The relationship between linoleic/linolenic acid ratio in the diet of periparturient cows, the activity of phospholipase A₂, superoxide dismutase, glutathione peroxidase and thiobarbituric acid reactive substances in placental tissues at calving

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SUMMARY

The studies were undertaken on examining the influence of different linoleic/linolenic acid ratios in the diet of pregnant cows on some parameters which are involved in metabolism of prostaglandins in their placentas. Phospholipase A₂ (PLA₂) which provides arachidonic acid (AA), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) the two being involved in neutralization of reactive oxygen species that appear indispensable during the metabolism of unsaturated fatty acids, thiobarbituric acid (TBA) reactive substances level, which indirectly reflects the level of peroxidative processes of unsaturated fatty acids, were all measured in the samples from the maternal and fetal part of placenta of cows fed with 2 different diets. Linoleate rich (sunflower group - linoleic/linolenic acid ratio 2,22) and linolenate rich (linseed group - linoleic/linolenic acid ratio 0,35) diets lasted 4 weeks before expected calving.

The activity of PLA₂, SOD and the content of TBA reactive substances were higher in linseed group in fetal part of placenta than in sunflower group. No significant differences were found in the maternal part of the placenta. No significant differences between experimental groups were observed according to GSH-Px activity.

It is concluded that the linoleic/linolenic acid ratio in the diet may influence PLA₂ activity and the level of TBA reactive substances. In result the metabolism of eicosanoids in the placenta may be modified.

KEY-WORDS : bovine placenta - diet - phospholipase A₂ - SOD - GSH-Px - TBA reactive substances.

RÉSUMÉ

Influence du rapport acide linoléique / linolénique de l’alimentation des vaches gestantes sur la phospholipase A₂, la superoxyde dismutase, la glutathion peroxydase et sur les substances sensibles à l’acide thiobarbiturique dans le placenta. Par M. KANKOFER, B. KEMP et M.A.M. TAVERNE.

Le but de cette étude était de déterminer l’influence du rapport acide linoléique / linolénique sur le métabolisme des prostaglandines placentaires.
Cette étude met en évidence une modification du métabolisme des éicosanoides dans le placenta.

MOTS-CLÉS : placenta - régime diététique - phospholipase A₂ - SOD - GSH-Px - vache.
Introduction

The bovine periparturient placenta is capable of synthesis and catabolism of eicosanoids via cyclooxygenase and lipooxygenase pathways [24, 9].

The first step in arachidonic acid (AA) cascade is the hydrolysis of phospholipids performed by phospholipases, mainly phospholipase A2 (PLA2, EC 3.1.1.4). AA - the product of this reaction is the main substrate for the synthesis of biologically active eicosanoids, in particular prostaglandins responsible for regulation of parturition and postpartum period in cows [5]. AA can also be obtained from the diet and/or by desaturation and elongation of linoleic acid [27]. That is why the diet rich in linoleic acid is supposed to deliver the precursors for prostaglandins of the 2 series synthesis. The pathway of linolenic acid metabolism may lead to eicosapentaenoic acid [2] which is a competitive inhibitor of enzymes involved in the production of prostaglandins of the 2 series [14].

The activity of AA cascade enzymes can be influenced by variety of agents and substances, as: inflammation, trauma, steroid hormones, free radicals and nutritional factors [22].

The free radicals and peroxides are generated during the metabolism of unsaturated fatty acids like: AA, linoleic and linolenic [27]. Their impaired balance leading to uncontrolled increase can modify metabolic pathways of AA cascade by changes in activities of enzymes involved in those pathways [25, 8] and NADPH + H+/NADP+ ratio [6].

The lipid peroxidation level can be evaluated indirectly by determination of the content of thiobarbituric acid (TBA) reactive substances such as for example malondialdehyde [20]. Glutathione peroxidase (GSH-Px, EC 1.11.1.9), superoxide dismutase (SOD, EC 1.15.1.1), catalase [7] and non enzymatic antioxidants like glutathione, ascorbate, urate, vitamin E, ubiquinone and beta carotene are natural defence mechanisms against free radicals [17, 23]. GSH-Px is also directly involved in AA metabolism [4] by catalysing the conversion of endoperoxide PGG2 to PGH2.

There is an evidence that in circumstances like the presence of certain substances in diet (like in presented study) including substrate and different medicines, the activity of some enzymes may be induced [19].

The aim of the following study was the determination of PLA2, GSH-Px, SOD and TBA in bovine placenta at term in order to test the hypothesis that changes in linoleic/linolenic acid ratio in the diet of cows during the last month before parturition [13], can influence the activity of these enzymes. Former plasma determinations in the same experimental animals [13] showed significant differences in linoleic/linolenic acids ratio which were supposed to be connected with the diet (1.00 ± 0.22 vs 4.41 ± 0.53).

Material and methods

Experimental Holstein Friesian x Dutch Friesian (HFxDF) cows were fed with two different diets from 4 weeks before expected calving.

Both diets consisted of sugar beet pulp, barley straw, grass silage and were similar in amounts of energy and protein. The first diet was supplemented by flaked sunflower seed (containing 65 % linoleic acid vs 0.4 % linolenic acid by weight in the total fatty acid fraction of sunflower meal) and resulted in

<table>
<thead>
<tr>
<th>Item</th>
<th>Diet 1</th>
<th>Diet 2</th>
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<tbody>
<tr>
<td>Linseed (kg)</td>
<td>-</td>
<td>1.4</td>
</tr>
<tr>
<td>Sunflower seed (kg)</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>Sugar beet pulp (kg)</td>
<td>2.4</td>
<td>1.4</td>
</tr>
<tr>
<td>Barley straw (kg)</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Grass silage (kg)</td>
<td>5.4</td>
<td>5.4</td>
</tr>
<tr>
<td>Calculated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Net energy (MJ)</td>
<td>52.8</td>
<td>53.0</td>
</tr>
<tr>
<td>Ileal digestible protein (g)</td>
<td>489</td>
<td>461</td>
</tr>
</tbody>
</table>

| Table I. — Composition of experimental diets. |
linoleic/linolenic acid ratio of 0.35. The second diet was supplemented by flaked linseed (containing 16 % linoleic acid vs 54 % linolenic acid by weight in the total fatty acid fraction of the linseed meal) and resulted in linoleic/linolenic acid ratio of 2.22 (for details see 13).

Placental tissues were collected from 9 animals of the linseed group and 5 animals of the sunflower group.

Placental tissues were obtained within one hour after spontaneous delivery of the calf and frozen in liquid nitrogen until analysed. No retained placenta were included.

After thawing placental tissues were separated manually into maternal and fetal parts, washed in cold 0.9 % NaCl, homogenized in phosphate buffer (0.1 mol/dm³ pH 7.4) using an Ultra Turrax T 25 (Ikawerk, Janke and Kunkel Inc., Staufen, Germany) for 5 min at the speed 10 000 rev/min and centrifuged for 20 min at 3000 g. The whole procedure was performed at 4°C.

**DETERMINATION OF ENZYME ACTIVITIES**

PLA₂ activity was estimated in supernatants of fetal and maternal part of placenta using a turbidimetric method based upon the clearing effect of lysolecithin [3] and measured by following decrease in optical density of reaction mixture at 400 nm in Specord M-40 UV-Vis double-beam spectrophotometer (Carl Zeiss, Jena, Germany). The reaction mixture contained 100 µmoles of lecithin, 2.48 µmoles of lysolecithin, 5.4 µmoles of sodium deoxycholate, 0.5 % albumin, 0.01 µmol HgCl₂ in 0.1 mol/dm³ TRIS-HCl buffer (pH 8.8) and 0.2 ml of enzyme source in a total volume of 3.2 ml. Incubation was at 37°C during 10 min. Optical density of this substrate emulsion against buffer blank at 400 nm was about 0.58-0.60.

Under conditions stated above, a change in optical density of 0.001 min/ml of sample x 1000 was taken as one turbidimetric phospholipase unit. Inter- 8.1 % (n=10) and intra- 7.7 % (n=10) assay variation were established.

GSH-Px activity was determined using a spectrophotometric method according to PAGLIA and VALENTINE [21]. The reaction mixture contained 100 µl of 8.4 mM NADPH, 10 µl glutathione reductase (GSSG-R, 100 U/mg protein/ml), 10 µl 1.125 mol/dm³ sodium azide, 100 µl 0.02 M glutathione (GSH) filled with phosphate buffer (0.05 mol/dm³, pH 7.0) up to the volume of 2.8 ml and 100 µl of supernatant. The enzymatic reaction was initiated by addition of 100 µl 0.022 mol/dm³ H₂O₂. The rate of GSSG formation was measured by following decrease in absorbance of the reaction mixture at 340 nm (Specord M-40, Carl Zeiss Jena, Germany), as NADPH was converted to NADP, between 2nd and 4th min after initiation of the reaction employing a cuvette with 1 cm light path, in accordance with the following reaction:

\[
2\text{GSH} + \text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{GSSG}
\]

\[
\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow \text{GSSG-R} \rightarrow 2\text{GSH} + \text{NADP}^+
\]

The activity was expressed as nanokatals (nkat) per protein content. Intra- 8.4 % (n=10) and inter- 8.7 % (n=10) assay variation were established.

SOD activity was determined using a spectrophotometric method according to MING SUN and ZIGMAN [18]. The method was based on the inhibition of the spontaneous degradation of adrenaline to adrenochrom at pH 10.2 by SOD. The reaction mixture contained 1.8 ml carbonate buffer (0.05 mol/dm³, pH 10.2), 100 µl of supernatant and 100 µl of adrenaline (18 mg in 10 ml of 0.1 mol/dm³ HCl). The increase in absorbance at 340 nm (Specord M-40, Carl Zeiss Jena, Germany) from zero during 10 min was compared with controls in which the supernatant was replaced by 0.9 % NaCl. The activity of SOD expressed as the percentage of inhibition of spontaneous degradation of adrenaline, was recalculated into SOD units per protein content. Under conditions stated above 50 % of inhibition was taken as 1 U SOD. Intra- 8.7 % (n=10) and inter- 9.1 % (n=10) assay variation were established. Enzyme activity determinations were performed using substrate saturation conditions. All parameters concerned with enzyme determinations were established as in previous papers [11, 12].

**TBA TEST**

The assay was performed as described by LEDWOZYW et al. [15]:

2.5 ml of 20 % trichloroacetic acid in 0.6 mol/dm³ HCl was added to 0.5 ml of supernatant. After 10 min 1.5 ml of 0.67 % TBA was added and the sample was incubated in boiling water bath during 20 min. After cooling 4 ml of n-butanol was added, shaked during 3 min and centrifuged (10 min, 4500 x g). The absorbancy of butanol layer was measured at 542 nm using spectrophotometer Specord M-40 (Carl Zeiss Jena). Standard curve was prepared with malondialdehyde. The results were expressed in µmol/dm³ of TBA reactive substances per protein content. The intra- 7.5 % (n=10) and inter- 7.9 % (n=10) assay variation were established.

**DETERMINATION OF PROTEIN CONTENT**

The protein content of supernatants was determined by the method of LOWRY et al [16] using bovine serum albumin as standard.

**STATISTICAL ANALYSIS**

Paired observations of all examined parameters were averaged and compared using Student’s t-test and Stat Graph 5.0 programme.

**Results**

The results of enzyme activity and TBA test determinations are presented in Table II.
The activity of PLA₂ was significantly higher (P < 0.05) in maternal than in fetal part of placenta in examined groups. Lower activity was observed in sunflower than in linseed group but statistically significant differences were only found in fetal part of placenta.

GSH-Px activity was higher in fetal than in maternal part of placenta but no significant differences between experimental groups were observed.

SOD activity was similar in maternal part of placenta in examined cows. The enzyme activity in the fetal part of the placenta was significantly higher (P < 0.05) in linseed group than in sunflower group.

The content of TBA reactive substances was similar in maternal part of placenta in examined groups. The contents of TBA reactive substances in fetal part were significantly higher (P < 0.05) in linseed than in sunflower group.

<table>
<thead>
<tr>
<th></th>
<th>Linseed group</th>
<th>Sunflower group</th>
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<tbody>
<tr>
<td></td>
<td>n = 9  (1.07±0.22)*</td>
<td>n = 5  (4.41±0.53)*</td>
</tr>
<tr>
<td><strong>PLA₂ (nkat/mg prot)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal</td>
<td>4.86 (±0.69)x</td>
<td>4.03 (±0.60)x</td>
</tr>
<tr>
<td>Fetal</td>
<td>1.57 (±0.21)y</td>
<td>0.97 (±0.09)y</td>
</tr>
<tr>
<td><strong>GSH-Px (nkat/mg prot)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal</td>
<td>111.33 (±11.66)x</td>
<td>114.02 (±10.50)x</td>
</tr>
<tr>
<td>Fetal</td>
<td>208.11 (±29.50)y</td>
<td>174.20 (±13.83)y</td>
</tr>
<tr>
<td><strong>SOD (U/mg prot)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal</td>
<td>3.40 (±0.58)</td>
<td>3.78 (±0.54)</td>
</tr>
<tr>
<td>Fetal</td>
<td>4.68 (±0.92)a</td>
<td>2.84 (±0.20)b</td>
</tr>
<tr>
<td><strong>TBA (µmol/dm³/mg prot)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal</td>
<td>0.531 (±0.043)</td>
<td>0.607 (±0.054)</td>
</tr>
<tr>
<td>Fetal</td>
<td>1.460 (±0.029)a</td>
<td>0.495 (±0.034)b</td>
</tr>
</tbody>
</table>

x, y: significant differences between maternal and fetal part of placenta (p < 0.05)

a, b: significant differences between diets (p< 0.05)

*: plasma linoleic/linolenic acid ratio cit. KEMP et al., 1998

The activity of PLA₂ was significantly higher (P < 0.05) in maternal than in fetal part of placenta in examined groups. Lower activity was observed in sunflower than in linseed group but statistically significant differences were only found in fetal part of placenta.

GSH-Px activity was higher in fetal than in maternal part of placenta but no significant differences between experimental groups were observed.

SOD activity was similar in maternal part of placenta in examined cows. The enzyme activity in the fetal part of the placenta was significantly higher (P < 0.05) in linseed group than in sunflower group.

The content of TBA reactive substances was similar in maternal part of placenta in examined groups. The contents of TBA reactive substances in fetal part were significantly higher (P < 0.05) in linseed than in sunflower group.

Discussion

Animals fed the sunflower diet with high linoleic/linolenic acid ratio presented lower enzyme activities and lower TBA-reactive substances levels in the fetal part of the placenta than in the same part of the placenta in linseed group.

Essential polyunsaturated fatty acids like linoleic (18:2) and arachidonic (20:4) acid are precursors of biologically active substances, as prostaglandins - important agents for parturition and the periparturient period.

The activity of PLA₂, which is essential for AA generation, was higher (fetal portion of the placenta) in linseed than in sunflower group. Higher PLA₂ activity in placental tissues of the linseed group were accompanied, (although not statistically significant) with lower level of PGFM (the metabolite of PGF₂α) in plasma than in sunflower group [13]. The linoleate rich diet obtained by adding sunflower seed, should

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increase the production of prostaglandins of series 2 and linolenate rich diet containing linseed should inhibit it and decrease the level of prostaglandins of the same series. Our results may suggest that the inhibitory effect of linolenate on prostaglandin metabolism probably may occur on a different step of the arachidonic acid cascade as the action of PLA2 is localised. Eicosapentaenoic acid which can be obtained during feeding with linolenate rich diet [2] is supposed to be a competitive inhibitor of enzymes responsible for prostaglandins production [14, 10] but the place of this inhibition has not been defined.

There is an evidence that extremely high levels of linoleic acid may reduce eicosanoid synthesis in endothelium probably by competition with AA for acylation into membrane phospholipids and thus inducing reduction of membrane AA [26]. The influence of dietary substances on biochemical changes in metabolism is very complex and may also depend on the diet duration and the amount of additions.

The diets applied in this study lasting 4 weeks before expected parturition had a clear and significant effect on linoleic/linolenic acid ratios in plasma of the examined cows (linseed diet 1.07 (SD±0.22), sunflower diet 4.41 (SD±0.53)) and resulted in an adequate tendency in PGFM levels [13].

The experiments of KANKOFER et al. [11] performed on cows fed the diet without any additions showed (although not statistically significant) lower activity of PLA2 in placental cows fed the diet without any additions. Relatively high activities of GSH-Px and SOD were performed in order to evaluate the antioxidant status of cows fed with diets containing different amounts of essential unsaturated fatty acids. Unsaturated fatty acids are susceptible for peroxidative changes [27] which, if uncontrolled, can alter their metabolism and may influence the level of eicosanoids.


