Title: The effect of ketamine and saffan on the β-endorphin and ACTH response to haemorrhage in the minipig.

Short Title: β-endorphin & ACTH response to haemorrhage

Authors: Therese Ruane-O'Hora*, W.J. Hall, F. Markos

Department of Physiology

University College Cork

Cork

Ireland

***Corresponding author:**

Dr. Therese Ruane-O'Hora

Department of Physiology

University College Cork

Cork Ireland

Tel. 00-353-214902235

Fax: 00-353-214272121

e-mail t.ruane-ohora@ucc.ie

Summary

The endocrine response is an important component of the physiological response to blood loss, and there is some variability in reported levels of certain hormones during haemorrhage such as the stress hormone adrenocorticotrophic hormone (ACTH). Therefore, the effect of two anaesthetic agents, ketamine and saffan, on ACTH and β-endorphin levels during haemorrhage was assessed in 12 mini-pigs. The animals were divided into 2 groups, group I saffan and group II ketamine (n=6). Pigs were subjected to a continuous fixed volume haemorrhage under one of the above anaesthetics while spontaneously breathing. Blood pressure and heart rate responses were recorded together with β-endorphin and ACTH levels both before and at 10, 20, 30, 40 minutes after commencement of bleeding. ACTH levels were higher in the ketamine anaesthetised pigs and rose significantly faster with falling blood pressure than ACTH measured in pigs under saffan anaesthesia. In contrast the haemorrhage induced β-endorphin increase was not significantly different between the two anaesthetic groups. These results indicate that choice of anaesthetic agent is important when investigating the hormone response to haemorrhage and may account for the variable hormone levels in the published literature to date.

Key Words: Anaesthesia, β-endorphin, ACTH

Introduction

In vivo animal models are essential in investigating the physiological response to blood loss (Bertolini et al., 1986; Yilmazlar et al., 2000; Kurita et al., 2007; Rivera-Chavez et al., 2007). Haemorrhage results in a variety of well known haemodynamic changes, such as increased cardiac output and heart rate, together with the less recognised but potentially important hormonal alterations (Schadt & Ludbrook, 1991). For example, increasing levels of β-endorphin, an opioid, have been shown during haemorrhage and this provided evidence that opioids may contribute to the pathophysiology of hemorrhagic shock (Lang et al., 1982; Chernow et al., 1986; Gurll et al., 1986; Tuggle & Horton, 1986; O'Benar et al., 1987). Further, naloxone an opioid receptor antagonist has been shown to have an anti-shock effect following circulatory shock (Holaday & Faden, 1978). Similarly, levels of adrenocorticotrophic hormone (ACTH) are also increased during haemorrhage (Bereiter, et al., 1984; Bereiter, et al., 1986; Darlington et al. 1986; O'Benar et al., 1987). Both hormones form two limbs of an important physiolological opioid/anti-opioid system in the body, with ACTH being the anti-opioid, and it is suggested that this is disrupted by haemorrhage and hence contributes to the pathophysiology of shock (Bertolini, 1995). In fact it has been shown that injection of ACTH aided resuscitation from haemorrhage in anaesthetised rats (Bertolini, 1995), and clinically in humans (Noera et al., 2001). As well as its anti-opioid effects treatment with ACTH has also been shown to suppress the inflammatory response after haemorrhage, together with activation of an anti-inflammatory reflex (Giuliani et al, 2007; Guarini et al, 2003 & 2004), further contributing to its potential therapeutic value.

However, the physiological β-endorphin and ACTH response is also variable, depending on the anaesthetic used, e.g. ketamine is an inhibitor of ACTH release in sheep (Powers & Wood, 2007), whereas halothane stimulates ACTH release more than isoflurane in rabbits (Gil et al., 2007). Finally, β-endorphin levels have been shown to increase in pigs under ketamine anaesthesia (Gerard et al 1996). It is possible that the variation in response to haemorrhage may be due to the choice of anaesthetic, since it is well known that the response of these two hormones during haemorrhage differs in the anaesthetised versus the conscious state (Schadt & Ludbrook, 1991). We examined the effect of general anaesthesia on the β-endorphin and ACTH response to haemorrhage in pigs under saffan and ketamine anaesthesia. Both of these anaesthetics are commonly used in experimental animals.

Methods

Ethical Approval

This study was carried out under licence issued by the Department of Health, Ireland as directed by the Cruelty to Animals Act Ireland and EU Statutory Instructions and local University ethics committee.

Twelve Vietnamese Yucatan hybrid pigs (19 – 24 Kg) bred and housed at the Biological Services Unit of University College Cork were used for these experiments. Animals were fed on a standard pig diet and allowed water ad libitum and were fasted for 24 hours before experiments. Pigs were pre-medicated with ketamine hydrochloride (10 mg/kg I.M.), and later assigned to two groups based on the anaesthetic to be used during bleeding. In both groups an intravenous catheter was inserted in the marginal ear vein.

Group I (n = 6):

Saffan (Pitman Moore). General anaesthesia was induced with saffan 2 mg/kg (12 mg/ml, 9 mg alphaxalone and 3 mg alphadolone acetate) administered via the ear vein, and maintained by continuous infusion at a rate of 12 mg/kg/hr, using a microprocessor controlled syringe pump (Harvard, Pump 22).

Group II (n = 6):

As ketamine is a dissociative anaesthetic it was decided to carry out the surgery using halothane 2-3% (Fluothane, Zeneca Ltd, UK) for induction, with nitrous oxide and oxygen, and maintenance of 1-2%. On completion of surgery, gaseous

anaesthesia was discontinued and a continuous infusion of ketamine (ketalar, 50 mg/ml) was commenced via the ear vein at a rate of 20 mg/kg/hr using a microprocessor controlled syringe pump (Harvard, Pump 22).

Depth of anaesthesia was assessed regularly by checking for response to a painful stimulus. Bi-lateral femoral artery cannulations were carried out in both groups, for subsequent blood pressure monitoring, blood sampling and bleeding. Blood pressure was monitored using a pressure transducer (Statham P23 AA) and an ECG was also recorded. Both the transducer and the leads were connected to a computerised amplifier unit (MacLab, ADInstruments, UK) allowing digitised recording of all data, which in turn was fed to a computer (Apple Mac, Power PC) where the data was recorded using ADInstruments software, UK. Following instrumentation, animals were heparinised with 250 units/Kg of heparin (Leo Laboratories) and transferred to a specially made hammock, which allowed the animal to be supported in a comfortable yet restrained normal horizontal position. At this stage animals were kept under observation, but left undisturbed for two hours, before commencement of bleeding. This 2 hour waiting period was partly to ensure that levels of halothane and nitrous oxide in the circulation of Group II animals would be minimal, and unlikely to interfere with the study in this group, where ketamine was the anaesthetic of interest. In a previous study using halothane in swine an end tidal concentration of just 0.12% was recorded just 45 minutes after discontinuing the drug, which was administered in a dose range of 0.6 - 1.63% (Brower & Merin, 1979).

After the 2 hr control period bleeding was commenced from a femoral arterial line using to a roller pump (Watson Marlow, Smith & Nephew) at a fixed rate of 1 ml/min/Kg body weight, until death occurred. Blood was collected into a graduated cylinder to measure volume lost. Control blood samples (10 ml) for measurement of ACTH and β-endorphin were taken 30 min before and at the start of bleeding (time zero min). Once bleeding commenced, blood was sampled at 10 min intervals. In all cases blood was replaced with an equal volume of saline. Samples were taken into plastic EDTA tubes and 1 ml of the protease inhibitor aprotinin (Sigma) was added. The tubes were immediately placed on ice, and later centrifuged (Sorvall, RT6000) for 15 minutes. Immediately afterwards plasma was drawn off into plain plastic tubes and frozen at - 40 ºC, for subsequent assay of ACTH and β-endorphin.

Adrenocorticotrophic hormone (ACTH) assay

This hormone was assayed using a commercially available kit (Nichols Institute Diagnostics). Samples were assayed in duplicate, 200μl of plasma required for each sample. Assay sensitivity was 1 pg/ml.

β-endorphin assay

A Nichols Institute assay kit was used also. Samples were assayed in duplicate, 200 μl of plasma required for each sample. Assay sensitivity was 14 pg/ml.

Statistical analysis

Results are expressed as means ± standard errors of the means (S.E.M). One way analysis of variance (ANOVA) was used to compare baseline values. In Figure 2 two way analysis of variance with repeated measures on both factors was used (2-way ANOVA) to assess the hormone response over the entire period of bleeding. This provides information on whether the entire response curve to haemorrhage differs significantly between the two anaesthetic groups. This is important, since we are not interested in identifying a particular time point at which the hormone response may be different. Finally, homogeneity of the regression lines obtained in figures 3 and 4 was compared using analysis of covariance (ANCOVA). $P < 0.05$ was taken as significant.

Results

Before haemorrhage commenced baseline values were as follows, all data presented as mean ± sem and n = 6 for both groups, for Group I saffan, heart rate averaged 109 ± 15 beats/min (range: 70 – 178), mean arterial pressure was 138 ± 6 mmHg (range: 114 – 156), neither values were significantly different to the corresponding heart rate and mean blood pressure in the group II, ketamine pigs which were 102 ± 9 beats/min (range: 75 – 133) and mean arterial pressure 145 ± 8 mmHg (range: 117 – 170), respectively.

Control ACTH levels averaged 11.4 ± 3.4 pg/ml (range: 6.7 – 27.7) in the saffan group, but this was significantly higher in the ketamine group giving a mean value of 63.3 ± 14.4 pg/ml (range: 31 – 108; P = 0.006 one way ANOVA). In contrast β-endorphin was 14.8 ± 1.8 pg/ml (range: 11 – 22.5) in group I which was not significantly different to the control levels measured in group II which averaged 12.1 ± 0.7 pg/ml (range: 10.6 – 15.1; P = 0.19 one way ANOVA).

Figure 1 shows that the heart rate (Figure 1A) and blood pressure (Figure 1B) response to haemorrhage was not significantly different between the two anaesthetic groups (P = 0.97 for heart rate and P = 0.47 for blood pressure, 2 way ANOVA).

The effect of choice of anaesthesia on the ACTH response to haemorrhage.

ACTH levels increased during haemorrhage in both groups (Figure 2A), as mentioned the control ACTH levels prior to haemorrhage were significantly higher in

the ketamine group. Despite this, ACTH levels increased significantly more in the ketamine anaesthetised pigs during haemorrhage than in the saffan anaesthetised pigs (Figure 2A, $P < 0.00001$ 2 way ANOVA). Further, the actual rate of increase of ACTH accompanying the fall in blood pressure was also significantly greater in the ketamine group (Figure 3), increasing at a rate of 5.5 pg/ml/mmHg for ketamine compared to an increase of 2.2 pg/ml/mmHg in the saffan ($P = 0.028$, ANCOVA) group.

The effect of choice of anaesthesia on the β-endorphin response to haemorrhage.

In contrast to ACTH, β-endorphin control levels were not significantly different between the two anaesthetic groups, in addition levels of the hormone were also not significantly different over the time course of haemorrhage (Figure 2B) ($P = 0.16$, 2 way ANOVA). Figure 2B shows that there was a large reading for β-endorphin in the saffan group at 40 minutes. We decided to use two-way ANOVA with repeated measures on both factors to analyse the data, this reducing the chances that aberrant data at a single time point would affect the conclusion, since, we are primarily interested in assessing the pattern of the response to haemorrhage over the entire period of blood loss, and not at a single time point. Finally, the rate of increase of β-endorphin (Figure 4) was not significantly different between the two groups, for ketamine β-endorphin increased at a rate of 0.44 pg/ml/mmHg and for the saffan group the rate was 0.98 pg/ml/mmHg, fig 4. ($P = 0.2$ ANOVA). In addition the adjusted means for the two groups in figure 4 were also not significantly different ($P = 0.34$ ANCOVA).

The survival time (i.e. the time in minutes after haemorrhage began until mean blood pressure fell below 20 mmHg was found to be statistically different between the two groups with mean values of 49.6 ± 1.5 and 41.8 ± 1.1 min for ketamine and alphaxalone/alphadolone respectively ($P < 0.05$, one way ANOVA).

Discussion

The main finding of this study was that haemorrhage under ketamine anaesthesia resulted in a greater rate of increase in ACTH with decreasing blood pressure than in pigs subjected to haemorrhage while under saffan (Figure 3). Furthermore the peak level we recorded after 40 minutes of bleeding, with ketamine 849 pg ml^{-1} approx (though not alphaxalone/alphadolone) was well above the peak level reported in conscious pigs 518 pg ml^{-1}, (O'Benar et al. 1987). The average blood loss as reported in these conscious pigs was 38.5 ml Kg^{-1}, whereas the average loss in our experiments was in fact less at 34-35 ml Kg^{-1}.

However, the increase in β-endorphin levels during haemorrhage did not differ between the two anaesthetic groups (Figure 4). There was no significant difference in the heart rate and blood pressure response to haemorrhage with either anaesthetic. Therefore, these results show that the choice of anaesthetic is an important factor to be considered when investigating hormonal responses to hemorrhagic shock using animal models, and this could potentially account for the variable results previously reported. A depressant effect of anaesthesia may provide a possible explanation for the lack of an effect of haemorrhage on the β-endorphin response in the present study, since higher levels have been shown in conscious pigs during haemorrhage (O'Benar et al., 1987). However, in other studies on haemorrhage carried out under anaesthetia, higher levels of β-endorphin have been reported Tuggle et al, 1986, than in the O'Benar et al. study (1987).

We did not carry out a set of experiments in conscious pigs as we felt this was both unnecessary and perhaps even un-ethical, in view of the data of O'Benar et al. 1987 which is quite clear, also there are many similarities between their experiments and ours. Both groups used pigs, both protocols used a fixed volume rather than fixed pressure model of haemorrhage and at the end of the haemorrhage period both the conscious pigs and our animals had lost similar though not identical amounts of blood. The major difference was that their animals were conscious while ours were anaesthetised.

Ketamine was chosen as one of the anaesthetics in the present study because it is widely acknowledged as an appropriate anaesthetic to use in clinical cases of hemorrhagic shock (Craven, 2007), and is also used as a research anaesthetic. (DeClue et al., 2008; Valentino et al., 2008). Although not used as an anaesthetic in humans, saffan is a commonly used general anaesthetic in *in vivo* animal research as well as veterinary medicine (McAllen & May, 1994; Gaudy et al., 1986; Mackway-Jones et al., 1999; Girolani et al., 2002; DeClue et al., 2008).

In summary, our results show greater changes in the ACTH response to haemorrhage under ketamine anaesthesia when compared with saffan. In the context of a physiological anti-opioid response this could be viewed as an additional endocrine benefit of ketamine, which would complement its well know cardiovascular advantages (Craven, 2007) in certain clinical situations, e.g. emergency surgery in patients where blood loss has or is likely to occur. However more robust clinical studies would be required to assess this possible anti-opioid benefit.

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Legends

Figure 1. Heart rate (A) and blood pressure (B) changes during progressive blood loss. Values shown are before (time 0) and at 10 min intervals during haemorrhage with ketamine or saffan anaesthesia. There was no significant difference in the response to haemorrhage between both anaesthetics.

Figure 2. The ACTH / β-endorphin response to haemorrhage. ACTH levels during haemorrhage (A), β-endorphin levels during haemorrhage (B). Values shown are the change from control at each time interval during haemorrhage, under ketamine and saffan anaesthesia. * $P < 0.05$ 2 way ANOVA with repeated measures on both factors.

Figure 3. Change in arterial blood pressure versus change in ACTH levels during haemorrhage under ketamine and saffan anaesthesia. Solid line is for ketamine ($r = 0.66$), and broken line shows the regression for saffan ($r = 0.73$). ACTH levels increased at a faster rate in pigs under ketamine anaesthesia compared to pigs under saffan anaesthesia ($P < 0.05$ ANCOVA).

Figure 4. Change in arterial blood pressure versus change in β-endorphin levels during haemorrhage under ketamine and saffan anaesthesia. Solid line is for ketamine ($r = 0.68$) and broken line shows the regression for saffan ($r = 0.52$). There was no significant difference between the endorphin levels with both anaesthetics (ANCOVA).