Neopterin and biopterin as biomarkers of immune system activation associated with castration in piglets

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ABSTRACT: Recent reports have shown that stressful situations may affect the production of unconjugated pterins (neopterin and biopterin). The aim of the study was to investigate the effect of castration on neopterin and biopterin plasma concentrations in piglets, using 2 groups of 12 piglets allocated to castrated and uncastrated (control) groups. Pterin concentrations were determined by HPLC with fluorescence detection. Blood samples were also analyzed for leukocyte profiles and plasma cortisol concentrations. A time × treatment interaction (P < 0.05) was detected for neopterin concentrations, such that neopterin was greater (P < 0.01) at 1 h after surgery in castrated piglets compared with precastration concentrations, and neopterin was greater (P = 0.05) in castrated than in control piglets at 1 h. Castration had no effect on biopterin concentration (P > 0.1). Time effects (P < 0.05) for neutrophil and lymphocyte concentrations and neutrophil-to-lymphocyte ratios were found. A time × treatment interaction (P < 0.01) was detected for plasma cortisol concentrations, such that cortisol was greater (P < 0.01) at 1 and 24 h after surgery in castrated piglets compared with precastration concentrations and was greater (P < 0.01) in castrated than in control piglets at 1 and 24 h. This study showed that castration activated the immune system of piglets as demonstrated by an increase in plasma neopterin concentrations.

Key words: cortisol, lymphocyte, neutrophil, pterin

INTRODUCTION

Neopterin and biopterin belong to a group of unconjugated pterins, derived from guanosine triphosphate by guanosine triphosphate cyclohydrolase I (Brown, 1971). Neopterin is synthesized mainly by activated monocytes/macrophages after stimulation by the cytokine interferon-gamma (IFN-γ), which is released by natural killer cells and T-lymphocytes (Hoffmann et al., 2003). Neopterin is a useful biomarker for the intensity of the immune response mediated by Th-1 type cells. Biopterin is produced by nonenzymatic oxidation of tetrahydrobiopterin. Synthesis also takes place in cells such as T-cells, B-cells, endothelium, smooth muscle cells, fibroblasts (Werner-Felmayer et al., 2002), and potentially in liver and kidney (Fujioka et al., 2008). Only recently has research focused on the use of pterins in human medicine (Murr et al., 2002; Longo, 2009), including the determination of pterin concentrations in body fluids as diagnostic markers and to monitor and assess treatment efficacy and prognostication of a host of diseases. Considerably fewer authors have investigated the use of pterins in veterinary medicine (Fujioka et al., 2008; Smutna et al., 2010). Breinekova et al. (2006, 2007) mention neopterin and biopterin as suitable markers of immune system activation under acute stress caused by transport of pigs to the slaughterhouse.

Because piglet castration may affect the immune system (Lessard et al., 2002) as well as behavior and stimulate activity of the adrenal axis (for review, see Prunier et al., 2006), the aim of the present study was to investigate the effect of castration on blood plasma concentrations of neopterin and biopterin in piglets where neopterin and biopterin were selected as biosensors of immune system activation. Neopterin and biopterin plasma concentrations were compared with...
cortisol as a marker of neuroendocrine activity, as well as to results of leukocyte profiles.

MATERIALS AND METHODS

All experimental procedures were approved by the Ethics Committee of the University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic.

Experimental Design

Twenty-four 7-d-old piglets were allocated into 2 groups [castrated and noncastrated (control)] of 12 piglets each. At treatment initiation, piglets to be castrated were hand-held upside down after disinfection of the scrotal area. Two parallel incisions were made on the scrotum, and the testes were caught and emasculated at the funiculus spermaticus by an emasculator. The incision was then disinfected. Blood samples (5 mL) were collected by venipuncture from the vena cava cranialis from both groups immediately before castration, 1 h after castration, and 24 h after castration of the experimental group. Samples for neopterin, biopterin, and cortisol determinations were collected in heparin-coated tubes, immediately centrifuged at 800 × g for 10 min at 4°C, and stored at −18°C. Samples for leukocyte profile examination were collected into tubes with EDTA and immediately examined. A total of 72 blood samples were examined.

Plasma Neopterin and Biopterin Determination

Measurement of neopterin and biopterin was based on HPLC with fluorometric detection (Carru et al., 2004). All samples and pterin solutions were protected against light when handled. For neopterin and biopterin analysis, 300 μL of trichloroacetic acid (5%) was added to 300 μL of standard or plasma. The samples were centrifuged at 800 × g for 10 min at 20°C. Supernatant was filtered through a 0.45-μm nylon filter (Millipore, Billerica, MA) and used for analysis. Elution was performed on a 150- × 4.6-mm, 5-μm Zorbax Eclipse XBD-C18 column (Agilent Technologies, Santa Clara, CA). Isocratic elution was performed at a flow rate of 1 mL/min with water/acetoniitride 96/4 (vol/vol) at 35°C. Fluorescence detection at 353 and 438 nm for excitation and emission, respectively, was used to selectively to detect pterins. The chromatographic analysis was accomplished by means of an Alliance 2695 chromatographic system with a PDA 2996 photodiode array detector (Waters Corp.). Cortisol was purchased from Sigma-Aldrich. All solvents were HPLC-grade purity (Chromservis). Cortisol determination was performed by HPLC with photometric detection (Blahova et al., 2007). The SPEC C18 AR cartridges (3 mL, 30 mg, Varian Inc., Palo Alto, CA) were used. Five hundred microliters of the sample was passed through a preconditioned cartridge (500 μL of methanol and 500 μL of water). The cartridge was then washed with 500 μL of acetone:water (10:90, vol/vol), allowed to dry for 5 min, and the analyte eluted with 1 mL of acetonitrile.

Plasma Cortisol Determination

Cortisol determination was performed by HPLC with photometric detection (Blahova et al., 2007). The SPEC C18 AR cartridges (3 mL, 30 mg, Varian Inc., Palo Alto, CA) were used. Five hundred microliters of the sample was passed through a preconditioned cartridge (500 μL of methanol and 500 μL of water). The cartridge was then washed with 500 μL of acetone:water (10:90, vol/vol), allowed to dry for 5 min, and the analyte eluted with 1 mL of acetonitrile.

The sample volume injected into the HPLC system was 20 μL. Cortisol was separated by an isocratic elution method with acetonitrile:water 60:40 (vol/vol) on a Polaris C18-A column (3 μm, 150 × 4.6 mm, Varian Inc.). The mobile phase flow rate was 1 mL/min, column temperature was 30°C, and UV detection was performed at 245 nm. The chromatographic analysis was accomplished by means of an Alliance 2695 chromatographic system with a PDA 2996 photodiode array detector (Waters Corp.). Cortisol was purchased from Sigma-Aldrich. All solvents were HPLC-grade purity (Chromservis). The detection limit for cortisol was 2.5 ng/mL. Limit of quantification for cortisol was 8.3 ng/mL. The intraassay CV was 4.1%.

Statistical Analysis

Statistical analysis was performed using Statistica (StatSoft Inc., Tulsa, OK). All data were checked for normality using a Kolmogorov-Smirnov test. A repeated-measures ANOVA was used to evaluate the effects of treatment, time, and time × treatment interaction on plasma neopterin, biopterin, and cortisol concentrations, and the leukocyte profile. When significant differences were found (P < 0.05), a Tukey’s test was conducted as a posthoc test to determine differences between individual groups.

RESULTS

Plasma neopterin concentrations in piglets are shown in Figure 1. An ANOVA revealed time (P < 0.01) and time × treatment interaction (P < 0.05) effects. Compared with the pretreatment concentration, plasma neopterin was greater at 1 h postsurgery in castrated piglets (P < 0.01). At 1 h, plasma neopterin was greater
(P = 0.05) in castrated than in noncastrated (control) piglets. At 24 h, concentrations tended to decrease in castrated piglets compared with those at 1 h after surgery (P < 0.1). Plasma biopterin (215 ± 40 nmol/L) was not influenced by castration or time, and there was no time × treatment interaction (P > 0.1).

Results of leukocyte profiles are shown in Table 1. No differences in leukocyte counts were found; however, time effects for neutrophil count (P < 0.05), lymphocyte count (P < 0.05), and neutrophil-to-lymphocyte ratios (P < 0.05) were found. No significant changes were found in monocyte, eosinophil, or basophil counts (P > 0.1).

There was a time × treatment interaction for plasma cortisol concentration (P < 0.01). Compared with the pretreatment concentration, plasma cortisol was greater (P < 0.01) at 1 and 24 h after surgery in castrated piglets and at 24 h in control piglets (Figure 2). At 1 and 24 h, plasma cortisol was greater in castrated than in control piglets (P < 0.01).

**DISCUSSION**

The present study was designed to investigate the effects of castration on blood plasma concentrations of neopterin and biopterin in piglets as possible biosensors of immune system activation. Neopterin is a product of monocytes/macrophages stimulated primarily by IFN-γ released by T-lymphocytes. Further studies with measurement of proinflammatory cytokines are needed.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>0 h treatment</th>
<th>1 h treatment</th>
<th>24 h treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Castrated</td>
<td>Control</td>
</tr>
<tr>
<td>Leukocyte, 10⁹/L</td>
<td>11.6 ± 4.1</td>
<td>10.3 ± 1.8</td>
<td>14.2 ± 6.4</td>
</tr>
<tr>
<td>Neutrophil, 10⁶/L</td>
<td>4.83 ± 3.41</td>
<td>3.16 ± 1.12</td>
<td>6.44 ± 5.51</td>
</tr>
<tr>
<td>Lymphocyte, 10⁷/L</td>
<td>6.48 ± 1.61</td>
<td>6.99 ± 1.24</td>
<td>7.07 ± 1.99</td>
</tr>
<tr>
<td>Monocyte, 10⁶/L</td>
<td>0.06 ± 0.12</td>
<td>0.03 ± 0.05</td>
<td>0.03 ± 0.05</td>
</tr>
<tr>
<td>Eosinophil, 10⁵/L</td>
<td>0.08 ± 0.08</td>
<td>0.07 ± 0.09</td>
<td>0.01 ± 0.03</td>
</tr>
<tr>
<td>Basophil, 10⁶/L</td>
<td>0.11 ± 0.14</td>
<td>0.02 ± 0.07</td>
<td>0</td>
</tr>
<tr>
<td>Neutrophil/lymphocyte</td>
<td>0.76 ± 0.51</td>
<td>0.46 ± 0.17</td>
<td>0.99 ± 0.71</td>
</tr>
</tbody>
</table>

¹Time effects were found for neutrophil and lymphocyte count and neutrophil-to-lymphocyte ratio (P < 0.05).
²Time relative to castration of treated group.
to fully characterize the effect of castration on neopterin and biopterin concentrations.

Although IFN-γ is the most potent inducer of neopterin synthesis in human cells, the production of neopterin is costimulated by tumor necrosis factor-α and lipopolysaccharides (Berdowska and Zwirska-Korczala, 2001). Moreover, published data show that IFN-γ is not necessary for neopterin production in vivo (Appay and Rowland-Jones, 2002). Biopterin is biotransformed from neopterin, and similarly increased during the IFN-γ stimulated immune response. Immune response-associated neopterin release is a consequence of the activation of the biopterin synthetic pathway (Huber et al., 1984).

In the present study, neopterin and biopterin concentrations in blood plasma of piglets before castration were in agreement with the values reported by Smutna et al. (2010) for piglets not subjected to stress (neopterin: 14.8 to 27.2 nmol/L, biopterin: 93 to 281 nmol/L). The previously reported blood serum neopterin concentrations in adult pigs of 5.22 ± 1.22 nmol/L are markedly less compared with piglets (Breinekova et al., 2007). Schrödl et al. (1998) reported the effect of a bacterial infection (Hemophilus parasuis) on the reduction of serum neopterin concentrations in piglets. Amann et al. (2001) demonstrated that neopterin concentrations in porcine serum were not a suitable biomarker of an acute cardiovascular event in pigs. Fujioka et al. (2008) found a 5-fold increase in biopterin concentrations in plasma of neonatal piglets in the acute stage of hypoxia-ischemia. Breinekova et al. (2006) reported an increase in biopterin concentrations in adult pigs associated with transport to the slaughterhouse. In contrast, results of the present study suggest that castration did not affect biopterin concentrations in experimental piglets.

The results obtained from leukocyte profile examinations indicated activation of the immune system. This is born out by the changes in neutrophil and lymphocyte counts, and the neutrophil-to-leukocyte ratio. Reporting on the effect of the immune system activation on pterin concentrations, other authors reported a decrease in lymphocytes counts, whereas neopterin concentrations increased (Stratigos et al., 2005; Smutna et al., 2010). Moreover, Smutna et al. (2010) reported a decrease in monocyte counts. Lessard et al. (2002), who also studied immune system response to castration in piglets, demonstrated an increase of T and B lymphocyte proliferation in piglets at 18 or 25 d after castration. In their study, Dalin et al. (1993) also reported a decrease in lymphocyte counts and an increase in neutrophil counts in pigs after stress induced by 10 and 30 min of transport. The effect of transport stress on an increase in the neutrophil-to-leukocyte ratio was also reported by Hicks et al. (1998).

Data from the current study confirm that cortisol release is stimulated by surgical castration, as previously demonstrated (Prunier et al., 2005, 2006; Carroll et al., 2006). Glucocorticoids may also play a role in influencing pterin synthesis. Glucocorticoids could cause suppression of cell-mediated immunity and consequently could result in decreased neopterin and biopterin concentrations (Atmaca et al., 2002; Kuloğlu et al., 2007). Brambilla et al. (1997) also reported that norepineph-

Figure 2. Concentrations of cortisol [mean ± SE (box) and SD (whiskers)] in blood plasma of noncastrated (control; n = 12) and castrated piglets (n = 12). **Means without a common letter differ (P < 0.01).
rime and hypothalamic-pituitary-adrenal axis activity might lead to the inhibition of cytokine secretion.

Repeated blood sampling and associated handling generates stress that may influence physiological variables (Breinekova et al., 2006; Carroll et al., 2006). For this reason, we used a control group of 12 uncastrated piglets to assess this effect. From our results, we conclude that repeated handling and vena cava puncture partially increased as a result of handling and vena cava puncture during individual blood sampling sessions.

In conclusion, these results show that castration may activate the immune system significantly. This is corroborated by an increase in blood plasma neopterin concentrations in piglets after castration. However, cortisol concentrations partially increased as a result of handling and vena cava puncture during individual blood sampling sessions.

LITERATURE CITED


