

The Role of Endotoxins in Methscopolamine Induced Ruminal Stasis in Calves

By S. Aiumlamai and H. Kindahl

Department of Obstetrics and Gynaecology and Department of Clinical Chemistry,
Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Aiumlamai, S. and H. Kindahl: The role of endotoxins in methscopolamine induced ruminal stasis in calves. Acta vet. scand. 1992, 33, 129-138. – Methscopolamine was used to induce ruminal stasis in calves. Clinical and blood biochemical parameters were studied to judge the possible role of gastro-intestinal endotoxins from Gram-negative bacteria. Two trials were carried out where one injection of 100 mg and 3 consecutive injections of 70 mg of methscopolamine were administered. The animals showed signs of ruminal stasis. General clinical signs and changes in blood biochemical parameters were similar to what is found in endotoxaemia or in induced ruminal acidosis. Relevant parameters such as prostaglandin $F_{2\alpha}$ metabolite, endotoxin, iron, zinc, calcium and glutamate dehydrogenase changed significantly indicating exposure of endotoxins.

prostaglandin; blood biochemical parameters.

Introduction

It is known that acute febrile diseases cause gastro-intestinal dysfunction in most animal species. In ruminants, marked inhibition of forestomach motility is a frequent complication in febrile diseases (*van Miert 1987*). Endotoxins from Gram-negative bacteria induce, in addition to fever, hypocalcaemia and provoke stasis of the forestomachs (*Fredriksson 1984, Aiumlamai et al. 1990, Aiumlamai & Kindahl 1990*). In cows suffering from milk fever (hypocalcaemia) the rumen contractions are weak or even lacking. During milk fever the cows show clinical signs resembling those seen after administration of endotoxins, and the animals respond to calcium therapy. On the other hand during endotoxin induced fever and hypocalcaemia, calcium therapy does not restore forestomach motility (*Lohuis et al. 1988*). Also the

ruminal lactic acidosis syndrome is always followed by ruminal stasis both in experimentally induced acidosis (*Crichlow & Chaplin 1985, Andersen & Jarløv 1990, Aiumlamai et al. 1992*) and in spontaneously occurring acidosis (*Blood & Radostits 1989*). All these disease complexes have clinical and biochemical changes similar to what is found in endotoxaemia. Therefore, it is of great interest to study the hypothesis that gastro-intestinal endotoxins from Gram-negative bacteria play a role in these disease syndromes. Ruminal stasis and hypomotility of the forestomach are typical signs of all above-mentioned diseases. Thus, the studies of clinical and blood biochemical changes during ruminal stasis are of interest to relate to the findings of endotoxaemia and other diseases such as ruminal acidosis or parturient paresis.

In the present study, methscopolamine was used to induce ruminal stasis. Methscopolamine is an anticholinergic agent such as atropine, scopolamine and benzetimide. It blocks ganglia in the gut and exerts antimuscarinic effects. Since methscopolamine is a more polar compound compared to the others, it does not cross the blood-brain barrier (Davis 1986) and is also less toxic. It has been reported that scopolamine has an effect as inhibitor of the prostaglandin synthesis (Dorigo & Gaion 1984, Sumitomo *et al.* 1986, Adewunmi & Ojewole 1987). Several reports also indicate that maintenance of smooth muscle tone, regulated by prostaglandins, is disturbed by scopolamine (Sanders & Ross 1978).

Materials and methods

Animals

Four healthy female calves (A,B,C and D), 2 Swedish Red and White and 2 Swedish Friesian breed, aged 6 months, weighing about 100-110 kg, were used in the 1st study (A1-D1). These four calves and an additional calf of Swedish Red and White breed (E2) were used in a 2nd trial (A₂-E₂). The time period between the 2 trials was one month. The animals were kept separated and fed with 0.1 kg/day of crushed oats, hay and water *ad libitum* throughout the study period.

Methscopolamine

Methscopolamine (Metylskopolamin without preservative, Apoteksbolaget, Umeå, Sweden) was prepared in a pharmaceutical preparation and considered as endotoxin free (in a Limulus test found to be less than 0.06 EU/ml (Fribberger 1985)). The compound was injected subcutaneously in a dose of 100 mg in each calf after a 60 h control period in

the 1st trial. In the 2nd trial, 3 s.c. injections of 70 mg were given at 0, 8 and 16 h to each calf after a 24 h control period.

Blood sampling

Jugular vein blood samples were taken by puncture every 2 h for 60 h before and after methscopolamine injection in the 1st trial. In the 2nd trial, blood samples were taken every 2 h for 24 h before and 84 h after the 1st of the 3 methscopolamine injections.

Five ml of jugular vein blood were withdrawn into heparinized Vacutainer tubes, 10 ml into plain Vacutainer tubes and 5 ml into EDTA Vacutainer tubes (Becton and Dickinson, England). Plasma samples were centrifuged immediately. For serum tubes 30 min elapsed before centrifugation. Serum was transferred, with special care to avoid contamination of endotoxins from the environment, to another plain Vacutainer tube for endotoxin analysis and to a plastic tube for clinical chemistry analyses. All samples were stored at -20°C until analyses were performed. Whole blood was immediately analysed for total white blood cell number (WBC) and blood was smeared for differential counts.

Analytical methods

15-Ketodihydro-PGF_{2α} (PG), serum endotoxin (ET), calcium (Ca), zinc (Zn), iron (Fe), bile acids (BA), glutamate dehydrogenase (GLDH), sodium (Na), potassium (K), total white blood cell count (WBC) and differential count were analysed by dividing the cells in polymorphonuclear (PMN) and mononuclear cells (MN) according to generally accepted methods and described in full in an accompanying paper (Aiumlamai *et al.* 1992). In the 1st trial in this study, endotoxin was not analysed and in the 2nd trial, total white blood cell and differential count were not evaluated.

Clinical examination

Rectal body temperature (BT) was measured every time when blood was collected. Ruminal contractions (RC) were recorded every 2 h in daytime in the 1st trial. In the 2nd trial RC was recorded every 2 h in the daytime and during the first 40 h after the 1st injection of methscopolamine.

Statistical analysis

In the 1st trial (1 injection of methscopolamine), differences of all parameters, in every 12 h interval (7.00 am-7.00 pm), from individual animals, were evaluated by analysis of variance (the Statgraphics Version 4.0, STSC, Inc.) and a confidence interval test was used to compare means, separately for the control and experimental periods. Then, the values from the experimental period for every 12 h interval were compared with the values of the respective control period by Student's *t* test. In the 2nd trial of 3 injections of methscopolamine, 24 h samples during the control period were used to compare with every 12 h interval samples during the experimental period (84 h) by analysis of variance in each individual animal and a confidence interval test was used to compare means. To be judged as significant changes, the values must be out of assay variation and significantly altered when compared to the control period of the respective time and/or to different parts of the experimental periods of the 1st trial. In the 2nd trial, the same criteria were used to compare the control period to the different parts of the experimental period. In addition, the normal physiological ranges in healthy cattle (reference values) of these parameters were also used to consider the biological importance. This procedure does not relate to pH, RC and BT, where the significance was judged from comparisons

between the control periods and the variation within the experimental periods.

Results

Control period

Both in the 1st and 2nd trials, during the control periods, all animals were clinically normal. In the 1st trial, the changes of all parameters were evaluated of each individual animal, in every 12 h interval during the control period (60 h). Rectal body temperature was significantly increased in 2 animals: animal A₁ showed increased BT in daytime compared to during the night and animal D₁ showed the highest BT 25-36 h after the control period started. There was no change of BT in the other animals. The levels of RC, PG, Ca, Zn, BA, Na and WBC were not significantly changed during the control period and Zn, Na and WBC as well as Ca values were within the reference range. However, Ca values in animal D₁ were higher than the reference range. As regards Fe there were no significant changes in animals A₁, C₁ and D₁. The values for animals A₁ and C₁ were however higher than the reference range. In animal B₁, Fe levels were significantly decreased during the long period of frequent blood sampling, but the levels stayed within the reference range. The changes of GLDH were inconsistent, animals A₁ and B₁ showed no significant changes, animal C₁ showed a significant increase and animal D₁ showed a decrease. The levels of K showed a tendency to increase during the 37-48 h period after the start of the collection of samples in all animals but all values were within the reference range.

Experimental period

In the 1st trial, after the injection of 100 mg of methscopolamine, ruminal contractions ceased in all animals (within 1 h) and the left

flank showed signs of ballooning like during bloat. They stopped eating within 2 h after injection, showed signs of discomfort, difficult breathing, they laid down and sometimes kicked at their belly. These clinical signs lasted about 10 to 12 h. About 20 to 24 h after injection, the animals were quite normal and started to eat and ruminal contractions were again normal. In the 2nd trial after the 1st injection of 70 mg of methscopolamine, all animals showed similar clinical signs as during the 1st trial. The signs remained until the 2nd injection after which the clinical signs became more severe. The signs remained until 12 to 14 h after the last injection. The animals started to eat and ruminal contractions became normal.

Tables 1 and 2 summarize the changes of all parameters seen in the 1st and 2nd trials.

Ruminal contractions were significantly decreased immediately after injection in both trials in all animals. In the 1st trial ruminal stasis lasted during 0-12 h after injection in all animals and in the 2nd trial, during 0-36 h in animals B₂, C₂, D₂ and E₂ and 0-48 h in animal A₂ calculated after the 1st injection.

In the 1st trial, rectal body temperature showed a significant increase in animal D₁ during 13-36 h after injection and a tendency to increase in animal B₁ during 25-36 h after injection. There was no change in the other animals. In the 2nd trial, BT was significantly increased in animals A₂, B₂ and E₂ during 49-72 h, 13-24 h and 25-60 h after the 1st injection, respectively.

Endotoxin was analysed in the 2nd trial, animal E₂ showed a tendency to increase during 0-24 h and was then followed by a significant increase during 37-48 h and 73-84 h after the 1st injection. Animals C₂ and D₂ showed a tendency to increase during 0-12 & 37-72 h and 13-24 & 73-84 h after the 1st injection, respectively. Animals A₂ and B₂ showed no significant changes, however animal B₂ had spurious elevations both in periods 13-24 h and 49-72 h after the 1st injection.

Prostaglandin levels were significantly increased in animal D₁ during 0-12 h and then showed a tendency to decrease 13-36 h after injection, in animal A₁ PG showed a tendency to increase during 13-24 h and a tendency to decrease during 37-48 h after

Table 1. A summary of the changes in clinical and blood biochemical parameters seen in experimentally induced ruminal stasis in calves (1 injection of methscopolamine, 100 mg s.c.).

| Parameters | A ₁ | B ₁ | C ₁ | D ₁ |
|------------|------------------|------------------|------------------|------------------|
| RC | - | - | - | - |
| BT | 0 | (+) | 0 | + |
| PG | (+)(-) | (-) | (-) | + (-) |
| Ca | 0 ^a | 0 ^a | 0 ^a | 0 ^a |
| Zn | (-) ^a | (-) ^a | (-) ^a | (-) ^a |
| Fe | - _b | - _b | - _b | - _b |
| BA | - | - | - | (-) |
| GLDH | (+) | + | (-) + | - |
| Na | 0 ^a | 0 ^a | 0 ^a | 0 ^a |
| K | (-) ^b | (-) ^a | (-) ^b | (-) ^b |
| WBC | (+) ^a | (+) ^a | (+) ^a | (+) ^a |
| MN | (-) ^a | (-) ^a | (-) ^a | (-) ^a |
| PMN | (+) ^a | (+) ^a | (+) ^a | (+) ^a |

Key for signs: Please see Table 2.

Table 2. A summary of the changes in clinical and blood biochemical parameters seen in experimentally induced ruminal stasis in calves (3 injections of methscopolamine, 70 mg s.c. in each injection).

| Parameters | A2 | B2 | C2 | D2 | E2 |
|------------|----------------|----------------|-------------------------------|-------------------------------|----------------|
| RC | - | - | - | - | - |
| BT | + | + | 0 | 0 | + |
| ET | 0 | 0 | (+) | (+) | + |
| PG | 0 | (+) | (+) | + | + |
| Ca | - ^a | - ^a | - ^a | - ^a | - ^a |
| Zn | - ^b | - ^b | - ^a | - ^a | - ^b |
| Fe | - ^b | - ^b | - ^b + ^b | - ^b + ^b | - ^b |
| BA | -+ | -+ | - | 0 | -+ |
| GLDH | (-) + | - | -+ | -+ | - |
| Na | 0 ^a | 0 ^a | 0 ^a | 0 ^a | 0 ^a |
| K | - ^b | - ^b | 0 | - ^b | - ^b |

+ Indicates significant increase ($p < 0.05$), - indicates significant decrease ($p < 0.05$) and 0 indicates no significant changes. () indicates a tendency to increase or decrease. Superscript "a" indicates that the values are in the normal reference range and superscript "b" indicates that the values are out of the normal range (if no superscript is given it means that no reference range is described for that parameter in cattle). Two signs in 1 cell mean that both events occurred. The reference ranges of these parameters are used in Department of Clinical Chemistry, Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences, Uppsala, Sweden.

injection in the 1st trial. In other 2 (B_1 and C_1), PG had a tendency to decrease during 25-36 h and 37-48 h, respectively, after injection. In the 2nd trial, PG was significantly increased in animals D_2 and E_2 during 0-12 h and 13-24 h after the 1st injection, respectively. In animals B_2 and C_2 , PG levels showed a tendency to increase during 49-84 h and 13-24 & 73-84 h, respectively, after the 1st injection. There was no change in animal A_2 .

The levels of Ca did not show any significant changes in the animals of the 1st trial. However, during 6-12 h after injection, low levels were recorded in all of them (a decrease of about 10%). In the 2nd trial, Ca levels were significantly decreased in all animals, starting immediately from the 1st injection to the end of the study in animals A_2 , C_2 and D_2 and during 0-24 h and 49-60 h after the 1st injection in animals B_2 and E_2 . The Ca values

were within the reference range in all animals in both studies.

In the 1st trial, the levels of Zn showed a tendency to decrease within the reference range in all animals during 13-36 h in animals A_1 and B_1 , 13-48 h and 13-24 h in animals C_1 and D_1 , respectively. In the 2nd trial, Zn levels were significantly decreased in all animals during 13-36 h in animals A_2 and C_2 , 0-36 h in animals B_2 and D_2 and 13-48 h in animal E_2 . The levels of Zn were below the reference range in animals A_2 , B_2 and E_2 , and within the normal range in animals C_2 and D_2 .

The levels of Fe were significantly decreased in all animals in the 1st trial and the values were below the reference range. The levels significantly decreased during 13-48, 0-48, 0-60 and 0-48 h in animals A_1 , B_1 , C_1 and D_1 , respectively. Similar findings were found in

the 2nd trial with lower levels found in all animals, the significant decrease occurred during 0-72 h in animals A₂ and E₂, 0-60, 13-48 and 0-60 h in animals B₂, C₂ and D₂, respectively, after the 1st injection. In animals C₂ and D₂ the levels of Fe also increased significantly later during 61-84 h after injection.

Serum bile acids showed a significant decrease in animals A₁, B₁ and C₁ and a tendency to decrease in animal D₁ during 13-24, 0-24, 0-36 and 0-24 h, respectively. In the 2nd trial, BA levels were significantly decreased and then increased in animals A₂, B₂ and E₂, animal C₂ showed only a significant decrease and there was no change in animal D₂. The decrease of the BA levels occurred during 25-36, 0-36, 0-36 and 0-12 & 25-36 h after the 1st injection, in animals A₂, B₂, C₂ and E₂, respectively.

In the 1st trial, the activity of GLDH was significantly increased in animals B₁ during 0-24 h and C₁ during 49-60 h and showed a tendency to increase in animal A₁ during 13-24 & 49-60 h but significantly decreased in animal D₁ during 13-60 h after injection. Animal C₁ showed also a tendency to decrease during 25-48 h after injection and then followed by the significant increase. In the 2nd trial, in animals C₂ and D₂, GLDH showed a significant decrease during 13-48 h and 13-24 h after the 1st injection then followed by a significant increase during 61-84 h and 25-60 h, respectively. Animal A₂ showed a tendency to decrease in the activity of GLDH during 25-48 h and followed by a significant increase during 49-84 h after the 1st injection. The activity of GLDH was significantly decreased in animals B₂ during 13-72 h and E₂ during 0-84 h after injection.

The levels of Na both in the 1st and 2nd trials showed no significant changes and the values were within the reference range. The levels

of K showed a tendency to decrease during 0-12 h after injection in all animals with the levels below the reference range, except in animal B₁ with levels still within the reference range. In the 2nd trial, K levels were significantly decreased in animals A₂, B₂, D₂ and E₂ during 0-12 h after the 1st injection and no change in animal C₂. The levels were below the reference range in all animals.

The changes of WBC, PMN and MN were evaluated only in the 1st trial, and all animals showed similar patterns. WBC showed a small increase during 6-14 h after injection and there was a shift in WBC in that PMN increased in levels concomitant with a decrease of MN during the same period.

Discussion

During the control period in the 1st trial, there were no significant changes in most of the studied parameters. A few parameters changed inconsistently e.g. in 1 animal GLDH and Fe showed a significant decrease during the long period of frequent blood sampling. In a previous study where the same blood sampling frequency was employed more pronounced changes were seen of some biochemical parameters in the control period as well as more obvious day and night differences in body temperature (*Aiumlamai et al.* 1992). The animals in that study were younger and the relative blood loss was greater. The changes during the control periods have been considered in the interpretation of the results from both trials.

In the 1st and 2nd trials, all animals behaved very similarly in most of the recorded changes both regarding clinical and blood biochemical parameters. No results from the 2 trials, except for prostaglandin metabolite levels, are contradicting. If 3 injections are used, a more pronounced and prolonged

response is seen as compared to 1 injection. Immediately after injection of methscopolamine, a ruminal stasis appeared in all animals in both trials during about 12 h after 1 injection and 48 h after 3 injections. This drug did not show any clinical side effects and was not harmful to the animals. They recovered and were clinically normal after the experiment had finished.

The general trends of clinical and blood biochemical changes, demonstrated in this study, showed evidence of that ruminal stasis is linked to resorption of endotoxins from the gastro-intestinal tract causing endotoxaemia/endotoxiosis. Leukocytosis and shift of WBC, an increase in number of PMN and concomitantly a decrease in the number of MN, were found in all animals. These changes accord well, with what has been demonstrated after injection of low doses of endotoxin (Yagoda *et al.* 1990 a,b). In calves overfed with oats, causing ruminal acidosis and stasis, similar changes in clinical and blood biochemical parameters were seen (Aiumlamai *et al.* 1992). It was concluded in that study that a low amount of endotoxins might have been resorbed from the gastro-intestinal tract. Most of the studied parameters discussed below support the hypothesis that endotoxins are resorbed in low amounts to the blood circulation also after methscopolamine induced stasis: increase in body temperature, endotoxins, prostaglandin metabolite levels and GLDH as well as decrease in iron, calcium, zinc and bile acids. Similar findings were found after endotoxin injection (Aiumlamai *et al.* 1990, Aiumlamai & Kindahl 1990). Furthermore, the decrease in ruminal contractions in this study caused by methscopolamine might later on have been enhanced by the effect of endotoxins. In the 2nd trial, the endotoxin levels showed a significant increase or a tendency to

increase in 3 out of 5 animals and 1 had some spurious elevations. The increase in the body temperature is also a strong support to that endotoxins have been resorbed, since endotoxins are known as strong pyrogens (Milton 1982, Dinarello 1983). Both trials showed significant or tendencies of increases of BT, 2 out of 4 after 1 injection and 3 out of 5 after 3 injections. Prostaglandin F_{2α} is a dominating endotoxin-mediated product and is a very reliable parameter for estimating the effect of endotoxins on the arachidonic acid cascade (Fredriksson *et al.* 1985, Aiumlamai *et al.* 1990). After 1 injection in the present study, PG levels increased in 1 animal and showed a tendency to increase in 1 animal followed by a tendency to decrease and in the 2 remaining ones it showed a tendency to decrease. Methscopolamine has been used as a muscarinic blocking agent causing relaxation of the gastro-intestinal tract but it has also been discussed as one in a group of inhibitors of prostaglandin synthesis (Dorigo & Gaion 1984, Adewunmi & Ojewole 1987). However, its potency to inhibit the prostaglandin biosynthesis is not known. This can explain the decrease of PG levels as seen in the 1st trial. However in the second trial PG showed an increase in four out of five animals. This might be explained by the fact that in the 2nd trial the ruminal stasis was both more severe and prolonged compared to the 1st trial, probably resulting in a more pronounced endotoxaemia. In that situation it is possible that the inhibiting effect of methscopolamine is not enough to block the synthesis of prostaglandin. Even though the animals were treated with a substance inhibiting PG-formation the net result will be increased levels of the PG-metabolite. Thus, from the finding of PG levels it can also be concluded that an endotoxaemic state has occurred. The levels were not highly

increased as found after injection of higher doses of endotoxin (Fredriksson *et al.* 1985, Kindahl *et al.* 1990) which also can impair reproductive performance. However, this low amount of PG release in ruminal stasis which was also found in acidosis/overfeeding (Aiumlamai *et al.* 1992) could possibly impair reproductive function when it turns into a circulus vitiosus.

The decreases of Fe, Zn and Ca accord well with the findings during experimentally induced endotoxaemia (Luthman *et al.* 1990, Aiumlamai & Kindahl 1990) as well as in experimentally induced acidosis (Andersen & Jarløv 1990, Aiumlamai *et al.* 1992). The clear profile of changes of these parameters makes it likely that endotoxins were resorbed from the alimentary tract to the blood circulation. The absence of changes in Na and the decrease in K levels are similar to the results obtained after endotoxin injection and induced ruminal acidosis (Aiumlamai & Kindahl 1990, Aiumlamai *et al.* 1992).

Bile acids decreased in the 1st trial and in the 2 trial BA first decreased and then increased above the basal levels in 3 of the animals. The low levels of bile acids found are probably due to the alimentary stasis and reflect the decreased enterohepatic circulation. This will lead to less bile acids available to the peripheral circulation (Olsson 1986, Aiumlamai *et al.* 1990). However, the reason for the increase later on is not known.

Two animals in the 1st trial and all in the 2nd one showed initially a decrease in the activity of GLDH. A decrease was also seen in experimentally induced acidosis/ruminal stasis (Aiumlamai *et al.* 1992). This decrease cannot be fully explained but seems to be linked to the induced stasis and/or some effect on the liver cells. In most of the animals an increase in the GLDH activity is seen later on. This can be due to an impaired

liver function and also to a more direct liver cell damage. Mullen (1976) described that GLDH is a good indicator for damaged hepatocytes although small increases of the enzyme were found.

Interestingly, the alimentary stasis in the calves demonstrated clinical and blood biochemical findings similar to what is found in endotoxaemia. Many disease syndromes such as acidosis and parturient paresis have signs of ruminal stasis as well as of endotoxaemia. During stasis of the gastro-intestinal tract, it might be possible that endotoxin from Gram-negative bacteria can be resorbed into the blood circulation, cause related pathophysiological changes and thus being a major component in these disease complexes. The changes of the parameters demonstrated in this study were less dramatic compared to experimentally induced endotoxaemia but more pronounced than during acidosis/overfeeding. The interpretation of the results can be complicated due to that methscopolamine has dual effects - induction of gastro-intestinal stasis and resorption of endotoxins versus inhibition of prostaglandin synthesis. The animals also showed significant changes in most of the relevant parameters such as prostaglandin, endotoxin, iron, zinc, calcium, GLDH which indicates the exposure of endotoxins. In conclusion, it showed that the ruminal stasis induced the clinical and blood biochemical profiles as found in endotoxaemia and thus it seems likely that endotoxins are resorbed from the alimentary tract during its stasis. These findings also show, that endotoxins complicate many disease complexes which have clinical signs of ruminal stasis/hypomotility of the alimentary tract, and as a matter of fact that endotoxins might be the major component in these diseases.

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Sammanfattning

Betydelse av endotoxiner?

II. Vid metylskopolamininducerad våmstas hos kalv.

Metylskopolamin användes för att inducera våmstas hos kalv. Kliniska och blodkemiska parametrar studerades för att utvärdera möjligheten av resorp-

tion av endotoxiner från Gram-negativa bakterier i mag-tarmkanalen. Två försöksserier utfördes med metylskopolamin: 100 mg gavs som engångsdos respektive 3 upprepade injektioner med 70 mg. Djuren visade tydliga tecken på våmstas. Den kliniska bilden och förändringar i blodkemiska parametrar är likartade dem som syns vid endotoxinämi eller vid inducerad våmacidos. Viktiga parametrar som prostaglandin $F_{2\alpha}$ metabolit, endotoxin, järn, zink, kalcium och glutamatdehydrogenas förändrades signifikant, vilket indikerar att djuren exponerats för endotoxiner.

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Reprints may be requested from: Hans Kindahl, Department of Obstetrics and Gynaecology, Swedish University of Agricultural Sciences, Box 7039, S-750 07 Uppsala, Sweden.