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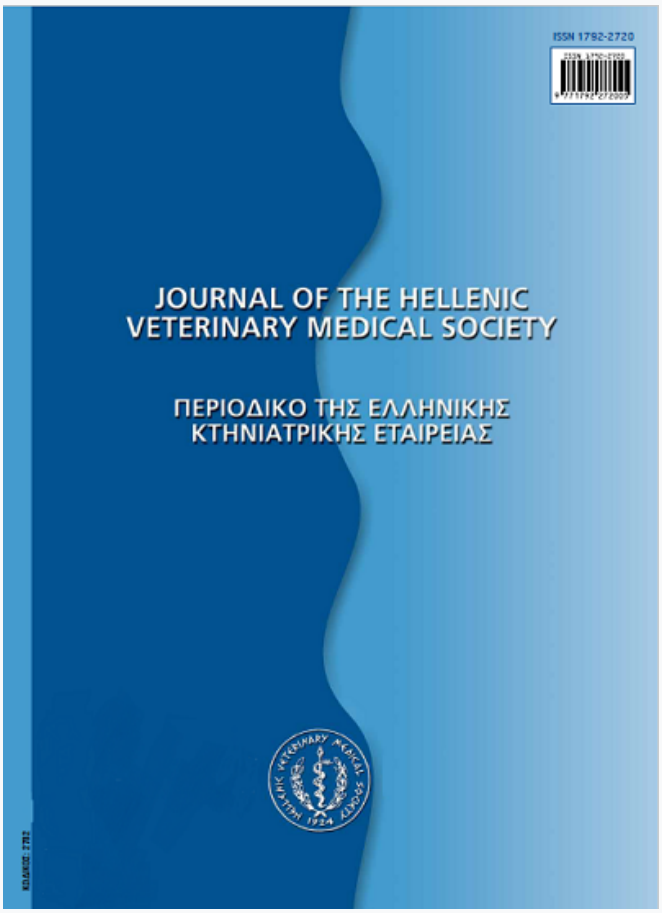

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Research article
Ερευνητικό άρθρο

Valuation of three different anaesthetic protocols on complete blood count and biochemical parameters on Wistar rats

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Αξιολόγηση της επίδρασης διαφορετικών αναισθησιολογικών πρωτοκόλλων στη γενική αίματος και στις βιοχημικές παραμέτρους σε αρουραίους Wistar

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ABSTRACT. The objective of the current study was to determine the impact of three different anaesthetic protocols on blood and biochemical parameters. Eighteen female Wistar rats (HsdOla:WI) 3 months old, weighting 197.09 ± 7.39 g were used. Baseline blood collection was performed in all animals from the lateral coccygeal vein for evaluation of glucose. The animals were then randomly allocated to receive one of three different anaesthetic protocols: dexmedetomidine/ketamine intramuscularly (0.25 mg/kg and 50 mg/kg respectively), or isoflurane 0.2 ml on cotton inside a syringe case, or isoflurane administered by vaporiser (5% induction and 2-3% maintenance of anaesthesia, delivered in oxygen flow 1 L/min). Blood samples were collected from caudal vena cava for complete blood count and biochemical analysis, while the lung and the liver were harvested for histological evaluation. Comparison between groups, as far as complete blood count parameters is concerned, revealed statistical significant differences in red blood cells, haematocrit and haemoglobin values with lower values being observed in vaporiser anaesthesia group. Furthermore, within group comparison revealed statistical significant differences for glucose in all three groups. No evidence of inflammatory, degenerative neoplastic or toxicity related lesions were observed during histological evaluation of the lung and liver.

Keywords: Anaesthesia; Rats; Blood parameters

ΠΕΡΙΛΗΨΗ. Ο στόχος της παρούσας μελέτης ήταν να προσδιοριστεί η επίδραση τριών διαφορετικών πρωτοκόλλων αναισθησίας στις αιματολογικές και βιοχημικές παραμέτρους. Για το σκοπό αυτό χρησιμοποιήθηκαν δεκαοχτώ θηλυκοί επίμυες Wistar (WI HsdOla) ηλικίας 3 μηνών, που ζύγιζαν 197,09 ± 7,39 g. Η αρχική αιμοληψία πραγματοποιήθηκε σε όλα τα ζώα από την πλάγια κοκκυγική φλέβα για την αξιολόγηση των επιπέδων γλυκόζης. Τα ζώα στη συνέχεια χωρίστηκαν τυχαία σε 3 ομάδες ώστε να λάβουν ένα από τα τρία διαφορετικά αναισθητικά πρωτόκολλα: δεξμεδετομιδίνη/κεταμίνη ενδομυϊκά (0,25 mg/kg και 50 mg/kg αντίστοιχα), βαμβάκι εμποτισμένο με 0,2 ml ισοφλουρανίου τοποθετημένο μέσα σε σύριγγα, ή ισοφλουράνιο χορηγούμενο με αναισθητική μηχανή (5% για εισαγωγή στην αναισθησία και 2-3% για συντήρηση , ροή οξυγόνου 1 L/min). Συλλέχθηκαν δείγματα αίματος από την οπίσθια κοίλη φλέβα για τη γενική αίματος και τις βιοχημικές αναλύσεις, ενώ συλλέχθηκαν ο πνεύμονας και το ήπαρ για ιστολογική αξιολόγηση. Η σύγκριση μεταξύ των ομάδων, όσον αφορά τις αιματολογικές παραμέτρους κατέδειξε στατιστικά σημαντικές διαφορές στις τιμές των ερυθρών αιμοσφαιρίων, του αιματοκρίτη και της αιμοσφαιρίνης με τις χαμηλότερες τιμές να παρατηρούνται στην ομάδα με τη χρήση αναισθητικής μηχανής. Η σύγκριση εντός της ομάδας κατέδειξε στατιστικά σημαντικές διαφορές στα επίπεδα γλυκόζης και για τις τρεις ομάδες. Δεν παρατηρήθηκαν ενδείξεις αλλοιώσεων σχετικών με φλεγμονή, εκφυλιστική νεοπλασία ή τοξικότητα κατά την ιστολογική αξιολόγηση του πνεύμονα και του ήπατος.

Λέξεις-κλειδιά: Αναισθησία, Επίμυες, Αιματολογικές παράμετροι

INTRODUCTION

Rat is one of the most commonly used animal models in biomedical research and often blood collection is a prerequisite procedure in the majority of experimental protocols. In many cases these procedures are conducted under anaesthesia. Criteria of choice are the ethical use of animals, safety for the animals and personnel, cost and equipment available in the laboratory setting as well as the impact of these compounds or techniques on blood parameters. The impact of one intramuscularly and two inhalant anaesthesia protocols were investigated. Intramuscular administration of dexmedetomidine and ketamine delivers adequate depth of anaesthesia while its cost is moderate (Wixson and Smiller, 1997). The administration of isoflurane through a precision vaporiser is safe for the animals and personnel, fast and its metabolic effect on the liver

and kidneys is minimal (Brunson, 1997). On the other hand, the acquisition cost of the necessary equipment is high and not available in all laboratory facilities, while the cost of the compound is moderate. When methoxyflurane was withdrawn for safety reasons, researchers were interested in finding a method which would be easier to apply and less time consuming than the well documented methods of anaesthesia (intramuscular or isoflurane though vaporiser). There are studies suggesting the use of isoflurane diluted in solvent or undiluted in an open-drop or nose-cone technique in small laboratory animals. In the present study a modification of the open-drop technique was investigated. Advantages of the procedure are its ease of applicability, low cost and time effectiveness. In an analogous setting the higher maximum volatile concentration was double the concentration needed for the induction of anaesthesia (Risling et al., 2012). Consequently, concerns arose about its possible irritant, adverse effects or interference with the scientific outcomes. The primary objective of the present study was to determine and compare the impact of those anaesthetic procedures on blood parameters in end stage evaluation.

MATERIALS & METHODS

Animals and housing

The study was performed in the Centre for Experimental Surgery of the Biomedical Research Foundation of the Academy of Athens. The experimental protocol was approved by the General Directorate of Veterinary Services, according to Greek legislation (Presidential Decree 160/1991, in compliance with the European Economic Community Directive 609/1986, and Law 2015/1992, in conformance to the European Convention for the protection of vertebrate animals used for experimental or other scientific purposes, 123/1986). Eighteen intact female Wistar rats (HsdOla:WI) 3 months old were obtained from the colony of the Biomedical Research Foundation of the Academy of Athens. In order to ameliorate the management of the population of our facility colony and to comply with National Institute of Health's suggestion that female animals need to be included in higher numbers into research, (Clayton 2014) female rats were selected. The animals were housed in pairs under positive pressure in polysulfone type III individual ventilated cages (Sealsafe, Tecniplast, Buguggiate, Italy). All cages were kept in the same room with HEPA filtered air supply (15 air changes per hour), at a room temperature of 24 ± 2 ^{o}C, relative humidity 55 ± 10 %, 12: 12-h light/dark cycle (07:00/19:00), and a positive air pressure of 0.6 Pa within the room. All animals in the facility are screened regularly according to the Federation of European Laboratory Animal Science Associations' recommendations and were free from the respective pathogens. All Wistar rats had ad libitum access to water and food. The pellets contained 18.5 % protein, 5.5 % fat, 4.5 % fiber, 6 % ash (irradiated vacuum packed, 2918, Harlan, Italy). The bedding in each cage comprised of approximately 350 g of corncob bedding (Rehofix MK 2000, J. Rettenmaier & So, Rosenberg, Germany) and cages were cleaned once a week.

Protocol design

After baseline body weight measurement, rats were randomly assigned into three groups: intramuscular anaesthesia (IA) (n = 6, mean body weight = 196.30 ± 6.78 g), nose cone anaesthesia (NCA) (n = 6, mean body weight = 198.03 ± 9.59 g) and inhalation vaporiser anaesthesia (VA) (n = 6 mean body weight = 196.93 ± 5.85 g). Blood collection for baseline glucose evaluation was conducted using a restrainer (MLA5022 Rodent Restrainer). Local anaesthetic cream (EMLA 5%, AstraZeneca, UK-Sweden) was applied on the rats' tail half an hour prior to the puncture. The lateral coccygeal vein was punctured with a needle (23G) and a drop of blood was immediately used for glucose measurement. Fifteen days after the first blood collection the animals were weighted (216.92 ± 9.52 g for IA, 217.88 ± 13.57 g for NCA and 223.83 ± 6.94 g for VA group) and anaesthetised using either dexmedetomidine (Dexdomitor, Orion Pharma, Finland) / ketamine (Imalgene, Merial, France) intramuscularly (0.25 mg/kg and 50 mg/kg respectively) or isoflurane 0.2 ml (IsoFlo®, Abbott, USA) on cotton inside a syringe (the total volume of the syringe was 65 ml while the cotton occupied 10 ml of the volume) or

isoflurane administered through a veterinary vaporiser (VME Small Animal Anaesthesia Machine, MDS Matrix, Orchard Park, NY, USA). For intramuscular anaesthesia each of the compounds was administered separately in the left and right anterior thigh in order to respect the maximum volume permitted per site (Bihun and Bauck, 2004).

As far as NCA is concerned, according to Risling *et al* (2012) in a similar setting (volume of chamber and agent, as well as ambient temperature), the volatile concentration of isoflurane (evaluated with the use of a precision gas analyser) represented a curve with its peak between 2 and 4 min after the application of the anaesthetic. Furthermore, the authors documented a gradual decrease, resulting to a concentration of 2-4% after 10 minutes (Taylor and Mook, 2009; Risling et al., 2012). According to our data, induction of anaesthesia in the NCA group was achieved during the time period of isoflurane's higher concentration, while the whole procedure was completed within 10 minutes. For each animal the cotton was replaced and the isoflurane renewed, while the distance between the cotton and the animals' nose (approximately 7 cm) was the same. All procedures were carried out in a hood. For VA, induction was performed with 5 %, while maintenance was achieved by 2-3 % isoflurane delivered in oxygen flow 1 L/min, gas delivery and removal was provided by a modification of the open circuit which was attached to a nose mask (Balafas et al., 2011). Isoflurane was stored at 4 ^{o}C while the ambient temperature was 24 ± 2 ^{o}C. An observer was recording the following parameters: time needed for the induction of anaesthesia, animals' resistance during administration (mild, moderate, and severe) and vocalisation, as well as any side effects. Additionally, the person restraining the animal recorded its resistance. During the performance of the anaesthetic protocols the observer and the person restraining the animals was not possible to be blinded of the procedure carried out. Time was defined as the period starting from the contact of either the needle (IA group) or the nose mask (VA and NCA groups) until an adequate depth of anaesthesia was achieved. Appropriate depth was defined as loss of corneal, palpebral and pedal withdraw reflexes. Animals were positioned in dorsal recumbency, the hair of the abdomen was clipped and a ventral midline incision was made in order to gain access to the caudal vena cava. Euthanasia was performed by exsanguination. Blood was collected and separated; 1 ml in a tube containing K3EDTA and 1 ml in an eppendorf tube.

In order to avoid discrepancies of glucose evaluation both samples (baseline and endpoint) were collected from the lateral coccygeal and glucose was immediately evaluated (ElementTM Infopia USA), while plasma was obtained after centrifugation at 3000 rpm for 15 min and stored in deep freezer (-78 ^{o}C). Plasma was inspected and there was no evidence of lipaemia or haemolysis. These observations were verified by the biochemical analyser. All interventions were carried out at the same procedure room between 11.00 and 13.00 hour, in order to avoid alterations due to circadian rhythm (Gärtner et al., 1980). At the end of the experiment the lung and liver were harvested. A complete blood count was performed (ADVIA 120 Hematology System, Siemens, Germany), while the biochemical parameters evaluated were creatinine (Cr), aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (ALKP), total proteins (TP), urea (Ur), albumin (Alb), glucose (Gluc), triglycerides (Tg), cholesterol (Chol) and gamma-glutamyl transpeptidase (γGT) (ADVIA 1200 Chemistry System, Siemens, Germany).

Tissue preparation

Tissues were fixated in phosphate buffered formalin 10% solution for 24 hours and were embedded in paraffin. A 3 μm sectioning was performed (Leica RM2255 - Fully Automated Rotary Microtome, Leica Biosystems GmbH Wetzlar, Germany) and the slides were stained with Hematoxylin and Eosin.

Statistical analysis

The first step in the statistical analysis was to evaluate the normal distribution of data (Kolmogorov-Smirnov test and P-P plots). In case of normal distribution, comparisons of the absolute values of variables between the three groups were performed using the one-way analysis of variance model. Pairwise multiple comparisons were performed using the Bonferroni test. In non-normal distribution com-

parisons were performed using the Kruskal-Wallis test. For glucose values which were estimated over two time-points, pairwise comparisons within group were performed using paired t-test. Between groups pairwise data analysis was performed using independent samples t-test. In the statistical evaluation $p < 0.05$ was defined as significant. All analyses were performed using the SPSS version 20.00 (SPSS Inc., Chicago, IL). All data are presented as mean or percentage ± standard deviation (SD).

RESULTS

General observation

Mean body weight of animals did not differ between groups ($p>0.05$) in both baseline and endpoint measurements. IA group needed a significantly longer period of time for the induction of anaesthesia compared to VA and NCA groups (543.67 ± 70.35 sec vs 89.33 ± 21.01 sec and 48.83 ± 15.64 sec respectively).

VA and IA administration were safe for the personnel while the NCA entailed the risk of inhaling anaesthetic's dispersed fumes. Restraining and administration of anaesthesia was always performed by the same person. The observer and the person restraining the animals recorded (independently) and agreed that a mild resistance was noted in all animals in VA group, while a small percentage (16.67 %) vocalised. All animals in NCA group resisted intensely at the beginning of the process but no vocalisation was observed. In IA group all animals vocalised, while 33.33 % exhibited mild, 16.67 % moderate and 50 % intense resistance. Adverse effects were observed in NCA group, where 50 % of the animals presented apnea, and 16.67 % gasping during the procedure. Depth of anesthesia was adequate in all groups

Complete blood count

The different anaesthetic protocols had no statistically significant effect on WBC, neutrophils, mononuclear, eosinophils, basophils and platelets, while their impact on RBC, haematocrit and Hb values was statistically significant ($p<0.05$).

Comparison between groups showed a statistically significant difference in RBC values between VA and IA groups as well as between NCA and IA groups. In both comparisons RBC values were increased in IA ($p<0.05$). Moreover, mean values of haematocrit in rats exposed to VA was significantly lower ($p<0.05$) compared to those exposed to NCA and IA. Haematocrit and haemoglobin were similar for NCA and IA however, in VA significantly lower haemoglobin values were observed when compared to NCA group ($p<0.05$). Values of all haematology parameters are displayed in **Table 1**.

Biochemical analysis

For Gluc comparison of mean percentage changes from baseline was performed. Gluc levels in rats anaesthetised using the vaporiser or the nose cone, exhibited a comparable percentage reduction of 15.18 ± 6.79 % and 15.62 ± 5.91 %. A statistically significant difference ($p<0.05$) was noted when compared to changes observed in IA group where glucose levels presented an increase (23.95 ± 8.83 %).

Pairwise comparison between groups was performed for biochemical parameters. No differences were observed for AST, ALT, ALKP, TP, Alb, Tg and Chol for the three groups ($p>0.05$). For endpoint measurements statistical significant differences were observed for Ur between VA and IA ($p<0.000$) as well as IA and NCA ($p<0.000$) groups, for Cr between IA and NCA ($p<0.045$), for γGT between VA and IA ($p<0.047$), and for Gluc between VA and IA ($p<0.002$) as well as IA and NCA ($p<0.001$) (**Fig. 2**). All endpoint biochemical parameters evaluated are presented in **Table 2**.

Histological evaluation

Liver histological appearance did not present any significant alterations between all three groups. Five out of six (5/6) animals of IA, 2/6 animals from NCA and 4/6 from VA group presented small foci of cytoplasmic vacuolation. In more detail, hepatocytes presented irregular and poorly defined clear spaces within their cytoplasm, with centrally located nuclei. This finding is consistent with glycogen accumulation, while its presence correlates with lack of fastening prior to euthanasia. Further degenerative, inflammatory or neoplastic lesions were not observed.

Table 1. Total blood count parameters measured at the endpoint of the protocol for all three anaesthetic regimens. (baseline values from: Baseline Hematology and Clinical Chemistry Values for Charles River Wistar Rats (1998))

	Treatment group			
	VA group	**NCA group**	**IA group**	**Reference range**
	(Mean ± SD)			
RBC (cells/μl)	7.97x10^6±0.15 ***	8.20x10^6±0.18	8.72x10^6±0.25 **	6.86-8.75x10^6
WBC (cells/μl)	5.27x10^3±0.51	5.27x10^3±1.02	5.30x10^3±1.79	4.77-12.08x10^3
Hb (g/dl)	13.58±0.18 *	14.48±0.26	14.40±0.89	14.1-17.1
Hct (%)	47.82±1.07	51.57±1.03 *	50.85±2.63 ***	35-44
Neu (cells/μl)	0.79x10^3±0.21	0.64x10^3±0.39	0.67x10^3±0.33	0.68-3.87x10^3
Lymph (cells/μl)	4.28x10^3±0.47	4.40x10^3±0.96	3.95x10^3±0.54	3.20-11.84.x10^3
Mono (cells/μl)	0.07x10^3±0.03	0.08x10^3±0.03	0.06x10^3±0,04	0-0.72x10^3
Eos (cells/μl)	0.08x10^3±0.03	0.09x10^3±0.03	0.06x10^3±0.03	0-0.36x10^3
Baso (cells/μl)	0.03x10^3±0.03	0.02x10^3±0.01	0.02x10^3±0.008	0-0.24x10^3
Plt (cells/μl)	884.00x10^3±128.36	898.94x10^3±94.87	950.33x10^3±151.35	500-1300x10^3

** Statistically significant difference (p<0.05) vaporiser vs nose cone anaesthesia.*
*** Statistically significant difference (p<0.05) intramuscular vs nose cone anaesthesia.*
**** Statistically significant difference (p<0.05) vaporiser vs intramuscular anaesthesia.*
All values are expressed as mean ± SD.

Lung histology also did not reveal any toxicity-mediated alterations. Two out of six (2/6) animals of VA and 1/6 of the NCA and IA group presented blood residing inside the tracheal lumen. This finding was considered to correlate with post mortem specimen handling, rather than representing haemorrhage. Finally, further degenerative, inflammatory or neoplastic lesions were absent, while Bronchus Associated Lymphoid Tissue (**BALT, Fig. 1/E**) appeared to be normal.

DISCUSSION

At the end of a protocol, euthanasia is commonly performed by exsanguination after anaesthesia administration. Every procedure in biomedical research must conform to two fundamental principles: compliance to the humane use of animals and at the same time avoidance of interfering or altering scientific data. Consequently, anaesthesia must be carefully chosen in order to fulfill these requirements. Secondary criteria that should be taken into account are easiness of administration, time needed and cost.

In humans undergoing sevoflurane or isoflurane anaesthesia abnormal AST and ALT values have been reported (Kharasch et al., 1997; Nishiyama et al., 1998). Similar results were found when patients were exposed twice to general anaesthesia. In a study

Table 2. Biochemical parameters' endpoint measurements for all three anaesthetic regimens. (baseline values from the Laboratory performed the analyses, Glucose baseline values from: Baseline Hematology and Clinical Chemistry Values for Charles River Wistar Rats (1998))

	Treatment group			
	VA group	**NCA group**	**IA group**	**Reference range**
	(Mean ± SD)	(Mean ± SD)	(Mean ± SD)	
Cr (mg/dl)	0.53±0.03	0.54±0.02 **	0.51±0.03	0.5-1
Urea (mg/dl)	36.83±3.38 ***	33.90±1.31 **	26.49±2.42	21.43-45
AST (IU/L)	142.77±35.47	106.74±18.87	113.14±55.57	39-84
ALT (IU/L)	57.74±15.61	46.23±4.34	43.59±5.36	35-80
ALKP (IU/L)	191.67±19.35	170.17±26.99	184.33±33.88	16-50
Gluc (mg/dl)	114.50±15.63 ***	110.00±10.56 **	157.83±20.88	85-132
TP (g/dl)	5.15±0.30	5.14±0.34	5.13±0.38	5.6-7.6
Alb (g/dl)	3.18±0.21	3.39±0.21	3.09±0.24	3.8-4.8
Tg (mg/dl)	161.00±27.46	211.06±57.98	158.46±21.27	16-175
Chol (mg/dl)	53.17±6.74	59.67±7.34	51.33±9.49	40-130
γGT (IU/L)	0.25±0.49 ***	0.42±0.23	0.68±0.41	0.5-1.5

** Statistically significant difference (p<0.05) vaporiser vs nose cone anaesthesia.*
*** Statistically significant difference (p<0.05) intramuscular vs nose cone anaesthesia.*
**** Statistically significant difference (p<0.05) vaporiser vs intramuscular anaesthesia.*
All values are expressed as mean ± SD.

Table 3. Glucose baseline and endpoint values for all three anaesthetic regimens. (Glucose baseline values from: Baseline Hematology and Clinical Chemistry Values for Charles River Wistar Rats (1998))

	Group			
	VA group	**NCA group**	**IA group**	**Reference range**
	(Mean ± SD)	(Mean ± SD)	(Mean ± SD)	
Baseline Gluc (mg/dl)	135±13.11	132.5±19.55	128.67±14,30	85-132
Endpoint Gluc (mg/dl)	114.50±15.63 ***	110.00±10.56 **	157.83±20.88	

*** Statistically significant difference (p<0.05) intramuscular vs nose cone anaesthesia.*
**** Statistically significant difference (p<0.05) vaporiser vs intramuscular anaesthesia.*
All values are expressed as mean ± SD.

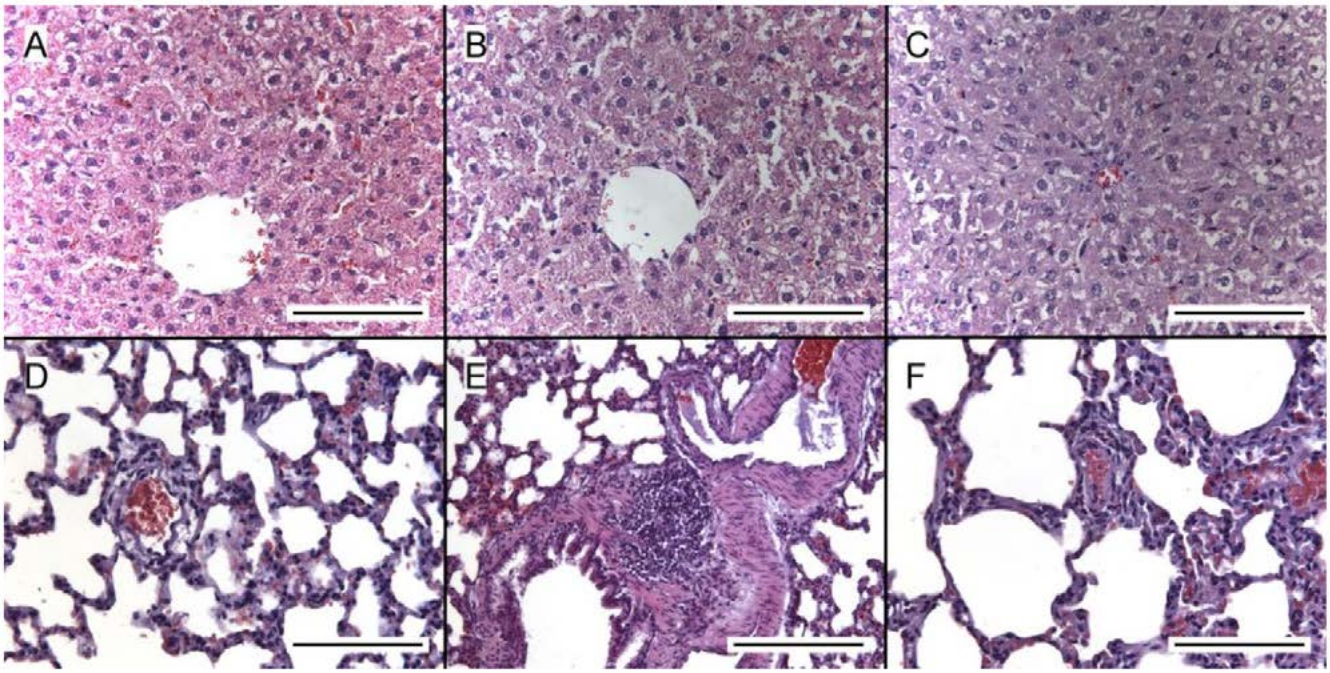

Figure 1. Liver (upper row; figures 1/A, 1/B and 1/C) and lung (lower row; figures 1/D, 1/E and 1/F) histology specimens. Specimens 1/A and 1/D belong to the inhalation vaporiser group; specimens 1/B and 1/E belong to the nose cone group, while figures 1/C and 1/F depict specimens from the intramuscular group. Note cytoplasmic vacuolation of the hepatocytes near the centrilobular (figs. 1/A and 1/B) or periportal (fig. 1/C) areas. Scale bars represent 0.1 mm in figures 1/A, 1/B, 1/C, 1/D and 1/F and 0.2 mm in figure 1/E.

conducted on rats, Deckardt *et al* (2007) reported that ALT was not altered under isoflurane anaesthesia. In the present investigation AST and ALKP values where higher when compared to reference values, while ALT values were between normal range. For VA and NCA groups this changes may be attributed to direct insult of the volatile agent on the liver, while for the IA group a contributing factor (as far as the AST values are concerned) can be attributed due to muscle irritation, necrosis, and fibrosis as a result of the intramuscular administration (Lugo-Roman LA et al., 2009). As far as comparison between groups is concerned there was no statistically significant changes in AST, ALT, and ALKP for any of the anaesthetic agents under study, while statistically significant difference was observed for γGT between the VA and IA groups, while this increase was not higher than reference values. (Levine BS, 1995)

In humans undergoing inhalation anaesthesia it has been observed that creatinine was within normal range, while BUN was affected in a small number of subjects with no clinical importance (Nishiyama, 2013) which is in agreement with our results. Male and female rats exposed to isoflurane anaesthesia through vaporiser presented decreased creatinine levels (Deckardt et al., 2007). In the same study by Decartd and colleagues (2007) urea levels were not altered which is in agreement with our findings as far as NCA and VA groups are concerned. The lower values of urea and creatinine observed in IA compared to NCA and VA groups may be attributed to an increase in the urinary output (Gonullu et al., 2014). The latter could be the result of polyuria and hyperglycaemia caused by dexmedetomidine/ketamine combination (Wixson and Smiller, 1997).

Increased glucose values can be the result of stress inflicted by painful or stressful stimuli, surgical procedures or even handling and restraining of the animals. Anaesthesia increases stress signals through catecholamines and cortisol excretion or may influence glucose homeostasis via insulin

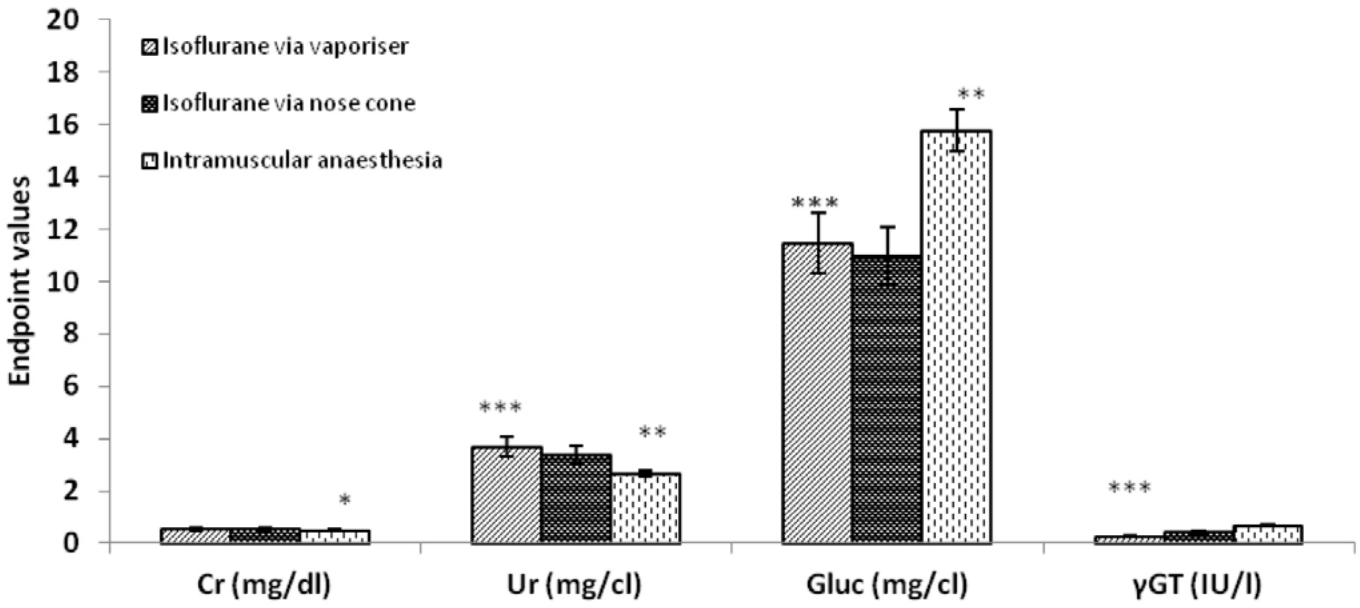

Figure 2. Pairwise comparison of endpoint values of biochemical parameters presenting statistical significant differences (Values of glucose and urea are expressed as mg/cl for better presentation). * Statistically significant difference ($p<0.05$) vaporiser vs nose cone anaesthesia; ** Statistically significant difference ($p<0.05$) intramuscular vs nose cone anaesthesia; *** Statisticlly significant difference ($p<0.05$) vaporiser vs intramuscular anaesthesia.

release. (Tanaka et al., 2005; Zuurbier et al., 2008). Studies on mice and rats have confirmed that a decrease in body temperature, as a result of anaesthesia, may further increase glucose levels (Tabata et al., 1998). It has been reported that hyperglycaemia, after the administration of isoflurane, is the result not only of increased production of endogenous glucose (Zuurbier et al., 2008) but also of decreased glucose clearance.(Tanaka et al., 2005) Isoflurane can affect the activation of the pancreatic KATP channels (Zuurbier et al., 2008; Tanaka et al.,2009). Intramuscular anaesthesia in rodents is usually conducted as combination of ketamine with other agents, usually an α2-adrenergic receptor agonist, such as xylazine or dexmedetomidine. It has been reported that ketamine combined with medetomidine reduces hexokinase activity binding on the mitochondria (Zuurbier et al., 2008) while α2-agonism is well known to exert decreased insulin release and consequent hyperglycaemia (Fish, 1997). Brown *et al.* (2005) reported that the use of different injectable anaesthetics significantly increased blood glucose levels in C57/Bl6J mice, while the combination of ketamine/xylazine was found to cause greater increase of glucose levels.

Baseline values of glucose did not differ significantly between groups (**Table 3)**. Interestingly, in our study, endpoint values in VA and NCA group were significantly lower compared to baseline. Although it is well documented that inhalant anaesthesia raises glucose levels, in the present study handling and restraining may have evoked significant dysphoria and stress to the animals which was reflected on glucose values. Our results are in disagreement with a study conducted by Tabata *et al.* (1998) which reports that in rats, handling had no effect on glucose levels. This discrepancy may be the result of differences between studies such as time, and method for blood collection (venesection vs vein catheterisation). In another study, Gärtner *et al.* (1980) reported that time of handling and restraining of the animals is a significant parameter interfering with plasma

profile, particularly as far as endocrine values are concerned. As it was expected, intramuscular anaesthesia revealed significantly higher endpoint values of glucose compared to baseline.

In the present investigation none of the interventions altered the haematolocial parameters out of the range of reference values. However, it has been reported that injectable and volatile anaesthesia can alter haematocrit and haemoglobin values due to changes evoked on plasma volume and/or on circulating red cells (Wilson et al., 2004; Gothelf et al., 2009). RBCs are sequestered in the spleen and under circumstances of stress they may be released back into circulation (Gothelf et al., 2009). In everyday veterinary practice, when blood is collected (without anaesthesia) from otherwise healthy dogs and cats, higher haematocrit values are attributed to fear or excitement and a consequent splenic contraction (Bush, 1991). Among volatile anaesthetics, halothane has been found to induce a significant reduction of haematocrit and plasma protein and increase in plasma volume in dogs and monkeys (Steffey et al., 1976). The same effect was observed when isoflurane anaesthesia was used in ferrets (Marini et al., 1994). This is in agreement with our observations where relative lower haematocrit values of the VA group were observed to IA groups. According to Risling *et al.* (2012), the maximum concentration of isoflurane, administered on gauze within a chamber, was 8.38 ± 1.60 %. Consequently, in NCA group the higher relative value of haematocrit, when compared to VA and IA groups, may be attributed to either stress or overdose. It could be assumed that stress was the major contributing factor and this hypothesis is further supported by the observers' remarks. As far as the IA group is concerned ketamine exerts a spasmolytic effect on spleen lowering Hct in cats (Bush, 1991), while dexmedetomidine exerts an opposite action (α2 adrenergic agonism causes vasoconstriction and hypertension in dogs) (Wilson et al., 2004). Additionally, their combination may affect haematocrit through polyuria and possible changes exerted on plasma volume (Wixson and Smiller, 1997). In the present study, we hypothesised that dysphoria observed in the NCA and IA groups may have been the main contributing factor for Hct's relative higher values.

Although there is evidence that haematological and biochemical parameters may be influenced by the phase of the oestrous cycle in many female mammals, this has been attributed to high circulating levels of progesterone. Typically, this occurs in those species with long dioestrous (such as dogs) and has been found to influence eosinophil counts, cholesterol, erythrocytic and AST values (Walter et al., 2013). Contrary, rats have a very short dioestrous period and there is evidence suggesting that female rodents display no more variability than males (Md Said and Abiola, 2014; Prendergast et al., 2014).

Although anaesthetics used in this study are metabolised by the liver, no related lesions were found on histologic evaluation. For the VA and IA groups this was expected, while concerns existed for the NCA group, where isoflurane concentration exceeded the recommended dosage. The absence of evidence of liver toxicity in the NCA group in our study is in agreement with documentation about isoflurane (Anonymous.2012), that hepatotoxicity is not being induced even in anaesthetic protocols, where its concentration is not strictly regulated (as in NCA group). Another concern was a possible irritant effect of the higher isoflurane concentration in the respiratory tract. According to lung histology there was no association with respiratory lesions in the NCA group.

CONCLUSIONS

All methods used had an impact on some of the parameters evaluated, even when no pharmaceutical compound was used. Restraining and handling elicited changes which altered glucose values through either endocrine, neurosympathetic or immunological responses (Gärtner et al., 1980). All anaesthetic protocols had a mild impact on some of the values recorded. Furthermore researchers should take into account that increases in AST and ALKP values may be the result of the anaesthetic agents used when evaluating their results. Histologically, no impact on the lungs and liver was observed. The safer method for the personnel was intramuscular administration, while nose cone possessed substantial perils. The higher percentage of adverse effect was observed in NCA group while its advantages were the easiness and inexpensiveness of the equipment needed

for its administration. According to our observation both IA and NCA appeared equally stressful for the animals. From all anaesthetic procedures, animals in VA experienced lower stress compared to IA and NCA groups. Furthermore, in the present study only the acute impact of the anaesthetic procedures was evaluated; therefore NCA could only be suggested as end-stage anaesthesia, since the overdose of isoflurane may elicit long-term consequences which have not been evaluated.

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