Original Article

In vitro mitochondrial test to assess haemodialyser biocompatibility

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Abstract

Background. This paper describes an in vitro mitochondrial test to assess the biocompatibility of haemodialysers.

Methods. We tested on isolated liver mitochondria the effect of solutions obtained by an aqueous rinse of different haemodialysers (cuprophane, cellulose acetate, Hemophan, polyacrylonitrile, polymethylmethacrylate, polysulphone, polyamide). Moreover, to determine the penetration into the cell and the cytotoxicity of these solutions from haemodialysers, we examined the effect of rinse solutions on HT29-D4 cells.

Results. Our results showed that rinse solutions from haemodialysers decrease the mitochondrial ATP synthesis. Cuprophane has the most marked effect, and the synthetic membranes exhibited only mild effects. Rinse solutions penetrated the cell and were cytotoxic by acting on mitochondria in the cell. In this respect, cellulotic membranes were the most toxic.

Conclusion. Taken together our findings lead to a classification of haemodialyser membranes which is identical to one based on criteria such as activation of complement (cuprophane > other cellulosics > synthetics). Moreover isolated mitochondria make it possible to differentiate among the synthetic membranes. Isolated mitochondria thus appear to be a good in vitro test to assess the biocompatibility of haemodialysers.

Key words: ATP; biocompatibility; haemodialyser; mitochondria

Introduction

Given the fundamental role of mitochondria, any alteration of their functional integrity will ultimately have a profound effect on cellular metabolism. Isolated mitochondria have been thus used in our laboratory to study adverse effects of some drugs [1–3]. This led to the idea that such a mitochondrial model could be used to assess the toxicity of rinse solutions from haemodialysers (RSH).

During haemodialysis, patients’ blood is exposed to many artificial compounds derived from the membrane, dialysate, blood lines, and residual materials resulting from the manufacturing process such as phthalate, polycarbonate, and polyurethane. Hence, a variety of biologically active substances that may induce pathophysiological events in the peripheral blood are generated. These substances include proinflammatory fragments which are generated as a result of activation of complement. Cell activation during haemodialysis has widely been assumed to be mediated by complement (for a review see [4] and [5]), but some reports point to the possibility of complement-independent blood-cell activation. For example it has been proved that contact of various cells with cuprammonium membranes causes an increase in cytosolic calcium (Ca²⁺), followed by release of PGE, TXB₂, and IL₁ independent of complement [6]. Also the degranulation of neutrophils by cuprophane membrane is partially dependent on complement activation and partially dependent on heat-stable plasma factors whose action is complement independent but dependent on calcium [7]. Also phagocytes can be activated by a complement-independent mechanism [8], and granulocyte activation during haemodialysis in the absence of complement activation seems to be mediated by calcium ions [9].

This calcium-dependent biocompatibility might originate at least in part in mitochondrial alterations. But to assign a biological relevance to the in vitro results obtained with isolated mitochondria it was necessary to verify that the RSH can penetrate the cell membrane to reach mitochondria. The aim of this investigation was thus to study the action of RSH on isolated liver mitochondria and on cells in culture. We show that aqueous rinses of haemodialysers fitted with different types of membranes can be classed into three groups according to their mitochondrial toxicity: cuprophane, cellulotic, and synthetic membranes. This classification corresponds exactly to those established on the basis of other tests [4,5]. Moreover, through a mitochondrial
impairment, RSH are cytotoxic to cells in culture. It thus becomes possible to propose that isolated liver mitochondria can act as an in vitro test of the biocompatibility of haemodialysers. Preliminary results of this study have already been published [10].

Subjects and methods

Reagents

Sterile and non-pyrogenic water was purchased from Fresenius Laboratory; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Dulbecco’s medium, and all the other reagents were Sigma Chemical products.

Obtaining rinse solutions from haemodialysers

We rinsed the ready-for-use haemodialysers twice by passing 2 litres of non-pyrogenic and sterile water through the blood compartment of the dialysers at 150 ml/min and at 20°C. The first single-pass rinse was then rejected and the second single-pass was lyophilized. The residue was redissolved in non-pyrogenic and sterile water and relyophilized twice, thus eliminating all the volatile substances such as ethylene oxide. The final residue was then dissolved in 3 ml of non-pyrogenic and sterile water for the mitochondrial study or in 3 ml of culture medium for the cellular study. This concentrated material (about 666 times) was called rinse solutions from haemodialysers (RSH). As a control, we obtained solutions from blood lines under the same conditions. Three to six dialysers of each type were thus extracted. We studied capillary haemodialysers fitted with seven different membranes (Table 1): cuprophane (CU), cellulose acetate (CA), Hemophan (HM), polyacrylonitrile (PAN), polymethylmethacrylate (PMMA), polysulphone (PS), and polyamide (PA).

Mitochondrial study

Preparation of mitochondria. Mitochondria were obtained from rat livers by using a modified version of the technique of Johnson and Lardy [3,11]. Briefly, male Wistar rats (170–220 g) were cervically stunned and decapitated. Mitochondrial isolation procedure was carried out at 4°C. Liver tissue (6–10 g) was rapidly excised, blotted, washed, and minced in extraction buffer (0.25 M saccharose, 10 mM Tris-HCl, 1 mM EGTA, pH 7.8, adjusted with HCl). Tissue was suspended in this buffer and homogenized. The suspension was centrifuged at 600 g for 10 min and the pellet discarded. The supernatant was then centrifuged at 15000 g for 5 min. The brown mitochondrial pellet was gently homogenized in 20 ml of washing buffer (0.25 M saccharose, 10 mM tris-HCl, pH 7.8, adjusted with KOH). The suspension was centrifuged twice at 15000 g for 5 min. The pellet was then homogenized with 100 μl of respiration buffer (0.25 M saccharose, 4 mM KH2PO4, 1 μM rotenone, pH 7.2, adjusted with KOH) and stored in ice until use. Mitochondrial protein was determined using Biorad Assay (Bio-rad laboratories).

Respiration rate measurements. Oxygen consumption was measured in a water-jacketed reaction chamber (1.8 ml) at 25°C by using a Clark microelectrode fitted to a Gilson oxygraph. Mitochondria (1.5 mg of protein) were suspended in respiratory medium (Saccharose 0.25 M, KH2PO4 4 mM, rotenone 1 μM, pH 7.2). The substrate was sodium succinate (6 mM final concentration). Oxidative phosphorylation was initiated by the addition of ADP to a final concentration of 0.10 mM.

Oxygen consumption rate during state 3 (V3) expressed as nmoles oxygen/min/mg of protein corresponds to the ATP synthesis (Figure 1). Oxygen consumption during state 3 allows calculation of the P/O ratio, which corresponds to the number of ADP molecules phosphorylated per oxygen atom consumed. P/O represents the yield of ATP synthesis. Oxygen consumption rate corresponding to state 4 (V4) is

![Fig. 1. Schematic representation of O2 consumption by mitochondria. a, Control; b, in the presence of RSH. Arrows indicate the addition of ADP.](image)

<table>
<thead>
<tr>
<th>Membranes</th>
<th>Dialysers</th>
<th>Sterilization agent</th>
<th>Area (m2)</th>
<th>Manufacturers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cuprophane (CU)</td>
<td>Disscap 160E</td>
<td>ETO</td>
<td>1.20</td>
<td>Hospal</td>
</tr>
<tr>
<td>Cellulose acetate (CA)</td>
<td>Altra Nova 140</td>
<td>ETO</td>
<td>1.40</td>
<td>Althin</td>
</tr>
<tr>
<td>Hemophan (HM)</td>
<td>FoCus 160 HG</td>
<td>ETO</td>
<td>1.60</td>
<td>National Medical Care</td>
</tr>
<tr>
<td>Polyacrylonitrile (PAN)</td>
<td>Filtral 12</td>
<td>ETO</td>
<td>1.15</td>
<td>Hospal</td>
</tr>
<tr>
<td>Polymethylmethacrylate (PMMA)</td>
<td>BK 1-6U</td>
<td>γ-ray</td>
<td>1.60</td>
<td>Toray</td>
</tr>
<tr>
<td>Polysulphone (PS)</td>
<td>Hemoflow F60</td>
<td>ETO</td>
<td>1.30</td>
<td>Fresenius</td>
</tr>
<tr>
<td>Polyamide (PA)</td>
<td>Polyflux 130</td>
<td>ETO</td>
<td>1.30</td>
<td>Gambro</td>
</tr>
</tbody>
</table>
reached when all ADP is phosphorylated into ATP. Respiratory control (RC) is the ratio $V_3/V_4$. The mitochondrial used exhibited a RC between 3.0 and 4.5. These values were low because the respiratory medium did not contain bovine serum albumin and EGTA in order not to modify the calcium concentration in the medium.

The effect of membranes was tested by adding increasing volumes of RSH to the reaction chamber. Each RSH was tested with at least three different mitochondrial preparations. Each measurement on a given volume of RSH was made in duplicate. In these conditions, each result represents the mean of at least six measurements. Similar experiments were performed in the presence of glycerol or rinses from blood lines.

Expression of results. The deleterious effects of the different RSH were expressed as percentage of alteration as follows:

$$\% \text{ of Alteration} = \frac{(V_3 \text{ or } P/O)(\text{Control}) - (V_3 \text{ or } P/O)(\text{RSH})}{(V_3 \text{ or } P/O)(\text{Control})} \times 100$$

Cellular study

Cells from human colon adenocarcinoma cell line, HT29-D4, were maintained in Dulbecco's modified Eagle's medium supplemented with 4.5 g glucose/L, 0.1 M sodium pyruvate plus 10% fetal bovine serum, l-glutamine, penicillin, and streptomycin [12].

Increasing volumes of RSH were added in wells containing $2.10^4$ HT29-D4 cells. The total volume was 200 µl. The cytotoxicity and cell penetration of RSH were assessed by the tetrazolium assay [13] as previously described (MTT test) [12]. Briefly, MTT was dissolved in phosphate-buffer solution at 5 mg/ml and filtered to remove insoluble residue. At the end of cell incubation with RSH (24 h), the MTT solution (10 µl per 100 µl of medium) was added to all wells. Plates were then incubated at 37°C for 4 h. Dimethylsulphoxide (150 µl) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. Then the optical densities (OD) of the wells were measured on a Dynatech MR 7000 apparatus at a wavelength of 550 nm. Before cell treatment, we established a linear relationship between MTT assay and cell number. Three independent experiments were performed in quadruplicate. Controls are values in the table correspond to one series of experiments. $V_3$, rate of oxygen consumption during state 3 corresponding to the ATP synthesis. $V_4$, rate of oxygen consumption during state 4 when all ADP was consumed. The rates are expressed as nmol of O$_2$/min/mg of protein. Respiratory control $RC = V_3/V_4$. $P/O$, number of ADP molecules phosphorylated per oxygen atoms consumed. It represents the yield of ATP synthesis.

Statistical analysis

Unpaired Student’s $t$ test and linear regression analyses were used. The null hypothesis was rejected at a value of $P<0.05$. Data are given as mean ± SEM [14].

Results

The rinses from blood lines had no effect on mitochondria or cells (not shown). Similarly, glycerol proved totally non-toxic (not shown).

Tables 2 and 3 show the deleterious effect of CU membrane on $V_3$, $V_4$, RC, and P/O. The effect was dose dependent; Figure 2 illustrates this dependence for $V_3$ in the case of the different membranes. Similar curves were obtained for P/O. The data were treated by linear regression analysis. In the first approximation the best relationship between $V$, the volume of RSH tested, and E, the effect observed, was given by the
equation:
\[
\log \frac{E}{100-E} = a \log V + \log K
\]

where \(E\) = observed effect expressed as percent, \(V\) = volume of RSH (\(\mu l\)), \(a\) = the slope value of the straight line and \(K = 1/(V_{50})^a\). From the regression thus obtained, we calculated the \(V_{50}\), which corresponds to the volume of RSH that yields the half-maximal effect (EC\(_{50}\)). The \(V_{50}\) values allowed us to compare the different membranes. Table 4 shows the \(V_{50}\) values calculated for \(V_3\) and P/O for each membrane. These values were compared two by two and differed significantly one from the others \((P<0.05)\) except for CA and HM.

In Table 5, are shown as an example the OD values obtained in the presence of various doses of cellulose acetate during an MTT assay.

Figure 3 shows the variation of proportion of killed cells as a function of RSH volumes. The effects of synthetic membranes (PAN, PMMA, PS and PA) were negligible in the range of volumes plotted in the Figure, we did not represent them. As above, the data were treated by linear regression analysis, and we calculated \(V_{50}\) values for each membrane. These values were compared two by two and differed significantly one from the others \((P<0.001)\) except for CA and HM (Table 6).

In Table 5, are shown as an example the OD values obtained in the presence of various doses of cellulose acetate during an MTT assay.

![Figure 3. Variations in the percentage of dead cells versus volumes of different RSH.](image)

**Table 4.** \(V_{50}\) values calculated for each membrane with regard to their effect on \(V_3\) and P/O

<table>
<thead>
<tr>
<th>Membranes</th>
<th>(V_{50}(\mu l))</th>
<th>(V_3)</th>
<th>P/O</th>
</tr>
</thead>
<tbody>
<tr>
<td>CU</td>
<td>68 ± 13</td>
<td>440 ± 57</td>
<td></td>
</tr>
<tr>
<td>CA</td>
<td>187 ± 22</td>
<td>537 ± 58</td>
<td></td>
</tr>
<tr>
<td>HM</td>
<td>220 ± 45</td>
<td>545 ± 64</td>
<td></td>
</tr>
<tr>
<td>PMMA</td>
<td>469 ± 72</td>
<td>803 ± 52</td>
<td></td>
</tr>
<tr>
<td>PAN</td>
<td>635 ± 92</td>
<td>892 ± 47</td>
<td></td>
</tr>
<tr>
<td>PS</td>
<td>1120 ± 145</td>
<td>1425 ± 112</td>
<td></td>
</tr>
<tr>
<td>PA</td>
<td>1531 ± 200</td>
<td>1778 ± 217</td>
<td></td>
</tr>
</tbody>
</table>

Cuprophone differs significantly from the others \((P<0.001)\). CA and HM do not differ significantly. The group of synthetic membranes differ significantly from the others \((P<0.001)\). In this group, each membrane is significantly different from the others \((P<0.05)\).

**Discussion**

Both in the mitochondrial model and on HT29-D4 cells neither glycerol, which is commonly used in the manufacturing of some haemodialyser membranes, nor rinses from blood lines had any effect under our experimental conditions. The aqueous rinses from haemodialysers were lyophilized three successive times to obtain the final RSH. It is thus possible to attest that ethylene oxide (ETO) is totally removed when the haemodialysers were sterilized with this compound. Therefore neither glycerol, nor ETO, nor rinses from blood lines interfere in our results.

RSH are uncoupling agents for isolated liver mitochondria; as an example we show the results with cuprophone (Table 2 and 3). Respiratory control is decreased in a dose-dependent manner and the uncoupling is particularly evidenced by a reduction in \(V_3\). In the same way P/O ratio was diminished. Consequently CU acts through a decrease in ATP synthesis, since \(V_3\) is related to the rate and P/O ratio to the yield of this synthesis. The curves in Figure 2 and results in Table 4 demonstrate that the mitochondrial toxicity of RSH depends on the type of membrane used.

Cellulose membranes appear to be more toxic on
mitochondria than do synthetic membranes. CU is the most potent as proved by its low $V_{50}$ values: $68 \pm 13 \mu l$ and $440 \pm 57 \mu l$ for $V_3$ and P/O ratio respectively. The difference between CA and HM is not statistically significant: about $200 \mu l$ and $540 \mu l$ for $V_3$ and P/O respectively. Moreover our results permit us to differentiate among the synthetic membranes and clearly, under our experimental conditions, PS and PA exhibited only mild mitochondrial toxicity: $V_{50} > 1000 \mu l$ for all the parameters.

The MTT test is based on the measurement of the dark blue intensity produced when the MTT is incubated with living cells. This colour appears because of the cleavage of the tetrazolium ring by active mitochondria, and occurs only in living cells. This test is thus classically used to determine the cytotoxicity of a substance [13]. Our results (Tables 5, 6 and Figure 3) demonstrate that RSH are cytotoxic depending on the type of membrane used. Cuprophane appears to be more potent than the others as proved by its low $V_{50}$ value: $45 \pm 6 \mu l$. Results in Table 6 show that membranes may be classed according to their cytotoxicity: first CU, then an intermediate group including CA and HM with a $V_{50}$ value of about $200 \mu l$ and a third group, the least cytotoxic, corresponding to the synthetic membranes, PMMA, PAN, PS, and PA, with $V_{50}$ value greater than $300 \mu l$.

The cytotoxicity of the different RSH can be related to their penetration into the HT29-D4 cells and their uncoupling effect on mitochondria. This assertion is supported by the correlation observed between the in vitro potencies of the different membranes as uncoupling agents on liver mitochondria (Table 4) and their cytotoxic properties on HT29-D4 cells (Table 6). Moreover our results are obtained with living mitochondria but may be more general and affect mitochondria from other cells. Mitochondria from different types of cells are not exactly the same. For example in the liver, kidney, lung, and smooth muscle, mitochondrial Ca$^{2+}$ movements are driven by a Na$^{+}$-independent pathway, whereas in the heart and brain, Ca$^{2+}$ mitochondrial release can be induced by Na$^{+}$ [15]. In contrast, whatever the origin of mitochondria, ATP synthesis is under the dependence of the same fundamental pathways. The decrease in ATP synthesis induced by RSH in liver mitochondria could thus occur in all types of mitochondria and affect cells other than hepatocytes like HT29-D4 cells.

Even if the precise relation between mitochondria and in vivo biocompatibility remains to be established, it now becomes possible to assign a biological relevance to our results obtained in vitro with a mitochondrial model. Indeed RSH enter the cells and alter their function. Moreover we observed a peroxidant effect on liver mitochondria could thus occur in all types of organs: an overview. Blood Purif 1987; 5: 100–111


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