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Concentrations of neopterin, biopterin, and cortisol associated with surgical castration of piglets with lidocaine

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ABSTRACT: The effect of surgical castration with local anesthesia using lidocaine on neopterin, biopterin, and cortisol blood plasma concentrations in piglets was studied. Three groups of 12 piglets were investigated: one group castrated without lidocaine, one group castrated with lidocaine, and one group left as an uncastrated control group handled in the same way as castrated piglets. Blood samples were collected 4 min before castration, and 1 h and 24 h after castration. The time × treatment interaction ($P < 0.01$) was detected for neopterin concentrations, yielding the result that neopterin was higher ($P < 0.01$) in castrated piglets without lidocaine 1 h after surgical castration compared with all other groups. The time effect ($P < 0.05$) was detected for biopterin concentrations. The time × treatment interaction ($P < 0.01$) was detected for plasma cortisol concentrations, yielding the result that neopterin was higher ($P < 0.01$) in castrated piglets without lidocaine and in castrated piglets with lidocaine 1 h after surgical castration compared with pre-treatment and concentrations 24 h after surgical castration. The study showed that the use of lidocaine for the surgical castration of piglets may significantly influence the activation of the immune system. This is corroborated by a significant difference in blood plasma neopterin concentrations between piglets castrated with anesthesia and those castrated without it. The use of lidocaine had no effect on cortisol concentrations in comparison with the group castrated without lidocaine.

Keywords: immune system; local anesthesia; blood; plasma

INTRODUCTION

Male piglets are surgically castrated because of elimination of boar taint in the meat and reduction of aggressive behaviour in pigs. It is carried out without anesthesia during the first week of life. However, the procedure causes concern with respect to animal welfare, because surgical castration is painful to piglets (Kluivers-Poodt et al. 2012). Our previous studies showed that stressful and painful procedures may cause activation of the immune system and significantly change the concentrations of blood plasma neopterin and biopterin. The castration of piglets without anesthesia caused an increase in neopterin concentration after castration (Marsalek et al. 2011), and transport to the slaughterhouse caused increases in neopterin and biopterin concentrations in adult pigs (Breinekova et al. 2006, 2007). Neopterin and biopterin belong to a group of unconjugated pterins derived from guanosine triphosphate by guanosine triphosphate cyclohydrolase I (Brown 1971). Neopterin is mainly synthesized by activated monocytes/macrophages following stimulation

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by interferon-gamma cytokine (IFN-γ), which is released by NK cells and T-lymphocytes (Hoffmann et al. 2003). Neopterin is a useful biomarker of the intensity of the immune response mediated by Th-1 type cells. Biopterin is produced by the non-enzymatic 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 probably in liver and kidney (Fujioka et al. 2008). Surgical castration without anesthesia and analgesia induces a strong activation of the hypothalamic–pituitary–adrenal axis and the result is a release of stress hormones in pigs (Prunier et al. 2005). Stress hormones could significantly affect cell-mediated immunity (Salak-Johnson and McGlone 2007) and consequently could result in changed neopterin and biopterin levels.

An intratesticular application of lidocaine was chosen for local anesthesia in the present study. Lidocaine has a low toxicity and rapid onset (Satas et al. 1997). The combination of lidocaine with norepinephrine was used because it prolongs anesthesia and reduces the risk of systemic reactions (Singi et al. 2001). The aim of the present study was to evaluate the effects of local anesthesia on piglets physiological responses after castration, using pterins as potential new indicators of the stress response to surgery. Cortisol was chosen as a useful stress marker.

MATERIAL AND METHODS

Experimental design. The piglets were housed in farrowing pens at a farrowing unit of large production swine farm in Újezd in the Czech Republic. Automated dry feeding system for sows and creep feeding for piglets was managed at the farm. Thirty-six 4-day-old piglets (Landrace × Czech Large White sows) were divided into 3 groups of 12 piglets each. Piglets from the first group were castrated without anesthesia (CAN), piglets from the second group were castrated with local anesthesia (CWL), and piglets from the third group were not castrated but only handled in the same way as castrated piglets (SHAM). Lidocaine 20 mg/ml with norepinephrine 0.02 mg/ml (Lidocaine 2%; FATRO S.p.A., Ozzano dell'Emilia, Italy) was used for local anesthesia in our study. A total amount of 0.5 ml was injected into each testicle. The lidocaine was administered at least three min before castration. During treatment, piglets were held upside down in the hands of the operator after disinfection of the scrotum area. Two parallel incisions on the scrotum were made, then each testis was caught and severed at the f*uniculus spermaticus* by an emasculator. The incision was disinfected. The castration without anesthesia was provided without rescue analgesia. Blood samples were collected by venipuncture from *vena cava cranialis* from CAN, CWL, and SHAM groups (1) 4 min before castration, (2) 1 h after castration, and (3) 24 h after castration. The blood was taken ca. 4 min before the surgical castration (intratesticular injection of lidocaine 1 min, waiting for the anesthetic effect 3 min). We maintained the same times in all groups. All 3 groups were not processed simultaneously. One piglet was processed at one time by the same operator and assisting personnel. We decided to use the same operator for all groups in order to induce comparable stress during the procedures. The time estimation between each castration was ca. 7 min. Blood plasma samples for neopterin, biopterin, and cortisol determination were collected in heparin-coated tubes, immediately centrifuged at 800 *g* at 4°C for 10 min, and stored at –18°C. A total of 108 blood plasma samples were examined. The procedures made with piglets during the study were approved by the Ethics Committee of the University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic.

Neopterin and biopterin. The neopterin and biopterin analysis was based on High Performance Liquid Chromatography 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%) were added to 300 µl of standard or plasma. The samples were centrifuged at 800 *g* at 20°C for 10 min. The supernatant was filtered through a 0.45-µm nylon filter (Millipore, Billerica, USA) and used for analysis. Elution was performed on a 150 × 4.6 mm, 5-µm Zorbax Eclipse XBD-C18 column (Agilent Technologies, Santa Clara, USA). Isocratic elution was performed at a flow rate of 1 ml/min with water/acetonitrile 96/4 (v/v) at 35°C. Fluorescence detection at 353 nm and 438 nm for excitation and emission, respectively, was used to selectively detect pterins. Chromatographic analysis was accomplished by means of an Alliance 2695 chromatographic system (Waters Corp., Milford, USA) with a FD 2475 fluorescent

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detector (Waters Corp.). Neopterin, biopterin, and trichloroacetic acid were purchased from Sigma-Aldrich (St. Louis, USA). All solvents were of HPLC-grade purity (Chromservis, s.r.o., Prague, Czech Republic). Detection limits for neopterin and biopterin were 0.23 ng/ml and 0.41 ng/ml, respectively. The limits of quantification for neopterin and biopterin were 0.75 ng/ml and 1.35 ng/ml, respectively. The coefficient of variation was 3.8%.

Cortisol. The cortisol analysis was performed using liquid chromatography tandem mass spectrometry (LC-MS/MS). Sample preparation was based on the method described by Blahova et al. (2007). SPEC C_{18} AR cartridges (3 ml, 30 mg) (Varian, Inc., Palo Alto, USA) were used. 500 µl of the sample were passed through a preconditioned cartridge (500 µl methanol and 500 µl water). The cartridge was then washed with 500 µl acetone/water (10/90, v/v) and allowed to dry for 5 min, and the analyte was eluted with 1 ml acetonitrile. A Thermo Scientific UHPLC Accela 1250 system was connected to a Thermo Scientific TSQ Quantum Access MAX Triple Quadrupole Instrument (Thermo Fisher Scientific, San Jose, USA) equipped with a heated electrospray ionization (HESI-II) probe. A Thermo Scientific Hypersil C_{18} column (2.1 mm × 50 mm, 1.9 µm) was used at a constant flow rate of 300 µl/min. The mobile phase consisted of water containing 0.1% formic acid (v/v) (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). Cortisol was separated by an isocratic elution method with solvent A/solvent B 40/60 (v/v). The full loop injection volume of the extract was set at 10 µl. The heated electrospray ionization was operated in positive-ion mode under the following conditions: capillary temperature 325°C, vaporizer temperature 300°C, sheath gas pressure 35 psi, auxiliary (drying) gas 10 a.u., spray voltage 3300 V. Cortisol was purchased from Sigma-Aldrich. All solvents were of HPLC-grade purity (Chromservis, s.r.o.). The detection limit for cortisol concentration was 2.5 ng/ml. The limit of quantification for cortisol was 8.3 ng/ml. The coefficient of variation was 4.1%.

Statistical analysis. Statistical analysis was performed using Statistica software (Version 8.0, 2007) for MS Windows. A normality check of all data sets of results obtained for the parameters investigated was performed with Kolmogorov–Smirnov. A repeated measures analysis of variance was used to evaluate the results of neopterin, biopterin, and cortisol. The effect of treatment was tested against between-subject variation and effects of time and time × treatment interaction against within-subject variations. When significant differences were found *($P < 0.05$)*, conservative Tukey's test was conducted as a *post hoc* test to determine differences between individual groups.

RESULTS

Plasma neopterin concentrations in piglets are shown in Figure 1. Analysis of variance revealed treatment, time, and time × treatment interaction ($P < 0.01$) effects. Compared with all other groups, plasma neopterin concentrations were higher in CAN piglets at 1 h after surgical castration ($P < 0.01$). Plasma biopterin concentrations in piglets are shown in Figure 2. Analysis of variance revealed a time ($P < 0.01$) effect. Compared with concentrations before surgical castration ($P < 0.05$) and concentrations 24 h after surgical castration ($P < 0.01$), plasma biopterin concentrations at 1 h after surgical castration were higher in CAN and CWL piglets. Compared with the concentration 24 h after surgical castration, plasma biopterin concentrations were higher at 1 h after surgical castration in SHAM piglets ($P < 0.05$).

Plasma cortisol concentrations in piglets are shown in Figure 3. Analysis of variance revealed treatment, time, and time × treatment interaction ($P < 0.01$) effects. Compared with concentrations before surgical castration and concentrations 24 h

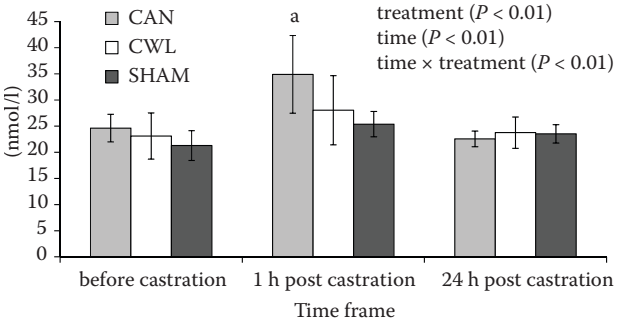

Figure 1. Concentrations (means ± SD) of neopterin in blood samples from piglet groups CAN, CWL, and SHAM ($n = 12$ each) before castration, 1 h after castration, 24 h after castration

CAN = castrated without anesthesia, CWL = castrated with local anesthesia, SHAM = not castrated but only handled in the same way as castrated piglets

[a]significant difference within time and treatment ($P < 0.01$)

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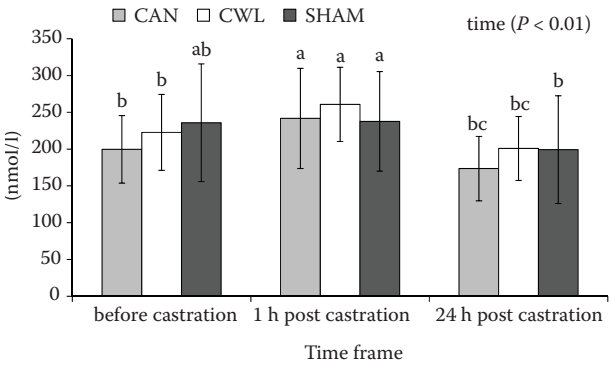

Figure 2. Concentrations (means ± SD) of biopterin in blood samples from piglet groups CAN, CWL, and SHAM (n = 12 each) before castration, 1 h after castration, 24 h after castration

CAN = castrated without anesthesia, CWL = castrated with local anesthesia, SHAM = not castrated but only handled in the same way as castrated piglets

[a–c]significant differences within treatment: [a]($P < 0.05$), [b]($P < 0.05$), [bc]($P < 0.01$)

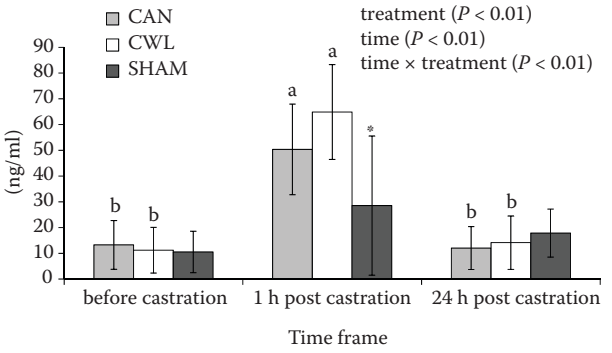

Figure 3. Concentrations (means ± SD) of cortisol in blood samples from piglet groups CAN, CWL, and SHAM (n = 12 each) before castration, 1 h after castration, 24 h after castration

CAN = castrated without anesthesia, CWL = castrated with local anesthesia, SHAM = not castrated but only handled in the same way as castrated piglets

[a,b]significant differences within treatment: [a]($P < 0.05$), [b]($P < 0.01$)

*significant differences within time ($P < 0.05$)

after surgical castration, plasma cortisol concentrations were higher ($P < 0.01$) at 1 h after surgical castration in CAN and CWL piglets. One hour after surgical castration, plasma cortisol concentrations were higher in CAN piglets and in CWL piglets compared to SHAM piglets ($P < 0.05$).

DISCUSSION

The present study confirmed the findings of our previous studies (Smutna et al. 2010; Marsalek et al. 2011), in which the mean concentration of neopterin in piglets from control groups not subjected to stress or surgical castration was 18 nmol/l and the mean concentration of biopterin was 215 nmol/l, while the concentration of neopterin in adult pigs was significantly lower (5.22 nmol/l) (Breinekova et al. 2007). There are no direct methods for measuring the intensity of pain (White et al. 1995). However, it has been shown that piglets react physiologically to pain, suggesting the existence of possible indicators. It is known that castration provokes specific pain-related behaviour. For instance huddling up, spasms, and trembling. Castrated pigs also walk less and avoid dog-sitting postures (Moya et al. 2008). Pain activates the sympathetic nervous system and is followed by several physiological changes. For example, there is a release of adrenocorticotropic hormone with the subsequent secretion of cortisol (Sjaastad et al. 2003).

Also, serum amyloid A (SAA), which is the major acute phase protein in pigs, increases quickly after stress or trauma and has already been used as an indicator of the health and welfare status of pigs (Petersen et al. 2004). For instance, Jacobson et al. (2001) found a 13–78-fold increase in the SAA values in the experimental pigs in the first two days after surgical castration.

The present study was designed to investigate the effects of castration with lidocaine local anesthesia on blood plasma concentrations of neopterin and biopterin in piglets, these suggested as possible markers of pain and stress associated with castration. The repeated blood sampling and handling associated with castration generates stress that may influence physiological parameters (Carroll et al. 2006). As a result, the group of SHAM piglets was handled in the same way as castrated piglets. Our findings showed that repeated blood sampling and handling influenced plasma concentrations of biopterin. Plasma concentrations of neopterin and cortisol were not influenced.

Based on the analysis of vocal parameters and plasma cortisol, castration with lidocaine is less painful and stressful compared with castration without anesthesia (White et al. 1995; Kluivers-Poodt et al. 2012). Neopterin and biopterin can be useful markers of acute stress in pigs (Breinekova et al. 2006, 2007). Our previous study showed that neopterin concentrations significantly increased

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in castrated piglets without anesthesia 1 h after castration (Marsalek et al. 2011). In this study, the use of lidocaine significantly modified neopterin concentrations at 1 h after castration. These results suggest that neopterin can be considered as a marker of pain and stress associated with castration in piglets. Although biopterin concentrations were elevated at 1 h after castration in CAN and CWL piglets, they did not differ from those of SHAM piglets at the same time points. Modified concentrations of biopterin are in contradiction to our previous study, in which biopterin was not influenced by castration (Marsalek et al. 2011).

Cortisol was included in this study because it is generally considered as a marker of stress and pain. Cortisol concentrations significantly increased at 1 h after surgical castration, however the use of lidocaine did not modify cortisol concentrations. These results are in contradiction to the findings of Kluivers-Poodt et al. (2012). They found significantly lower increase of cortisol concentration in piglets castrated with lidocaine 20 min after castration compared with piglets castrated without local anesthesia. However, we performed blood sampling 1 h after castration and the action time of lidocaine is about 60 min (Ranheim et al. 2005). Cortisol could cause suppression of cell-mediated immunity and consequently could result in decreased neopterin and biopterin concentrations (Atmaca et al. 2002). Prunier et al. (2005) showed that the increase of plasma cortisol concentration started 15 min after castration of piglets and returned to the pre-castration level within 3 h with peak values occurring between 30 and 60 min after castration.

CONCLUSION

The results show that the use of lidocaine for the surgical castration of piglets may significantly affect the activation of the immune system. These findings are corroborated by a significant difference in blood plasma neopterin concentrations between CAN and CWL piglets. The use of lidocaine did not influence biopterin concentrations. Although concentrations of biopterin increased at 1 h after castration in CAN and CWL piglets, there were not significant differences compared to SHAM piglets. The castration significantly increased cortisol concentration at 1 h after the procedure, however the use of lidocaine did not lead to a reduction of cortisol concentration.

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