well. A substrate that is specific to the conjugated enzyme is finally added to the system. The binding of the substrate to the conjugated enzyme starts a reaction that will produce some color in the solution. The more sample present in the well and bound to the antibody, the less conjugate will react with the substrate and thus result in less color production.

Figure 4 Competitive ELISA mechanisms

MATERIALS AND METHODS

Materials and Instruments

The animals used were 26 male F344 rats (F344/Sca), obtained from Scanbur B&K, Sollentuna, Sweden, weighing 246±3.6 grams (mean±sem). Instruments that were used were macrolone type III cages, surgery equipment, catheter (DiLab, Lund, Sweden), Accusampler®, ELISA reader, and centrifuge.

Materials used were Nutella® hazelnut and chocolate cream, buprenorphine (Temgesic®, Schering-Plough Europe, Brussel, Belgium), iodine (Jodopax vet®, Pharmaxin AB, Helsinborg, Sweden), Corticosterone Enzyme Immunoassay kit (Assay-Design Inc., Ann Arbor, MI, USA), and pentobarbital 100 mg/mL.

Research Design and Methods

Experimentation (days 0-4)

Twenty six adult male Fischer 344 rats with almost the same weight were used in this study. Two rats were used in pilot project using Buprenorphine 0.1 mg/kg. The rest of the animals (24 rats) were divided into four treatment groups with six animals in each. Group 1 was the Control and untreated group, Group 2 was the Nutella® treated group (vehicle), Group 3 were the Buprenorphine 0.4 mg/kg BW treated group, and Group 4 were the Buprenorphine 1.0 mg/kg BW treated group. Six rats died during the surgery and four rats were excluded due to the technical difficulties with blood sampling. Fourteen rats were thus included in this study.

Blood sampling started immediately after surgery when rats regained consciousness, followed by another sample at 2 pm, then every 4th hour until 18 hours post-operative (day 1). In the morning of day 1 and day 2 the rats were treated with Buprenorphine or Nutella® according to the table. In the morning (8 am) day 2, the blood sampling continued with a blood sample every 4th hour to investigate the effect of the treatment on the levels of corticosterone unrelated to the surgery. At 8 am day three, blood samples were collected every 4th hour until the experiment was finished at 8 am day 4 to investigate the physiological levels of corticosterone unrelated to analgesic treatment.

One day prior to surgery, rats were put in a clean cage for sampling of pre-operative fecal samples. Twenty four and forty eight hours after surgery, feces were collected for post-operative samples. After completion of blood sampling day 4, rats were euthanized with 1 ml pentobarbital 100 mg/mL.

Table 1 Treatment groups of rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control (no analgesia)</td>
</tr>
<tr>
<td>2</td>
<td>Control (vehicle) [Nutella 2g/kg]</td>
</tr>
<tr>
<td>3</td>
<td>Buprenorphine 0.4 mg/kg in Nutella</td>
</tr>
<tr>
<td>4</td>
<td>Buprenorphine 1.0 mg/kg in Nutella</td>
</tr>
</tbody>
</table>

Animals

After arrival, the rats were housed in Macrolone type IV cages in groups of two animals per cage and kept for seven days acclimatization in animal rooms under standardized conditions: Diurnal rhythm was regulated with a 12 h light: 12 h dark cycle with lights on from 06.00 to 18.00; temperature was kept at 20 ± 2 °C; relative humidity was 30-60%; air was changed approximately 15 times per hour; and cages were cleaned twice a week. Aspen chips were used as bedding material. The animals had free access to food pellets (R36 Laktamin, Stockholm, Sweden) and tap water at all times. Food pellets were placed on the bedding to
improve accessibility after surgery. One week before surgery, the rats were transferred to single housing in Macrolone type III cages and moved to a designated laboratory with similar environmental conditions, where the experiments were commenced. Initially, the rats were transferred already after two days, which is sufficient when using Sprague-Dawley rats, as shown by Goldkühl et al. (2008) and Dahlin et al. (2008). However, it was observed in the present study that the F344 animals required a longer habituation period after translocation and switch from group to single-housing. After translocation, all rats, except from Control animals, were allowed to ingest Nutella® hazelnut and chocolate cream for habituation to the flavor, and to facilitate future administration of analgesia. All rats were handled regularly each day to habituate to the experimenter. The body weight and water consumption of all animals were registered daily during the entire experiment, starting when the animals were transferred to the laboratory one week prior to surgery until the end of blood sampling.

Surgery (day 0)

All surgery was commenced and completed before noon. One hour before surgery, rats from group 3 and 4 were treated for pre-emptive analgesia with Buprenorphine (Temgesic®, Schering-Plough Europe, Brussels, Belgium), 0.4-1.0 mg/kg dissolved in Nutella® (2 g/kg body weight) for oral administration by voluntary ingestion. The dose is based on that recommended in the literature (Dobromylskyj et al., 2000; Flecknell et al., 1999; Hedenqvist and Hellebrekers, 2003) and on experience of this route of administration (Goldkuhl et al., 2008; Royo et al., 2004; Siswanto et al. 2008). Rats were placed in an induction chamber and anesthesia was induced with 5% isoflurane delivered in 100% oxygen. Once the paw withdrawal reflex was absent, the rats were shaved at the incision sites and attached to a Simtec anesthetic mask for spontaneous respiration. Isoflurane was maintained at an appropriate level to ensure adequate anesthesia. The shaved parts were washed with iodine (Jodopax vet®, Pharmaxin AB, Helsingborg, Sweden). An incision was made in the skin of the neck and an arterial cannulation was performed by catheterization of the common carotid artery for blood sampling with catheters (DiLab, Lund, Sweden) filled with heparinised saline. The catheters were secured in the vessel and led subcutaneously through a DiLab Dacron button attached to the dorsal region of the neck. The catheters were led further through a metal spring and connected to an AccuSampler® for automated blood sampling.

Corticosterone Extraction

Corticosterone metabolites were extracted from fecal samples using the method described by Eriksson et al. (2004). In brief, all of the fecal pellets samples were dried in 30 °C for 2 hours. After that, all of the samples were weighed and MiliQ water was added (4 g per 1 g sample), followed by thorough homogenization. Then, 1 g of fecal homogenates was weighed and placed in a glass tube. Five mL of dichloromethane was added to each tube, and tubes were vortexed for 5 seconds 7 times. Each tube was covered by parafilm and centrifuged for 15 minutes at 1200 g. The reminder of the extraction procedures were commenced at a temperature of 4OC. After centrifugation, the hydrophilic phase was removed by vacuum. The organic phase was transferred to a clean tube. One mL of NaOH (0.1 M) was added, tubes were vortexed 3 times, and the tubes were covered with parafilm. The tubes were again centrifuged for 15 minutes at 1200 g. The hydrophilic phase was removed and the organic phase was washed two times using MiliQ water. One ml of each sample was transferred to a clean glass tube, and the dichloromethane was evaporated with nitrogen. The samples were dissolved in 500 μl assay buffer for the ELISA kit described below, and kept at -20 °C until analysis.

Corticosterone Analysis

Blood samples were collected in cooled tubes at 4°C, after which they were centrifuged to remove blood cells and obtain serum. Serum was stored at -20°C until analysis. Fecal pellets were homogenized and corticosterone metabolites were extracted as described above. Serum corticosterone and fecal corticosterone metabolites were quantified with enzyme-linked immunosorbent assay (ELISA), using a commercial Correlate-ELISA kit for corticosterone (Assay-Designs Inc., Ann Arbor, MI, USA).

The samples were thawed in room temperature and 10 ul of samples were pipetted to new tube and were diluted 100 times with assay buffer 15 for blood sample and 5 times for fecal samples. After that, 100 ul of samples and standard were pipetted to ELISA
plate. Then, 50 ul of conjugate and 50 ul of antibody were added to each well and the plate was incubated for 2 hour in room temperature at 200 rpm. After that, the plate was washed 3 times by washing buffer and evaporated in room temperature. Each well was added with 200 ul of pNPP substrate and incubated for 1 hour. Finally, 50 ul of stop solution was added to each well and the absorbance of each well were read in ELISA reader at 405 nm.

Statistical Analysis
The data are presented as mean. Analysis of variances (ANOVA) was used to calculate differences between treatment group on serum, and fecal corticosterone levels at different time point, differences in water consumption, and differences in mean body weight changes. P<0.05 were considered significant.

RESULTS AND DISCUSSION

Blood samples were taken in 3 phases. The first phase (0-18 h post-op) was to determine the post-operative corticosterone levels of Fischer 344 rats and to see the effect of pre-emptive analgesia on post-operative corticosterone. The second phase, which was taken on 8 am day 2 until 8 am day 3, was to determine the effect of post-operative treatment with analgesic on corticosterone release and compare it with Control. The last phase, which was taken on 8 am day 3 until 8 am day 4, was to determine the normal corticosterone level on each group after all treatments were stopped. Serum corticosterone levels during 18 hours after surgery are shown in Figure 5. The serum corticosterone levels when animals in Control group regained consciousness were 250 ng/ml. After that, serum corticosterone declined between 0-6 h, followed by an increase that reached maximum at 10 h. After that, the corticosterone levels decreased until it reached minimum level, which was 30 ng/ml, at 18 h. The Nutella® group showed similar profile as the Control group. The highest level was 300 ng/ml at 0 h post-operative and the lowest was 150 ng/ml at 18 h post-op. Buprenorphine 0.4 mg/kg BW group also showed similar profile as the Control group. The highest level was 250 ng/ml at 0 h post-operative and the lowest was 100 ng/ml at 18 h post-op. Serum corticosterone in the Buprenorphine 1.0 mg/kg BW group also showed similar profile as the other groups, but there were differences on 0 h post-op. The level at 0 h was 150 ng/ml and significantly lower than other groups (ANOVA, F(3,10)=12.991 ; p<0.05). After that, no differences could be observed untill 18 h post-op. Serum corticosterone level of this group at 18 h was 10 ng/ml and was significantly lower than Nutella® group (ANOVA, F(3,10)=4.112 ; p<0.05).

The serum corticosterone levels during 44-92 h after surgery are shown in Figure 6. All groups showed the same diurnal rhythm with highest level in the evening and the lowest in the morning. This may indicate that the adrenal cortex performed normally after surgery. The level in Buprenorphine 1.0 mg/kg group is significantly lower than Control group in the beginning of dark period in day 2 (ANOVA, F(3,8)=4.947 ; p<0.05). After that, no differences could be observed between groups.

Surgical stress can cause rapid increase in serum corticosterone levels. This is due to surgical tissue damage and anesthetic agents during surgery, including isoflurane that is used in this study, which can lead to stress and elevated corticosterone levels in the post-operative phase (Whitten et al. 1998; Martini et al. 2000). Pre-emptive analgesia is associated with reduced post-operative pain and attenuated production of pro-inflammatory cytokines. These cytokines are associated with hyperalgesia during the post-operative phase (Shavit et al. 2005).

The high serum corticosterone level at the beginning of post-operative phase (0 h post-op) on Control, Nutella® and Buprenorphine 0.4 group may indicate that these groups were suffering from post-operative pain. The levels of corticosterone, when the animals regained consciousness, were very high in these groups compared to Sprague-Dawley rat (with maximal levels at approximately 200 ng/ml) (Royo et al. 2004; Abelson et al. 2005; Goldkuhl et al. 2008). This finding confirms that F344 is more responsive to stress and have high corticosterone serum level after stressful event. The serum corticosterone declined 0-6 h after surgery. This profile shows the negative feedback mechanism in reducing corticosterone release from adrenal cortex. When serum corticosterone release increased, corticosterone will perform a binding with its receptor in adrenal cortex which will stop corticosterone synthesis. The serum corticosterone increase at 10 h post-operative show normal diurnal rhythm as the animal entering its active phase, but could also indicate lack of sufficient analgesia (Goldkuhl et al 2008).