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Original Research

Physiologic Effects of Prolonged Terminal Anesthesia in Sheep (*Ovis gmelini aries*)

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The ruminant alimentary tract and its effects on blood homeostasis complicate prolonged terminal studies conducted under general anesthesia in sheep. We therefore studied 15 healthy female white alpine sheep that were undergoing prolonged anesthesia (> 30 h) for an unrelated terminal study. In the current study, all sheep developed a decreased hematocrit and hemoglobin concentration after induction of anesthesia, which fell further, along with a significant decrease in white blood cell count, over the course of anesthesia. Sheep also showed an initial hyponatremia, a persistent hypokalemia, hypocalcemia, and a progressive hyperchloremia. A significant drop in blood pH developed over time despite normal values of blood lactate and a marked decline in partial pressure of carbon dioxide over the course of the experiment. The latter consequently reduced the efficacy of mechanical ventilation, as reflected in a reduced oxygen partial pressure. A significant increase in lactate dehydrogenase and creatinine kinase was observed. Arterial blood pressure and heart rate significantly decreased over time, but remained within normotensive and normocardic limits. Central venous pressure rose significantly over the course of anesthesia. In conclusion, prolonged anesthesia in sheep is associated with a wide range of complex physiologic changes. An in-depth understanding of all metabolic compensatory mechanisms and their underlying cause during prolonged anesthesia is necessary for interpreting data from the primary study, with special considerations to account for ruminant-specific physiology.

Abbreviations: ABP, arterial blood pressure; CVP, central venous pressure; CK, creatinine kinase; $etCO_2$, end tidal CO_2; Hct, hematocrit; Hgb, hemoglobin; LDH, lactate dehydrogenase; pCO_2, partial pressure CO_2; pO_2, partial pressure O_2

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Introduction

Terminal studies that are conducted under continuous anesthesia[28] and perhaps extend over many hours and days without a return to consciousness are often conducted in rodents in the field of neurosciences. Such studies are also conducted for a variety of reasons in large animal translational research. First, Directive 2010/63/EU requires assigning prospective and retrospective severity grading to procedures carried out on animals for scientific purposes.[16] Factors such as the invasiveness of the procedure and the duration over which the animal may experience pain, suffering, or distress must be considered.[51] Maintaining the animal under general anesthesia for the duration of the experiment reduces the severity grade. Second, medical devices designated for market approval as bridging therapeutic options are often not chronically implantable and are intended only for the sedated or anesthetized patient in the intensive care unit. Such devices require a follow-up time exceeding the longest potential duration of use to confirm safety and efficacy.[58] Third, novel methodologies for improving donor organ grafting and reducing hyperacute graft rejection are tested over a period of up to 48 h and provide no scientific justification for waking the animal from anesthesia prior to euthanasia.

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Sheep are a commonly used large animal model in a variety of these research fields due to the similarity of their anatomic structures and physiologic functions to those of humans.[60] Prolonged mechanical ventilation is frequently associated with alveolar atelectasis, pulmonary perfusion heterogeneity, and gravitational inhomogeneity, which cause pulmonary shunting in many species including both sheep and humans.[23,24,25,56] However, anesthetic management in ruminants is further complicated by their specialized alimentary tract, problems related to aspiration of saliva and rumen contents, and low lung gas volume during recumbent body postures.[8,52] Moreover, the rumen contains a highly diverse population of anaerobic microorganisms.[47] These bacteria are thereby predominantly responsible for microbial protein synthesis and carbohydrate digestion and the production of volatile fatty acids (VFA) through fermentation of plant-based glucose.[28] Carbon dioxide and methane are produced in quantities that directly depend on the type of fatty acid (for example, acetate, propionate, and butyrate) that is produced.[33] Ruminal fermentation continues in anesthetized animals; however, sedatives and anesthetics inhibit gastro-intestinal motility, often leading to gas accumulation and rumen bloating, especially in nonfasted animals. The consequent increase in intraabdominal pressure compresses the diaphragm and great vessels such as the posterior vena cava, compromising the cardiopulmonary system.[41] Fasting prior to anesthesia reduces the amount of gas produced by fermentation.[41] However, the ovine rumen also has a significant role in fluid and electrolyte homeostasis.[39] Food deprivation leads to

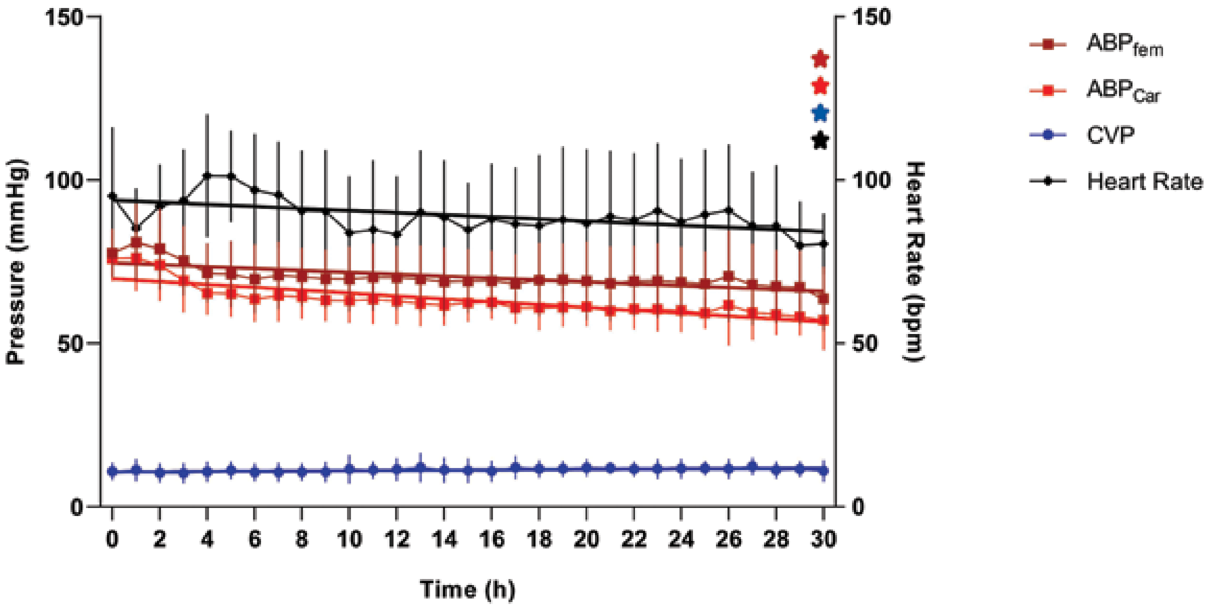

Figure 1. Hemodynamics (*n* = 15). Trend of mean arterial pressure in the femoral artery (ABP_{fem}) and in the carotid artery (ABP_{car}), as well as heart rate and central venous pressure (CVP) over the course of 30 h of anesthesia. Pressures were continuously recorded and averaged over one hour. All values are shown as mean ± SD. An asterisk indicates a significant nonzero slope.

the loss of large amounts of bicarbonate, sodium and phosphate, which are normally secreted into the rumen via saliva during food intake.[50] This situation is thought to cause an aldosterone-like response that preserves the blood sodium concentration and subsequently depletes potassium.[57] Further physiologic peculiarities in ruminants include the use of ruminal VFA for approximately 70% of caloric requirements[3] and recycling of urea for endogenous protein production.[48] Preoperative fasting, prolonged anesthesia, alteration in ruminal tonicity, reduced production of VFA, and continuous loss of urea due to lack of saliva may result in significant metabolic derangement.

The goal of this study was to document the effects of prolonged anesthesia under mechanical ventilation and the associated changes in physiologic homeostasis during prolonged terminal studies in sheep.

Materials and Methods

Ethical statement. Animal housing and all experimental procedures were approved by the local Committee for Experimental Animal Research (Cantonal Veterinary Office Zurich, Switzerland) under the license numbers ZH41/2017 and ZH51/2020 in conformity with the European Directive 2010/63/EU of the European Parliament and the Council on the Protection of Animals used for Scientific Purposes, and the *Guide for the Care and Use of Laboratory Animals* procedure.[16,28]

Animals, housing, and husbandry. Fifteen adult female white alpine sheep (*n* = 15), 2 to 6 y of age, with a body weight of 91 ± 4 kg, were included in this study because they were independently assigned to experience prolonged anesthesia for a performance test of an intravascular device. The intravascular device may have influenced hemodynamics but would not be expected to influence other measured parameters. Upon arrival at the facility, sheep underwent an acclimation period of at least 1 wk. All sheep were regularly screened under the national surveillance program for foot and mouth disease, ovine rinderpest, sheep pox, ovine brucellosis, contagious agalactia, rabies, scrapie, tuberculosis, paratuberculosis, pseudotuberculosis, blue tongue, sheep pulmonary adenomatosis, and foot rot. Upon arrival, sheep received a clinical examination that assessed general behavior and appearance, posture, gait, and parameters such as body temperature, heart rate, respiration, and rumen motility. Sheep were housed in groups of 3 to 4 animals at all times. Pens were scattered with straw and sheep had free access to an automatic water trough, meadow hay, and a mineral licking stone for small ruminants (Blattin®, Profuma, Germany). Sheep were kept at a room temperature of 19 °C with a relative humidity of 45% to 55%. Lights were on in all animal rooms from 7:00 AM to 5:00 PM. A skylight provided natural daylight. The general condition of each animal was checked twice daily by the animal caretakers and regularly by a veterinarian.

Anesthesia and analgesia. Prior to surgery, sheep were fasted for approximately 16 to 18 h. Water was available ad libitum during the fasting period. On the day of surgery, an intravenous catheter (BraunüleMT Luer Lock, 14 G, B. Braun Medical, Melsungen, Germany) was placed in the jugular vein of the conscious sheep. Anesthesia was induced by an intravenous injection of ketamine hydrochloride (Ketasol-100 ad us.vet.; Dr. E. Graeub, Berne, Switzerland; 3 mg/kg BW) in combination with midazolam (Dormicum, Roche Pharma [Switzerland], Reinach, Switzerland; 0.3 mg/kg BW) and propofol (Propofol - Lipuro 1%, B. Braun Medical; Sempach, Switzerland; 1 mg/kg BW). Sheep were orotracheally intubated with a size 11 endotracheal tube and connected to a semiclosed ventilator circuit (Dräger Fabius GS, Dräger Medical, Lübeck, Germany).

During animal preparation, anesthesia was maintained by inhalation of isoflurane (Attane Isoflurane ad.us.vet., Piramal Enterpr. India; Lyssach, Switzerland) (1.5 to 3 vol%) in combination with 50% oxygen under spontaneous respiration. An orogastric tube (Rüsch 12 mm, Teleflex Medical Enterprise, Athlone, Ireland) was placed, and eye ointment containing vitamin A (Vitamin A Blache, Bausch and Lomb Swiss, Zug, Switzerland) was applied. All sheep received tetanus serum (Tetanus Serum Intervet, MSD Animal Health, Lucerne, Switzerland; 3 mL, SC) and perioperative antibiosis in form of either amoxicillin/clavulanic acid (4:1) (Co-Amoxi-Mepha, Mepha Pharma, Basel, Switzerland; 20 mg/kg BW IV) or oxytetracycline (Enge-

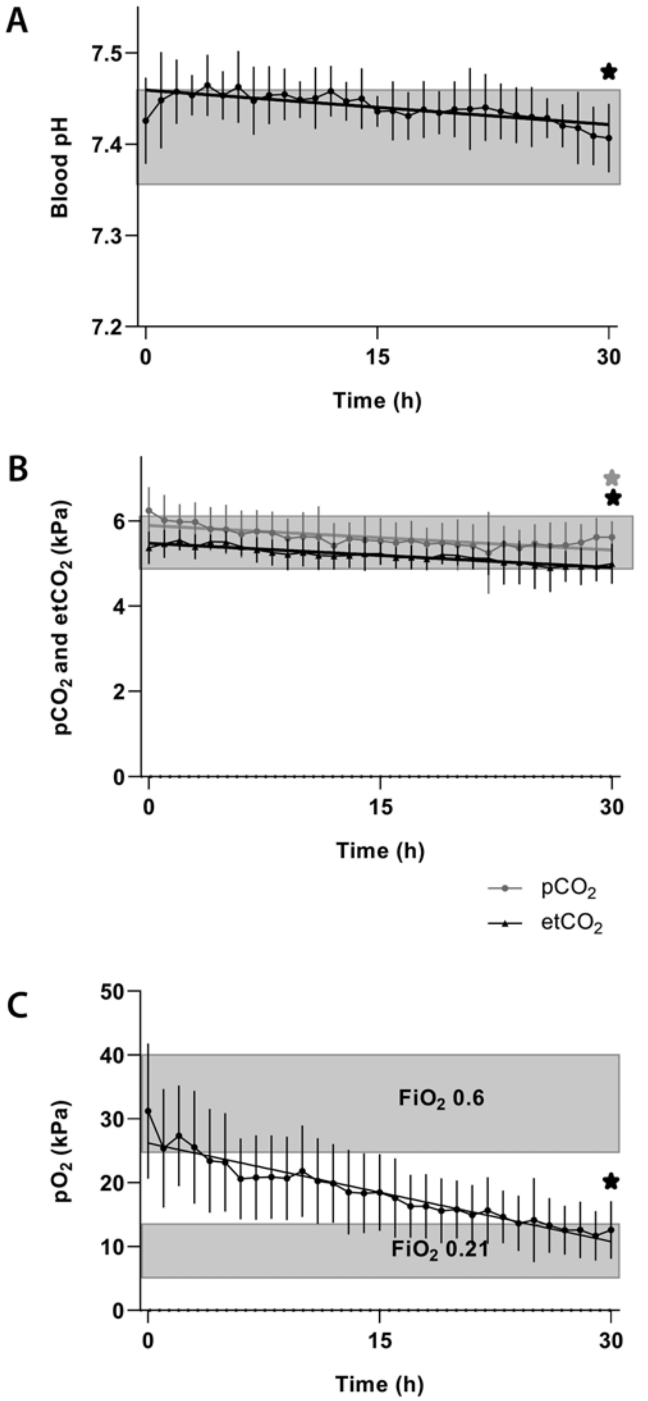

Figure 2. Blood gases (*n* = 15). A) Trend of arterial blood pH over the course of 30 h of anesthesia; B) Trend of end tidal CO_2 ($etCO_2$) and partial pressure of CO_2 (pCO_2) over the course of 30 h of anesthesia; C) Trend of partial pressure O_2 (pO_2) over the course of 30 h of anesthesia. Samples were collected every hour. All values are shown as mean ± SD. The gray area indicates normal values in sheep. An asterisk indicates a significant nonzero slope.

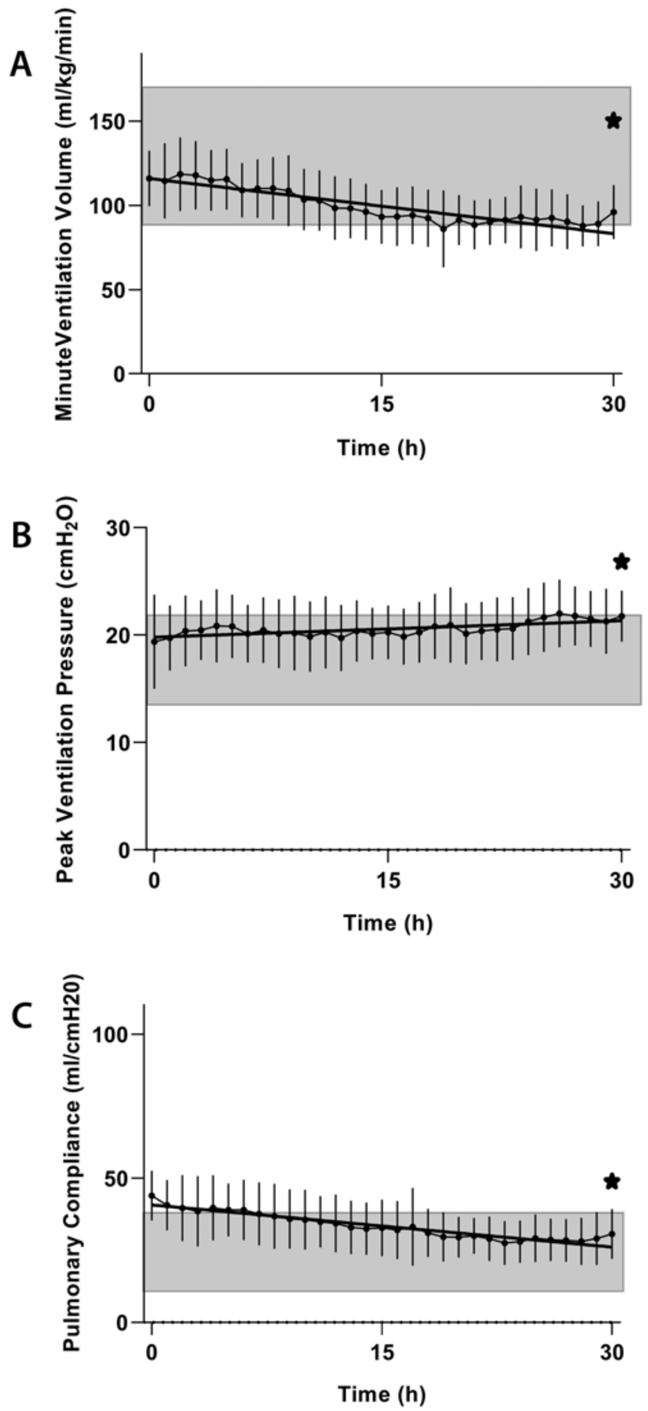

Figure 3. Ventilation (*n* = 15). A) Trend of minute volume over the course of 30 h of anesthesia, continuously adjusted to maintain an $etCO_2$ of 4.6 to 6 kPa; B) Trend of peak ventilation pressure; and C) static pulmonary compliance over the course of 30 h of anesthesia. Values were recorded every hour. All values are shown as mean ± SD. The gray area indicates normal values in sheep. An asterisk indicates a significant nonzero slope.

mycin 10% ad us. vet, MSD Animal Health, Lucerne, Switzerland; 8 mg/kg BW IM).

After sheep were transferred to the operating room, they were placed in a supine position and the large vessels were catheterized as described below. For the duration of the experiment, sheep were in right lateral recumbency on a memory foam mattress with a heating mat. Anesthesia was maintained with 1% to 1.5% isoflurane in an oxygen/air mixture, in conjunction with a continuous infusion pump administering propofol (Propofol- Lipuro 2%, B. Braun Medical; Sempach, Switzerland 2 to 5 mg/kg/h) and ketamine-midazolam at an infusion rate of 0.24 mL/kg/h (Ketasol-100 ad us.vet.; Dr. E. Graeub, Berne, Switzerland, 500mg, Dormicum, Roche Pharma [Schweiz], Reinach, Switzerland, 15mg, diluted with 0.9% saline to 50 mL). Throughout the procedure, the sheep also received a continuous intravenous infusion of sufentanil (Sufenta Forte, Janssen-Cilag,

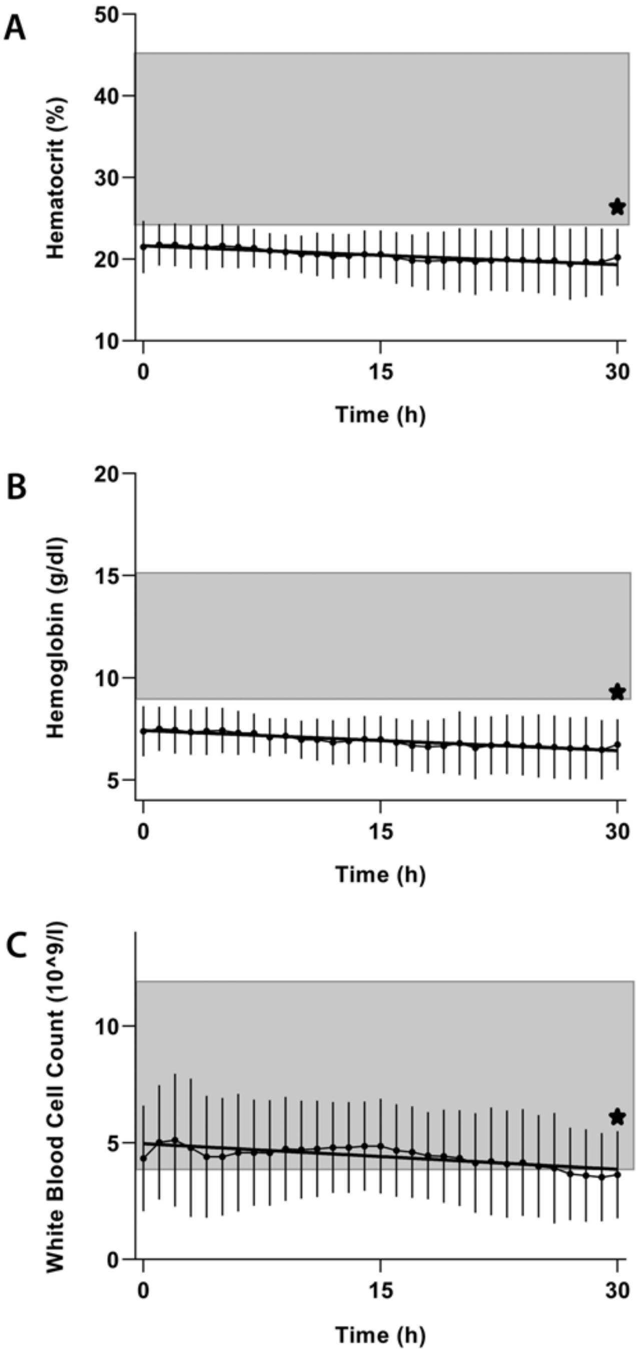

Figure 4. Hematology (*n* = 15). A) Trend of hematocrit; B) hemoglobin concentration C) white blood cell count (WBC) over the course of 30 h of anesthesia. Samples were collected every hour. All values are shown as mean ± SD. The gray area indicates normal values in sheep. An asterisk indicates a significant nonzero slope.

Zug, Switzerland; 0.02 to 0.05mg/kg/h) and warmed Ringers lactate solution at an infusion rate of 5 mL/kg BW/h. All sheep were anticoagulated with sodium heparin (B. Braun Medical, Sempach, Switzerland) to achieve a stable Activated Clotting Time (ACT) of 180 to 200 s.

Catheterization and monitoring of hemodynamics. Ultrasound guided percutaneous placement of a femoral arterial introducer sheath (Check-Flo Performer Introducer, 14 F, Cook Medical, Bloomington IN), a femoral 2-lumen venous catheter (HighFlow Dolphin Catheter, 13 F, Baxter International, Deerfield IL), a carotid arterial line (Avanti+ Introducer, 5F, Cordis, Miami Lakes FL) and a multilumen jugular vein catheter (AeroGuard Blue, Arrow, Teleflex Medical Europe, Ireland) was performed by means of the Seldinger method.

Carotid and femoral arterial blood pressure (ABP_{car} and ABP_{fem}) as well as central venous blood pressure (CVP) and heart rate (HR) were continuously recorded using the DSI Ponemah System V.5.1. (DataScience International, St. Paul, MN).

The intravascular device that was tested in this experiment was inserted transfemorally through the 14 F arterial sheath, as previously described.[58]

Blood gases and ventilation. Sheep were ventilated under positive pressure with volume control by using a semiclosed-circuit ventilator (Dräger Primus, Dräger Schweiz, Liebefeld, Switzerland). Fresh gas flow was set to 1 to 1.5 L/min with an FiO_2 of 0.6, 16 to 20 breaths/min, a tidal volume of 5 to 8 mL/kg BW, and a maximum peak ventilation pressure (P_{max}) of 30 cmH_2O to maintain an end tidal CO_2 of 4.7 to 6 kPa (35 to 45 mm Hg). 15 mg of the bronchosecretolytic bromhexine hydrochloride (Bisolvon ad. us. vet., Boehringer Ingelheim, Basel, Switzerland) was administered intramuscularly to prevent excessive mucus formation in the respiratory tract. Mucus formation was further attenuated by humidifying the inhaled air with an auto-feed chamber (VentStar, Dräger Schweiz, Liebefeld, Switzerland). Respiration parameters (end tidal CO2 [etCO2], tidal volume, minute volume [MV], respiration rate, peak ventilation pressure and patient static compliance) were documented every hour. Arterial blood gases were analyzed every hour on the epoc Blood Analysis System (Siemens Healthcare, Erlangen, Germany).

Hematology, electrolytes and clinical chemistry. Blood was sampled from the carotid artery catheter; excess blood was returned via the venous catheter. Baseline blood samples were collected immediately after placement of the catheters. A total of 254 mL of blood, equaling approximately 4.5% of the total blood volume, was collected from each sheep during the 30-h procedure. ACT was determined using 1 mL of blood sampled every 30 min. Blood gas analysis used 0.5 mL of blood sampled each hour, hematologic analysis used 0.3 mL of blood collected every hour, and clinical chemistry analysis used 17 mL of blood collected every 4 h (a 10-mL heparin tube for urea, creatinine, lactate dehydrogenase [LDH], creatinine kinase [CK] and myoglobin; a 4-mL fluoride coated tube for lactate, and a 3-mL tube for free hemoglobin).

A complete hematologic analysis was performed every hour using a VETSCAN HM5 Hematology Analyzer (Zoetis Schweiz, Delémont, Switzerland). Hematology analysis included the following parameters: white blood cell count with automatic differentiation, hematocrit (Hct) (incl. mean corpuscular volume [MCV], mean corpuscular hemoglobin [MCH], mean corpuscular hemoglobin concentration [MCHC]), and hemoglobin (Hgb). Analysis was performed using EDTA-treated whole blood. Electrolytes (potassium, sodium, ionized calcium, chloride) and metabolites (glucose, lactate) were also analyzed on the epoc Blood Analysis System (Siemens Healthcare, Erlangen, Germany). A lithium-heparin and a fluoride coated blood collection tube were submitted to the inhouse laboratory every 4 h for clinical chemistry to analyze the following parameters: urea, creatinine, lactate dehydrogenase (LDH), creatinine kinase (CK), myoglobin and lactate.

Urinary output. A urinary balloon catheter (Rüsch, Ch 12, Teleflex Medical, Belp, Switzerland) was placed transurethrally in all sheep and was connected to a urinary drainage bag with a release valve. Urinary output was monitored every hour and maintained at 1 to 2 mL/kg BW/h. If urine excretion rate was below 1 mL/kg BW/h for more than 3 h, furosemide (Lasix,

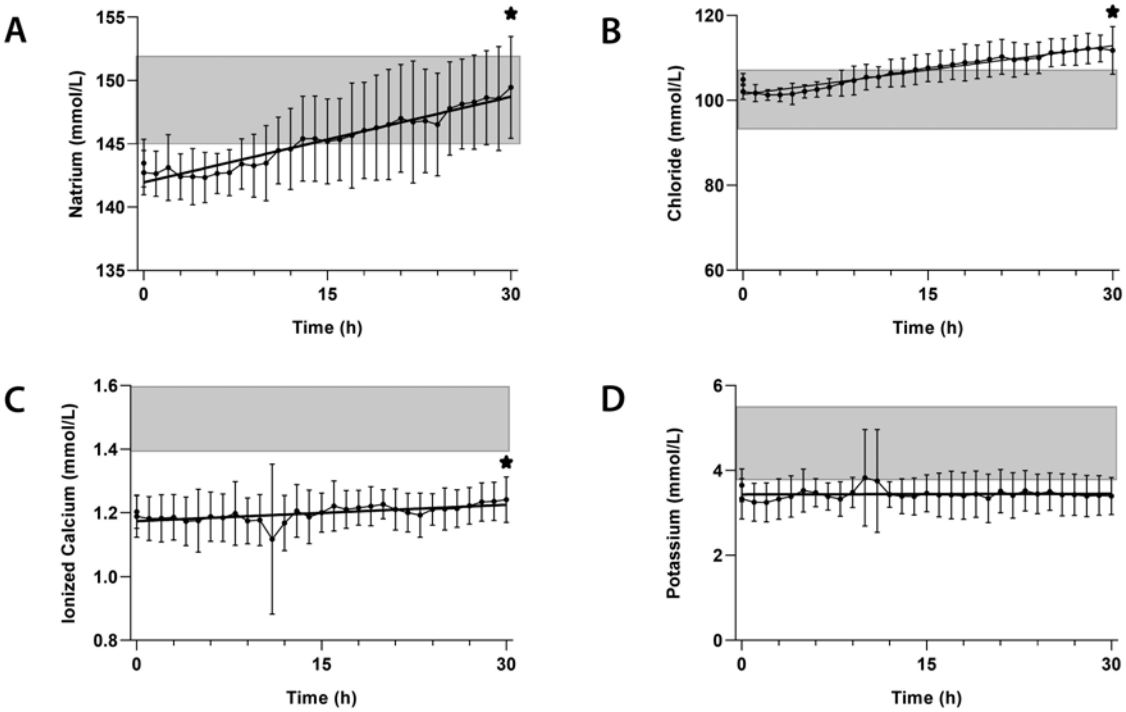

Figure 5. Electrolytes (*n* = 15). A) Trend of sodium; B) chloride; C) ionized calcium and D) potassium over the course of 30 h of anesthesia. Samples were collected every hour. All values are shown as mean ± SD. The gray area indicates normal values in sheep. An asterisk indicates a significant nonzero slope.

Sanofi-Aventis (Suisse), Vernier, Switzerland; 0.5 mg/kg BW) was administered intravenously.

Body temperature. Sheep were placed in right lateral recumbence on a heating pad for the duration of the study. If needed, sheep were in addition warmed with an air-blower (3M Bair Hugger, St. Paul, MN).Body temperature was continuously monitored via an esophageal temperature probe and maintained between 38.3 to 39.9 °C.

Euthanasia. Upon completion of the experimental protocol, sheep were euthanized without recovery from deep, general anesthesia by intravenous administration of sodium pentobarbital (Eskonarkon ad.us.vet., Streuli Pharma, Uznach, Switzerland) (75 mg/kg BW).

Statistics. All analyses were performed with GraphPad Prism software version 8.0.0. (GraphPad Software, San Diego, CA). All data are shown as mean ± SD. Significant deviation from zero for the best-fit slope from linear regression line was determined by using the mean value for each y-point. Significance for all statistical tests was established at $P \leq 0.05$.

Results

Hemodynamics. At the beginning of anesthesia, the mean ABP was 77 ± 7 mm Hg in the femoral and 76 ± 7 in the carotid artery, with no significant difference between them (Δ6.8 ± 1.8 mm Hg, $P = 0.577$). Both ABP_{fem} and ABP_{car}fell significantly over time (m = −0.276 and m = −0.356, respectively; $P< 0.0001$). Mean CVP was 10.8 ± 2.6 at baseline and increased significantly over time (m = 0.02644, $P< 0.0001$). Baseline HR was 95 ± 20 bpm and showed a significant decrease over time (m = −0.3063, $P< 0.0001$) (Figure 1).

Blood gases and ventilation. Mean arterial blood pH was 7.4 ± 0.1at the beginning of anesthesia (Figure 2a), with a pCO_2 of 6.2 ± 0.5 kPa, and a pO_2 of 31.2 ± 10.2 kPa (Figure 2b and c). All 3 values fell significantly over time (m = 0.0013, m = 0.0019 and m = 0.5119 respectively, $P< 0.0001$), with pH remaining within physiologic range but pO_2 falling out of range, considering an FiO2 setting of 0.6. pCO_2 was actively maintained through mechanical ventilation. All sheep were mechanically ventilated with a MV of 116 ± 16 mL/kg BW/min distributed to a respiration frequency of 18 ± 1 brpm (Figure 3a) Ventilation was adjusted to maintain an $etCO_2$ of 4.6 to 6 kPa (baseline mean value: 5.0 ± 1.3 kPa). Mean peak ventilation pressure was 19.6 ± 4.2 cmH_2O at baseline (Figure 3b) with a mean pulmonary compliance of 43.9 ± 8.0 mL/cmH_2O. (Figure 3c)

The MV and pulmonary compliance both fell significantly over time (m = −1.041, m = −0 to 477; $P < 0.0001$). MV remained within the recommended tidal volume range of 8 to 10 mL/kg bodyweight for mechanical ventilation in sheep.[13] Static pulmonary compliance also remained within normal ranges found in the literature.[10] Peak ventilation pressure significantly increased over time (m = 0.052, $P < 0.0001$) but remained within physiologic range (13 to 22 cmH_2O).[13]

Hematology, Electrolytes and Clinical Chemistry. Baseline Hct and Hgb in all sheep were slightly below normal physiologic values with mean values of 24.0 ± 1.9% and 8.5 ± 0.9 g/dL respectively (physiologic range: Hct: 27 to 45%, Hgb: 9 to 15 g/dL[30]) (Figure 4a and b). Hct and Hgb fell significantly over time (m = −0.076, m = −0.447; $P < 0.0001$). MCV, MCH and MCHC remained within physiologic ranges (data not shown). At the beginning of anesthesia, the mean WBC count was 5.5 ± 1.9 × 10^9/L and thus was within normal range (4 to 12 × 10^9/L[30]) but fell significantly over time to end up slightly below the physiologic range (m = −0.037, $P < 0.0001$)(Figure 4c).

Both sodium (m = 0.226, $P < 0.0001$) (Figure 5a) and chloride (m = 0.381, $P > 0.001$) (Figure 5b) showed significant increase over time. Calcium and potassium were supplemented but remained below physiologic range (Figure 5c and d).[30]

Lactate concentrations did not change significantly over time ($P = 0.271$) (Figure 6a). Glucose levels fluctuated but remained

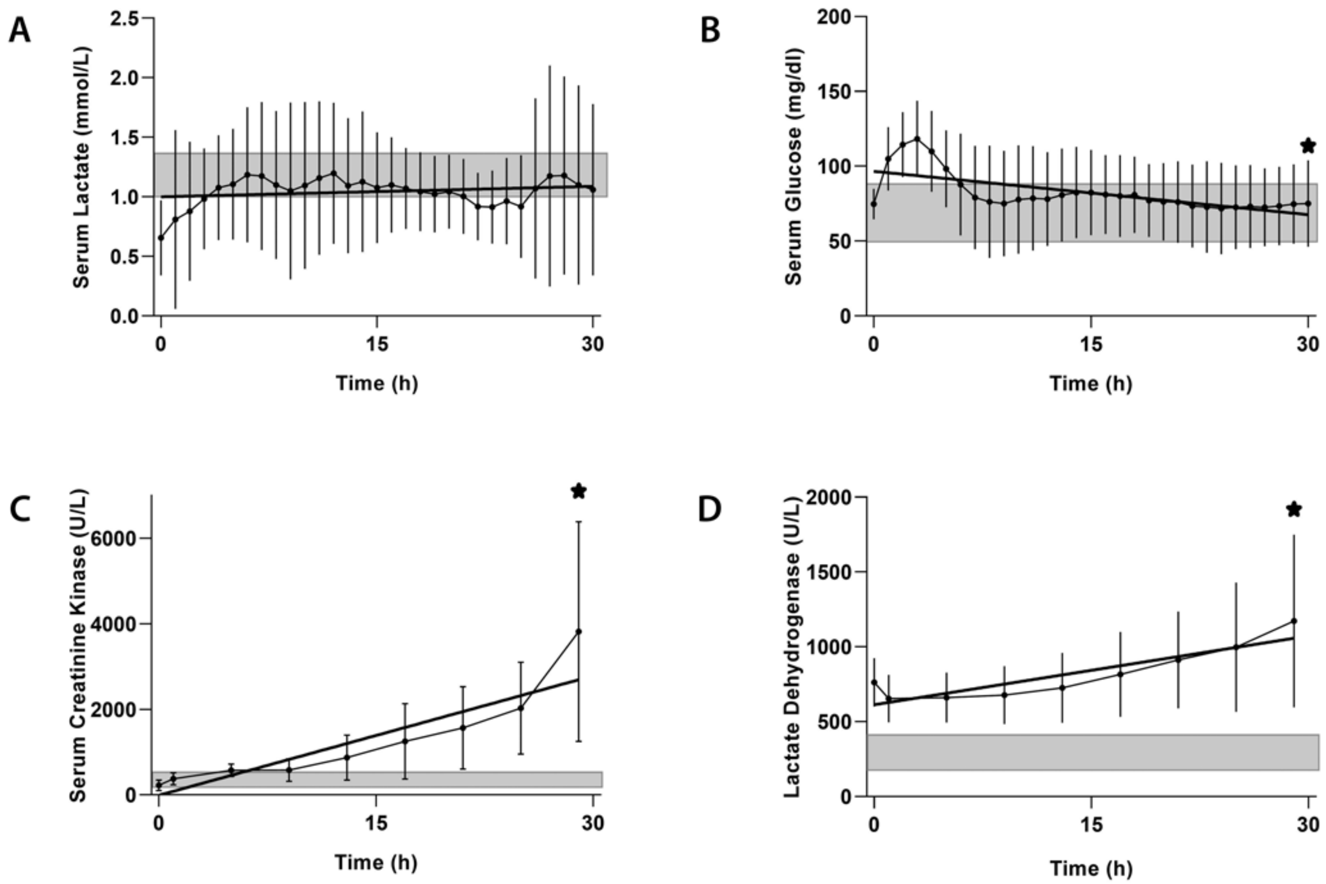

Figure 6. Metabolism. A) Trend of lactate (*n* = 15); B) glucose (*n* = 15); C) creatinine kinase (CK) (*n* = 8); and D) lactate dehydrogenase (LDH) (*n* = 15) over the course of 30 h of anesthesia. Samples for lactate and glucose were collected every hour, CK and LDH were measured every 4 h. All values are shown as mean ± SD. The gray area indicates normal values in sheep. An asterisk indicates a significant nonzero slope.

within the physiologic range (50 to 80 mg/dL[30]) (Figure 6b). CK and LDH showed rapid and significant increases (respectively, m = 97.26, m =14.99; $P = 0.0009$, $P = 0.0015$) (Figure 6c and d) with no concurrent increase in myoglobin. Urea also showed a significant decrease (normal range 3 to 10 mmol/L; m = –0.049, $P < 0.0001$) (Figure 7a), but creatinine did not change significantly (70 to 105 µmol/L) ($P = 0.096$) (Figure 7b); both values remained within the physiologic range for sheep.[30]

Discussion

In this study, we examined the wide range of complex physiologic changes in 15 healthy female white alpine sheep that were undergoing prolonged anesthesia for an unrelated terminal study. After induction of anesthesia, all sheep presented with a decreased hematocrit and hemoglobin concentration that fell further, along with a significant decrease in white blood cell count over the course of anesthesia. Sheep further developed a variety of electrolyte imbalances such as an initial hyponatremia, a persistent hypokalemia, hypocalcemia and progressive hyperchloremia. A significant drop in blood pH occurred over time despite normal values of blood lactate and a marked decline in partial pressure of CO_2 over the course of the study. The fall in pCO_2 latter reduced the efficacy of mechanical ventilation, as reflected in a reduced oxygen partial pressure. A significant increase in lactate dehydrogenase and creatinine kinase also occurred. Arterial blood pressure and heart rate fell significantly over time but remained within normotensive and normocardic limits. Central venous pressure rose significantly over the course of anesthesia.

The specialized alimentary tract of ruminants complicates their anesthetic management for a variety of reasons.[8,52] Ruminal distention, which reduces lung capacity and thus hampers adequate ventilation,[54] along with the regurgitation and aspiration of saliva and ruminal fluid, are the most common challenges in sheep anesthesia. Fasting sheep prior to anesthesia will reduce the amount of gas produced by fermentation and minimize ruminal bloating.[30] However, preoperative fasting in sheep may itself create a negative starting point for long-term anesthesia by disrupting electrolyte balance. All sheep in the current study had pre-existing hyponatremia and hypokalemia at the beginning of anesthesia, probably due to the loss of bicarbonate, sodium and phosphate, which are normally secreted into the rumen via saliva during food intake.[51] This drain of bicarbonate and sodium from the blood induces an aldosterone-like response that aims to preserve blood sodium concentration by promoting excretion of potassium in the urine.[57] Anesthetic induction with ketamine, which is known to cause hypersalivation,[22] will therefore aggravate this preexisting electrolyte deficit. Calcium circulates in the extracellular space in fractions of ionized calcium, protein-bound calcium and calcium complexed to anions such as bicarbonate, citrate, sulphate, phosphate, and lactate.[6] Ionized calcium was low throughout the experiment in all sheep. Potential underlying reasons for this include perianesthetic fluid infusions causing a relative fall in plasma albumin levels, resulting in altered measurements of total calcium levels,

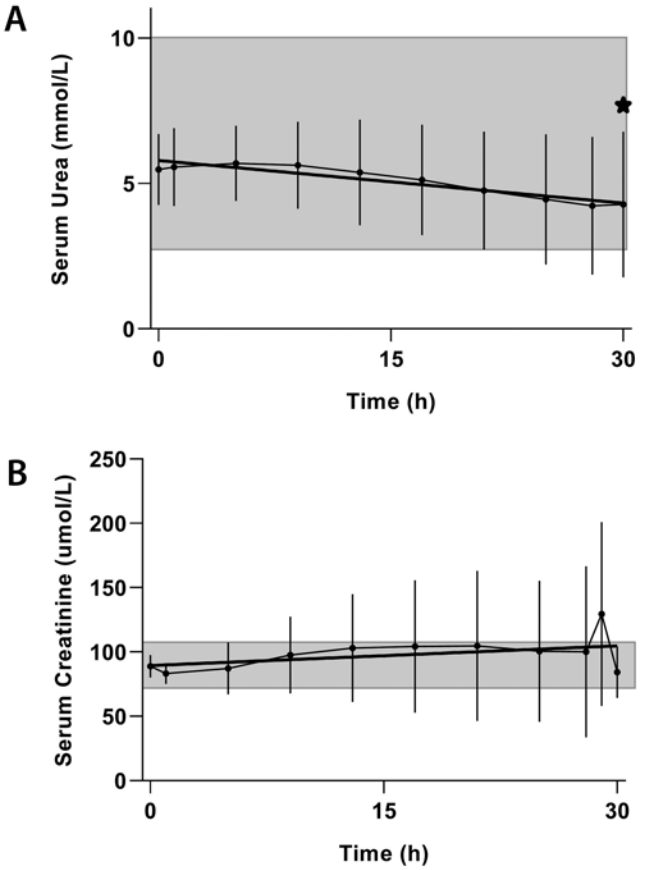

Figure 7. Kidney. A) Trend of urea and B) creatinine over the course of 30 h of anesthesia. Samples were collected every 4 h. All values are shown as mean ± SD. The gray area indicates normal values in sheep. An asterisk indicates a significant nonzero slope.

despite ionized calcium remaining within physiologic range.[20] Neither albumin concentrations nor total calcium levels were assessed in this study. Conversely, hypomagnesemia and hyperphosphatemia can lead to reduced production and release of parathyroid hormone, consequently causing hypocalcemia.[43] A study in New Zealand White rabbits showed a significant increase in blood phosphate after the injection of ketamine and diazepam.[18] Phosphate and magnesium are not commonly assessed during anesthesia. A variety of drugs such as antibiotics[12] and anticoagulants[55] may also cause an iatrogenic reduction in ionized calcium.[55] Finally, in a previous study, sheep showed a significant decrease in ruminal pH during a 9-h anesthetic procedure.[21] Sheep also showed a marked increase in ruminal calcium concentration,[21] raising the possibility that the hypocalcemia seen in our study was due to movement of calcium from the circulation to the rumen.

All sheep in our study had low hematocrit and hemoglobin values at the start of anesthesia. This could be due to hemodilution caused by excessive water intake (possibly during the fasting period) and by liberal fluid management during anesthesia. The significant increase in central venous pressure over the course of 30 h would further support this theory. However, due to its relatively large volume, the rumen can act as a water reservoir during prolonged dehydration and may also temporarily sequester of large volumes of water for rehydration.[29] Therefore, even large amounts of water added to the rumen would not be expected to affect the baseline hematocrit.[15,37] Nonetheless, the hematocrit may remain low due to our fluid management strategy. True anemia in nonspecific pathogen free (non-SPF) sheep is often a clinical symptom of endo- or ectoparasite infestations;[4,9] these have become increasingly difficult to treat in small ruminants due to their high anthelmintic resistance.[44] We did not examine parasitic infestation in our sheep. Anesthesia may also induce splenic relaxation, which results in an apparent anemia due to sequestration of up to 30% circulating red blood cells in the spleen.[14,38,59] The 2 anesthetic agents used throughout our study, midazolam and ketamine, cause a significant drop in hemoglobin, packed cell volume and red blood cell count in a variety of species.[1,26,34] A similar drop in these analytes was also seen in dogs anesthetized with propofol.[11] In cats, ketamine-midazolam administration also leads to a marked drop in white blood cells,[26] which was also found in the current study. The blood volume of sheep is estimated to be around 63 mL/kg bodyweight.[19] Approximately 4.5% of the total blood volume was collected from each sheep during the 30-h procedure, markedly below the largest allowable collection volume of 10%, after which alteration in cardiac output and hemodynamics are expected.[7] Frequent blood sampling throughout the 30-h course of anesthesia should not have further decreased hematocrit and hemoglobin concentrations in these sheep, although sampling frequency and volume should always be considered. Free hemoglobin was not detected in the sheep in our study (data not shown). Increased free hemoglobin would indicate hemolysis.

Aspiration of saliva and ruminal fluid can be prevented in unfasted sheep by intubation with a cuffed endotracheal tube. However, this practice has been associated with bacterial colonization of the lung and impairment of tracheal mucus clearance due to the breach of the anatomic barrier of the larynx and possibly injury to the tracheal mucosa with the cuff of the endotracheal tube.[40] Prolonged mechanical ventilation is also often associated with alveolar atelectasis, pulmonary perfusion heterogeneity and gravitational inhomogeneity, consequently causing pulmonary shunting and impaired oxygenation.[23-25,56] Sheep in our study were ventilated with an FiO_2 of 0.6 throughout the course of anesthesia. Nonetheless, pO_2 fell significantly over the 30-h anesthesia, suggesting that oxygenation could be inadequate if anesthesia persisted for an even longer period. All sheep showed significantly higher peak ventilation pressure and lower pulmonary compliance with a slight ventilation-perfusion mismatch (discrepancy between pCO_2 and $etCO_2$ > 0.7 kPa) throughout the course of anesthesia. The observed increase in airway resistance and impaired oxygenation is most likely an accompanying effect of atelectasis in the lower lung lobes due to right lateral recumbence and increased mucus accumulation in the lower respiratory tract and the endotracheal tube. In human patients, hypocalcemia is also associated with bronchospasm, which may further impair ventilation and oxygenation.[49] We observed a persistent significant decrease in pCO_2 and $etCO_2$, despite adapting the ventilation volume; this outcome was initially thought to be associated with the changes in ventilation efficacy. However, when considering these data along with the other parameters we measured, another possibility is that prolonged infusion of balanced fluids, such as Ringers Lactate, caused a drop in the physiologic strong ion difference (SID) based on the Stewart Acid-Base Approach[53] Thus, metabolic acidosis due to infusion-induced hyperchloremia[35] and the concurrent intracellular displacement of bicarbonate occurs. Because the bicarbonate/carbon dioxide (HCO_3/CO_2) equilibrium system is the major pH buffer system in the body, CO_2 in these sheep is continuously converted to HCO_3 to counteract the metabolic acidosis. The continuous decrease in minute ventilation volume and the decrease in oxygenation indicate that this equilibrium is unstable. Balanced fluids with a higher in-vivo SID, such as Plasmalyte, may be more suitable during long-term anesthesia in sheep. In New Zealand White rabbits, blood sodium and chloride increased after sedation with ketamine-diazepam[18] This effect was not confirmed in a study of cats sedated with the same anesthetic mixture.[14] In buffalo calves, blood chloride increased after administration of ketamine-diazepam, with no increase of blood sodium.[36] These mechanism by which these 2 agents affect blood sodium and chloride in sheep remains unclear.

Although serum myoglobin was not increased in any of the sheep, increased serum levels of CK and LDH are common indicators of damage to muscle membrane and other tissue structures.[5] However, important electrolyte abnormalities associated with rhabdomyolysis, such as hyperkalemia, hypocalcemia, hyperphosphatemia, and hyperuricemia, were only partially present or not assessed in this study. Myoglobinuria was not seen in any of the sheep. An increase of creatinine and urea was found in calves after detomidine-midazolam-ketamine anesthesia, with values returning to baseline 24 h after drug administration.[34] A significant increase of LDH was also found in New Zealand White rabbits undergoing ketamine-diazepam anesthesia.[18] The effects of the anesthetic combination used in our sheep remain unclear. However, prolonged lack of movement during anesthesia-induced recumbence is a major contributor to muscle ischemia due to compression of blood vessels.[33]

In conclusion, the body's ability to maintain homeostasis over a prolonged period of anesthesia is remarkable. To improve data quality in accordance with the 3R principles established worldwide as the ethical approach to the use of animals in research requires, conducting prolonged terminal studies in sheep requires an in-depth understanding of all metabolic compensatory mechanisms and their underlying cause, with special considerations of ruminant-specific physiology. Failing to do so can markedly alter and confound the results of the study. The need for preoperative fasting in sheep should be reconsidered as it may pose a risk for early homeostatic imbalances, particularly in sheep predominantly fed a pelleted diet.[31] Anesthetic agents can cause clear alterations in hematologic and biochemical parameters that should be considered when choosing agents. The concept of combining different anesthetic drugs is often referred to as balanced anesthesia, which refers to the simultaneous administration of various anesthetic agents to lower the risk of overdose of one single agent.[42] Synergistic and additive effects allow the use of lower doses of each individual agent, contributing to fewer undesirable effects of a higher doses of a single agent on blood homeostasis. Throughout anesthesia, infusion fluids with a higher in-vivo SID may attenuate metabolic acidosis and the consequent depletion of CO_2, which in return may cause inadequate ventilation and thus oxygenation. Although the consensus in the field of veterinary anesthesia is that fluid substitution should be performed at an infusion rate of 5 to 10 mL/kg/h during prolonged anesthesia,[17] evidence in humans supports the benefits of a goal-directed therapy over the liberal or restrictive intraoperative fluid therapy approach.[2] Examining urine concentration and specific gravity during prolonged anesthesia in sheep would allow better management of fluid requirements. The rumen's ability to act as a water reservoir should be considered by regular assessment of ruminal water and electrolytes.

Based on the hemodynamic, hematologic, and biochemical parameters, one may be tempted to assume that these sheep, if necessary for the experiment conducted, could regain consciousness after this prolonged duration of anesthesia, as mechanical ventilation over days is not uncommon in humans. However, prolonged mechanical ventilation in patients is often associated with tracheal injuries, infection, cardiovascular failure, and lung injury, and also appears to cause a rapid onset of diaphragmatic atrophy in humans and animals,[45,46] leading to difficulty in weaning from the respirator. To avoid unnecessary suffering and to follow the principle of experimental refinement, actions that may lead to insufficient respiration and oxygenation after surgery should first be excluded, if sheep are intended to regain consciousness. Muscle and tissue damage remains a significant problem in sheep kept in prolonged recumbence, particularly if the animal cannot be repositioned during the procedure. Nonetheless, prolonged terminal studies under general anesthesia are feasible in sheep if they are termed optimal for studying the scientific question asked.

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Competing Interests

The author(s) declare no competing interests.

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