

Effects of buprenorphine in the adrenal, thyroid, and cytokine intra-operative responses in a rat model (*Rattus norvegicus*): a preliminary study

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ABSTRACT

Objective(s): Buprenorphine is a common analgesic in experimental research, due to effectiveness and having few side-effects, including a limited influence in the immune and endocrine systems. However, how buprenorphine affects cytokine levels and the adrenal and thyroid response during general anesthesia and surgery is incompletely understood. This study aimed to assess whether buprenorphine modulated significantly those responses in rats submitted to general anesthesia, mechanical ventilation, and surgical insertion of intravascular catheters.

Materials and Methods: Animals were anesthetized with isoflurane, mechanically ventilated, and surgically instrumented for carotid artery and the femoral vein catheter placement. The test group (n=16), received buprenorphine subcutaneously before surgery, whereas the control group (n=16) received normal saline. Blood sampling to determine plasma levels of adrenocorticotropic hormone (ACTH), corticosterone (CS), total thyroxine (TT4), total triiodothyronine (TT3), thyroid-stimulating hormone (TSH), TNF- α , IL6, IL10, TNF- α , IL6, and IL10 mRNA was performed at 10 min after completion of all surgical procedures and at 90, 150, 240, and 300 min thereafter, with the animals still anesthetized and with mechanical ventilation.

Results: Buprenorphine-treated animals had higher levels of ACTH, CS, and TT4 at several time points ($P<0.05$) and TSH and TT3 at all-time points ($P<0.05$). They also had increased IL10, TNF- α , and IL10 mRNA levels.

Conclusion: In this model, buprenorphine significantly modulated the intra-operative cytokine and endocrine response to anesthesia, mechanical ventilation, and surgical placement of intravascular catheters. The mechanism and significance of these findings remain undetermined. Researchers should be aware of these effects when considering the use of buprenorphine for analgesic purposes.

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Introduction

General anesthesia (GA) and surgery-associated tissue injury induce a stress response with activation of the hypothalamic-pituitary-adrenal (HPA) axis and production and secretion of glucocorticoids by the adrenal glands (1, 2). They also induce increased production and secretion of cytokines such as TNF- α , IL6, and IL10 (2-4) and affect hypothalamic-pituitary-thyroid axis (HPT). The latter is manifested by a decrease in serum levels of free triiodothyronine, total triiodothyronine (TT3) and thyroid stimulating

hormone (TSH). Total thyroxine (TT4) may be decreased or not be affected at all (2).

These stress and cytokine responses constitute a compensatory protective mechanism to GA and surgery to restore homeostasis in the host. However, if these responses become exaggerated and/or last longer than expected, there is potential for detrimental effects (5, 6). Consequently, there is interest in adopting approaches that maintain the benefits of this response but at the same time prevent it from becoming abnormal (7). One of the approaches which has been

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suggested is the use of preemptive analgesia because intense and uncontrolled perioperative pain constitutes a powerful stimulus for exaggerated stress and cytokine responses (7-9). Experimental studies have confirmed the benefits of preemptive analgesia in this regard. For example, the use of opioid analgesia was shown to decrease surgery-induced immunosuppression in rats (10).

Unfortunately, most commonly used perioperative analgesics, in particular opioids, are also known to affect the immune and endocrine systems (11). These effects can in their own way increase post-operative morbidity (6, 12-14). Thus the ideal analgesic for the post-operative period would be one which effectively controls peri-operative pain, preserves the beneficial effects of the stress and cytokine peri-operative responses, and prevents the latter from becoming exaggerated. Buprenorphine, one of the most common analgesics used in experimental animal research (15) has properties that in many aspects fulfill these premises.

Buprenorphine has a particular pharmacology, possessing both μ and nociceptin opioid peptide (NOP) agonistic and κ and δ antagonist activities (16, 17). Its good analgesic properties and low incidence of side-effects have been well recognized in several species (18, 19). In addition, it is believed that the influence of buprenorphine in the immune and endocrine systems is more limited than other opioids (17, 20, 21).

The effects of buprenorphine on the immune and endocrine systems depend on several factors including species, dosage, route of administration, and other experimental conditions. For example in rats, intracerebral (IC) administration of buprenorphine was shown to decrease the secretion of ACTH and corticosterone (CS) (20). However, in the same species but in non-operated animals, its administration did not lead to changes in CS levels (22). It is also known that in rats, buprenorphine affects TSH secretion in a dose-dependent manner (22). However, no studies are available which describe if this influence is also manifest during the peri-operative period. Finally, most studies suggest that buprenorphine does not affect (20) or has a minimal influence on immune function (23, 24) although there are also reports that describe its immunosuppressive (25-27) and immuno-enhancing effects (28, 29).

Several studies have shown that preemptive administration of buprenorphine provides effective analgesia and attenuates the post-surgical stress response in rats (8, 9, 30). The latter has been largely attributed to the control of post-operative pain. Data is also available that demonstrates a modulation of the immune response in the postoperative period by buprenorphine (30-32). Some studies actually have shown that this immune modulation was associated

with beneficial effects on the host. These include a decreased lethality in a cecal-ligation-puncture septic model (31) and attenuation of surgery-induced immunosuppression (28).

How analgesics affect the stress and cytokine responses during surgery and especially their interactions with general anesthetics in this regard is still incompletely understood. For example, it is known that opioids concur with general anesthetics to promote perioperative immunosuppression (14) and that the preemptive administration of opioids such as remifentanyl affect the intra-operative stress and cytokine response (33). A better understanding of the effects of analgesic drugs in this context is important because an early modulation of the perioperative stress and cytokine responses is believed to decrease post-operative morbidity (34). In addition, in the experimental setting, one should be aware how preemptive analgesia affects these intra-operative responses because in experiments which measure endocrine and immunological variables the latter can be a source of variation and error.

To our knowledge, there is only one study which describes the influence of buprenorphine in the intra-operative endocrine and cytokine responses. In this study, the administration of buprenorphine to rats with burn injury led to increased IL6 levels, when compared with animals that did not receive it (29).

The aim of this study was to evaluate how buprenorphine affected the intra-operative HPA, HPT, and cytokine responses to GA, mechanical ventilation (MV), and surgical instrumentation for catheter placement in rats. These procedures were chosen because they are commonly used in biomedical research. Our hypothesis was that buprenorphine would not significantly affect the intra-operative endocrine and cytokine responses.

Materials and Methods

All experiments were conducted in compliance with the Portuguese legislation for the use of animals for experimental purposes (decreto-Lei nº 129/92 and Portaria nº 1005/92, DR nº 245, série I-B, 4930-42) and with the European Union legislation (directive n. 86/609/EEC, from 24th October 1996) and The Council of Europe Convention ETS 123. Experimental protocols were approved by the Institutional Animal Care and Use Committee (CEBEA – Comissão de Ética e Bem-Estar Animal). Some authors are also accredited as FELASA category C scientists or equivalent.

Experimental animals

Twelve-week-old Wistar male rats (Charles Rivers, Barcelona, Spain), weighing 250–450 g were used. Animals were housed three per cage, in a climate-controlled room. The temperature was set at 21°C +/- 3°C and relative humidity at 55% +/- 15% and light intensity at 60 Lux with a 12 hr light/dark

cycle. The noise was kept under 45 dB. All animals were acclimatized for seven days before the experiment. Water was provided *ad libitum* and food consisted in rat chow (Harlan Teklad 2014®). Paper sheets were used as nest material. One day before the procedure, the animals were moved to the place of experiments, which had the same environmental and housing conditions. Only one person handled the animals to avoid stress.

Study groups

Rats were randomly assigned to two body weight-matched groups: G0 (control group, 16 rats) and G1 (buprenorphine group, 16 rats). Both groups were submitted to GA, MV, and surgery.

Experimental procedure

The experimental procedure was similar in both groups with exception of buprenorphine administration in G1. The experiment began around 9:30 AM and surgical procedures for catheter placement and institution of MV were always complete before 11:00 a.m. All animal experiments ended around 17.00 p.m. In G1, buprenorphine (Budale®, Dechra, UK) was administered SC at 0.05 mg/kg (35), 20 min before inducing GA. Once its dose was calculated, buprenorphine was added to sterile saline in a syringe to obtain a final volume of 0.2 ml. In G0 the injection consisted only in 0.2 ml of sterile saline.

The animals were placed in an induction chamber (World Precision Instruments, UK) previously saturated with isoflurane 5% (IsoFlo®, Abbott, USA) and 100% oxygen. Once anesthetized, animals were moved and placed in dorsal recumbence over a water-based heating plate (World Precision Instruments, UK) and maintained with isoflurane anesthesia and 100% oxygen delivered through a face mask, until placement of the tracheostomy tube. During the entire experiment, isoflurane concentrations varied between 1.5–2%. Body temperature was always kept between 37–38 °C. ECG trace was registered continuously through lead wire probes (ECG-ML136 Animal Bio Amp, ADInstruments, UK). A respiratory sensor was placed on the thoracic wall to measure respiratory rate. A rectal probe (MLT1403, ADInstruments, UK) and pulse oximetry tail sensor (ADInstruments, UK) were placed to record temperature and pulse oximetry, respectively. The pedal withdrawal reflex was used to help in assessing the depth of anesthesia which was considered adequate if a toe pinch did not induce the reflex. The left femoral vein was cannulated with an intravascular catheter (Introcan®, 26 Gauge, B. Braun Medical, Portugal) for fluid administration. The right external carotid artery was cannulated with an intravascular catheter (Introcan®, 26 Gauge, B. Braun Medical, Portugal). The catheter was then connected to a three-way stopcock and used for collection of arterial blood samples and arterial blood pressure measurement. The

latter was achieved by connecting the three-way stopcock to a fluid-filled pressure transducer (MLT844, ADInstruments, UK) and blood pressure amplifiers (ML221 Bridge Amp, ADInstruments, UK). Thereafter, a tracheostomy was performed to insert a tracheostomy tube previously made from an intra-vascular catheter (Introcan®, 16 Gauge, B. Braun Medical, Portugal). This was connected to an anesthetic circuit and animals were mechanically ventilated to maintain normocapnia (defined as 35–45 mmHg) and PaO₂ >85 mmHg, by measuring arterial blood gas analysis. Arterial blood pressure, ECG, rectal temperature, pulse oximetry, and respiratory rate data were transmitted to amplifiers and subsequently to a data acquisition unit (PowerLab®, ADInstruments, UK). Data was then analyzed with specific software (LabChart Pro®, ADInstruments, UK). Once MV initiated, a 10 min stabilization period was established before blood sampling began. This allowed recovering the stability of hemodynamic parameters as these were affected by the initiation of MV. Blood sampling began after the end of the recovery period, defined as T0. At T4, after the collection of the last blood sample, all animals were euthanized with a lethal injection of pentobarbital at 100–150 mg/kg IV (Eutasil®, Ceva, Portugal), administered through the femoral vein catheter.

Sample collection and anesthesia monitoring

At five time points (T0, T1, T2, T3, and T4), blood samples (500 µl) were collected in both groups. In every time point, after blood sampling, 1 ml of saline was administered through the femoral vein to prevent hemodynamic changes and consequently, activation of the HPA axis, caused by blood collection. At the same time points, temperature, ECG, ventilator settings, and the presence/absence of the pedal withdraw reflex were also recorded. T0 was set at the end of the 10 min recovery period. T1, T2, T3, and T4 were set at 90, 150, 240, and 300 min post-T0, respectively.

Immunological (TNF-α, IL6, IL10) and endocrine (ACTH, CS, TT3, TT4, and TSH) variables

Plasma levels of TNF-α, IL6, IL10, ACTH, and CS were determined at all-time points. Thyroid hormones and TSH were determined only at T0, T1, T2, and T3. To obtain cytokine and hormone plasma levels, blood was collected into sterile heparinized tubes with 200 µl capacity (FactorMed, Portugal) and then centrifuged at 12000 rpm for 15 min. Plasma was then stored at -20°C until further analysis. ACTH and CS (Rat stress hormone panel Millipore, Arium Laboratórios, Portugal), TT3, TT4, TSH (Rat Thyroid Three Plex, Millipore, Arium Laboratórios Portugal), and TNF-α, IL6, IL10 (Rat Cytokine/Chemokine, Millipore, Arium Laboratórios, Portugal) plasma levels were determined by Multiplex/Luminex technology, according to manufacturer's instructions (36). The minimal detectable concentrations (MinDC) of TNF-α, IL6, and

IL10 were 0.48 pg/ml, 14 pg/ml, and 0.53 pg/ml, respectively. The MinDCs of ACTH and CS were 15.22 pg/ml and 2914 pg/ml, respectively. The intra-assay and inter-assay precision (% CV) for TNF- α , IL6, and IL10 were 4.6 % and 8.1%; 5% and 10.9%, and 3.9% and 19.7%, respectively. The intra-assay and inter-assay precision (% CV) for both ACTH and CS were <7% and <15%, respectively. Finally, the MinDCs of TSH, TT3, and TT4 were 7.4 pg/ml, 99 pg/ml, and 1074, respectively and their intra-assay and inter-assay precisions (%CV) were <10% and <5%, respectively.

Molecular biology variables

Sample collection, mRNA extraction, and cDNA synthesis

Blood mRNA expression of TNF- α , IL-6, IL-10, and β -actin genes (chosen as the reference gene) was also determined at all-time points. For this, blood was collected into 200 μ l capacity tubes with RNA stabilizer adapted to extract RNA from the blood of rats ("RNA protect animal blood tubes", Qiagen®). Following collection, samples were refrigerated at 4°C for 24 hr until further processing. This began by extracting RNA from blood ("Q-Amp RNA blood mini kit" Qiagen®) according to manufacturer's instructions. Once RNA was obtained, it was dissolved in diethylpyrocarbonate-treated water and stored at -70°C until further analysis. The latter was initiated by DNA digestion with an RNase-free DNase Set (Qiagen GmbH, ref. 50979254), according to the manufacturer's instructions. RNA's concentration and purity were determined spectrophotometrically at 260 and 280 nm. cDNA was obtained ("Transcriptor High Fidelity CDNA Synthesis Kit", Roche, Portugal) as described by others (37). Primer pairs were selected from different exons, using specific murine sequences. Primers for β -actin, TNF- α , IL-6, and IL-10 genes were designed accordingly to sequences obtained from the GenBank Data Bank (Table 1).

TNF- α , IL-6, IL-10, and β -actin (reference gene) mRNA were quantified by cPCR (38) accordingly to MIQE guidelines (39). Amplicons for TNF- α , IL6, IL10, and β -actin were obtained using the Applied Biosystems 7300 Real Time PCR (Perkin Elmer, Portugal) following manufacturer's instructions and as described by others (37). The data of relative mRNA quantification was analyzed with the real-time PCR Miner algorithm (40).

Table 1. Primer sequence for interleukin-6, tumor necrosis factor- α , interleukin-10, and β -actin used for cPCR, obtained from GenBank data bank

IL6 forward	GGAAATGAGAAAAGAGGAAATTTGC
IL6 reverse:	TGACTCTGGCTTTGTCTTTCTTGT
TNF- α forward	GAAGTCCAGGCGGTGTCTGT
TNF- α reverse	TGGGAAGTCTCTCCTTGTG
IL10 forward	CCCTGGGAGAGAAGCTGAAGA
IL10 reverse	CACAGCTTGCTTTTATTCTCACA
β -actin forward	GTGAAAAGATGACCCAGATCATGT
β -actin reverse	CACAGCTGGATGGCTACGT

Relative quantification of TNF- α , IL6, IL10 and β -actin mRNA expression

Statistical analysis

All data was expressed as median and interquartile range (IQR, 25th, and 75th percentiles). Correlation analysis among different variables was performed using the Spearman rank correlation, considering the set of all five-time points. Differences between time points (T0 to T4) at the same group (G0 and G1) and at each time point between groups were determined by one-way analysis of variance with the nonparametric Kruskal-Wallis test. Statistical analysis was conducted with the *Statistica*® software. Statistical significance was established at P -value<0.05.

Results

All animals survived the experiments until T4. No immediate effects due to buprenorphine were found in any of the studied animals.

Endocrine variables

Statistical analysis of endocrine variables is shown in Figures 1 and 2. ACTH levels were higher in G1 than in G0 at all time points although this only reached statistical significance in T1 ($P=0.025$), T3 ($P=0.005$), and T4 ($P=0.01$) (Figure 1). ACTH concentration varied

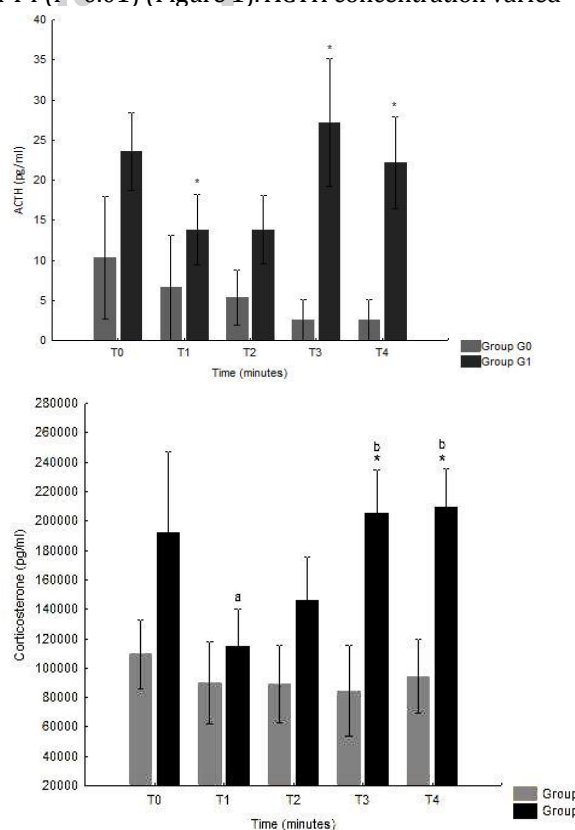


Figure 1. Variation of adrenocorticotrophic hormone and corticosterone from T0 to T4 in G0 and G1. Data are expressed as mean \pm SE of 6 \leq n \leq 8 rats for each group. *indicates P <0.05 versus the corresponding time point in the control group. Different lower case letters indicate statistically significant different values between times within the same group

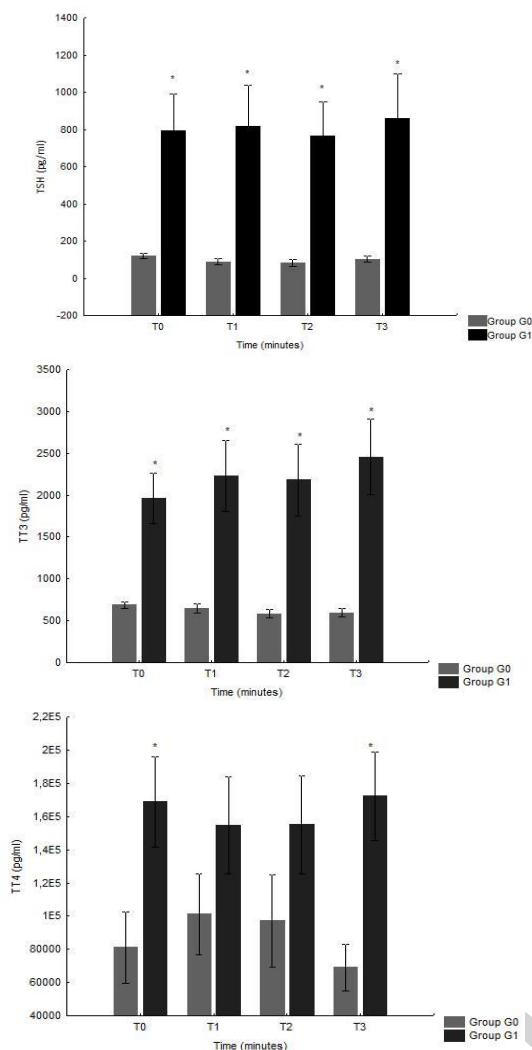


Figure 2. Variation of thyroid stimulating hormone, total triiodothyronine, and total thyroxine from T0 to T3 in G0 and G1. Data are expressed as mean \pm SE of 6 \leq n \leq 7 rats for each group * indicates $P < 0.05$ versus the corresponding time point in the control group. Different lower case letters indicate statistically significant different values between times within the same group.

along the experiment in a different way between groups. In G0, ACTH levels decreased continuously from T0 to T4. In contrast, in G1, after a first decrease from T0 to T1, they increased from T1 to T3 and remained elevated until the end of the experiment. CS levels were higher in G1 than in G0 at all-time points, although this was only statistically significant in T3 ($P=0.022$) and T4 ($P=0.016$) (Figure 1). In G1, following a first decrease from T0 to T1, CS levels increased continuously until the end of the experiment. In G0, CS levels remained always stable. TSH, TT3, and TT4 levels were higher in G1 than in G0 at all time points (Figure 2). This was statistically significant at all time points for TSH (T0, $P=0.025$; T1, $P=0.005$; T2, $P=0.010$; T3, $P=0.025$) and TT3 (T0, $P=0.010$; T1, $P=0.022$; T2, $P=0.007$; T3 $P=0.007$), but only in T0 ($P=0.022$) and T3 ($P=0.022$) for TT4.

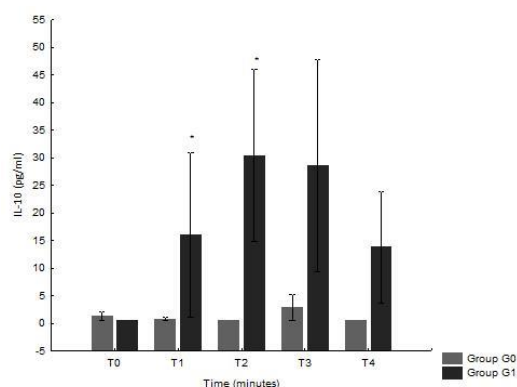


Figure 3. Variation of interleukin10 from T0 to T4 in G0 and G1. Data are expressed as mean \pm SE of 5 \leq n \leq 7 rats for each group * indicates $P < 0.05$ versus the corresponding time point in the control group. Different lower case letters indicate statistically significant different values between times within the same group.

Immunological variables

The only immunological variable which differed significantly between groups was IL10 (Figure 3). Buprenorphine-treated animals had always higher IL10 levels although this increase was only statistically significant at T1 ($P=0.022$) and T2 ($P=0.003$).

mRNA expression

In both groups, molecular biology variables had a large variation (Figure 4). Groups only differed significantly between $TNF-\alpha$ and $IL10$. $TNF-\alpha$ was significantly higher in G1 at T3 ($P=0.025$) and T4 ($P=0.032$). $IL10$ was significantly higher in G1 at T4 ($P=0.049$).

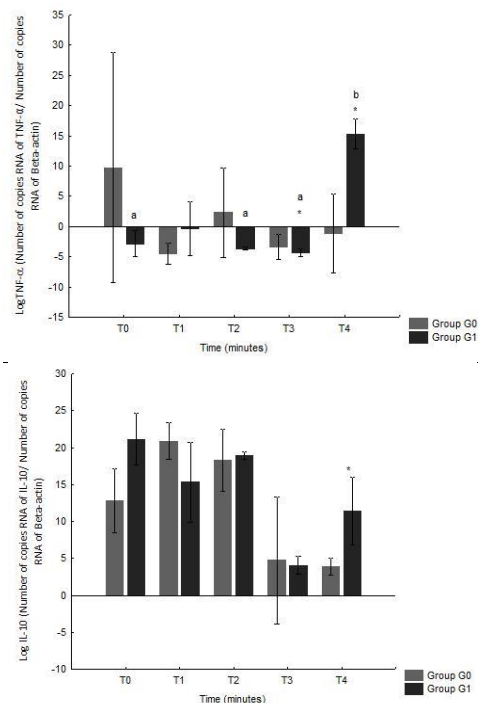


Figure 4. Variation of log $TNF-\alpha$ and log $IL10$ from T0 to T4 in G0 and G1. Data are expressed as mean \pm SE of 2 \leq n \leq 5 rats for each group * indicates $P < 0.05$ versus the corresponding time point in the control group. Different lower case letters indicate significantly different values between times at the same group

Table 2. Statistically significant correlations between the immune and endocrine variables.

	ACTH (pg/ml) (n=34)		CS (pg/ml) (n=34)		TSH (pg/ml) (n=28)		TT3 (pg/ml) (n=28)		TT4 (pg/ml) (n=28)		TNF- α (pg/ml) (n=34)		IL6 (pg/ml) (n=30)		IL10 (pg/ml) (n=30)	
	r	P	r	P	r	P	r	P	r	P	r	P	r	P	r	P
TNF- α (pg/ml) (n=34)					-0.505	0.006			-0.442	0.019			-0.590	0.001	-0.479	0.004
IL6 (pg/ml) (n=30)					0.538	0.003					-0.590	0.001				
IL10 (pg/ml) (n=30)											-0.479	0.004				
TSH (pg/ml) (n=28)							0.510	0.006	0.650	0.000	-0.505	0.006	0.538	0.003		
TT3 (pg/ml) (n=28)	-0.543	0.003			0.510	0.006			0.495	0.008						
TT4 (pg/ml) (n=28)	-0.438	0.020	0.524	0.004	0.650	0.000	0.495	0.008			-0.442	0.019				

Correlations were performed by the Spearman rank correlation. ACTH: adrenocorticotropic hormone; CS: corticosterone; TNF- α : Tumor Necrosis Factor - α ; IL6: Interleukin 6; IL10: Interleukin 10; TSH: thyroid stimulating hormone; TT3: total triiodothyronine; TT4: total thyroxine; r- correlation coefficient; P-level of statistical significance, which was established at P -value<0.05

Correlation analysis

The results of correlation analysis are described in Table 2. No correlation with statistical significance was found between ACTH with CS and between ACTH, CS, and the different cytokines. ACTH was negatively correlated with TT3 ($P=0.003$, $r=-0.543$) and TT4 ($P=0.02$, $r=-0.438$), and CS positively correlated with TT4 ($P=0.004$, $r=0.524$). TNF- α was negatively correlated with IL6 ($P=0.001$, $r=-0.590$), IL10 ($P=0.004$, $r=-0.479$), TT4 ($P=0.019$, $r=-0.442$) and TSH ($P=0.006$, $r=-0.505$). IL6 was positively correlated with TSH ($P=0.003$, $r=0.538$). TSH was positively correlated with TT3 ($P=0.006$, $r=0.510$) and TT4 ($P=0.000$, $r=0.650$). TT3 and TT4 were positively correlated with each other ($P=0.008$, $r=0.538$). Correlation analysis between mRNA expression and other variables was not performed due to the limited amount of data.

Discussion

In this study, the preemptive administration of buprenorphine to rats submitted to GA, MV, and surgical placement of intravascular catheters was associated with significantly higher intra-operative levels of ACTH, CS, TT3, TT4, TSH, IL10, TNF- α , and IL10 mRNA compared to the control animals. Thus buprenorphine seemed to modulate significantly the intra-operative stress and cytokine response to those stressors when compared to the control animals.

Effects of buprenorphine in ACTH and CS

The finding of increased ACTH and CS levels in buprenorphine-treated animals was unexpected. According to the literature, buprenorphine does not affect or actually decreases HPA axis activity in rats (8, 9, 20). In non-operated rats, the administration of buprenorphine did not change CS levels (22). In addition, several experimental models which used

buprenorphine to provide peri-operative analgesia reported a decrease in CS levels in the post-operative period (8, 9, 28). These changes were largely attributed to the ability of buprenorphine to decrease post-operative pain. To our knowledge, there is only one study which described the influence of buprenorphine in HPA activity intra-operatively (20). In this, when buprenorphine was administered by IC route, a decrease in ACTH and CS levels occurred. The authors of the study attributed these findings to buprenorphine's partial agonist activity in the opioid receptors.

We think that buprenorphine was associated with increased ACTH and CS levels based on several assumptions. First, buprenorphine was the only factor where both groups differed. The type of surgical technique (41), severity of injury, blood collection and total operating time (9, 41-44), all known activators of the HPA axis, were similar between groups. It has been shown that when the volume of blood collected is less than 15% of body weight and this is replaced with normal saline there is no increase in HPA activity (45). This was the approach adopted in this study. Second, the time points where increased ACTH and CS levels were found are compatible with buprenorphine pharmacokinetics. Buprenorphine's serum half-time in rats is estimated to be 2.8 hr although its effects can extend up to 8 hr in this species, due to the presence of an extensive enterohepatic circulation (46, 47). In addition, the results of a recent study with rats submitted to jugular catheterization and where buprenorphine and CS serum concentrations were measured at several time points supports our findings (9). In this study, buprenorphine was also administered at the same dose, by SC route, 30 min before GA and surgery. It was found that when buprenorphine reached its highest serum

concentrations, an increase in CS levels also occurred. Interestingly, the peak CS levels reported in that study were similar to the ones we found.

How buprenorphine was able to increase ACTH and CS levels remains unclear, but it may be related to its particular pharmacology. Buprenorphine possesses agonistic activity in the μ and NOP receptors and antagonistic activity in the κ and δ receptors (17). In addition, some of its metabolites, such as norbuprenorphine, are also pharmacologically active (48). It is known that ACTH release is modulated by endogenous and exogenous opioids through action in several opioid receptors (20, 49). The nature of this modulation varies with species, type of opioid (in particular its specific opioid receptor agonist/antagonist activity), dosage, and route of administration (50-53). Based on what was described for other opioids, buprenorphine could have increased ACTH levels through its μ and possibly δ agonist activity at the HPA axis. Whether its NOP agonistic activity played a role remains unknown. In rats, it is known that the endogenous NOP system is involved in the acute HPA axis response to stressful stimulus (53, 54). However, there are also reports demonstrating that the administration of nociceptine does not lead to CS elevation in mice (55).

Increased CS levels in buprenorphine-treated rats could have resulted from buprenorphine's agonist activity in μ and to a less extent, κ receptors present in the adrenal cells of zone fasciculata, similar to what has been reported with other opioids (56). An action of buprenorphine in both the central and peripheral levels of the HPA axis could explain why we did not find a correlation between ACTH and CS levels.

It may be also hypothesized that buprenorphine interacted with isoflurane to increase HPA activity. Isoflurane can be associated with increased CS levels in rats when administered for extended periods of time (57). How this occurs is still unclear, but the mechanism may be species-specific. In anesthetized rabbits, isoflurane increases intra-operatively CS but not ACTH levels (58). In contrast in mice anesthetized with isoflurane and administered pre-operatively with dexamethasone to prevent ACTH secretion, CS levels decrease, implying a role for ACTH in isoflurane's modulation of CS levels in this species (59). In Humans, whether isoflurane stimulates the HPA axis is still being debated because there is supporting evidence against this hypothesis (60, 61). Based on these studies, a possible role of buprenorphine in facilitating isoflurane's ability to increase CS levels is a possibility, which to our knowledge has not yet been investigated.

How these findings can be conciliated with the well-demonstrated ability of buprenorphine to decrease post-operative stress responses? There are

several possible explanations. One is that because pain is one of the most potent stimuli of HPA axis activation in the post-operative period, the analgesic effects of buprenorphine are predominant over other endocrine effects in the postoperative period. Another possibility is the potential interaction between buprenorphine and isoflurane. Finally, variable drug dosages and routes of administration used in the different studies could explain these differences (9, 20, 28).

Effects of buprenorphine in the thyroid axis

Buprenorphine-treated animals had increased levels of TSH, TT3, and TT4, which suggests that the opioid counteracted the effects of GA and surgery in the thyroid axis. An increase in HPT axis' hormones following buprenorphine has already been described, but only in non-operated animals (22). Based on what has been described for other opioids, buprenorphine could have increased the release of TSH through its κ and NOP-R antagonist and agonist activity, respectively (62, 63). The positive correlation found between TSH, TT3, and TT4 also suggest that increased TSH levels led to the increased TT3 and TT4 levels. In addition, it is possible that buprenorphine acted directly in the thyroid gland to stimulate TT3 and TT4 release. Although to our knowledge this effect has never been reported, it has been described for morphine (64). How morphine stimulates the release of TT3 and TT4 from the thyroid gland is still poorly understood. Nevertheless, because this effect is completely prevented by naloxone pre-administration, it most likely involves the ligation of morphine to opioid receptors (64).

An interaction between the HPA and the HPT axes remains also possible because ACTH and CS were found to be negatively and positively correlated with TT4, respectively. It is known that thyroid hormones participate in the HPA axis's central regulation (65). In addition, experimentally induced hyperthyroidism in rats increases ACTH and CS's production and release (66, 67). It cannot also be ruled out that buprenorphine facilitated the interaction between the HPA and HPT axis.

Effects of buprenorphine in the cytokine response

To our knowledge, this is the first study that reported an increase in IL10 and cell-free circulating $TNF-\alpha$ and $IL10$ mRNA levels associated with buprenorphine administration. In our experiment, the cytokine response was evaluated both at the protein and genetic levels. This approach is similar to what has been described in another study where buprenorphine was associated with decreased production of IL4 and $IL4$ mRNA by human T-lymphocytes (68). We used cytokine mRNA measurement in the evaluation of the cytokine

response because cPCR has several advantages. cPCR is considered the most reliable method to evaluate cytokine networks due to its sensibility and accuracy (69). When compared with the measurement of cytokine levels in plasma, it can demonstrate evidence of cytokine production even when the former is inconclusive, especially if cytokine levels are under the limit of detection (38, 70). cPCR is also less limited by the volume or size of the sample. Finally, it can demonstrate evidence that opioids such as buprenorphine exert their immune effects at a genetic level, leading to a better understanding of their mechanism of action regarding the immune system.

Surgery induces a cytokine response which has both pro and anti-inflammatory components (4). In this study, buprenorphine seems to have modulated both components, by increasing both TNF- α and IL10 mRNA levels. However, because it also increased the intra-operative levels of IL10 it seems that this modulation was more pronounced in the anti-inflammatory component.

Most studies suggest that buprenorphine is deprived (20) or has minimal effects on immune function (23, 24, 27, 28, 31). However, there are also reports that have demonstrated anti-inflammatory effects by buprenorphine (25, 26, 30), including in the post-operative period (30, 32, 71). The reason why there is such conflicting evidence regarding its immune effects is not completely understood. It may result from methodological differences between studies, including the species and gender of the animal (72). Buprenorphine has also an unusual pharmacology which may lead to different immune effects, depending on the underlying context.

The mechanisms responsible for the immune effects of buprenorphine found in this study were not determined. There are however several possibilities including a direct influence of the drug in immune cell activity (73) or an indirect mechanism, with buprenorphine modulating HPA or sympathetic nervous system axis activity, by peripheral or central mechanisms (11, 20, 74).

Several studies have shown that buprenorphine is able to influence directly immune cell activity. Once ligating to opioid receptors present in the immune cell's surface, buprenorphine can then lead to changes in the levels of cytokines such as TNF- α and IL10 (68, 75). How buprenorphine and other opioids interact with opioid receptors to regulate cytokine expression and secretion is still incompletely understood. A common mechanism seems to involve the activation of several intracellular signaling pathways and transcription factors (73), which in turn lead to changes in the expression of genes such as TNF- α and IL10 (76-78). Which opioid receptors are responsible for buprenorphine's immune effects is still unclear. The

stimulation of μ -receptor seems to be a major mechanism of immunosuppression induced by opioids such as morphine (79). Because buprenorphine is a high-affinity partial μ -agonist, it might be expected that the ligation to this receptor leads to immunosuppressive effects. A study performed in rats seems to corroborate this hypothesis. It reported that naltrexone, a μ -receptor antagonist counteracted buprenorphine's anti-inflammatory effects (26). Buprenorphine is also a κ antagonist. It is unlikely that this activity could have justified our findings because the stimulation of this receptor leads mainly to anti-inflammatory (80) and even immunosuppressive effects (81). The immune effects of δ -receptor stimulation are less understood. Most studies suggested that its stimulation is pro-inflammatory (82, 83). More recently, evidence for an anti-inflammatory role in specific contexts such as hypoxia, have also emerged (84). It has also been suggested that δ -receptor overstimulation leads to decreased cytokine production and release (85). To our knowledge, the role of buprenorphine's δ -receptor antagonism to cytokine expression and production has not yet been studied. Consequently, its contribution to our results remains unknown. Finally, buprenorphine's agonist activity in the NOP-receptor could have at least in part justified our findings, because the NOP receptor is involved in immune system modulation at several levels (86). Interestingly, an increase in NOP levels which paralleled increases in IL10 have been demonstrated in humans admitted to ICU with sepsis and post-cardiopulmonary bypass (87).

Alternatively, the increased IL10 and IL10 mRNA levels could have resulted from buprenorphine's effects in the HPA axis. As discussed previously, buprenorphine-treated animals had increased levels of CS and glucocorticoids are known to increase IL10 levels in rats (88) and to upregulate IL10 mRNA transcription (89). Interestingly the opposite may have also occurred, with the late increase in ACTH secretion being the result of the high levels of IL10, because this cytokine can induce CRH and ACTH secretion by hypothalamic neurons and the pituitary gland, respectively (90, 91). Although the interaction between ACTH, CS, and IL10 seems plausible, we were not able to find a correlation between these variables, making this hypothesis less likely.

A final hypothesis is that buprenorphine and isoflurane interacted together to change the intra-operative cytokine response. This has been described before when rats submitted to burn injury and which had buprenorphine associated to isoflurane anesthesia had a different cytokine response than those with isoflurane alone (29). There is conflicting data regarding a direct influence of isoflurane in IL10. Some studies reported a lack of influence of isoflurane in IL10 production (92)

whereas others reported a suppressive effect (93). In contrast, more recent studies have shown that emulsified isoflurane actually leads to increased production of IL10 in rats (94).

Interaction between the immune and thyroid axis?

Our correlation analysis also suggests that some type of cross-talk may have occurred between the immune system and the thyroid axis. We found a negative correlation between TT4 and TT3 and TNF- α levels, which has already been described in the literature (95). However, the positive correlation between IL6 and TSH was unexpected, as previous studies in rats have shown that the administration of this cytokine decreases TSH levels (96). It is possible that the stimulatory action of buprenorphine in the thyroid axis counteracted IL6's and TNF- α 's inhibitory effects on TSH secretion. Alternatively, the opioid itself modulated the action of these cytokines in the thyroid axis through unclear mechanisms.

Our study had some limitations. The first is that we only used male Wistar rats of young age. It is known that the activity of opioids, including buprenorphine, can be affected by age, sex, and genotype of the animals (30, 71, 97, 98). Thus our findings may be specific to our experimental model. Another limitation was the limited number of samples where it was possible to obtain mRNA for measurement. Finally, our findings may be specific to the isoflurane/buprenorphine combination. Further studies are necessary to evaluate if those findings are confirmed with other anesthetic and analgesic combinations.

Conclusion

In this preliminary study, buprenorphine was found to modulate significantly the intra-operative stress and cytokine response in rats submitted to GA, MV, and surgical instrumentation for catheter placement. In particular, it was associated with increased activation of the HPA and thyroid axes, increased IL10 levels, and upregulation of several cytokine genes. Its immune effects were considerably less significant than those reported with other opioids. The implications of these findings remain undetermined at this moment, including their consequences for the post-operative period and/or if these properties can be used therapeutically. However, they should be considered if buprenorphine is used as an analgesic in experiments where immune and endocrine variables are evaluated, to prevent errors of data interpretation. We suggest that future studies should be undertaken to confirm and clarify the mechanisms underlying our findings. These may include the use of specific agonist and antagonist drugs to the different opioid receptors and other anesthetic/ analgesic combinations. Ideally, they should also include the evaluation of different

transcription factors to evaluate how buprenorphine can change cytokine gene expression.

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