The hypothesis of the present study is that pre-operative treatment of Buprenorphine administered by oral voluntary ingestion can reduce stress and suppresses the post-operative serum corticosterone levels in F344 rats kept single-housed and connected to an automated blood sampling device. The study investigates both immediate effects during the first 24 hours and later effects two and three days after surgery. The study also aims to validate the oral treatment as a sufficient post-operative treatment by investigating its impact on clinical signs such as body weight gain and water consumption. The research significance is to give information about pain and stress response in laboratory rats and to serve as important guidance for better analgesic strategy and increased animal welfare.

LITERATURE REVIEW

Pain, Stress and Corticosterone

The ability to experience pain is vital for the survival of all animals. Pain is a subjective experience and serves as a warning signal to make an escape or evasive action possible. The physiological and pharmacological activities that lead to a painful sensation are denominated nociception. The nociceptive pathway can be described as a three-neuron chain that transmits the nociceptive information from peripheral tissue to cerebral cortex.

The first order neurons are transferring the nociceptive signal from periphery tissue to the spinal cord. This is caused by degeneration of cell membranes that lead to accumulation of phospholipids in the tissue, which are converted to arachidonic acid, which in turn is converted to prostaglandins. The latter sensitizes and activates the nociceptors and elicits a transmission of information to the spinal cord. From the spinal cord, the nociceptive information ascends via the second order neurons to the thalamus, hypothalamus, and other regions of the brain. (The second order neuron). This results in activation of most brain structures, including the Hypothalamus-Pituitary-Adrenal (HPA) axis which causes release of corticosterone. From the thalamus, the nociceptive information is transmitted to the cerebral cortex via the third order neurons (Abelson 2005).

Corticosterone is a steroid hormone that consists of 21 carbons and excreted by the glamorous zone and the fasciculate zone of adrenal gland. Corticosterone is a glucocorticoid hormone that regulates the conversion of amino acids to glucose (gluconeogenesis) and glycogen (glycogen synthesis) in the liver and also stimulates glycogen synthesis in other tissue (Turner & Bagnara 1976). This class of hormone (Figure 1) also regulates cardiovascular, immune, and behavioral processes. Glucocorticoids are steroid hormones that have the capability to perform a binding with glucocorticoid receptors (GR), a 94 kD cytosolic protein. This complex will bind to specific DNA motifs termed glucocorticoid response elements (GREs) in the promoter region of glucocorticoid responsive genes and regulate the expression of this hormone through interaction with transcription factors (Smith & Vale 2006).

Corticosterone release is induced by brain response to stressful stimuli. In stress conditions, stressful stimuli cause activation of the paraventricular nucleus (PVN) in the hypothalamus to release Corticosteroids Releasing Factors (CRF). CRF contains Corticosteroids Releasing Hormone (CRH), Vasopressin, and some unknown factors. CRF will in turn induce the release of Adrenocorticotropic hormone (ACTH) from the pituitary gland. ACTH will recognize its receptor (melanocortin type 2 receptor) on parenchymal cells of fasciculata zone of adrenal cortex and cause activation of adenylyl cyclase. This results in the accumulation of cAMP inside the cell and activates the phosphorylase enzyme. This enzyme will catalyze glycogen catabolism and therefore increase in full (NADPH) concentration inside the cell. NADPH is the main substrate for steroid synthesis (glucocorticoid) by adrenal cortex. Specifically ACTH promotes the conversion of cholesterol into δ-5 pregnolone during the initial step of glucocorticoid biosynthesis (Moberg & Mench 2000; Gordon et al. 1982; Smith & Vale 2006; Cohen et al. 2006).
the adrenal cortex releases corticosterone, the corticosterone will inhibit the secretion of the CRF and ACTH via negative feedback mechanism.

The newly synthesized corticosterone will penetrate through cell membranes and bind with its receptor inside the cell and make a receptor-ligand complex that will penetrate the cell nucleus. Inside the nucleus, the complex will bind to GREs located in the promoter site of gene. This will cause activation of appropriate hormone-responsive gene (Tsigos & Chrousos 2002; Gordon et al. 1982).

**Buprenorphine as Analgesic**

Analgesic drugs can be divided into 2 major classes, which are non-narcotic analgesic drugs and narcotic analgesic drugs. The latter is the oldest and most common analgesic for experimental use and as pain reliever. The analgesic effect of narcotic drugs is hypothetically caused by interaction between the drug and specific receptors which are located in the brain, the spinal cord, and the heart. Receptors that attract particular interest from scientists are μ (mu) and κ (kappa) opioid receptors. Activation of mu receptors causes sedation, addiction, and have been found to cause analgesia. Activation of kappa receptors causes sedation but not addiction like the mu receptors (McCurnin & Bessert 2006). Buprenorphine is a lipophilic (Cowan et al. 1977), semi-synthetic derivative of thebaine. It is primarily used in the management of moderate to severe pain and is also known to reduce opiate consumption in individuals physically dependent on cocaine and heroin (Gomez-Flores & Webber 2000; Sacerdote 2006). Physical properties of Buprenorphine are water solubility at room temperature, stable over a wide pH range and at 55 °C, and it will become unpalatable for rats if the concentration, dissolved in gelatin, is more than 5 mg/kg (Martin et al. 2001).

Most frequently, Buprenorphine is classed as mu receptor agonist with antagonist effect at kappa receptor. But in higher dose (1 mg/kg by subcutaneous administration), it has been shown that the antagonist effect of Buprenorphine predominate and the analgesic effect decline, making a plateau profile (Roughan & Flecknell 2002). It takes longer time (compared to morphine) to bind with its receptor, but once it binds, it is difficult to displace it (with naloxone) and the dissociation of the drug from the receptor is very slow, making it an analgesic drug with long duration (Cowan et al. 1977). Buprenorphine doesn’t immediately activate HPA axis (like μ-agonist opioid, i.e. morphine) but repress corticosterone production early after its administration. Since glucocorticoids have been associated with immunosuppression, Buprenorphine can overcome the disadvantages of morphine in natural killer cell activity and tumor metastasis, making it a suitable post-operative treatment for minor to major surgery (Franchi et al 2006). Study of efficacy of Buprenorphine using analgesiometric tests in rats indicates that at equi-analgesic dosage, Buprenorphine is 6-10 times more potent than morphine if given orally (Roughan & Flecknell 2002). Buprenorphine has been proved to attenuate post-operative behavioral effect such as reduction in food and water consumption, body weight (Goldkuhl et al. 2008), and generalized locomotor activity (Roughan & Flecknell 2002).

**Automated Blood Sampling**

Automated blood sampling is a very convenient method for measuring stress marker molecules, such as corticosterone in conscious rats. Although the surgical procedure for catheterizing animal is associated with stress, since the surgery causes painful tissue damage which activates the HPA axis, the blood sampling itself is less stressful to the animal compared to manual blood sampling (Vachon & Moreau 2001) and the corticosterone level in rats will recover within 18 hour after the surgery. This will make it more accurate than manual blood
sampling for measuring stress marker molecules, like corticosterone, as the result will not be biased with the stress caused by handling during manual sampling (Abelson 2005; Royo et al. 2004). An example of an automated blood sampler is AccuSampler®.

The AccuSampler® (DiLab, Lund, Sweden) is a computerized, fully automatic blood sampler that enables administration of drugs into blood circulation of conscious rats, as well as collection of blood samples, without any human interception except for the surgical insertion of catheters in the jugular vein and/or common carotid artery (Figure 3). The ability to withdraw blood without disturbing the animal by handling makes the AccuSampler® a very applicable tool for physiological and biological studies in laboratory animals in vivo. One advantage of Accusampler® is that it allows repeated blood sampling at some interval without causing loss of body fluid, since the withdrawn blood volume is immediately replaced by saline. However, too much blood withdrawal and too high frequency of blood sampling with this equipment will affect the corticosterone level due to hemorrhagic shock (Abelson et al. 2005).

F344 has been extensively used as a model for aging research and model of stress response and for several stress-related psychiatric diseases (together with inbred Lewis strain). This strain is a good model for stress research and it is often compared to inbred Lewis, the genetically histocompatible inbred strain which is more hyporesponsive to stress, and outbred Sprague-Dawley strain, the origin of the F344 and Lewis strains. F344 has been shown to be more responsive in stress and corticosterone release due to environmental stressor (such as novelty environment or single housing) and human interaction (such as surgery or handling) compared to the other two strains. Due to this robust stress corticosterone level, F344 is protected from autoimmune diseases but more vulnerable to viral and bacterial diseases and also cancer because of the immunosuppressive effect of corticosterone (Baumann et al. 1999; Dhabhar et al. 1993; Gomez et al. 1998).

Enzyme-Linked Immunosorbent Assay

Enzyme-Linked Immunosorbent Assay (ELISA) is one of the most commonly used immunoassay nowadays. The method was first introduced in 1971 by Engvall and Perlman (Lequin et al. 2005). The method is still expanding in all fields of basic and applied biology. A recent application is in detection of contaminants in quality assessment of food. The key to all ELISA is the use of antibodies. Immunoassays involve the use of antibodies or antigen as reagent. Enzyme immunoassay makes use of enzymes attached to one of the reactants in the assay to allow quantification through the development of color after the addition of a suitable substrate/chromogen. ELISA involves the stepwise addition and reaction of reagents to a solid-phase bound substance, through incubation and separation of bound and free reagents by washing. An enzymatic reaction is utilized to yield color and to quantify the reaction through the use of an enzyme-labeled reactant.

There are several kinds of ELISA. One of them is the competitive ELISA (Figure 4). In this kind of ELISA, the sample is added to the well that contains an antibody specific for the sample. After that, an antigen that is conjugated with an enzyme (usually alkaline phosphatase), called the conjugate, is added to the system. This antigen has the same binding specificity as the sample and competes with the sample of binding to the antibody in the
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