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Evaluation of Endothelium Loss by in vitro Vessel Perfusion Using Millipore Filters

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Summary

Endothelial loss of isolated rabbit femoral artery and renal artery was evaluated during *in vitro* vessel perfusion. Desquamated endothelial cells were captured on millipore filters from the perfusion solution outflow of the vessel in 3 intervals lasting 5 minutes each. In the first 5 minutes of perfusion the endothelial loss was 1 289.2 ± 166.5 cells: in the interval after a 30 minute perfusion 4 967.9 ± 1 428.3 cells were caught on the filters, 3.9 times more than in the first interval. During and after the 2 minutes air bubble perfusion the endothelial catch was 5.5 times greater as compared to the second interval with the average of 27 473 ± 6 209.6 cells. The present method of quantification of the endothelial cell loss in the *in vitro* vessel perfusion experiment makes it possible to obtain informations about the actual state of the endothelial lining and to contribute to more precise evaluation of the modulatory effect of the endothelium on vessel reactivity to pharmacological agents.

Key words
Vessel perfusion – Endothelium loss – Millipore filter

Introduction

Damage of the endothelial lining of blood vessels modifies the vasomotor reaction to different stimuli (Furchgott and Zawadzki 1980, Angus and Cocks 1989/. Thus, interpretation of experimental data requires the knowledqe of the endothelial lining status in tested vessel specimens. Evaluation of the vessel segment contractility related to the histologically verified endothelial status (Kriška *et al.* 1989) does not enable assessment of endothelial loss in relation to the quality or quantity of the stimulus, during the experiment, but the final condition. Determination of the number of cells caught on the millipore filter from the perfusion medium is a possible approach to the dynamic evaluation of the endothelium loss. Almost complete deendothelization by perfusion with air bubbles (Ralevic *et al.* 1989, Kriška *et al.* 1989) can render the total referential amount of endothelial cells in the vessel segment.

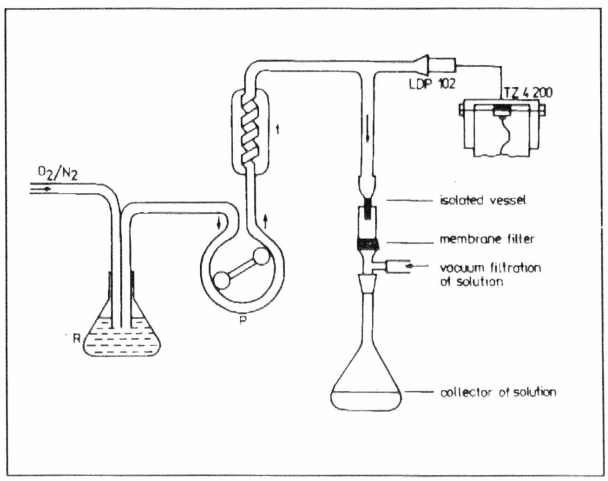

Fig. 1
The method of endothelial cell collection during perfusion of isolated vessels. R – reservoir, P – peristaltic pump, t – thermostatic heater, LDP 102 – pressure transducer, TZ 4200 – recorder. Perfusion solution from isolated vessels was collected through the millipore apparatus on membrane millipore filters under conditions of vacuum filtration of the solution.

Material and Methods

Male rabbits (Cincilla)(6-12 months old) were sacrified by cervical dislocation. A segment of the abdominal aorta together with the renal arteries, iliac

and femoral arteries was carefully removed up to knee level. Cannulae 1.0 mm in diameter were introduced into 5 femoral and 3 renal arteries through the branching from the aorta with a 5 mm long free segment. The cannulated vessel segments were placed into the perfusion system (Fig. 1) and perfused with Tyrode's solution at 37 °C, pH = 7.3–7.4 with a constant flow 20 ml per min on the average and monitoring of pressure changes in the system (Kriška and Kovalčík 1973). Basic pressure in the perfusion system was 15–20 mm Hg. The circulating medium with a total volume of 500 ml was saturated with room air during the experiment.The perfusate was collected in a container over the filter with 3 lm pores (Millipore, FRG) on a Millipore apparatus (FRG) in three intervals lasting 5 minutes each:

I. during the first 5 minutes of vessel perfusion
II. after 30 minutes of perfusion
III. during and after 2 minutes air bubble perfusion immediately following the II.interval. Bubbles were generated by a perfusion pump and were drawn through the whole lumen, where they broke up the endothelial lining.

Filter preparation:

The filters were fixed for 2 hours in 10 % formalin, washed in tap water, stained with haematoxylin and eosin and mounted in artificial resin Solakryl (Synthesia, ČSFR) and covered by cover glass (Frost *et al.* 1967).

Cell counting:

Each filter was divided into quarters and cells in one quarter were counted under a light microscope using 10 x objective and a micrometer disk with a 5 x 5 square grid. The paired Student t-test was used for statistical analysis.

Histology :

After finishing the experiment the vessels were filled with 1 % Bacto-agar (Difco laboratories, USA) at 40 °C to preserve endothelium *in situ* and processed by a routine paraffin method. Perpendicular slices were stained with haematoxylin and eosin.

Results

During the first 5 minutes of perfusion there was a pressure decrease in the system because of relaxation of the vessel segment. In this interval, only some isolated cells were desquamated with an average 1 289.2 ± 166.5 (Tab. 1). Endothelial cells caught on the filter had elongated shapes with oval longitudinally oriented nuclei (Fig. 2). The microphotographs were taken from a millipore filter preparation which causes a cloudy background and the cells represent three-dimensional objects often distorted by the experimental procedure itself.

The number of captured cells in the second interval was 4 967.9 ± 1 428.3 representing a 3.9 fold increase. The endothelial cells were predominantly isolated. In some groups up to 20 cells were found.

In the third interval during and after the perfusion of air bubbles there was massive desquamation of the endothelial lining with groups of up to 300 cells present (Fig. 3). The average number of captured cells on the filter was 27 473 ± 6 209.6 representing an increase 5.5 times greater than that in the second interval.

Histological specimens disclosed complete deendothelization of the vessels with an intact nude basal membrane.

Discussion

In our former *in vitro* vessel perfusion experiments, it was evident that hypoxia and application of various vasoconstrictive agents led to considerable changes in the endothelium characterized as a loss of endothelial cells and electronmicroscopically detected dystrophic changes (Babál *et al.* 1990). The histological evaluation of the perfused vessels did not provide information about the endothelial losses at different stages of the perfusion experiment. In contrast, evaluation of the endothelial loss during different time intervals using the millipore filter system allowed a continual follow-up of the state of the endothelial lining in perfused vessel segments. The number of desquamated endothelial cells in the first interval was low and represented mostly cells dislodged during the surge of the perfusion solution. To low desquamation of endothelial cells might contribute considerably to the relaxation of the vessel segment during the first 5 minutes which is followed by increased flow velocity. This may particularly diminish "the mechanical force" acting on endothelial cells associated with the flow. During the following 30 minutes there was a 3.9 fold increase of captured cells. The finding of solitary cells suggested disseminated scattered desquamation of endothelial cells during the perfusion. This corresponded to our previous histological observations of maximal loss of as much as 20 % of cells during this interval (unpublished data). The 5.5 fold increase of desquamated cells by the perfusion with air bubbles, representing the total remaining endothelium in the vessels, confirmed that the loss during period II. was about 20 %. Such endothelial loss could be explained by the nonoptimal conditions existing during the experiment.

Furchgott *et al.* (1984) demonstrated that the presence of at least 1/3 of endothelial lining was sufficient to indemnify from the modulatory effect of

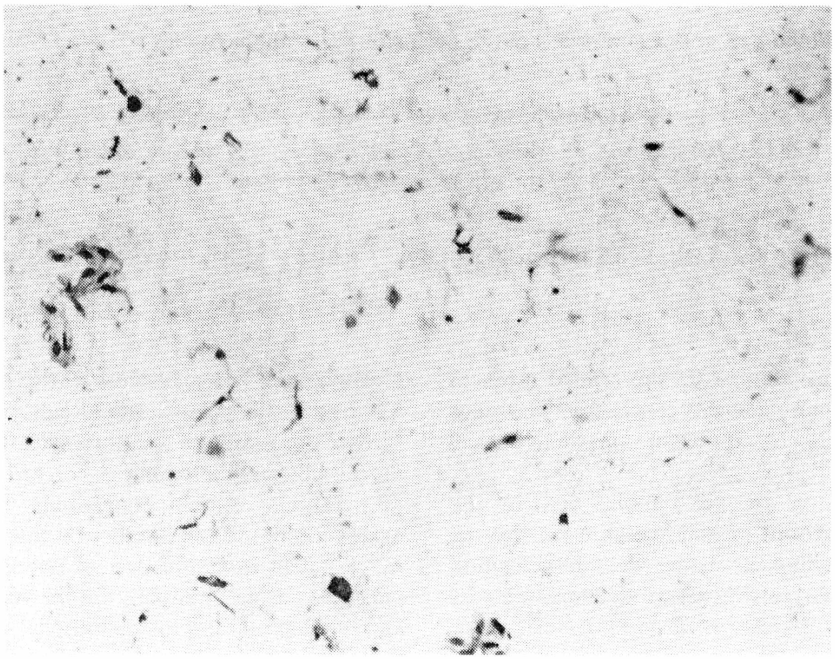

Fig. 2
Millipore filter with captured endothelium desquamated during the first interval of the experiment. The endothelial cells are mostly isolated having an elongated shape with oval longitudinally oriented nuclei. Haematoxylin and eosin (HE). 200 x.

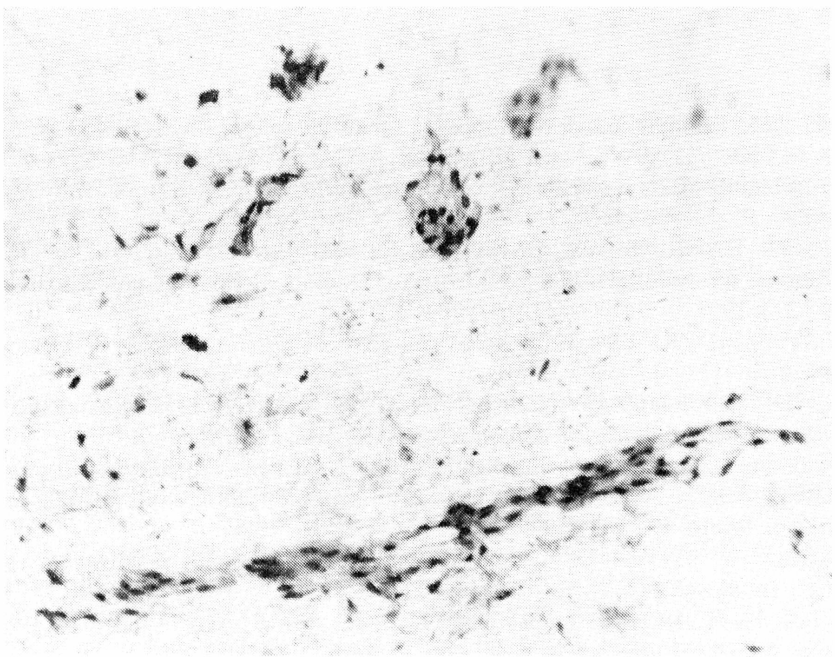

Fig. 3
Millipore filter with desquamated endothelial lining after air bubble perfusion with the presence of groups of up to 300 cells (HE). 200 x.

Table 1

Evaluation of endothelial loss at three intervals of the perfusion experiment (n=8)

I.	II.	III.
1 289.2±166.5	4 967.9±1 428.3*	27 473±6 209.6**

Means ± S.E.M., comparison I.-II., II.-III.interval * $p<0.05$, ** $p<0.01$

the endothelium. This represents the crucial moment of reliability in evaluation of the constrictive reactions by perfused vessels to different pharmacological stimuli.

The perfusion by air bubbles led to the dislodging of large groups of endothelial cells. Passing air bubbles represented a strong deendothelization stimulus which has also been documented by histological findings (Krišska *et al.* 1989, Ralevic *et al.* 1989).

Quantitative evaluation of the endothelial losses has not been used routinely. Hladovec (1978) evaluated *in vivo* endotheliaemia by counting the cells in a Burker's counting chamber. A very effective way of evaluating the endothelial cell loss of *in vitro* perfused vessels could be the use of an electronic cell counter. However, the latter, because of the presence of several hundreds cell groups, would not be useful whithout further processing of the perfusate. The application of morphometric photoanalysis for cell counting on filter preparations should contribute to making the evaluation of the cell number on filters more effective.

The quantification of endothelial cells in the perfusate using millipore filters represents a simple and reliable method for endothelial cell loss evaluation during perfusion experiments. It provides information about the actual status of the endothelial lining in perfused vessel segments during the experiment. Application of this method can contribute substantially to a critical evaluation of *in vitro* studies of vasomotor regulatory mechanisms and their pharmacological modulation.

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