Is there Circadian Variation in Cortisol Levels in Young Sows in Heart Catheterization?

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Abstract: Cortisol is the main glucocorticoid (GC) hormone in pigs associated with stress response. It is well known that GCs levels are not stable during the day; their concentration is a circadian variable with the peak in the morning and the nadir in the night (in diurnal animals). Circadian variation is present during postnatal ontogeny. The onset of the circadian fluctuation occurs in pigs at the age of 3 to 20 weeks (according to the literature). The aim of our pilot study was to determine if young sows (used in cardiosurgical experiments) already developed the circadian variation. Twelve-week-old sows were used in the heart catheterization experiment. Cortisol was measured during four different stages of the experiment at two different times of the day (the operation was performed in the morning or afternoon). To determine circadian variation the Mann-Whitney test was used; to determine changes in cortisol levels within the experiment the Friedman test was performed. We didn’t find any circadian variation (p>0.05) or

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statistical significant variation in the Friedman test (p>0.05). We assumed that our pigs are too young to have circadian rhythm present. Our findings are in accordance with many authors.

Introduction
Glucocorticoids (GCs) are steroid stress hormones produced by the adrenal cortex. Production of these hormones increases in response to the stress stimuli (stressor). The main GC in pigs is cortisol. GCs play an important role in metabolism, especially glucose, and its control (Selye, 1936; Greenberg et al., 2002; Möstl and Palme, 2002).

GCs are small lipophilic molecules with a hydroxy group on C-11 (cortisol). The lipophilic character of the GCs allows free entry to the target cells through cell membranes to the cytoplasm where they are bound to specific receptors. The GC-receptor complex then enters the nucleus and modulates the transcription process (Seckl, 1997). GCs with another group on C-11 (e.g. keto-group; cortisone) couldn’t enter the cell and bind to cytoplasm receptors.

GCs production depends on circadian and circannual biorhythms. The peak of GCs level is just before awaking (in nocturnal animals at sunset, in diurnal animals at sunrise) while the nadir of GCs levels is before sleep (Barnett et al., 1981; Becker et al., 1985a, b; Bradbury et al., 1994; Kramer and Sothern, 2001). Pigs are diurnal animals, therefore their GCs peak production is in the morning. In some studies two peaks were found (in the morning and in the afternoon) corresponding to the feeding time (Geverink et al., 2003; Hillmann et al., 2008).

Circadian rhythm develops in ontogeny; young animals have no detectable rhythm as the real onset of the rhythm comes in puberty, in pigs in the age of their 15th week (von Borell and Ladewig, 1992; de Jong et al., 2000), adult animals already have fully stable rhythm (since the age of 20 weeks) (Ruis et al., 1997).

Heart catheterization is a minimally invasive intervention, but many studies didn’t find differences in elevated GCs levels between invasive open surgery and minimally invasive operations in pigs (Mansour et al., 1992; Bessler et al., 1994; Burpee et al., 2002; Margulis et al., 2005; Matsumoto et al., 2005; Duchene et al., 2008). This means that even minimal intervention can cause a rise in GCs levels; therefore it can be considered a stressor.

Anesthesia influence on GCs production is known. But there are differences in used medicaments. In pigs there were tested differences between inhalation of anestheticum sevoflurane (Ledowski et al., 2005; Marana et al., 2008) or halothan (Brasil et al., 2006) and intravenous anesthesia with propofol in combination with midazolam or remifentanil; lower GCs levels were found in combination with propofol medication.

Circadian rhythm in cortisol concentration is well known. Circadian variation is connected with the ontogeny of the organism. The aim of this pilot study was to determine circadian rhythm in cortisol levels in young sows (12 weeks old). In the
literature the onset of this rhythm varies between three (Salfen et al., 2003) and fifteen (von Borell and Ladewig, 1992; Ruis et al., 1997; de Jong et al., 2000) weeks of age in the pigs. Our pigs are in the middle of this range; therefore we tested if their age is sufficient for the onset of the circadian rhythm. According to our hypothesis the eventual circadian rhythm of young sows can be influenced by the catheterization procedure and so we tested also the differences in cortisol levels during the catheterization procedure.

**Material and Methods**

**Animals**

In our experiment young sows (*Sus scrofa domestica*) were used, crossbred (*Landrace × Large white*) 12 weeks old. The pigs were too young to have a sexual cycle; we could exclude influence of the oestrus cycle on cortisol levels. AGRO Jesenice, a.s. (breeding farm Radějovice; RČH CZ-21045103) was their home farm. The pigs were maintained at the farm under conventional conditions (room temperature about 20 ºC, humidity between 40 and 70%, natural lighting and regulated ventilation). They were fed a balanced diet for fattening pigs (Čos II, Velaz, Czech Republic) in accordance with the feeding standard. Drinking water was available *ad libitum*.

**Ethical note:** The experiment was performed in accordance with Czech law and corresponding EU regulations and was approved by the Institutional Animal Care and Use Committee.

**Experiments**

Our study was divided into two experiments. Both of them employed identical experimental procedures: the same cardiac catheterization and the same blood collection protocol were used. We tested two different parameters; the first one was circadian variation during the procedure in young sows (Experiment 1) and the second one differences in cortisol levels in various stages of catheterization in two different day periods (morning and afternoon) (Experiment 2). For these two substudies different statistical analyses were used and due to this fact, two different samples of sows were used.

**Experiment 1:** Circadian rhythm was determined in this experiment. For the first experiment 23 female pigs were used. The sows were divided into two groups (in accordance with day time catheterization): 1) catheterization in the morning (n=13), and 2) catheterization in the afternoon (n=10).

**Experiment 2:** In the second experiment, changes in cortisol levels during heart catheterization in four defined stages of experiment were tested. Eight pigs in the morning and five in the afternoon group in catheterization were used. In both experiments the same experiment and blood collection protocols were used.

**Heart catheterization:** The heart catheterization was bilateral (sinistral and dextral) performed using a standard catheterization procedure (through arteria and vena
The catheterizations were carried out as part of electrophysiological projects, in which electrical stimulation and radiofrequency ablation were performed. In all tested animals of both groups cortisol levels were assessed in the blood serum.

Anaesthesia and medication: Stresnil (dose: 5 mg/kg), Atropin (dose: 0.05 mg/kg) and Narcetan (dose: 14 mg/kg) by an intramuscular injection for pre-medication and sedation were used. To obtain intravenous (IV) access an 18 G or 20 G IV cannula was inserted into the marginal ear vein. IV anaesthetic introduction was performed with Propofol (bolus dose 2 mg/kg). For intubation (under direct laryngoscopic control) with 7 or 7.5 mm orotracheal tubes was used (depending on the size of the sow). Anaesthesia was maintained with an average of 4 mg/kg/h propofol IV infusion, and as analgesic with morphine 0.2 mg/kg (IV bolus) every hour. Ventilation was sustained at an average volume of 8 to 10 ml/kg and respiratory rates of 15 per minute. Continuous monitoring of mean arterial pressure (MAP), heartbeat rate (HR), O2 saturation (SO2) and exhaled capnometry (PCO2) was observed by a multiparameter bed-side monitor.

Blood collection: Blood was collected from jugular vein (v. jugularis) in four defined stages of the experiment. The first (1) was collected at the farm, in non-stress domestic conditions (control sample, basal levels of cortisol), other samples were collected 10 minutes after the presumed stress event: the second sample (2) 10 minutes after intubation and introduction to anesthesia, the third sample (3) 10 minutes after heart stimulation or ablation and the last (4) at the end of the experiment. Total amount of blood (10 ml) was collected in a 10 ml serum Vacutainer system tube (BD Vacutainer, SST II Advance), after 30 minutes incubation in room temperature the tubes were centrifuged (2000×g) for 15 minutes and then the serum was stored at –20 °C till further analyses.

Laboratory analysis: The serum samples were twice extracted and afterwards a high performance liquid chromatography (HPLC) system from Dionex Softron (Germering, Germany) was used for hormone separation. HPLC separation was carried out with the reverse phase EC 250/4 NUCLEOSIL® 100-5 C18 column (MACHEREY-NAGEL, Düren, Germany). To avoid possible column contamination, the Phenomenex SecurityGuard system with C18 cartridge (Phenomenex, Torrance, CA) was used. The solvents Merck (Darmstadt, Germany) as mobile phase for HPLC were used. Serum cortisol concentrations were determined according to a calibration curve using UV/VIS detection. The method was published elsewhere (Šimůnková et al., 2008).

Statistics
Data were divided into two groups, according to the time of day of the experiment (morning or afternoon hours). For each group an independent statistical test to evaluate the mean and standard deviation of cortisol levels at each stage of the experiment was performed.
Experiment 1: The Mann-Whitney test for testing difference between morning and afternoon samples was used. A non-parametric test was used since our data didn’t show standard Gaussian distribution. The Mann-Whitney test was counted for two independent samples (morning and afternoon catheterization) for cortisol concentration in each of the four stages of experiment.

Experiment 2: For testing difference in cortisol levels within the experiment non-parametric Friedman test was used. Friedman test was designed for four dependent samples (different stages of catheterization: 1–4; see above in this section). The test was performed for two independent samples (accordance to day time of intervention, as in Experiment 1).

Results

Experiment 1
For the first stage of experiment (non-stress conditions) the cortisol level mean was higher in the morning (255 nmol/l) than in the afternoon (205 nmol/l) (but not significantly), according to premise circadian rhythm in diurnal animals. For all following stages of the experiment, the morning cortisol levels were surprisingly lower alternative to afternoon levels (mean values are shown in Table 1).

Statistical differences between morning and afternoon cortisol levels were not significant in any tested stage of the procedure (Mann-Whitney, p>0.05). That means we didn’t find any circadian rhythm in cortisol levels. Results are shown in Table 1.

Experiment 2
For the morning group, the highest cortisol level was assessed in the first stage of the procedure (279 nmol/l), then cortisol concentration decreased through second and third stage of experiment to the minimum value at the end of the experiment.

<table>
<thead>
<tr>
<th>Stage of the experiment</th>
<th>Group</th>
<th>Mean (nmol/l) ± standard deviation</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>morning</td>
<td>255 ± 248</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>afternoon</td>
<td>205 ± 110</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>morning</td>
<td>216 ± 115</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>afternoon</td>
<td>278 ± 113</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>morning</td>
<td>215 ± 98</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>afternoon</td>
<td>238 ± 91</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>morning</td>
<td>175 ± 96</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>afternoon</td>
<td>234 ± 122</td>
<td></td>
</tr>
</tbody>
</table>

*stage of the experiment: 1) the baseline level sampled at non-stress conditions at home farm; 2) sampled 10 minutes after intubation and introduction to anaesthesia; 3) sampled 10 minutes after cardiac stimulation or conducting tissue ablation; 4) sampled at the end of experiment
(186 nmol/l). Differences in cortisol levels among the groups operated in the morning were not significant (Friedman test, \(p>0.05\)).

For the afternoon group cortisol levels dynamic was slightly different. The lowest level was at the first stage of the procedure (237 nmol/l), the highest at the second (307 nmol/l), then cortisol concentration decreased back to the baseline level at the end of the experiment (237 nmol/l). Friedman test was not significant for afternoon group (\(p>0.05\)), as for morning group.

Therefore we could conclude that the heart catheterization procedure did not influence cortisol concentrations in any day time. Results are shown in Table 2.

**Discussion**

The aim of the presented study was to determine circadian rhythm of serum cortisol concentration in young sows (12 weeks old) undergoing minimally invasive heart catheterization. It is well known that pigs have circadian rhythm of cortisol levels (Barnett et al., 1981; Becker et al., 1985a, b; Griffith and Minton, 1991). Pigs are animals with diurnal biorhythm; this means the highest cortisol levels are in the morning, the lowest are in the evening. Compared to this premise, no circadian rhythm in GCs was found in our experiment at any stage of the experiment. It is known that the circadian rhythm could be disrupted e.g. due to immaturity of HPA axis in very young animals or by stressful events (e.g. transport, fasting, social instability, surgery, etc.).

In the presented study, twelve-week-old sows were chosen. We wanted to find out if the circadian rhythm is present at this age. Twelve weeks is in the middle of age for the onset of circadian variation presented in the literature; the earliest circadian rhythm occurrence was found in three-week-old piglets (Salfen et al., 2003), but the majority of studies registered circadian rhythm onset a little bit later – from 8 weeks (Ekkel et al., 1996) to 20 weeks (Ruis et al., 1997; de Jong et al., 2000). Many authors (von Borell and Ladewig, 1992; Ruis et al., 1997;

<table>
<thead>
<tr>
<th>Group</th>
<th>Stage of the experiment*</th>
<th>Mean (nmol/l) ± standard deviation</th>
<th>(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>morning</td>
<td>1</td>
<td>279 ± 275</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>242 ± 107</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>198 ± 90</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>186 ± 120</td>
<td></td>
</tr>
<tr>
<td>afternoon</td>
<td>1</td>
<td>237 ± 131</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>307 ± 98</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>266 ± 89</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>237 ± 89</td>
<td></td>
</tr>
</tbody>
</table>

*stage of the experiment: 1) the baseline level sampled at non-stress conditions at home farm; 2) sampled 10 minutes after intubation and introduction to anaesthesia; 3) sampled 10 minutes after cardiac stimulation or conducting tissue ablation; 4) sampled at the end of experiment
de Jong et al., 2000) observed an absence of cortisol circadian rhythm in pigs younger than 15 weeks of age.

As it was mentioned, circadian rhythm could be affected by stress events (references in following text). On the home breeding farm, the pigs were used to living in natural groups. The day before heart catheterization, the experimental pig had been separated from its home pen and moved to other pen, where it was isolated from its pen mates. It can represent a stress resulting from moving and from social deprivation. Moving and exposure to new territory (Dantzer and Mormède, 1983; Becker et al., 1985a; Grandin, 1997; Désautès et al., 1999) and social instability, less or none social contacts (Janssens et al., 1994; Ekkel et al., 1997; Ruis et al., 1997) increase GCs levels. Moving experiments were carried out by Becker et al. (1985a) and they have proved that moving can disrupt diurnal rhythm.

The day before the experiment, after moving, the pig had to fast (due to anesthesia performed the next day). Food intake can influence circadian rhythm. In some studies two peaks of corticosterone levels were found, in the morning and in the afternoon; which was the time when the animals expected to be fed (Geverink et al., 2003; Hillmann et al., 2008). Fasting can elevate cortisol levels in pigs (Salfen et al., 2003; Kojima et al., 2009), but according to Salfen et al. (2003) it doesn’t disrupt the circadian rhythm in cortisol. In our study cortisol levels were slightly (not significantly) higher in alternative to baseline level, but no circadian rhythm was present.

Transport from the farm to the laboratory could be also a stressful event (Becker et al., 1985b; Dalin et al., 1993; Averos et al., 2007).

The influence of these events was tested in Experiment 2. We designed four different stages of experiment in which cortisol concentration was assessed (for details see the section Material and Methods). We didn’t find any statistical difference in the whole process; therefore we could suppose stress events hadn’t disrupted circadian rhythm in our sows. Due to this fact, we suppose that the circadian rhythm absence was primarily caused by HPA axis immaturity in our young sows.

In this pilot study only preliminary results and conclusions were presented as only small number of animals was tested (particularly in the second experiment), in further analyses we are planning to test more sows and also more stress markers (e.g. cortisone, dehydroepiandrosteone, etc.) in order to bring more compact view to problem of stress in pigs undergoing heart catheterization.

**Conclusion**

We assume that the absence of cortisol circadian rhythm in our experimental young pigs was caused particularly by the immaturity of the HPA axis. Moreover, circadian rhythm could be theoretically affected by the procedures held on the day before the experiment or by the experiment itself (e.g. transport from breeding home farm to our laboratory, social isolation from known pen mates, premedication procedures and heart catheterization). To avoid the influence of stress on determining circadian
rhythm, we tested differences between stages of catheterization. We didn’t find any differences in cortisol levels in tested situations, therefore we can conclude that the handling of sows was not stressful and probably did not influence circadian rhythm absence.

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References

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