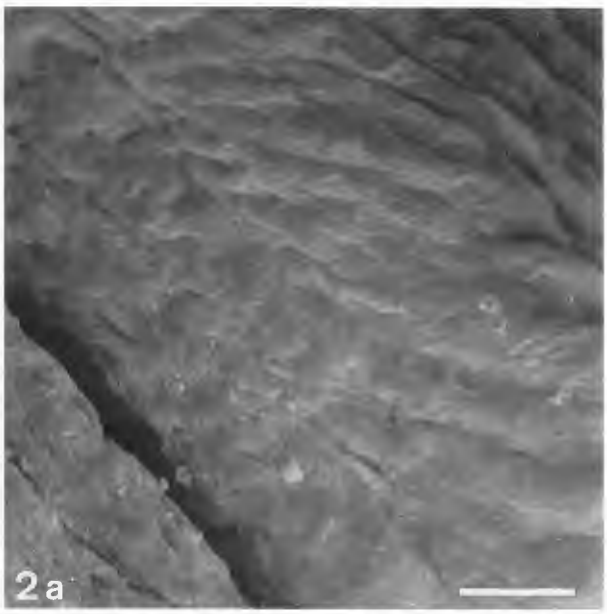
tudinal axis of the vessels. Some of these cells were elongated, but most were ovoid in shape. The surface was generally smooth: microvilli were seldom seen; spikes or blebs were never seen. Deposits were rarely noted. Apart from rare deposits of single platelets, in general, no microaggregates were detected. The morphology of the control veins was normal, given the mean age of the patients.

Heparinized whole blood (Fig. 3): Compared to the controls, these specimens showed a slightly higher degree of dissociation of endothelial cells and heterogeneity. However, endothelial cell loss was rarely detected. In general, the surfaces appeared smooth, with occasional spikes of approximately 0.5 μm in length. Depositions of cell remnants and microaggregates were found much more frequently than in the controls despite the heparinization.

Bretschneider's HTK (Fig. 4): The luminal surfaces were continuously covered with endothelial cells. Endothelial cell loss and dissociation, as well as deposition of cell remnants, were seldom seen. However, a high degree of heterogeneity as well as numerous spikes and blebs were seen in the majority of the endothelial cells in nine out of ten specimens, indicating major changes in the cytoskeleton and in membrane topology and, thus, impairment of function.



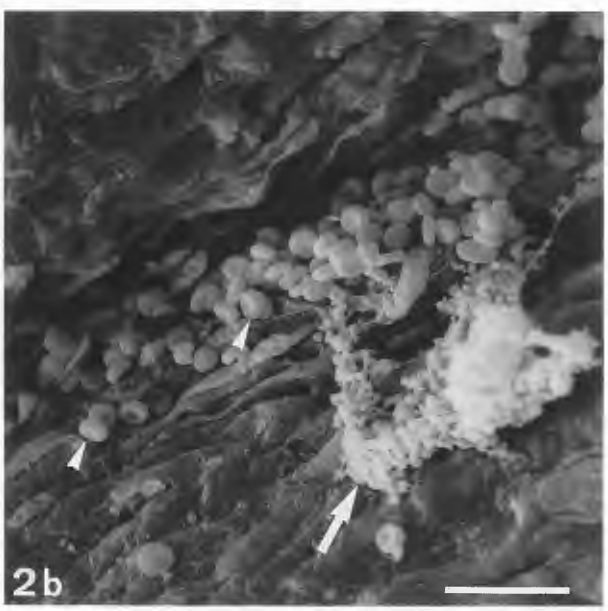
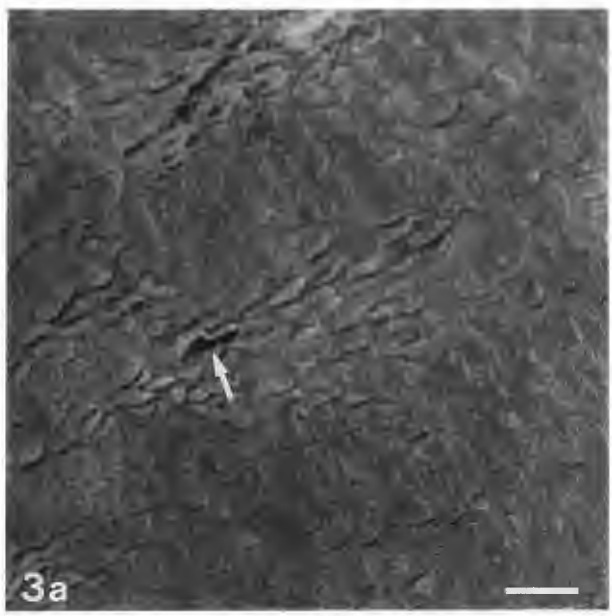
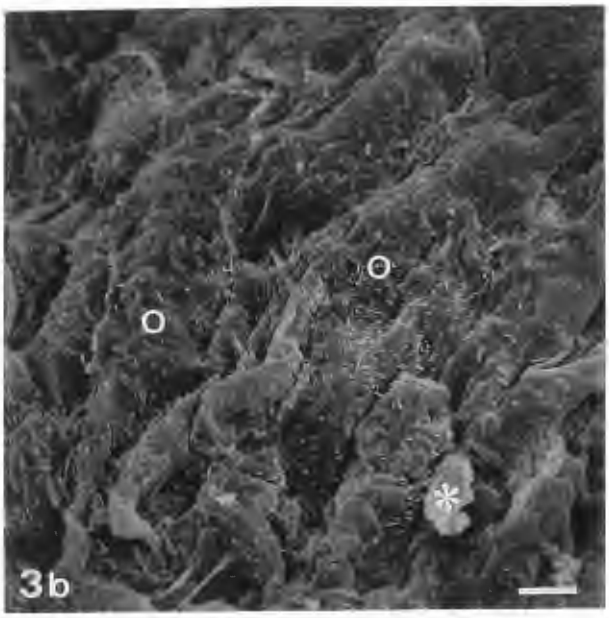
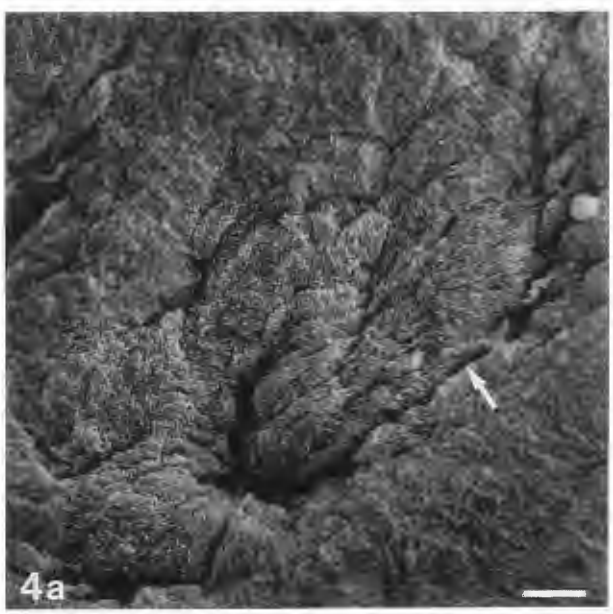


Figure 2. Control specimen (group I) with flat, continuous endothelium. Most of the cells are ovoid in shape (**a**). Deposits of red blood cells (arrowheads) or platelets (arrows), as shown in (**b**), are rarely seen. Bars = 30 μm (in a) and 20 μm (in b).

Human albumin (Fig. 5): A common feature of specimens rinsed with human albumin was the comparatively large extent of endothelial cell loss, accompanied by significant dissociation of cells. In four out of ten specimens, intimal fractures were also seen. Deposits were occasionally detected, especially in denuded areas.







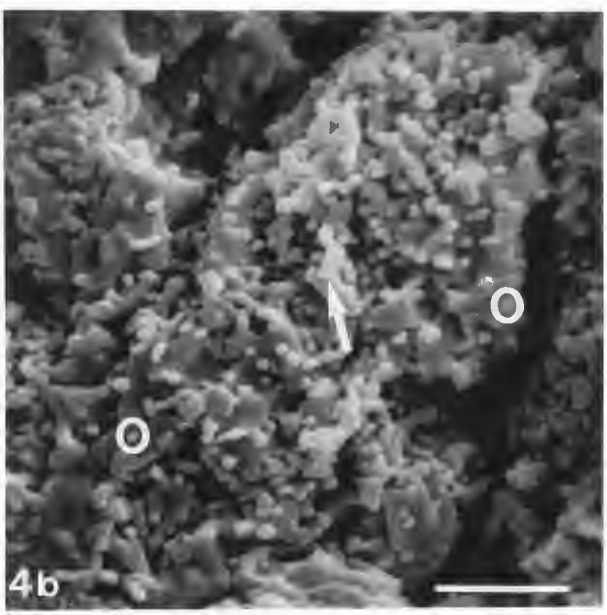


Figure 3 (a and b). Specimen rinsed with heparinized whole blood (group II) showing a higher degree of dissociation of endothelial cells (arrow). By comparison to controls, the endothelial cell surface is rougher and the shapes of cells vary. In places, remnants of detached endothelial cells are visible (*). Note the increased number of spikes (o). Bars = 30 μm (in a) and 10 μm (in b).

Figure 4 (a and b). Specimen rinsed with Bretschneider's HTK (group III), showing dissociation of endothelial cells (arrow) and a rough surface caused by membrane alterations. Both blebs (thick arrow) and spikes (o) are frequently seen phenomena. Bars = 20 μm (in a) and 3 μm (in b).

The endothelial cells were heterogeneous in terms of shape. Abnormally large numbers of spikes were seen at times.

Ringer's solution (Fig. 6): All specimens had an extremely altered surface structure with severe endothelial cell loss and dissociation. The endothelial cells

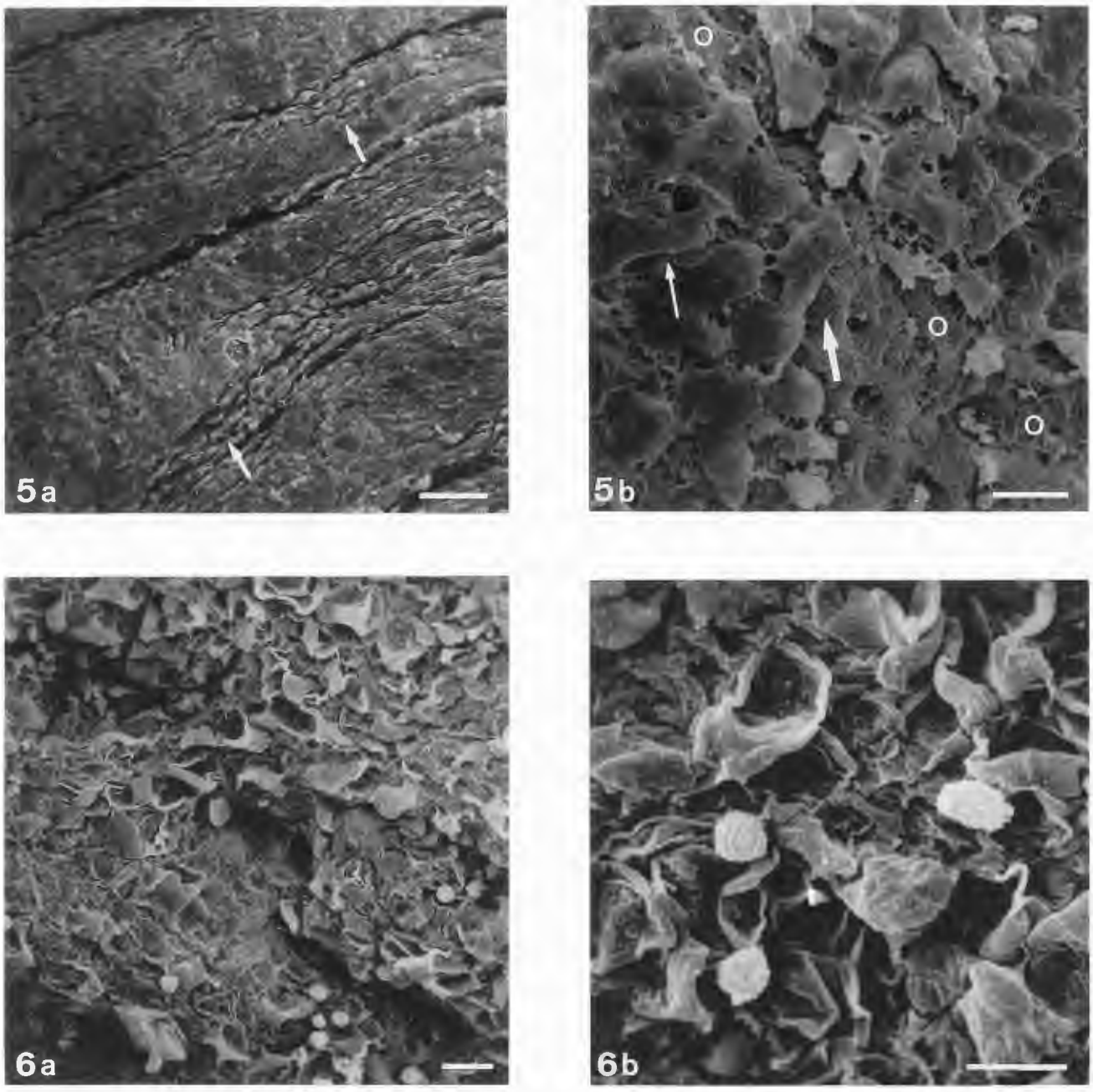


Figure 5 (a and b). Segment of a vein incubated with human albumin (group IV) showing a higher degree of endothelial cell loss (o) and dissociation (arrows) than controls. Frequently, the subendothelial layer and bundles of collagenous fibers are exposed (thick arrow). Bars = 50 μm (in a) and 15 μm (in b).

Figure 6 (a and b). Segment of a vein incubated with Ringer's solution (group V) with an extremely heterogeneous and rough endothelial cell surface. Numerous folds and clefts, as well as detached or dissociated endothelial cells, predominate. Bars = 30 μm (in a) and 10 μm (in b).

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were no longer typically flat and extended in shape but, instead, numerous folds and clefts created a rough surface. Spikes and blebs were seen to the same extent as in the group rinsed human albumin.

Semiquantitative scoring (Tables 1 and 2)

The analysis using the parameters suggested by Gundry *et al.* (1980), gave the lowest values for the controls (group I: 10). The specimens rinsed with

Table 1. Scoring of the endothelium of autologous vein grafts by the method suggested by Gundry *et al.* (1980). Group I, controls; group II, heparinized whole blood; group III, Bretschneider's HTK; group IV, human albumin; group V, Ringer's solution. All values represent group scores.

Parameter	Group I	Group II	Group III	Group IV	Group V
extent of coverage of intima with endothelial cells	1.5	2	1	11	7
separation of endothelial cells	4	10	11.5	23	13.5
loss of endothelial cells	0.5	2	4	14	11.5
exposed bundles of collagen fibers	2	5	2	7	6
intimal fracture	2	0	1	4	1
cumulated group score	**10**	**19**	**19**	**59**	**39**

Table 2. Result of the suggested scoring system for the endothelium of autologous vein grafts using parameters that reflect both gross damage to endothelial cells and integrity of the endothelial cell surface. Group I, controls; group II, heparinized whole blood; group III, Bretschneider's HTK; group IV, human albumin; group V, Ringer's solution. All values represent group scores.

Parameter	Group I	Group II	Group III	Group IV	Group V
separation of endothelial cells	4	10	11.5	23	13.5
loss of endothelial cells	0.5	2	4	14	11.5
amount of deposits	6	10.5	8.5	9	6
surface homogeneity of endothelial cells	9	20	30	31	28.5
frequency of spikes	7	10.5	26	17	10
frequency of blebs	0	5	8	7	4
cumulated group score	**26.5**	**48**	**88**	**101**	**73.5**

heparinized blood (group II: 19) and Bretschneider's HTK (group III: 19.5) did not clearly differ. However, the difference to the controls is obvious. Similar results were obtained for specimens incubated with human albumin (group IV: 59) and Ringer's solution (group V: 39). Thus, these parameters allow us to discriminate between controls and test groups. However, differences between test groups were not reflected by the scores even though the morphology revealed clear differences.

The evaluation of additional parameters, such as homogeneity of endothelial cell shape and frequency of spikes and blebs, resulted in a more differentiated scoring that better reflected the morphological changes (Table 2).

Discussion

Numerous studies suggest that the accumulated patency rate of aorto-coronary bypasses depends on vessel damage that is present before implantation (Lüscher, 1991), mechanical irritation during removal (Grondin, 1984), hypoxia and stretching (Hofer *et al.*, 1981), as well on the solutions used for rinsing, distension and short-term storage of the vein grafts (Angelini *et al.*, 1989; Chiavarelli *et al.*, 1992; Lawrie, 1990; Mattila *et al.*, 1987; Sottiurai *et al.*, 1983; Wagner, 1990). Apart from pre-existing vessel damage, all of these parameters can be positively affected by proper surgical handling. However, no clear rationale for the choice of the rinsing media has yet been proposed. Currently, a variety of crystalloid and cardioplegic solutions as well as heparinized blood are used but all of them may have disadvantages; for example, they are cytotoxic to or functionally impair the endothelial cells (Angelini *et al.*, 1989; Cantinella *et al.*, 1982; Chiavarelli *et al.*, 1992; Dhein *et al.*, 1991; von Oppel *et al.*, 1990; Welz *et al.*, 1991).

This problem has been approached by morphological examinations of the damage induced by different incubation media. However, the results have often been contradictory. Bush *et al.* (1986), for example, found that normothermic whole blood preserved both the biochemical function, as well as morphological appearance, of the endothelium significantly better than hypothermic saline solution. Welz *et al.* (1991), by contrast, regarded Bretschneider's HTK as the best preservation medium, whereas Mattila *et al.* (1987) reported dramatic alterations to the endothelium after exposure to cardioplegic solution. Haudenschild *et al.* (1981) as well as LoGerfo

et al. (1981) concluded that both adventitial *in situ* application of the smooth-muscle relaxant papaverine and extracorporeal maintenance of the vessel at body temperature are of higher importance for the preservation of an intact endothelium than the incubation medium used. However, in most clinics today, the vein grafts are rinsed and stored at room temperature.

Against this background, in our own morphologic studies, we focused on a comparison of the most frequently used incubation media. According to our results, heparinized blood as the rinsing and incubation medium is superior to the other crystalloid or cardioplegic solutions investigated since only limited damage occurred in this medium.

In order to facilitate comparisons of the influence of incubation media on endothelial preservation, semiquantitative scoring systems of different morphological parameters have been introduced. A widely accepted system is that of Gundry *et al.* (1980). However, our preliminary studies have shown that this scoring system does not reflect the extent of morphological changes. Therefore, the aim of our study was to examine both morphologically and semiquantitatively the effects of various incubation media on the endothelium.

The double-blind assessment of the specimens showed that all controls could be positively identified as controls. The specimens in the test groups showed a wide range of alterations. However, the grading of the endothelial alterations by the system of Gundry *et al.* (1980) did not reflect the extent of damage. For example, the group score for Bretschneider's HTK did not differ from that of group III. However, the morphologic examination revealed major changes in the specimens treated with Bretschneider's HTK, whereas those rinsed with heparinized blood had only slight alterations. In our opinion, this discrepancy is due to the fact that most of the parameters suggested by Gundry *et al.* (1980) do not encompass the morphological or functional impairment of single endothelial cells, for example, alterations in the cytoskeleton or membrane topology but, instead, they are mainly focused on the morphological consequences of endothelial cell death. None of the parameters mentioned by Gundry *et al.* (1980) allow predictions to be made as to the fate of damaged but not (yet) dead endothelial cells.

At least two parameters scored by Gundry *et al.* (exposure of bundles of collagen fibers and intimal fractures) appear to be of little value since they do not contribute to a better discrimination between the test groups. Furthermore, the parameters exposure of bundles of collagen fibers, exposure of the basement membrane and coverage of the intima with endothelial cells describe on principle direct consequences of the parameter loss of endothelial cells. They should, therefore, be omitted.

By contrast, the evaluation of parameters that relate to membrane and cytoskeletal changes, for example, the frequency of spikes and blebs, reflects the morphological findings much more effectively and results in a more differentiated scoring. When these parameters were used, the scores for the individual test groups showed significant differences. In particular, the parameter "frequency of spikes and blebs" contributed to large differences between the group scores. Increases in the frequency of spikes and blebs need not be associated with any alterations in the parameters suggested by Gundry *et al.* (1980).

In conclusion, we regard a combination of the parameters listed in Table 2 as being suitable for scoring of damage to vein grafts. However, it should be kept in mind that this scoring system reflects static damage only. Functionality can only be assessed indirectly from these scores. However, the results presented in this morphological study widely parallel the results of isometric tension recordings in an organ bath (Zerkowski *et al.*, 1993). In this study on 30 patients, vein segments were incubated in the same solutions. All controls responded to noradrenaline; in heparinized whole blood, Bretschneider's HTK, human albumin and Ringer's solution, the numbers of responders were 29, 25, 13 and 12, respectively. Of those, 21, 11, 0 and 1, respectively, showed an endothelium-dependent relaxation after administration of acetylcholine, whereas all controls showed a relaxation. From these data, storing and rinsing in heparinized whole blood is far better than the other examined solutions. The results of the functional tests of Bretschneider's HTK and human albumin paralleled the morphological results. The lower score of Ringer's solution compared to group III and IV was, however, not reflected in the functional test results.

Hence, we suggest that any morphological analysis of the intima of vein grafts should be accompanied by pharmaco-physiological testing of the endothelial function, for example, as described by Zerkowski *et al.* (1993).

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Discussion with Reviewers

R. Christofferson: The only relevant end-point should be the accumulated patency rate for the grafts treated according to different protocols. How did your patients do?

Authors: This study gives only answers on the influence of different incubation media on the endothelium *in vitro*. The vein segments used for bypass surgery were mainly stored in heparinized whole blood or Bretschneider's HTK. Since we used in our experiments four test groups, we cannot compare the outcome of the patients to our results. For a clinical trial, a far higher number of patients is required because of the huge variety of patient inherent factors. Therefore, we did not present data on the patients treated.

S. Aharinejad: Your results show that patient's own blood is the best incubation medium for venous grafts, with least damage to the endothelial cells after 1 hour incubation. How would the same grafts (and their endothelial cells) look like, if they would be implanted and reperfused? I think reperfusion along with a longer follow up would be more conclusive, particularly because endothelial cells of venous grafts will undergo morphological changes due to (high) arterial blood pressure anyway.

Authors: Vein grafts used for bypass surgery undergo a variety of morphological changes including hypertrophy of the media. Little is known on the mechanisms of endothelial cell adaptation processes, but it is generally accepted that the endothelial damage should be kept as low as possible. We can only speculate on the mor-

phological appearance of endothelial cells of reperfused vein grafts used for bypass surgery, since any re-explantation is ethically and medically unfeasible. Animal experiments on this item, which are on principle possible, were not yet carried out to our knowledge.

S. Aharinejad: An implanted venous graft will release many endothelial factors. Endothelial cells would behave totally different once they are under shear stress. Would intravital microscopy be a good tool to focus on this tissue?
Authors: We believe that the thickness of the arterialized vessel wall will limit the outcome of this approach. Intravascular ultrasonography could give some information on the vessel's behavior and physical properties in an animal experiment, but no or little information on cellular level. However, it should be kept in mind the devices used for intravascular ultrasonography may damage the endothelium themselves.

W.R. Wagner: What are the functional implications of spikes and blebs? Has any work demonstrated this morphology in association with an inflammatory state or in response to an inflammatory mediator?
Authors: Spikes and blebs on the cellular and subcellular level are indicative for alterations of the cell skeleton. In part, they may be reversible. Inokuchi *et al.* (1993) showed blebs as early ultrastructural changes in glomerular epithelial cells in acute puromycin aminonucleoside nephrosis and Moreira *et al.* (1992) pointed out the high occurrence of endothelial blebs and cytoplasmic projections in capillary blood vessels after experimental injection of snake venom. According to Faa *et al.* (1992), multiple blebs are regularly found as early changes during thioacetamide-induced apoptosis.

G. Pasquinelli: Have you any experience with the use of the University of Wisconsin (UW) cold storage solution?
Authors: We did not include the UW solution in our study since it is (at least in Europe) not frequently used in coronary artery bypass grafting.

G. Pasquinelli: Why did you not perform matched experiments hypothermically? Endothelial cells when exposed to intracellular electrolyte equivalent preservation solutions (e.g., B-HTK) at normothermia or room temperature undergo cytotoxic changes.
Authors: Of course hypothermia slows metabolism and is therefore used for extracorporeal preservation of entire organs. However, Haudenschild *et al.* (1981) pointed out that this rationale is not applicable to venous grafts, since the time span is relatively short, and the contracted smooth muscle cells have difficulties to relax in hypothermia. They found less damage in grafts stored at body temperature. We have chosen room temperature to reflect as close as possible the clinical situation.

Additional References

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