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IMPACT OF ANESTHESIA, ANALGESIA AND EUTHANASIA TECHNIQUE ON THE INFLAMMATORY CYTOKINE PROFILE IN A RODENT MODEL OF SEVERE BURN INJURY

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Abstract

Background—Anesthetics used in burn and trauma animal models may be influencing results by modulating inflammatory and acute-phase responses. Accordingly, we determined the effects of various anesthetics, analgesia, and euthanasia techniques in a rodent burn model.

Methods—Isoflurane, ketamine-xylazine (KX), or pentobarbital, with or without buprenorphine, were administered prior to scald-burn in 72 rats that were euthanized without anesthesia by decapitation after 24 hours, and compared to unburned shams. In a second experiment, 120 rats underwent the same scald-burn injury using KX, and 24 hours later were euthanized under anesthesia or carbon dioxide (CO₂). Additionally, we compared euthanasia by exsanguination versus decapitation. Serum cytokine levels were determined by ELISA.

Results—In the first experiment, isoflurane was associated with elevation of cytokine-induced neutrophil chemoattractant (CINC)-2, and monocyte chemotactic protein-1 (MCP-1), and KX and pentobarbital with elevation of CINC-1 and 2, interleukin (IL)-6 and MCP-1. Pentobarbital also decreased IL-1. Interleukin-6 increased significantly when isoflurane or pentobarbital were combined with buprenorphine. In the second experiment, euthanasia performed by exsanguination under isoflurane was associated with reduced levels of IL-1, CINC-1 and 2, and MCP-1, while KX reduced CINC-2 and increased IL-6 levels. Meanwhile, pentobarbital reduced levels of IL-1 and MCP-1, and CO₂ reduced CINC-2 and MCP-1. Additionally, decapitation following KX, pentobarbital or CO₂ decreased IL-1 and MCP-1, whilst we found no significant difference between isoflurane and controls. Euthanasia by exsanguination compared to decapitation using the same agent also led to modulation of several cytokines.

Conclusions—Differential expression of inflammatory markers with the use of anesthetics and analgesics should be considered when designing animal studies and interpreting results, as these appear to have significant modulating impact. Our findings indicate a brief anesthesia with isoflurane immediately prior to euthanasia by decapitation exerted the least dampening effect on the cytokines measured. Conversely, KX with buprenorphine may offer a better balance during longer procedures to avoid significant modulation. Standardization across all experiments that are compared and awareness of these findings is essential for those investigating the pathophysiology of inflammation in animal models.

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Keywords

Isoflurane; ketamine; xylazine; pentobarbital; buprenorphine; animal model; sacrifice techniques; IL-1 ; IL-6; TNF- ; CINC-1; CINC-2; MCP-1

INTRODUCTION

Experimental factors should ideally have minimal and uniform influence on study parameters such as cytokines when these are used as outcome measures to elucidate molecular mechanisms and evaluate interventions. In animal model studies, such factors include anesthetics and analgesics administered to perform surgical procedures, and also used at the time of euthanasia.

An array of inflammatory mediators are released during the acute response to severe burn trauma. Severe burns of 40% total body surface area (TBSA) and greater in the clinical setting are characterized by the release of both pro- and anti-inflammatory cytokines (1). Major burn injuries lead to a large increase in basal metabolic rate, with consequent hypermetabolism, protein breakdown and profound muscle catabolism (2). These complex responses are mirrored in animal models to study biological mechanisms and interventions to modulate these detrimental features and enhance the treatment of thermal injuries. In our established rodent model, we create full-thickness scald burns of the back for a 40% TBSA burn, and back and abdomen for a 60% TBSA burn, and used the more severe burn in this study to allow enhanced detection of subtle changes due to the different agents and conditions.

A variety of anesthetic agents have been shown to significantly modulate inflammatory changes in models of endotoxemia (3–10), septic peritonitis (11, 12), and ischemia-reperfusion (12–15). These models share features with severe burns, although modulation of burn-induced inflammatory changes associated with anesthetics has not been thoroughly investigated to our knowledge. We, therefore, aimed to characterize the influence of factors including choice of anesthetic, supplemental opioid analgesia, and euthanasia method on the inflammatory cytokine response post-burn. Study findings could enhance future experimental designs, assist in validating previous study results and data interpretation, as well as have potential implications for choice of anesthetic in the clinical setting. These factors were investigated in an established rodent model of severe burn injury.

Isoflurane, ketamine, and pentobarbital are anesthetics in wide clinical use as well as in animal studies. Isoflurane, a halogenated ether inhalational anesthetic, interacts at GABA-, glutamate-, and glycine-receptors, and exhibits a rapid rate of induction and recovery at a minimum alveolar concentration of around 1.3% in humans and animals. Ketamine, an N-methyl D-aspartate (NMDA) receptor antagonist, has activity at opioid receptors at high doses, and therefore, good analgesic as well as anesthetic properties. It is widely used as a veterinary anesthetic, as well as in the pre-hospital care and battlefield setting. In humans, it causes dissociative anesthesia with hallucinogenic effects. In animal studies, it is often used in combination with the sedative xylazine. Xylazine, an α_2 receptor agonist with potent sedative properties, originally developed as an anti-hypertensive drug, has muscle relaxant and analgesic properties, and aids in the smooth emergence from anesthesia with ketamine. Pentobarbital, a short-acting barbiturate, causes non-selective depression of the central nervous system and is used as a hypnotic and anticonvulsant. Buprenorphine, a semi-synthetic opiate, is a high affinity μ -receptor partial agonist. A recent review into rodent experiments found the reported use of isoflurane and ketamine-xylazine (KX) to be on the increase, KX being the most frequently used, whilst the use of pentobarbital has decreased

over the period 2000–6 (16). Buprenorphine remains the most commonly reported analgesic administered in rodent studies (16).

Aims

The study determined whether inflammatory responses following severe burn injuries may be influenced by the administration of different anesthetic and analgesic drugs. Different agents were investigated in a rodent model and administered to perform the burn procedure, and also examined separately when used immediately prior to euthanasia following an earlier burn. Additionally explored were the modulating effects of analgesia given at the time of the burn, as well as any influence of euthanasia technique used.

MATERIALS AND METHODS

Several experimental factors that may be modifying the inflammatory response, as measured by serum pro-inflammatory cytokine levels, were examined. The influence of various classes of anesthetics and supplemental analgesia, administered for animal manipulations and surgical procedures, were investigated in the first arm of the study, experiment A. Experiment B meanwhile investigated the effect of anesthetics, as well as carbon dioxide (CO₂), when given immediately prior to euthanasia by two alternative methods, following an earlier burn procedure. Euthanasia using CO₂ as an adjunct is commonly used in animal models, and therefore, its influence was determined. This animal study protocol was approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch, Galveston, Texas. Care and handling of animals were in accord to the National Institute of Health's *Guide for the Care and Use of Laboratory Animals*.

Scald-burn procedure

The procedure follows an established rodent scald-burn model (17) and the procedure described was used for all burned animals and performed by the same group of experienced researchers. Comprehensive guidelines were followed to monitor the animals throughout the study, with precautions taken to minimize any pain or distress. All experiments were performed on adult male Sprague-Dawley rats (Harlan Sprague Dawley, Houston, TX) weighing 300–350g which were housed in a climate-controlled room with a 12-hour light-dark cycle. All animals were acclimatized for seven days prior to experimental procedures, receiving rat chow and water *ad libitum* throughout the study.

Analgesia and anesthesia was administered according to group randomization. Supplemental analgesia was given before and after the scald-burn to half the animals in experiment A and to all animals in experiment B. The back and abdomen was shaved with clippers, and the animal secured in a prefabricated mold to expose a fixed surface area of skin of the dorsal or ventral aspect. Special care was taken to create a tight seal at the margins to avoid exposure of the head and neck, limbs, genital region and tail. Satisfactory depth of anesthesia was carefully and individually checked for each animal with the toe-pinch test, and confirmed to reach surgical depth immediately prior to the scald-burn. Additional anesthesia was administered if required to achieve satisfactory depth.

To create a burn wound of 60% total body surface area, exposed skin was immersed in scalding water (96–98°C) for 10 seconds on the back, and 2 seconds on the abdomen. All animals received fluid resuscitation with 40 ml/kg Ringer's Lactate solution IP. After a period of monitoring and recovery in a customized area with high-flow oxygen, the animals were returned to individual cages to recover in a warm environment. This protocol has been shown to produce consistent full-thickness burn wounds without injury to deeper structures.

Animals continued to be monitored for signs of distress, and if exhibited, a further dose of analgesia following the procedure was given to animals assigned to receive it, according to our standardized pain protocol. Early euthanasia was proposed for animals in distress not randomized to receive analgesia, but this did not become necessary. All animals were euthanized the following day after a 24-hour recovery period.

Experiment A

The first study arm investigated the effects of different anesthetics administered to perform the burn procedure, as well any modulating effects of supplemental analgesia. Isoflurane (Hospira Inc., Lake Forest, IL) 2–3% in air (ISO), Ketamine HCl (KetaVed™, Vedco Inc, Saint Joseph, MO) 40–80 mg/kg IP plus Xylazine (TranquiVed™, Vedco Inc., Saint Joseph, MO), 5–10 mg/kg IP (KX) or Pentobarbital sodium (Nembutal®, Ovation Pharmaceuticals Inc., Deerfield, IL) 30–50 mg/kg IP (PEN) were administered to a different group (n=12) of randomized animals (Fig. 1A).

Supplemental buprenorphine HCl (Buprenex®, Reckitt Benckiser Pharmaceuticals Inc., Richmond, VA) analgesia (0.025–0.05 mg/kg SC) was given to half the groups receiving each class of anesthetic to investigate any synergistic influence. A dose of analgesia was given prior to the burn procedure and an additional dose given post-operatively to any animal showing signs of discomfort. A standardized pain protocol was used to uniformly assess discomfort and administer supplemental analgesia accordingly.

The standard scald-burn procedure was performed as described above. Following a 24-hour recovery period, all animals in experiment A were euthanized by decapitation using a standard small animal guillotine device and blood collected for serum analysis. Thirty sham animals that did not undergo the burn procedure, or receive an anesthetic or analgesia, were used for comparison.

Experiment B

The second study arm investigated the effects of different agents administered immediately prior to euthanasia. All animals initially underwent the same scald-burn procedure described using KX anesthesia and buprenorphine analgesia, and were monitored during a 24-hour period of recovery. Animals were randomized into eight groups (n=12) to receive different agents immediately prior to euthanasia. Burned animals euthanized concurrently by decapitation without anesthetic were used as a control group (n=24). The anesthetics administered were ISO, KX, or PEN at the previously stated doses, with a further group receiving carbon dioxide (CO₂) 100% inhalation. Satisfactory depth of anesthesia was carefully checked for each individual animal with the toe-pinch test and confirmed to reach surgical depth immediately prior to euthanasia.

In order to investigate the possible influence of euthanasia technique, animals receiving one of the four agents were euthanized by laparotomy and exsanguination by venipuncture of the inferior vena cava (Fig. 1B), and a further four groups receiving an agent euthanized by decapitation (Fig. 1B).

Cytokine analysis

Serum cytokine levels were determined using commercially available ELISA kits according to manufacturer's instructions. Six different inflammatory cytokines were chosen based on previous studies (1, 18) and measured using samples collected at euthanasia: interleukin (IL)-1, IL-6, tumor necrosis factor-alpha (TNF- α), cytokine-induced neutrophil chemoattractant (CINC)-1 and CINC-2 (Quantikine® Rat Immunoassay, R&D Systems Inc., Minneapolis, MN) and monocyte chemotactic protein-1 (MCP-1) (BioSource™ International

Inc., Camarillo, CA). Plates were analyzed using a micro-plate reader at a wavelength of 450 nm.

Statistical analysis

Analysis was performed using the Sigmastat statistical package (v3.5, Systat Software Inc, San Jose, CA) by unpaired Student's t-test, and Kruskal-Wallis one-way analysis of variance (ANOVA) on ranks, using Dunn's method for pairwise multiple comparisons in experiment A, and multiple comparisons versus controls in experiment B, as appropriate, with significance set at $p < 0.05$.

RESULTS

Experiment A

Isoflurane, KX, and pentobarbital were three classes of anesthetic compared when administered to perform a burn procedure. These were delivered individually, with or without supplemental buprenorphine analgesia. Comparison was made on samples obtained at euthanasia with unburned sham animals and analyzed according to groups receiving analgesia, groups not receiving analgesia, and groups receiving the same anesthetic with or without supplemental analgesia (Fig. 2A–F, Table 1).

Anesthetic without supplemental analgesia

Comparing only between groups receiving anesthesia without buprenorphine prior to the burn procedure and sham animals: anesthesia with ISO was associated with significantly elevated levels of CINC-2, and MCP-1 relative to sham ($p < 0.05$); KX was associated with significantly elevated levels of CINC-1, CINC-2, and MCP-1 relative to sham ($p < 0.05$), as well as higher levels of IL-6 relative to ISO and sham ($p < 0.05$); and PEN was associated with lower levels of IL-1 relative to KX and sham ($p < 0.05$), although also higher levels of CINC-1, CINC-2, IL-6 and MCP-1 relative to sham ($p < 0.05$).

Anesthetic with supplemental analgesia

Comparing between groups receiving buprenorphine with anesthesia for the burn procedure and sham animals: ISO was associated with higher levels of CINC-2 and MCP-1 ($p < 0.05$); KX was associated higher levels of CINC-1, CINC-2 and MCP-1 ($p < 0.05$); PEN was associated with reduced levels of IL-1 ($p < 0.05$), and increased levels of CINC-1, CINC-2 and MCP-1, relative to sham ($p < 0.05$), as well as increased levels of IL-6 relative to both ISO and sham ($p < 0.05$).

Analgesia versus no analgesia

Comparing between groups when the same anesthetic agent was administered, with or without supplemental analgesia, only levels of IL-6 were significantly increased when ISO or PEN were administered in combination with analgesia than when omitted ($p < 0.05$).

Experiment B

Isoflurane, KX, PEN, and CO₂ were administered immediately prior to euthanasia and sample collection. This followed a standardized burn procedure performed 24 hours earlier on all animals, including controls, using KX and buprenorphine. Two different methods of euthanasia were assessed: laparotomy with exsanguination, and decapitation. Burned animals euthanized without anesthetic by decapitation served as concurrent controls for comparison. Relative cytokine responses were analyzed according to certain factors: laparotomy with agent versus controls; decapitation following agent versus controls; and laparotomy versus decapitation when the same agent was used (Fig. 3A–F, Table 2).

Laparotomy with exsanguination

Comparing groups euthanized by laparotomy and exsanguination versus controls, ISO was associated with significantly reduced levels of IL-1, CINC-1, CINC-2, and MCP-1 ($p<0.05$); KX was associated with reduced levels of CINC-2 ($p<0.05$), although also significantly increased levels of IL-6 ($p<0.05$). Pentobarbital was associated with lower levels of IL-1 and MCP-1 ($p<0.05$). Carbon dioxide administration was associated with lower levels of CINC-2 and MCP-1 ($p<0.05$).

Decapitation only

Groups were administered an agent immediately prior to euthanasia by decapitation and compared to controls euthanized without an agent. Administration of KX, PEN and CO₂ were all associated with lower levels of IL-1, and MCP-1 ($p<0.05$). Interestingly, no significant differences were found between ISO and controls when comparing euthanasia by decapitation only.

Laparotomy with exsanguination versus decapitation

Examining the influence of euthanasia technique on the cytokine response when the same agent was administered, laparotomy with ISO was associated with increased levels of TNF ($p<0.05$), whilst laparotomy with KX was associated with increased levels of both TNF and MCP-1 ($p<0.05$). Pentobarbital and CO₂ were also associated with increased levels of MCP-1 when used for laparotomy ($p<0.05$). Meanwhile, decapitation using CO₂ was associated with elevated levels of CINC-2 ($p<0.05$), whilst ISO was associated with increased levels of MCP-1 ($p<0.05$).

Summary

Each of the three anesthetics administered (ISO, KX, PEN) to perform the burn procedure were associated with specific modulation patterns of the cytokine response measured after 24 hours. These patterns varied slightly in their relationships when analgesia was administered or withheld, with statistically significant differences found in levels of IL-6. When administered immediately prior to euthanasia (ISO, KX, PEN, CO₂) following an earlier standardized burn, they also modulated patterns in the inflammatory cytokine response. When the same agent was administered, certain cytokines were also found to vary significantly depending on the method of euthanasia, compared to controls euthanized by decapitation without anesthesia.

DISCUSSION

Significant variation in the inflammatory cytokine response was found in a rodent model of severe burn injury following the use of different anesthetics during the burn procedure and at euthanasia. Additional influence and modulation was found with supplemental opiate analgesia and euthanasia technique. These modulating factors were investigated in parallel to enable a number of different comparisons, although interpretation of the findings is more complex. The response pattern varied according to the type of anesthesia used, with agents having a variable impact on cytokine levels, although no single agent uniformly depressed the response in relation to other agents.

Experiment A investigated the effects of anesthesia and analgesia administered at the time of the scald-burn procedure, and showed that anesthetics had a significant and diverse impact on serum cytokine levels measured 24 hours later at euthanasia. After isoflurane anesthesia, statistically significant differences between burned and unburned sham animals were found in only two out of six measured cytokines. Therefore, compared to ketamine-xylazine or

pentobarbital, it may be considered that isoflurane dampened the inflammatory response following burn injury to a level closest to that of unburned animals.

Another key finding from experiment A was that buprenorphine analgesia administered at the time of the procedure had a differential effect on IL-6 levels. Combining isoflurane or pentobarbital with buprenorphine led to significantly greater IL-6 levels than without analgesia, although this effect was not found with ketamine-xylazine. This potentially may lead to significant non-uniformity in an experiment where individual animals receive buprenorphine as required rather than on a standardized schedule.

In experiment B, when anesthetics or carbon dioxide were administered immediately prior to euthanasia, we found all agents exerted a mostly inhibitory influence on measured cytokine levels, in comparison to burned animals euthanized without anesthesia. Interleukin-1, CINC-2, and MCP-1 in particular were widely influenced, showing a dampened cytokine response in comparison to control animals with at least three of the agents administered. The method of euthanasia utilized appeared to modulate MCP-1 in particular, with significantly reduced levels following decapitation using ketamine-xylazine, pentobarbital or carbon dioxide, as well as significantly reduced levels following laparotomy using isoflurane.

Isoflurane immediately before euthanasia by decapitation had comparatively the least dampening effect in experiment B, with no significant difference in cytokine results compared to controls decapitated without anesthesia, although did tend to reduce the overall response. Other agents used were found to significantly depress IL-1 and MCP-1 levels. We speculate that this likely occurred due to the fast onset of action of the inhaled anesthetic, in comparison to the other agents, enabling a brief anesthetic with a shorter exposure time and reduced systemic effect. We would therefore suggest isoflurane be used prior to decapitation if an anesthetic is administered.

However, in contrast to these findings at euthanasia, isoflurane appeared to have the widest dampening effect on the inflammatory response when used during the burn procedure, in line with previous findings from sepsis and ischemia-reperfusion models. This difference may be due to the longer exposure required to perform a procedure compared to euthanasia. Although we would be cautious about making definitive recommendations regarding anesthesia and analgesia from this single study, we would suggest ketamine-xylazine with analgesia appears to offer the best balance for performing procedures without significant exaggeration or dampening of inflammation, although this remains relatively tentative, as a control group without anesthesia is not possible although is the clinical situation with regards to burn injury.

A number of studies have found both IL-1 and TNF- to be significantly attenuated with isoflurane exposure in models of sepsis and ischemia-reperfusion injury (5, 12, 14, 19). Pretreatment with isoflurane in combination with pentobarbital and fentanyl anesthesia in a model of endotoxemia inhibited levels of IL-1 and TNF-, with spontaneous nitrite release from isolated alveolar macrophages also significantly attenuated, measured by activity of inducible nitric oxide synthase (iNOS) (5). Isoflurane also conveyed protection against cell-death induced by TNF- and IL-1 in rodent vascular smooth muscle and human umbilical vascular endothelial cells, potentially mediated via protein kinase C and ATP-sensitive potassium channels (20). Isoflurane also suppressed release of these cytokines following stimulation of peripheral blood mononuclear cells (21). Enhanced macrophage cell viability was demonstrated in a recent study using 2% isoflurane pretreatment, through inhibition of the expression of nitric oxide synthase, although cell viability was not enhanced with 1 or 3% isoflurane (22). This effect was also postulated to be mediated via protein kinase C.

Isoflurane was recently compared with pentobarbital during three hours of anesthesia, and shown to improve survival and protect against renal and hepatic injury following septic peritonitis in mice (12). Survival following LPS injection was also improved in mice following one-hour continuous isoflurane anesthesia, associated with a delay in the inflammatory response, and decreased activity of nuclear factor- κ B (NF- κ B), as well as decreased levels of TNF- α , IL-6 and IL-10 (3). This study also compared isoflurane with pentobarbital and ketamine-xylazine, showing the greatest reduction of serum TNF- α and IL-6 with ketamine-xylazine, in contrast to our findings, although in a different model. Isoflurane has also been shown to inhibit the activation of NF- κ B, and to be associated with decreased production of TNF- α and IL-6 in exposed whole blood (23).

Ketamine has also been shown to modulate the inflammatory response in models of endotoxemia. Ketamine has been found to attenuate LPS-induced iNOS expression, NF- κ B and activator protein-1 (AP-1) DNA binding activity (24), and induce heme-oxygenase-1 (HO-1) expression, with the gastro-protective effects of ketamine suggested to be mediated via HO-1 (24). Ketamine was shown to significantly reduce IL-6 levels in LPS-induced lung injury in a rodent model (10). Ketamine was also found to reduce hemodynamic changes in comparison to isoflurane in a porcine model of hemorrhagic liver injury (25), although levels of IL-6, IL-8, and TNF- α did not vary significantly, though TNF- α mRNA was lower with ketamine. However, animals were also more acidotic with ketamine IV anesthesia (25).

Pentobarbital in the presence of LPS was reported to lower TNF- α in both *in vivo* and *in vitro* settings, through suppression of both NF- κ B and AP-1 activities (9). Conversely however, pentobarbital has also been shown to increase cardiac dysfunction in a trauma model, via induction of iNOS (26). In addition to IL-6, TNF- α , and IL-1 have been shown to be up-regulated and involved in burn-induced cardiac dysfunction in a rodent model (27).

Different opioid drugs have also been found to have variable immune-modulating effects. Buprenorphine, a partial opiate agonist, is an extremely effective analgesic and has been shown to induce a beneficial immune profile (28) following surgical stress (29), improved cardiac and metabolic parameters in various models of sepsis (30, 31), and did not impair renal function in an ischemia-reperfusion model.(32) The combination of buprenorphine with isoflurane or pentobarbital significantly increased measured levels of IL-6 in our study, although other cytokines did not appear significantly influenced. This may have been an indirect effect resulting from an increased recovery time with regards to pentobarbital in particular, and this finding may warrant further study.

Most of the current literature report findings from models of sepsis, trauma, or ischemia-reperfusion, rather than the burn model reported here, which therefore may limit direct parallels. The findings from our study may reflect differences in the cytokine response that result from burn injury. Other possible explanations for dissimilarity with previously reported studies include mechanism of action of the agent, differing analgesic qualities, total dose administered and half-life, timing and length of exposure, rapidity and duration of action; all of which potentially exerted a varied influence on inflammatory signaling cascades. Additionally, modulating effects of anesthesia on the cytokine response when delivered immediately prior to euthanasia, as in experiment B, may vary in comparison to pre-treatment effects demonstrated 24 hours following the procedure in experiment A.

The mechanisms responsible for the varied effects seen with different anesthetic agents and euthanasia methods remain to be fully determined. From previous literature, we would speculate that specific differences in immune-modulating and anti-inflammatory effects due to the agents, as well as induced hemodynamic changes and differences in recovery times may be responsible for the effects observed. These include both a prolonged or knock-on

effect at the time of the burn procedure, as well as an immediate impact at euthanasia. The present study was primarily established to determine whether differences in the cytokine response can be observed in our burn model in order to enhance future studies. Further studies would be needed to confirm these findings and investigate the mechanistic aspects underlying these observations.

All animals were resuscitated with IP administered lactated ringers during the burn procedure and then subsequently self-resuscitated from cage water bottles. Recovery time from anesthesia clearly varied depending on the agent and route of administration, with animals receiving isoflurane recovering most quickly, whilst animals receiving both pentobarbital and buprenorphine taking longer to recover. This may have led to differences in self-resuscitation, although the degree to which this ultimately affected overall total fluid input would require further study.

Other limitations of this study may include the confirmation of similar depth of anesthesia with associated anesthetic dosing. Agents were delivered according to animal body weight and depth of anesthesia was confirmed by the toe-pinch withdrawal reflex. This test is simple to perform and is commonly used to determine adequate anesthesia in rodent experiments, and we felt represented the most relevant test in this setting. Levels of injected and inhaled agents could have been more accurately determined by serum analysis or gas sampling, although the experimental setup used reflected common practice.

Measured TNF levels were at or below the ELISA assay limit of detection in several samples throughout the different groups in both experiments A and B, which limited analysis and interpretation. Levels of IL-1 and TNF- in models of endotoxemia appear to demonstrate a greater response than that reported here following burn injury at 24 hours.

Modulation of the inflammatory response is occurring due to anesthetic agents, although results of our study may remain open to interpretation and require further validation in different models and species. It is therefore difficult to suggest a standard as each lab may have its own protocols, although better standardization is required both within experimental protocols and especially when comparing results from different animal model studies.

CONCLUSIONS

Differential effects and attenuation of inflammatory patterns following anesthetics should be considered when designing animal studies and interpreting results, as these appear to have significant modulating impact. Effects of anesthesia should be considered both at the time of euthanasia as well as during procedures, as agents used during a procedure led to modulation of cytokine levels measured 24 hours later at euthanasia. We recommend particular attention to the choice of anesthetic and analgesic drugs used in experiments studying inflammatory responses, as the pro-inflammatory cytokine response following burn injury is influenced and modulated by these factors. Due to the significant variation of effect, we recommend a standardized anesthesia, analgesia and euthanasia regimen be used across all experiments that are compared.

Although it remains difficult to recommend an optimal choice of drugs and euthanasia technique in different models and animal species, our findings suggest that a brief anesthetic with isoflurane immediately prior to euthanasia by decapitation may exert the least dampening effect on the cytokines we measured. In contrast however, more prolonged anesthesia with isoflurane during the burn procedure exerted a dampening effect. When used to perform longer procedures, ketamine-xylazine with buprenorphine appears to offer a better balance to avoid significant exaggeration or dampening of inflammation, in comparison to either isoflurane or pentobarbital. Additionally, we noted particular

augmentation of the IL-6 response when buprenorphine was used in combination with isoflurane or pentobarbital during the burn procedure, which may warrant further investigation. These findings may also have implications and potential for further investigation both in a clinical setting, with regards to anesthetic choice for surgical procedures following burn and traumatic injury, as well as novel mechanisms to modulate the stress-response.

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Figure 1.**A)** Experiment A – Flowchart. **B)** Experiment B – Flowchart.Isoflurane (ISO), Ketamine-Xylazine (KX), Pentobarbital (PEN), Carbon Dioxide (CO₂), TBSA (Total Body Surface Area).

Figure 2.

Experiment A - Cytokine results. Various anesthetics used for the burn procedure, with or without supplemental analgesia, and a standardized euthanasia method (decapitation). Unburned Sham (SHAM) receiving no agents, Isoflurane (ISO), Ketamine-Xylazine (KX) and Pentobarbital (PEN). Data displayed as mean $\pm$ SEM (error bars), with statistically significant results as indicated.

A) * Significant difference between PEN no analgesia and SHAM groups by one-way ANOVA on ranks – Dunn's method ($p < 0.05$). † Significant difference between PEN and KX no analgesia groups by one-way ANOVA on ranks – Dunn's method ($p < 0.05$). ‡ Significant difference between PEN analgesia and SHAM groups by one-way ANOVA on ranks – Dunn's method ($p < 0.05$).

B) † Significant difference between both KX and PEN no analgesia groups and SHAM by one-way ANOVA on ranks – Dunn's method ($p < 0.05$). ‡ Significant difference between both KX and PEN analgesia groups and SHAM by one-way ANOVA on ranks – Dunn's method ($p < 0.05$).

C) † Significant difference between ISO, KX and PEN no analgesia groups and SHAM by one-way ANOVA on ranks – Dunn's method ($p < 0.05$). ‡ Significant difference between ISO, KX and PEN analgesia groups and SHAM by one-way ANOVA on ranks – Dunn's method ($p < 0.05$).

D) No significant differences.

E) † Significant difference between KX no analgesia and SHAM groups by one-way ANOVA on ranks – Dunn's method ($p < 0.05$). * Significant difference between both KX and PEN no analgesia groups and ISO by one-way ANOVA on ranks – Dunn's method ($p < 0.05$). ‡ Significant difference between PEN analgesia and SHAM groups by one-way ANOVA on ranks – Dunn's method ($p < 0.05$).

§ Significant difference between PEN and ISO analgesia groups by one-way ANOVA on ranks – Dunn’s method ($p < 0.05$). ¶ Significant difference between ISO and PEN no analgesia versus analgesia groups by Student’s t-test ($p < 0.05$).

F) † Significant difference between ISO, KX and PEN no analgesia groups and SHAM by one-way ANOVA on ranks – Dunn’s method ($p < 0.05$). ‡ Significant difference between ISO, KX and PEN analgesia groups and SHAM by one-way ANOVA on ranks – Dunn’s method ($p < 0.05$).

Figure 3.

Experiment B - Cytokine results. Various agents and methods used at euthanasia (laparotomy plus exsanguination or decapitation) following a standardized burn procedure. Controls (CON), Isoflurane (ISO), Ketamine-Xylazine (KX), Pentobarbital (PEN) and Carbon Dioxide (CO₂). Controls underwent burn using KX and buprenorphine analgesia, similar to other groups, and were euthanized without anesthesia by decapitation. Data displayed as mean $\pm$ SEM (error bars), with statistically significant results as indicated.

A) † Significant difference between ISO and PEN laparotomy groups and CON by one-way ANOVA on ranks – Dunn's method ($p < 0.05$). ‡ Significant difference between KX, PEN and CO₂ decapitation groups and CON by one-way ANOVA on ranks – Dunn's method ($p < 0.05$).

B) † Significant difference between ISO laparotomy group and CON by one-way ANOVA on ranks – Dunn's method ($p < 0.05$).

C) † Significant difference between ISO, KX and CO₂ laparotomy groups and CON by one-way ANOVA on ranks – Dunn's method ($p < 0.05$). ¶ Significant difference between CO₂ laparotomy versus decapitation groups by Student's t-test ($p < 0.05$).

D) ¶ Significant difference between ISO and KX laparotomy versus decapitation groups by Student's t-test ($p < 0.05$).

E) † Significant difference between KX laparotomy group and CON by one-way ANOVA on ranks – Dunn's method ($p < 0.05$).

F) † Significant difference between ISO, PEN and CO2 laparotomy groups and CON by one-way ANOVA on ranks – Dunn’s method ($p<0.05$). ‡ Significant difference between KX, PEN and CO2 decapitation groups and CON by one-way ANOVA on ranks – Dunn’s method ($p<0.05$). ¶ Significant difference between ISO, KX, PEN and CO2 laparotomy versus decapitation groups by Student’s t-test ($p<0.05$).

Table 1

Experiment A - Summary table.

Anesthesia	Isoflurane		Ketamine-xylazine		Pentobarbital	
	No Analgesia	Analgesia	No Analgesia	Analgesia	No Analgesia	Analgesia
Cytokine						
IL-1					*	*
CINC-1			*	*	*	*
CINC-2	*	*	*	*	*	*
TNF						
IL-6			*			*
MCP-1	*	*	*	*	*	*

Arrows , , , indicate greater/lower/similar cytokine levels compared to SHAM group, respectively.
* Significant differences versus SHAM group by one-way ANOVA on ranks – Dunn’s method (p<0.05) as highlighted. (Refer to results and figure 2 for further details)

Table 2

Experiment B - Summary table.

Anesthesia	Isoflurane		Ketamine-xylazine		Pentobarbital		Carbon Dioxide	
	Laparotomy	Decapitation	Laparotomy	Decapitation	Laparotomy	Decapitation	Laparotomy	Decapitation
Cytokine								
IL-1	*			*	*	*		*
CINC-1	*							
CINC-2	*		*				*	
TNF								
IL-6			*					
MCP-1	*			*	*	*	*	*

Arrows , , , indicate greater/lower/similar cytokine levels compared to CONTROL group, respectively.
* Significant differences versus CONTROL group by one-way ANOVA on ranks – Dunn’s method (p<0.05) as highlighted. (Refer to results and figure 3 for further details)