The Isolated Perfused Bovine Uterus as a Model for Mucous Membrane Irritation and Inflammation

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Summary
Isolated perfused bovine uteri were used to study the irritation potential of antiseptics frequently used in veterinary practice for the treatment of endometritis. Comparable to the isolated perfused bovine udder (Kietzmann et al., 1993), the viability of uteri obtained directly after slaughtering of cows, was tested with the help of biochemical parameters. These parameters of the viability were nearly unchanged over five hours. Thereafter, different antiseptics were tested for their irritation potential on the mucous membrane with the help of the MIT-assay. Here the irritant effect of Lugol’s iodine solution is demonstrated exemplarily. An additional study was performed to establish a hemoperfused uterus. An injection of arachidonic acid into the serosa induces a visible inflammatory reaction and a marked increase of the prostaglandin E2 synthesis. In conclusion, the isolated perfused bovine uterus seems to be a promising in vitro model for mucous membrane irritancy as well as for inflammatory reactions.

Keywords: isolated perfused bovine uterus, irritation, inflammation

1 Introduction
Based on experiences with the isolated perfused bovine udder which is used as an ex vivo model in different areas of experimental pharmacology (Kietzmann et al., 1993; Eshraghi and Zeitlin, 1997; Ehinger and Kietzmann, 2000; Bäumer and Kietzmann, 2000), the isolated perfused bovine uterus was supposed to be established. In contrast to the udder which is predominantly used for skin irritation and penetration experiments, the isolated (hemo)perfused uterus was taken to examine mucous membrane irritation and inflammatory processes. Ex vivo perfusions of uteri are already described for sheep (Peirce et al., 1970), rats (Suzue, 1992), mice (Suzue, 1994) and man (Bulletti et al., 1986; Tojo et al., 1972). These studies served as a model to establish the isolated perfused bovine uterus.

To determine the viability of the organs, glucose consumption, lactic acid production and the LDH liberation were determined. Additionally, the viability of the mucous membrane was determined with the help of the MTT (methythiazol tetrazolium) assay. In the following experiments different antiseptics were tested for their irritation potential on the mucous membrane. The origin of this study was the point at issue whether a treatment of chronic endometritis with antiseptics is useful or due to massive destruction of tissue contraindicated (Kietzmann, 1999). Therefore, antiseptics which are widely used in veterinary practice for treatment of chronic endometritis were instilled into uteri. The influence of these antiseptics on the viability of the mucous membrane, measured by means of the MTT assay, is demonstrated here exemplarily for a 5%
Lugol’s iodine solution. Apart from this question, directly connected with the medical treatment of the bovine uterus, further studies were made to transfer the already established inflammation model of the udder (Bäumer and Kietzmann, 2000; 2001) to the uterus. For this study the uteri were hemoperfused and a serositis was induced by arachidonic acid. The increase of prostaglandin E$_2$ and a visible reddening were taken as endpoints.

2 Material and methods

2.1 Obtaining the uteri
All studies were performed with uteri from Holstein Friesian dairy cows. The uteri were taken from slaughtered cows older than two years. The uteri were cut off the gut 10 minutes after bleeding out. The uterine horns had to be two to three fingers thick, the ovaries had to have a corpus luteum and there had to be no sign of inflammatory processes (endometritis). Furthermore the vessels to be cannulated had to be uninjured. To avoid clot formation, the organs were perfused with 300 ml heparinised Tyrode solution via the A. uterina directly after the severance. For studies on irritancy potential the organs were perfused with tyrode solution, for the study on inflammatory processes a hemoperfusion using homologous blood was performed.

2.2 Tyrode solution perfusion
Directly after transport to the laboratory the uterus was connected to the perfusion apparatus. This results in a perfusion 30-45 minutes after slaughtering. The uterus was weighted and put on a fixation mattress (CP-Pharma, Burgdorf) into a prewarmed (39°C) incubator (Incubator S.L60, Stuart Scientific). For studies on irritancy potential the cervix was lift up and fixed to avoid an outflow of administered substances. After fixation the Aa. uterinae were cannuled and perfused. Additionally, the main efferent vessel, the V. ovarica was cannuled at each side. The perfusion medium was a carbogen gassed (95% O$_2$, 5% CO$_2$), blood isotone tyrode solution. The tyrode solution was prewarmed and reached the organ with 39°C. A flux of 0.7 to 1 litre per hour (depending on the size of the organs) was achieved by a peristaltic pump (Colora Messtechnik, Lorch/Württ.). The flux and the viability were determined via the venous drainage obtained by silicone tubes. The organ was covered with a humid bandage to avoid drying up.

2.3 Hemoperfusion
The hemoperfusion used for studies on inflammatory processes was realised by bovine blood (heparinised with 18,000 IU/l) taken from cows slaughtered at the same time. Logistically it was not possible to obtain blood and organ from the same animal. For the following studies the organs were laid in a bath filled with tyrode solution. The uteri were perfused with tyrode solution to remove remaining blood. Following this the organs were perfused with a blood-tyrode mixture (4:1). The blood mixture was oxygenised by means of the counter-current-principle with a dialysis (Braun, Melsungen) circle (Fig. 1). As an additional parameter of viability a periodic contraction and relaxation occurred during blood perfusion.

2.4 Determination of the viability of isolated perfused bovine uteri
The experiments concerning the viability of the tissue were made to demonstrate that the isolated perfused bovine uterus is vital over a sufficient time period. Therefore, seven uteri were perfused with tyrode and hemoperfused respectively for five hours. Every hour, glucose consumption, lactate generation and LDH liberation (Boehringer, Mannheim) were measured in the perfusate. After receiving the results of tyrode perfused uteri samples were taken after three and five

Fig. 1: Schematic portrayal of the isolated hemoperfusion of uteri.
hours from hemoperfused uteri. To estimate a developing edema by a weight difference the organs were weighted before and after the perfusion. The biochemical parameters of hemoperfused uteri were determined in centrifuged plasma. For comparison, the blood mixture was also taken directly before reaching the organ. It was demonstrated that the organs are vital over a time period of five hours, the already mentioned studies concerning irritancy potential and inflammation were started.

2.5 Irritancy potential of test substances

The target of the first study was to examine a possible irritancy potential of widely used antiseptics. Exemplary, the results of Lugol’s iodine solution are shown here (further investigations of antiseptics can be found at Mertens, 2001).

For comparison four uteri were treated by instillation of 100 ml tyrode solution and four uteri were not treated at all.

Lugol’s iodine solution (WDT, Garbsen) was obtained as an usual concentrate. 1 ml of the concentrate consists of 100 mg iodine and 200 mg potassium iodide. To get a customary solution (1:20) 50 ml of the concentrate were diluted with 950 ml distilled water. After an equilibration time of 30 minutes Lugol’s iodine solution (100 ml) was instilled (as usual in veterinary practice) with an insemination pipette through the cervix into the uterus. Directly before as well as 1h and 3h after instillation, biopsies of the mucous membrane were taken through the cervix with the help of a biopsypincers (Olympus, Deutschland). The biopsies were taken from the area of the big curvature of the left and right horn, shock frozen at -196°C and stored at -80°C. Three small biopsies were taken at each time point. The viability of the tissue was measured by means of the MTT-assay (methylthiazoletetrazolium).

2.6 MTT-assay

The biopsies of the mucous membrane were weighted and placed in 1 ml TKM buffer containing 0.1 ml MTT solution. After an incubation period of 2h (37°C) the samples were homogenised. 1 ml 0.1 N HCl in 2-propanol was added to the homogenate and the samples were centrifuged. After centrifugation the supernatant of each homogenate was measured photometrically (570 nm) in triplicate (MRX, Dynatech Deutschland GmbH, Denkendorf).

2.7 Induction of an inflammatory reaction by arachidonic acid (hemoperfusion)

5 mg arachidonic acid (Sigma, Deisenhofen) were solved in 40 µl acetone. Directly before the injection arachidonic acid was taken in 200 µl tyrode solution (vehicle) and injected under the serosa. Three hours after administration of arachidonic acid, biopsies of 6 mm diameter were taken and frozen at -196°C.

2.8 Determination of PGE

The biopsies were homogenated and the centrifuged supernatant collected. After a dilution (1:5), PGE was measured directly in the supernatant by ELISA according to manufacturers protocol (R&D Systems, Wiesbaden).

2.9 Statistical evaluation

The determination of significant differences between the different time points of treatment (irritancy study) and between vehicle treated and arachidonic acid treated areas (inflammation study) was done with the help of the Wilcoxon test (rank sum) for paired matches.

3 Results

3.1 Viability of the uterus

The results concerning the examination of the viability are shown in Table 1. The glucose consumption increases markedly during the first hour and lies between 0.13 and 0.21 g/h. The hemoperfused uteri are characterised by a higher glucose consumption (0.26-0.51 g/h; Tab. 1).

The lactate production decreases significantly within the first hour to values between 0.17-0.22 g/h (Tab. 1). At

<table>
<thead>
<tr>
<th>time of perfusion</th>
<th>glucose consumption (g/h)</th>
<th>lactate production (g/h)</th>
<th>LDH liberation (U/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>tyrode</td>
<td>blood</td>
<td>tyrode</td>
</tr>
<tr>
<td>0 min</td>
<td>0.1 ± 0.01</td>
<td>0.26 ± 0.05</td>
<td>0.29 ± 0.03</td>
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<tr>
<td>60 min</td>
<td>0.13 ± 0.01</td>
<td>not determined</td>
<td>0.22* ± 0.03</td>
</tr>
<tr>
<td>120 min</td>
<td>0.14 ± 0.03</td>
<td>not determined</td>
<td>0.22* ± 0.03</td>
</tr>
<tr>
<td>180 min</td>
<td>0.13 ± 0.03</td>
<td>0.37 ± 0.06</td>
<td>0.17* ± 0.03</td>
</tr>
<tr>
<td>240 min</td>
<td>0.17* ± 0.02</td>
<td>not determined</td>
<td>0.19* ± 0.01</td>
</tr>
<tr>
<td>300 min</td>
<td>0.21* ± 0.02</td>
<td>0.51 ± 0.06</td>
<td>0.21* ± 0.02</td>
</tr>
</tbody>
</table>

Tab. 1: Comparison of glucose consumption, lactate production and LDH liberation between tyrode solution and blood perfusion during a perfusion period of 5 hours. Mean and standard error of seven uteri; *p<0.05 (significant difference compared to 0 min).
the beginning of the hemoperfusion the lactate production is comparable to tyrode solution perfused uteri. But the production increases slightly at the end of the perfusion (0.16-0.31 g/h; Tab. 1).

The LDH liberation lies between 3 and 5 U/h. During the hemoperfusion the LDH liberation lies under the detection limit of 1 U/h (Tab. 1).

Five hours after perfusion of the seven uteri with tyrode solution the mean increase in tissue weight is approximately 20%. The hemoperfusion results in a similar increase in tissue weight.

3.2 Irritancy potential of Lugol's iodine solution

Untreated, or sham treated uterine mucous membrane consists of a white, smooth, shining surface. The uterine tissue has a soft consistence. After treatment with Lugol's iodine solution the mucous membrane shows a brown and rough surface.

As far as the MTT-turnover is concerned there is no significant difference between untreated and vehicle treated uteri. During three hours of perfusion a slight but not significant decrease of the MTT turnover is obvious (Fig. 2).

After intrauterine application of Lugol's iodine solution (5%) the MTT turnover decreases significantly (n=4). One and three hours after treatment with Lugol's iodine solution, the MTT turnover is reduced to an average of 18% compared to the beginning of the experiments (Fig. 3).

The treatment has no influence on the parameters of viability measured in the perfusate (glucose consumption, lactate production, LDH liberation), which indicates that the massive irritation is limited to the mucous membrane.

3.3 Arachidonic acid induced inflammation

Approximately 30 minutes after injection of arachidonic acid a distinct reddening at the site of injection is obvious. This reddening is absent in vehicle treated sites. This reddening lasts for 2 to 3 hours. Three hours after injection the PGE2 concentration in arachidonic acid treated areas is significantly increased. The amount of the response to the arachidonic acid treatment differs markedly (Fig. 4).

4 Discussion

4.1 Viability of the organ

Many different isolated perfused organs are described for studies on absorption, penetration and irritation. Most of these are models working with isolated perfused skin. To give some examples, there is the isolated perfused porcine skin flap (Monteiro-Riviere et al., 1985), the isolated perfused porcine ear (De Lange et al., 1992), in vitro perfused human skin (Hiernickel, 1983) and the isolated perfused bovine udder (Arens, 1991; Maass, 1993; Sterl, 1998). Ex vivo perfusions of uteri are described for sheep (Peirce et al., 1970), rats (Suzue, 1992), mice (Suzue, 1994) and man (Bulletti et al., 1986; Tojo et al., 1972).

The first aim to establish this model was to examine the irritancy potential of antiseptics which are widely used in veterinary practice. These antiseptics are instilled into the uterus for the treatment of endometritis. The used antiseptics irritate the mucous membrane. The following acute inflammatory process is said to heal previous chronic inflammatory processes (De Kruijf, 1999). The treated cows often show signs of pain like bended backs, lifted tails and groaning (Grüßel and Busch, 1998). The sense and purpose of this treatment is often questioned and should also be considered critically in respect to protection of animals (Gilbert and Schwark, 1992; Paisley et al., 1986). As invasive examinations on a living cow are ethically not to justify, studies on an isolated perfused organ were regarded as an alternative. In this publication the effect of one antiseptic (Lugol's iodine solution) is demonstrated exemplarily.

The performed pretrials proofed the viability of the model. In analogy to other isolated perfused organs the glucose consumption increases during five hours of perfusion from an average of 0.1 to 0.21 g/h (tyrode solution) and from 0.26 to 0.51 g/h (hemoperfusion) respectively.

For the isolated perfused bovine udder, Maass (1993) describes a quiet constant glucose consumption during six hours of perfusion (0.81-0.84 g/h). Sterl (1998) measures a doubling of the glucose consumption (0.5-1 g/h) and gives the reason in a recovery of the organ after preparation and transport of the udder. Monteiro-Riviere (1990) postulates a glucose con-

![Fig. 2: MTT turnover (μg formazan/mg) in mucous membrane biopsies of 4 untreated (1) and vehicle treated (2; 100 ml tyrode solution) uteri. There is no significant change of the MTT turnover.](image-url)
assumption of at least 0.01 g/h for the isolated perfused porcine skin flap. De Lange et al. (1992) measure a glucose consumption of 0.015 g/h for the isolated perfused porcine ear. Compared to the quoted skin models the isolated perfused bovine uterus reveals a high glucose consumption in relation to its weight.

The production of lactate in the perfusate decreases (tyrode solution) or increases (hemoperfusion) during five hours of perfusion. The initial drop of the lactic acid production can be explained by an adaptation to the circumstances of the laboratory experiment. After preparation and tyrode solution perfusion at the slaughterhouse and the transport to the laboratory (without perfusion) there is a transient anaerobic metabolism. After perfusion with oxygenated tyrode solution the metabolism switches obviously from anaerobic to aerobic glucose consumption, explaining the decrease of lactate production. These results correspond to findings of Bulletti et al. (1986) for the isolated perfused human uterus. In analogy to the bovine uterus there was a distinct decrease of the lactate production during three hours of perfusion with a Krebs-Ringer-glucose solution. The lactate production is nearly constant for the first six hours of perfusion but increases during the next six hours of perfusion to amounts of 0.5 mg/100 ml. Maass (1993) and Sterl (1998) also describe a decrease of the lactate production for the isolated perfused bovine udder and justify it with a compensation of initially anaerobic metabolism. The increase measured for the hemoperfused uteri has to be regarded with respect to a more distinct increase for the glucose consumption compared to tyrode solution. Both results may be explained by a generally higher metabolism in hemoperfused uteri. The average LDH liberation does not indicate a cellular damage. For the isolated perfused human uterus Bulletti et al. (1986) describe a decrease of the LDH liberation from 15 to 7 U/h. The values stay nearly constant until the end of perfusion (12 hours). Maass (1993) measures during a perfusion period of six hours values between 37 and 49 U/h for the isolated perfused bovine udder. Sterl (1998) measures a significant decrease of the LDH liberation from 40 to 30 U/h during four hours of perfusion. Monteiro-Riviere (1990) postulates a LDH activity of less than 10 U/h for the isolated perfused porcine skin flap whereas Riviere et al. (1986) postulate less than 30 U/h. The LDH activity measured in own experiments has to be estimated as low. Values under the detection limit in hemoperfused uteri can be interpreted as a sign for an intact organ during five hours of perfusion.

The degree of edema was determined by means of increase in tissue weight and histological examination after the end of perfusion. Within five hours of perfusion with tyrode solution, the weight of the uteri show an average increase of 20%. A moderate edema in the Lamina propria mucosae is apparent in histological sections (Mertens, 2001). An increase in tissue weight is also documented for other isolated perfused organs. Isolated hemoperfused porcine ears show an increase of approximately 6% within four hours of perfusion (de Lange et al., 1992). Maass (1993) describes an increase of 17% for isolated perfused bovine udders. Riviere et al. (1986) determine an increase in weight of 25–42% for the isolated perfused porcine
skin flap. The increase of weight is therefore comparable to other isolated perfused organs. A hemoperfusion of the uterus does not result in a diminished formation of edema.

Studies on mucous membrane irritancy have been performed to show early effects of antiseptic agents on the endometrium. The MTT assay was taken as endpoint as this assay offers the possibility to discriminate (mild) irritative from corrosive substances. After 1 and 3 hours the MTT turnover of the uterus horn treated with Lugol's iodine solution was decreased to less than 20%. This decrease lies distinctly below the limit of 50% for cellular viability, postulated by Swischer et al. (1988), confirming a massive cytotoxic effect of Lugol's iodine solution (1:20). Such a massive irritative, or more exactly, corrosive effect undoubtedly goes along with pain, explaining signs like bended backs, lifted tails and groaning.

The increase of prostaglandin E₂ and the reddening observed in this study confirm results of topical application of arachidonic acid onto the bovine udder skin (Bäumer and Kietzmann, 2000). Due to the hemoperfusion, the isolated perfused uterus is even more similar to in vivo conditions (Opas et al., 1985). Other mediators of inflammation, like interleukin 1β, interleukin 8, TNFα and nitric oxide are being determined right now in our laboratory. The dependence of the arachidonic acid model on the cyclooxygenase 1 (COX-1) pathway (Puigneré and Queralt, 1997) suggests further experiments to establish a more COX-2 mediated model of inflammation in the uterus using Carragenan (Harada et al., 1996) and LPS (Mafteier et al., 1990). Such a model could be utilised to examine antiinflammatory agents for their inhibitory selectivity on the COX-1 and COX-2 pathways simultaneously in an ex vivo model.

In conclusion the isolated hemo- or tyrodeperfused uterus seems to be a suitable model for different fields of research, which is confirmed by first results from studies on mucosal irritancy and inflammation.

References


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Sondermeldungen aus aktuellem Anlass:
**CH: Ergebnisse der Vernehmlassung zur Revision des Tierschutzgesetzes vorgestellt**


Generell stossen Kann-Bestimmungen in Tierschutzkreisen auf Widerstand, welche den Bundesrat ermächtigen aber nicht bindend verpflichten, einen bestimmten Gegenstand zu regeln. Es kann daraus ein gewisses Misstrauen bzw. die Angst vor einem realen Abbau des bestehenden Schutzniveaus abgeleitet werden.

Die an verschiedenen Stellen des Vorentwurfes enthaltenen Regelungen bezüglich Ausbildung und Weiterbildung von Personen, die mit Tieren umgehen oder mit dem Vollzug des Tierschutzrechts befassen sind, werden positiv gewertet.

Kontrovers werden die Verwaltungsmaßnahmen diskutiert, vorab das von einem Kanton ausgesprochene Tierhalteverbot, das neu für die ganze Schweiz gelten soll. Während das Prinzip an sich begrüsst wird, herrscht bei der Zugriffsberechtigung auf die Liste der Halteverbote noch keine Einigung. Auch die Strafbestimmungen sind Gegenstand von Forderungen von Seiten der Tierschutzkreise, die durchgehend eine Verschärfung des Katalogs der Handlungen und der Strafmasse verlangen.

Neben den im Vorentwurf vorgeesehenen (hierarchischen) Kommissionen, die sich auf kantonaler und auf Bundesebene mit Tierversuchen befassen, verlangen Tierschutzkreise eine Eidgenössische Tierschutzkommission, und zusätzlich eine Tierschutzkommission für jeden Kanton; aus den Kantonen kommt zudem die Forderung nach einer Bundeskommission, welche die Zuchttöne zu beurteilen hätte. Die neuen Vollzugsinstrumente (Zielvereinbarung und Mitwirkung Dritter) werden positiv aufgenommen.
