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Blood Sampling in the Rat: Current Practices and Limitations*

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Abstract. Historically, blood samples have been taken by puncture of the orbital sinus under light ether anaesthesia. In the last decade, there was concern over the use of ether as an anaesthetic. Furthermore, in recent years, more attention has been focused on the need to improve current laboratory practices for the benefit of animal welfare. Exposure for 2 min to a CO_2/O_2 mixture produced acute hyperkalaemia and changes in a range of haematological parameters. Hyperkalaemia in some individuals reached pathological levels when compared to values normally obtained from ether-anaesthetised animals. Together with published data, our findings indicate that the use of a 70:30 mixture of CO_2/O_2 as an anaesthetic in rats is questionable. With isoflurane, the only consistent findings following exposure for 5 minutes were a slight downward trend in the red blood cell parameters and in potassium, and an increase in glucose. These changes probably represent an effect of prolonged exposure to isoflurane. Therefore, to avoid variation in these parameters, the duration of exposure should be limited to 3 min. When comparing sublingual to retro-orbital blood sampling after 2 min exposure to 4% isoflurane, no major differences were found in the plasma chemistry parameters examined. However, interference with plasma amylase levels following sampling from the sublingual vein has been suspected in one toxicity study. The acute effect of blood removal over 24 h on the main haematological parameters consisted of decreases in red blood cells (RBC), haemoglobin (HGB) and haematocrit (HCT), which were similar for blood removal exceeding 7.5% but below 15% of circulating blood volume, and were positively related to the amount of blood loss beyond 15%. There were no biologically significant effects on these parameters below 7.5%. Time to recover from the effects of the bleed was also proportional to the volume taken, and was estimated to range from 48 h for amounts between 5% and 7.5%, 12 days for 7.5% to 20%, and 19 days for amounts above 20 %. The collection of up to 20% of the circulating blood volume over 24 h did not affect the welfare of rats, as indicated by the absence of mortality and clinical signs and the lack of effects on body weight and food consumption.

Keyword: Anaesthesia; Blood removal; Clinical pathology; Rats

INTRODUCTION

Clinical pathology testing and toxicokinetics are routinely performed during the course of toxicology studies. Appropriate bleeding sites and techniques are needed, and these should cause minimal harm or stress to the animal whilst affording accurate and repeatable results.

Historically, at Pfizer Amboise, blood samples were taken by puncture of the orbital sinus under light ether anaesthesia.

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However, in the last two decades there has been concern about the use of ether as an anaesthetic. Further, in more recent years, attention has been focused on the need to improve current laboratory practices for the benefit of animal welfare. Such improvements were also intended to improve the quality of scientific research.

Blood removal from animals is recognised as stressful for the animals, simply because of the handling, the type of anaesthetics or the discomfort associated with a particular technique.

Recently, the European Federation of Pharmaceutical Industry Associations / European Centre for Validation of Alternative Methods (EFPIA/ECVAM- Diehl et al, 2001) has issued a guideline on blood removal volumes and sites for blood sampling in laboratory animals. In this presentation, the most frequently used anaesthesia techniques, sites of blood sampling and the effects of blood sampling on rats will be reviewed. We have focused mainly on bleeding in rats, since rat studies represent more than 80% of our activity, and anaesthesia in mice is normally administered only terminally (i.e., recovery not needed, therefore the conditions are different). The major part of this paper is based on data generated in our facilities, the remainder on published data.

ANAESTHESIA TECHNIQUES

Ether is still used as an anaesthetic, as judged by a number of recent publications. However, in addition to its explosive nature, ether is an irritant to mucous membranes and causes copious secretions which may obstruct the airway of animals (Tarin and Sturdee 1972). The ideal alternative to ether should be non-flammable, non-toxic and possess similar anaesthetic properties; that is, ease of use, rapid onset of action and low toxicity. Injectable anaesthetics were therefore excluded for routine studies. In recent years, a number of new volatile anaesthetics used in veterinary or human anaesthesia have been used for the light or surgical anaesthesia of laboratory rodents (Molello and Hawkins 1968; Sebesteny 1971; Tarin and Sturdee 1972; Carvell and Stoward 1975; Spencer 1976; Green 1982; Raper et al. 1987).

CO_2 has anaesthetic properties when mixed with oxygen (O_2) in sufficient proportions. Therefore, for use in routine toxicology studies, it was thought that CO_2/O_2 could be a possible alternative to ether. This CO_2 mixture is volatile, non-flammable, odourless, and colourless. Fenwick and Blackshaw (1989) described a method for short-term anaesthesia in rats with subclinical respiratory disease using CO_2/O_2 (80:20). Lukas (1994) reported the use of a 60:40 mixture to produce light anaesthesia for blood sampling in rodents. Urbanski and Kelley (1991) reported the use of a 50:50 mixture to produce anaesthesia before decapitation of rats. However, published data on the effects of CO_2/O_2 anaesthesia on plasma chemistry and haematology parameters routinely assessed in toxicology studies are sparse, and refer to a limited range of parameters, which need to be updated (Fowler et al. 1980).

Isoflurane is actually being tested or used in a number of toxicology centres, and results are qualified as 'satisfactory' (personal communications). Isoflurane is also the anaesthetic recommended for surgical procedures in the veterinary profession. To our knowledge, the evaluation of isoflurane as an anaesthetic in rats and its effects on clinical pathology parameters has not been yet published.

As a result, during the past 5 years we evaluated isoflurane and CO_2 as alternatives to ether anaesthesia. This evaluation is summarised below.

Materials and Methods

Ethical considerations. The protocols for the studies were approved by the Pfizer Amboise ethics committee. All technicians involved in this work were qualified and well trained to perform anaesthesia and blood sampling in rodents.

Animals. Male and female albino rats (Crl:COBS-VAF-CD(SD)BR (France)) were obtained from Charles River (Saint-Aubin-Les-Elbeuf, France) and were acclimatised to our laboratory conditions for one week. At the start of the experiments rats were aged 9 weeks.

Housing conditions. Rats were housed individually in autoclaved polycarbonate cages ($425 \times 266 \times 180$ mm) on autoclaved wood

chip bedding and maintained on a 12 h light – 12 h dark cycle. Room temperature and humidity were maintained at 20 ± 2 °C and 60 ± 20% relative humidity respectively. HEPA filtered air was provided with 14 air changes per hour.

Feeding. Animals had free access to tap water, filtered at 0.2 m, and to commercial certified laboratory animal food (UAR A04C, Usine d'Alimentation Rationnelle, Villemoisson-Sur-Orge, France).

Anaesthesia Apparatus. The anaesthetic apparatus was supplied by Minerve Veterinary Equipment (77185 Lognes, France) and delivered a constant flow rate of a CO_2/O_2 mixture or oxygen. The induction chamber consisted of a transparent plexiglas cage. The CO_2/O_2 mixture (70:30) and medical-grade oxygen were supplied by Air Liquide, France (37300 Joué-les-Tours).

CO_2/O_2 (70:30) was delivered at a flow rate of 1 l/min. Isoflurane was delivered at various flow rates and concentrations as described in the isoflurane anaesthesia section below. The atmosphere in the apparatus was allowed to stabilise for about 3 min prior to introducing the first animal into the induction chamber.

Clinical Pathology Determinations. Rats were fasted overnight prior to blood sampling. Heparinised blood samples were taken. All plasma chemistry analyses were performed on an automated centrifugal analyser (Monarch 2000, Instrumentation Laboratory, France) using the analytical procedures recommended by the manufacturer, and commercially available reagents. A routine list of 16 assays was performed on each rat plasma sample: sodium, potassium, chloride, calcium, phosphates, urea, creatinine, glucose, cholesterol, triglycerides, albumin, total proteins, bilirubin, alkaline phosphatase, aspartate and alanine amino transferase.

Blood samples were collected in tubes containing complexone (EDTA, K3). Red blood cell (RBC) count and distribution width, haemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, white blood cell count and differential (absolute) and platelet count were determined using a Technicon H*1 haematology analyser (Bayer, France).

Anaesthesia with CO_2/O_2

Experimental Design.

1. *Anaesthesia parameters in rats*. To determine the optimal exposure time, 10 animals/sex were exposed to CO_2/O_2 for 3 min. The animals were then removed from the chamber and a blood sample (1.8 ml, corresponding to about 10% of total blood volume) equivalent to that obtained for clinical pathology determinations was taken by orbital sinus puncture (OSP) and discarded. The same animals were subjected to the same procedure one week later, but with only 2 min exposure to the gas mixture. Mortality and clinical signs were monitored as general indices of toxicity. The apparent reaction to orbital sinus puncture and the time to recover from anaesthesia relative to the end of OSP, were recorded as indicators of pain perception and depth of anaesthesia.

2. *Effects of CO_2/O_2 in rats submitted to repeated anaesthesia*. To simulate the conditions of a pharmacokinetic study in rats, 5 rats/sex were used. On days 1 and 9 of the experiment, rats were anaesthetised and blood was sampled by orbital sinus puncture. On each day the procedure was repeated at 1, 3, 5 and 7 hours after the initial anaesthesia. After 2 min of exposure to the gases (duration determined from the previous experiment), the animals were removed from the chamber and a blood sample equivalent to that obtained for pharmacokinetic determination (– 1 ml each time, corresponding to about 20% of total blood removed over 24 h) was taken and discarded.

3. *Effects of a single exposure of rats to CO_2/O_2 on clinical pathology parameters*. To compare the effects of ether and CO_2/O_2 on plasma chemistry and haematology parameters, 10 male rats were anaesthetised with ether and 10 other males were exposed to CO_2/O_2 for 2 min. A blood sample ($\sim$ 1.8 ml) was taken for laboratory determinations.

Results.

1. *Anaesthesia parameters in rats*. One rat died 2.5 min after the initiation of exposure and another was found dead 1.5 min after OSP. There were no clinical signs, particularly none of acute anaemia, in the rats which died.

There was no reaction to the OSP procedure in any of the rats following exposure for 3 and 2 min to CO_2/O_2. The mean recovery

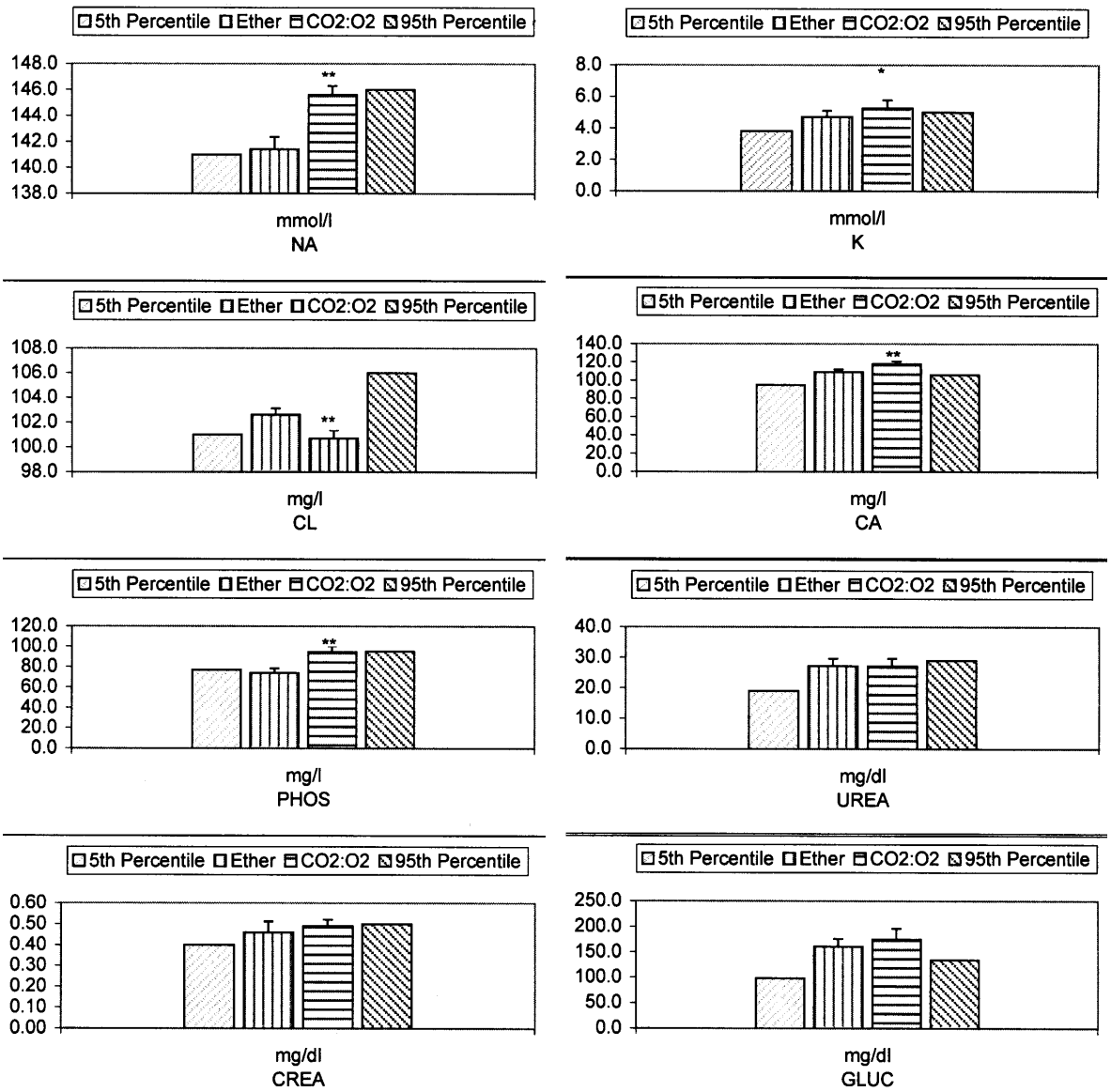

Fig. 1a Plasma chemistry parameters in male rats after exposure to ether or CO_2/O_2. Abbreviations: NA, sodium; K, potassium; CL, chlorine; CA, calcium; PHOS, phosphate; CREA, creatine; GLUC, glucose; *,**, statistically significant difference from the ether group with $p = 0.05$ and 0.01, respectively.

time was 40 ± 7 and 68 ± 24 s after 2 and 3 min of exposure, respectively.

2. *Effects of CO_2/O_2 in rats submitted to repeated anaesthesia*. There were no deaths or clinical signs and no animals reacted to the OSP procedure following repeated exposure.

3. *Effects of a single exposure of rats to CO_2/O_2 on plasma chemistry and haematology*. A number of statistically significant differences were present when CO_2/O_2 was used. The most striking finding was an increase in potassium values as shown in Fig. 1a and b. A number of plasma samples were haemolysed, but there was no clear correlation with the corresponding potassium levels. Sodium and total protein values also tended to be elevated as they were around the upper limit (95% percentile) of their respective historical ranges. This change was accompanied by an upward trend in calcium values.

For haematology, when compared to the ether controls, increases in HGB, RBC, HCT and MCV and decreases in mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and mean red cell distribution width (RDW) were observed, as shown in Fig. 2a and b. In addition, MCHC and RDW values were

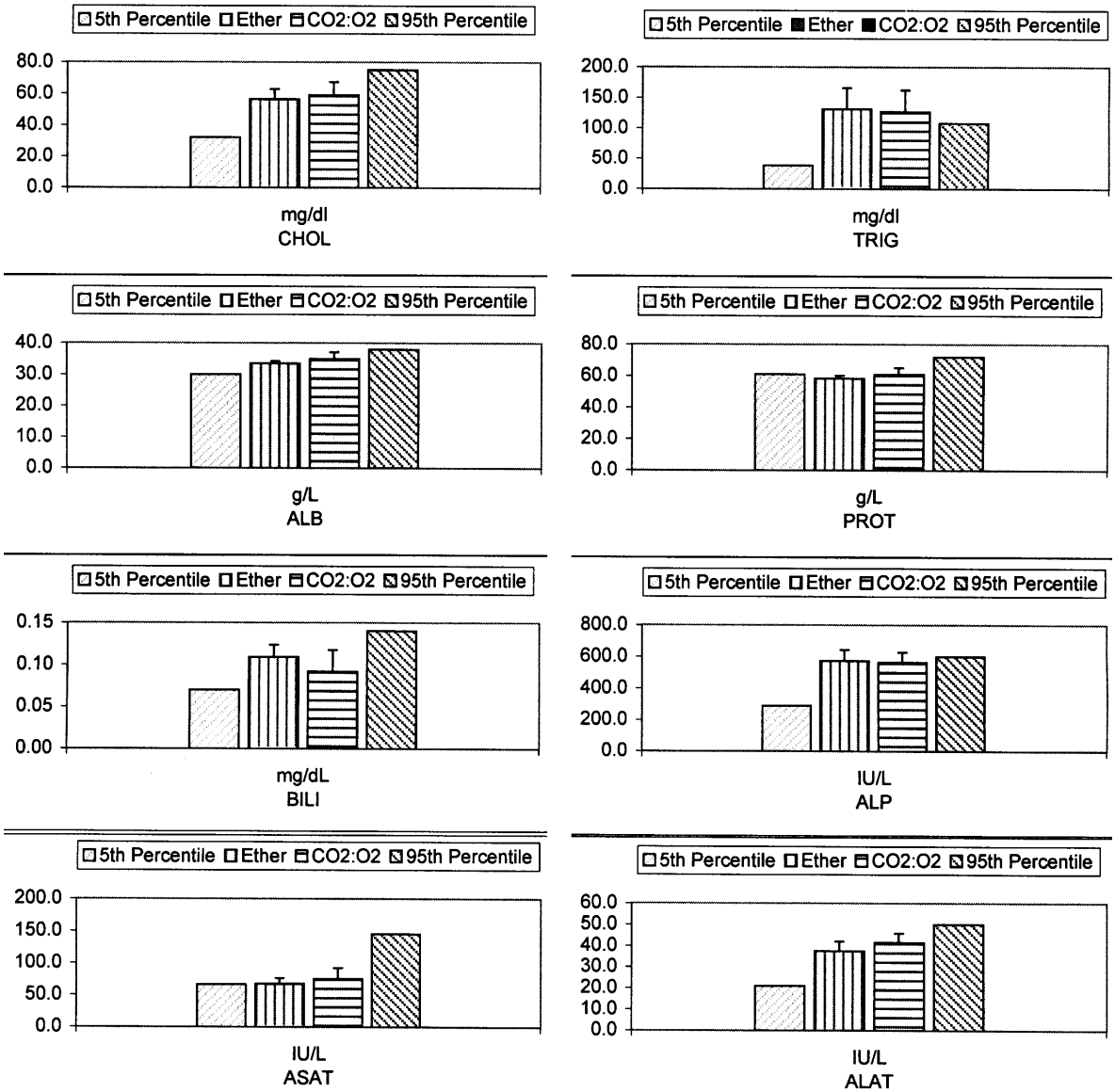

Fig. 1b. Plasma chemistry parameters in male rats after exposure to ether or CO_2/O_2. Abbreviations: CHOL, cholesterol; TRIG, triglycerides; ALB, albumin; PROT, protein; BILI, bilirubin; ALP, alkaline phosphatase; ASAT, aspartate aminotransferase; ALAT, alanine aminotransferase. *,**, statistically significant difference from the ether group with $p = 0.05$ and 0.01, respectively.

below the 5th percentile of our historical range. Variations in the white cell parameters and platelets were inconsistent.

Discussion. Exposure for 2 min produced acute hyperkalaemia and changes in a range of haematological parameters. Hyperkalaemia in some individuals reached a pathological level as compared to values normally obtained from ether-anaesthetised animals. In the absence of a direct correlation with haemolysis or renal changes, this increase is likely due to an impaired acid–base status (namely acidosis) as a result of an increased carbon dioxide plasma concentration (Duncan and Prasse 1986; Thier 1986). This increase in plasma CO_2 was probably produced by the direct administration of CO_2 and/or the reduction of pulmonary ventilation. The increase in measured potassium precludes the use of CO_2/O_2 as an anaesthetic for clinical pathology blood sampling in rats.

Haematological changes concerned red blood cell parameters. Increase in RBC and HCT without a concurrent increase in MCV is likely to reflect a splenic contraction following anaesthesia with CO_2/O_2, i.e. more

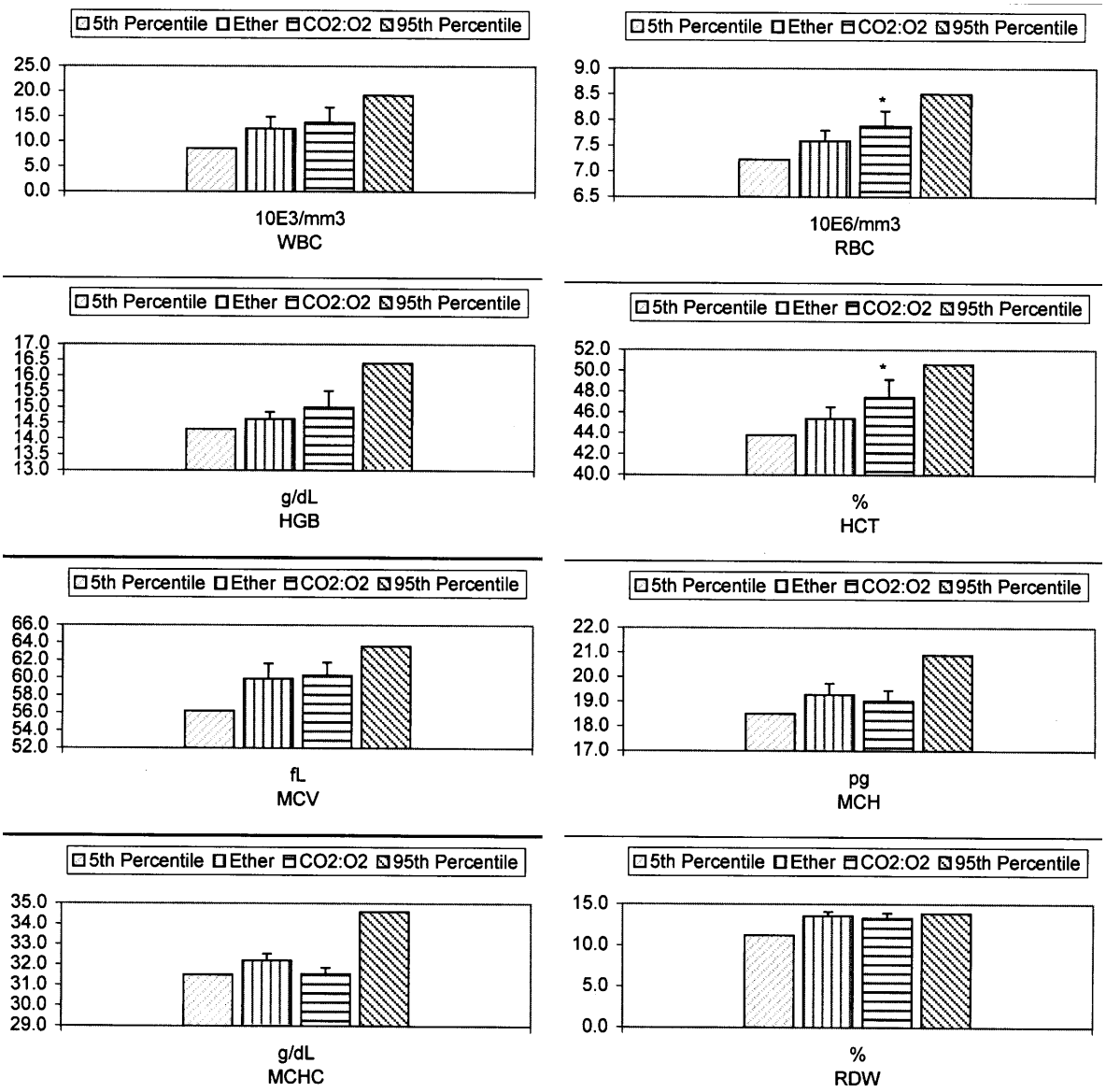

Fig. 2a. Haematology parameters in male rats after exposure to ether or CO_2/O_2. Abbreviations: WBC, white blood cells; RBC, red blood cells; HGB, haemoglobin; HCT, haematocrit; MCV, mean corpuscular volume; MCH, mean corpscular haemoglobin; MCHC, mean corpscular haemoglobin concentration; RDW, mean red cell distribution width; *,**, statistically significant difference from ether group with $p = 0.05$ and 0.01, respectively.

mature erythrocytes coming into the bloodstream (Walter 1999) and likely reflect some degree of stress. Increases in lymphocytes were reported in rats after CO_2/O_2 anaesthesia (Walter 1999). Although these haematological changes are of no toxicological significance, the routine use of CO_2/O_2 would require the constitution of a new historical database.

Fowler et al. (1980), following repeated blood sampling in rats using a mixture of CO_2/O_2 (3:1) and 2 min exposure, reported that the choice of anaesthetic did not affect packed cell volume, urea, total protein and alamine aminotransferase (ALAT), but increased blood glucose levels. However, in their protocol, electrolytes and other red blood cell parameters were not assessed. On the other hand Walter (1999), using 66:34% CO_2/O_2, obtained the same effects on haematology and serum chemistry, with the exception of potassium, as in our study, thus confirming our results.

Our data contribute to the controversy on the use of CO_2 for anaesthesia or euthanasia in laboratory rats and mice. There are contra-

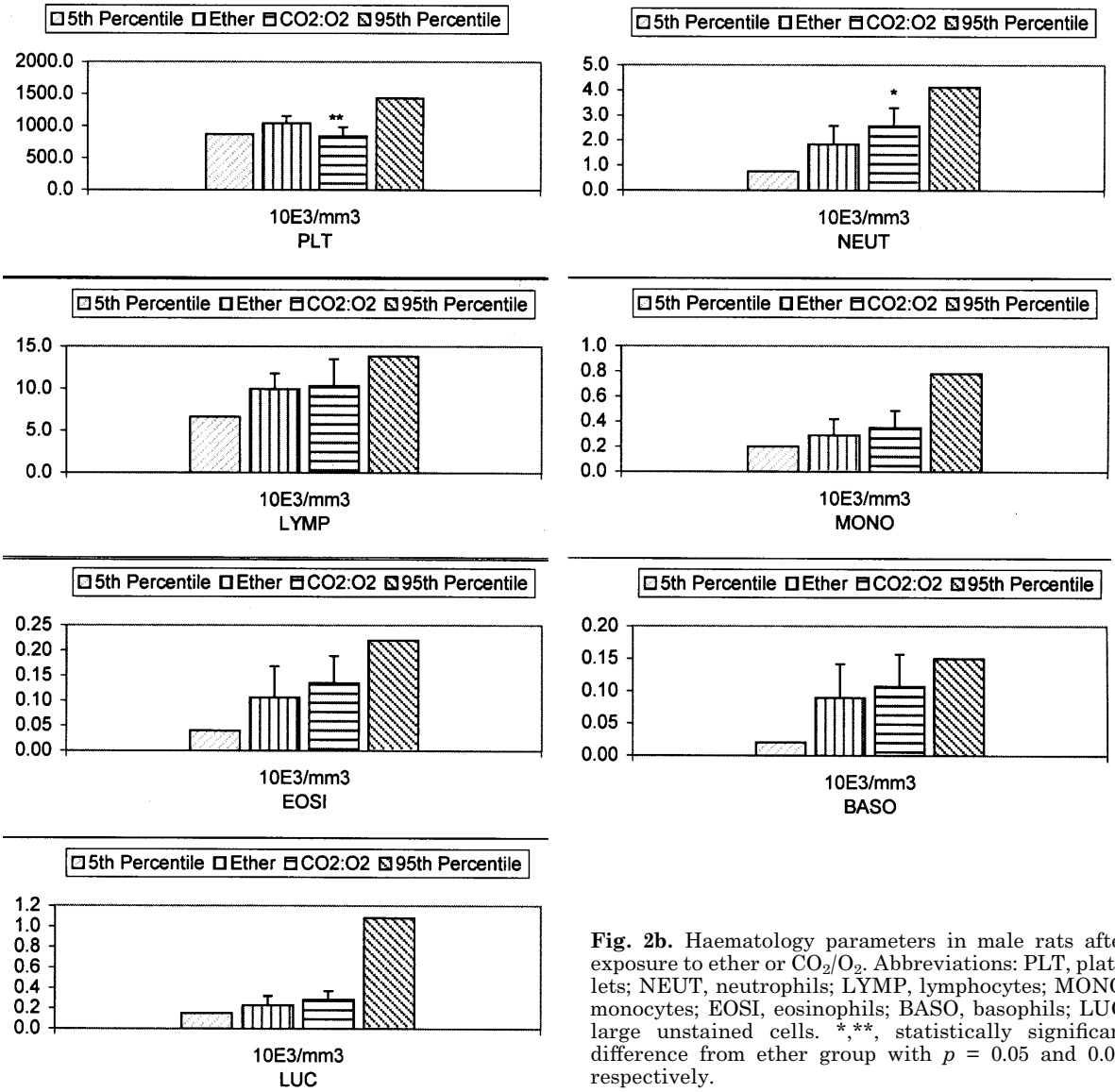

Fig. 2b. Haematology parameters in male rats after exposure to ether or CO_2/O_2. Abbreviations: PLT, platelets; NEUT, neutrophils; LYMP, lymphocytes; MONO, monocytes; EOSI, eosinophils; BASO, basophils; LUC, large unstained cells. *,**, statistically significant difference from ether group with $p = 0.05$ and 0.01, respectively.

Table 1. Experimental conditions for isoflurane in rats

Oxygen Flow rate (l/min)	Isoflurane concentration (%)	Duration of exposure (min)	Number of rats	Remarks
1	3	2	5M+5F	Reaction during sampling 3/10; sampling missed 1/10
1	3	3	10F	Reaction: during sampling 5/10; end of sampling 1/10
1	3	5	5F	Reaction: during sampling 3/5; end of sampling 1/5
1	5	3	3M+7F	–
1	5	5	5M	–
2	3	3	5F	Reaction during sampling 1/5
2	4	2	3F	No anaesthesia 1/3
2	4	2.5	5F	–
2	4	3	5F	–
2	4.5	2.5	10M+10F	–
2	5	3	10F	

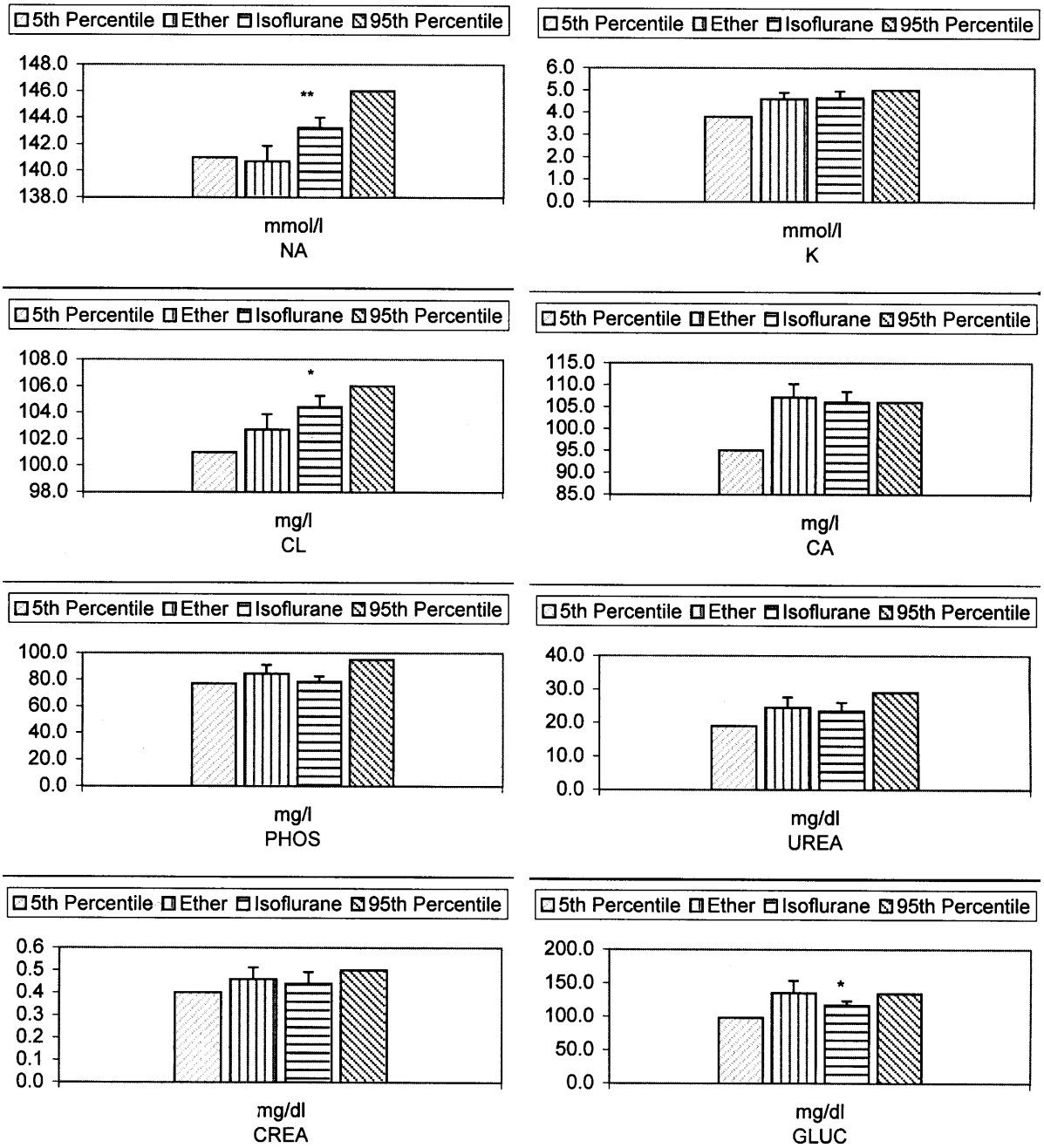

Fig. 3a. Plasma chemistry parameters in male rats after exposure to ether or isoflurane. For abbreviations see Fig. 1. *,**, statistically significant difference from ether group with $p = 0.05$ and 0.01, respectively.

dictory reports on pain or distress in animals being anaesthetised or euthanised with CO_2 (Mikeska and Klemm 1975; Feldman and Gupta 1976; Fenwick and Blackshaw 1989; Urbanski and Kelley 1991; Hewett et al. 1993; Berger-Sweeney et al. 1994; Coenen et al 1995; Danneman et al. 1997). Danneman et al. (1997) have reported that the concentrations that are least likely to cause pain and distress are associated with histological changes in the lungs. These published data, together with our findings, indicate that the use of a 70:30 mixture of CO_2/O_2 as an anaesthetic in rats is questionable.

Anesthesia with Isoflurane

Experimental Design. At the start of the evaluation with isoflurane, the orbital sinus puncture was used as it was our routine,

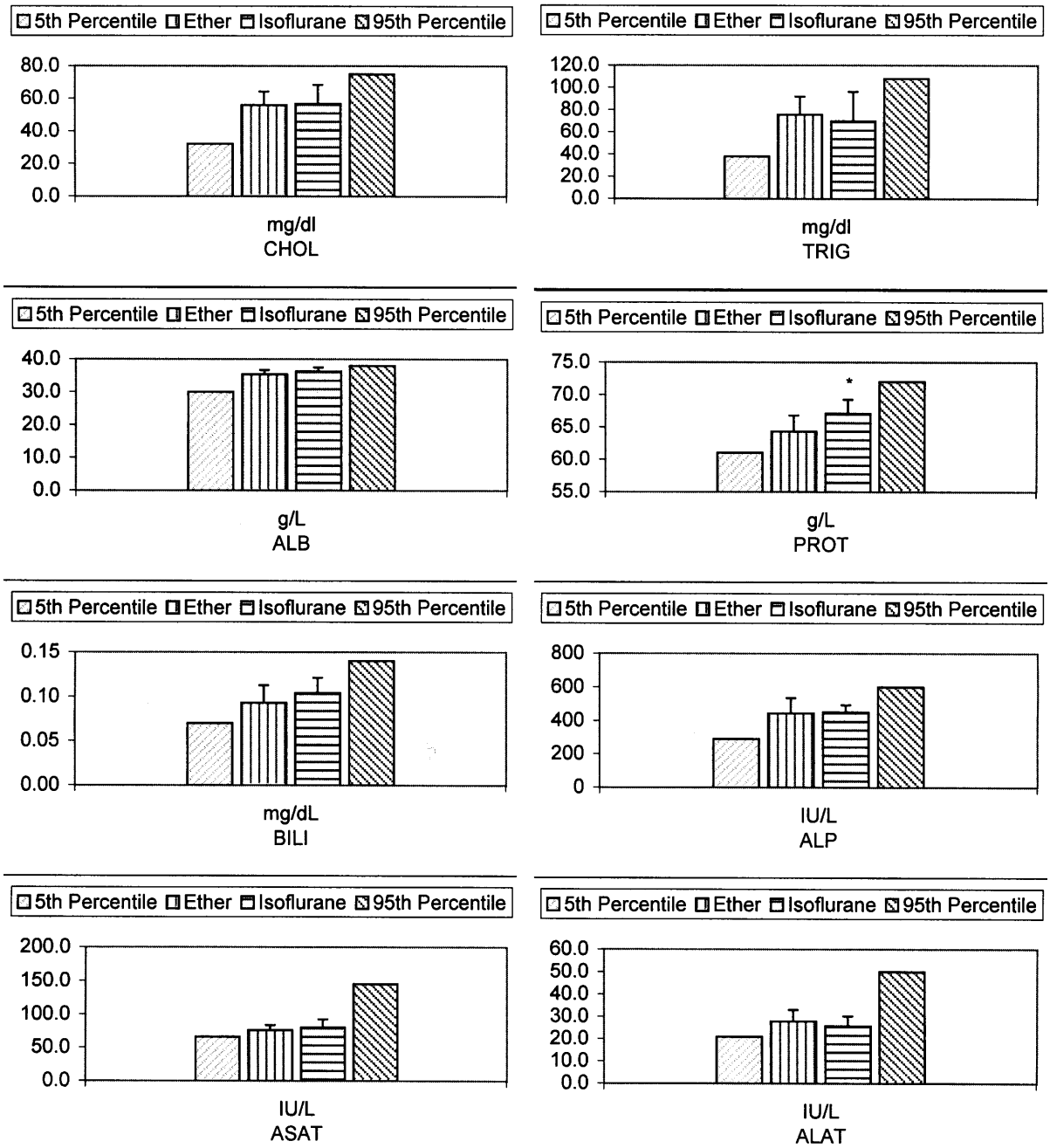

Fig. 3b. Plasma chemistry parameters in male rats after exposure to ether or isoflurane. For abbreviations see Fig. 1. *,**, statistically significant difference from ether group with $p = 0.05$ and 0.01, respectively.

standard sampling site at that time. However, during the course of evaluation, we switched from orbital sinus to sublingual sampling. Thus, some of the experiments were performed using orbital bleeding and the others with sublingual.

1. *Anaesthesia parameters in rats*. A number of assays was performed on stock male and female rats using various flow rates, isoflurane concentrations and duration of exposure (Table 1) . Blood samples were taken from the orbital sinus and the depth of anaesthesia was assessed as already described for CO_2.

2. *Effects of isoflurane in rats submitted to repeated anaesthesia*. The experiment to evaluate the effects of blood removal in rats was used to simulate a regimen similar to that used for blood sampling in pharmacokinetic studies (see later).

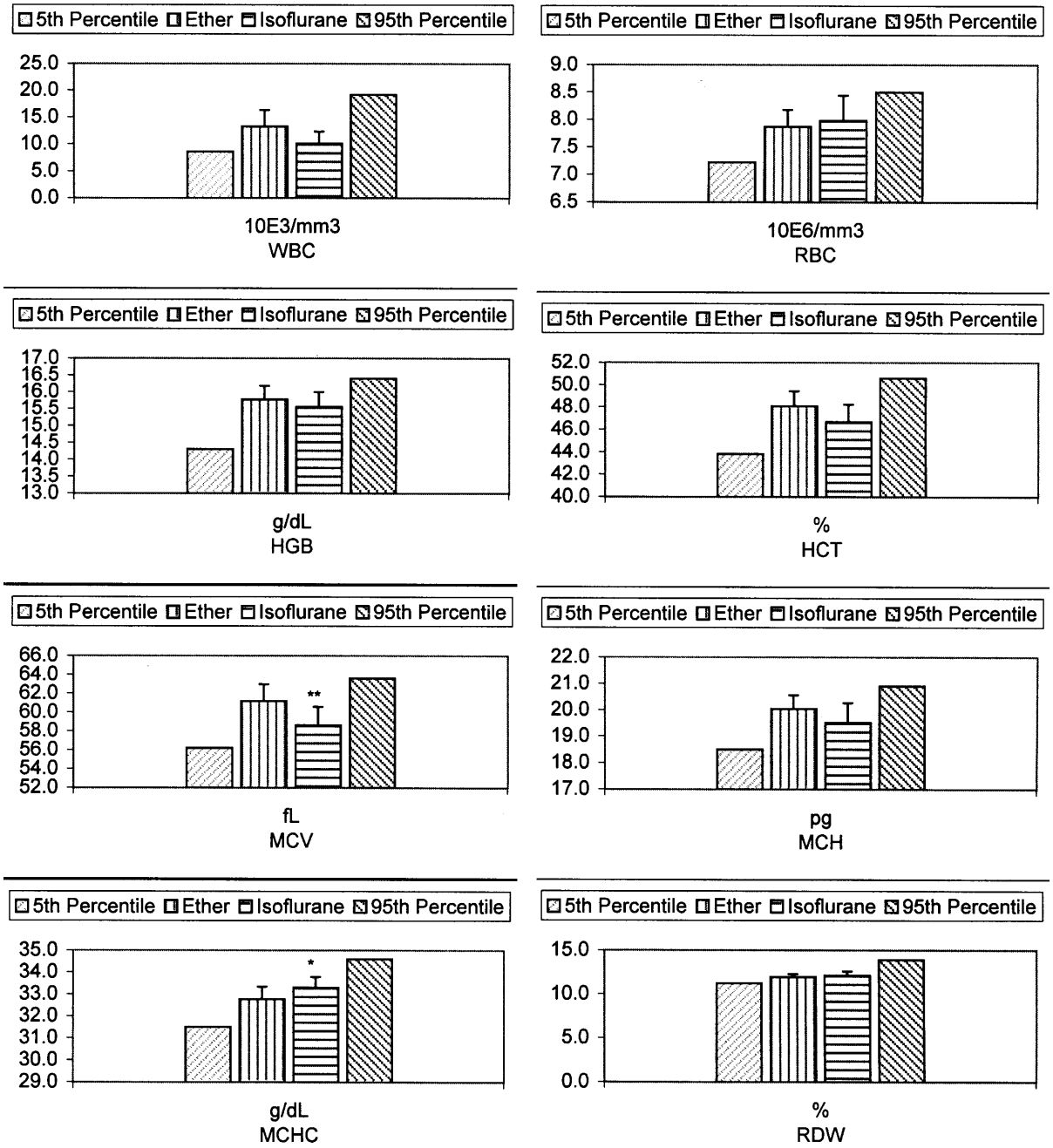

Fig. 4a. Haematology parameters in male rats after exposure to ether or isoflurane. For abbreviations see Fig. 2. *,**, statistically significant difference from ether group with $p = 0.05$ and 0.01, respectively.

3. *Effects of a single exposure of rats to isoflurane on clinical pathology parameters.* Two assays were performed. The first aimed to compare the effects of single exposure to isoflurane under optimal conditions of use. Ten male rats were anaesthetised using ether and 10 using isoflurane (2 l/min, 4%, 2.5 min). Blood samples were taken from the orbital sinus.

In the second experiment 10 rats/group were exposed to isoflurane (2 l/min, 4%) for 2, 3 and 5 min. Blood samples were taken from the sublingual vein for clinical pathology determinations.

Results.

1. *Anaesthesia parameters in rats.* Flow rates, isoflurane concentrations and duration of exposure are shown in Table 1. None of the conditions tested produced mortality or respiratory distress. The optimal conditions which better fit our objec-

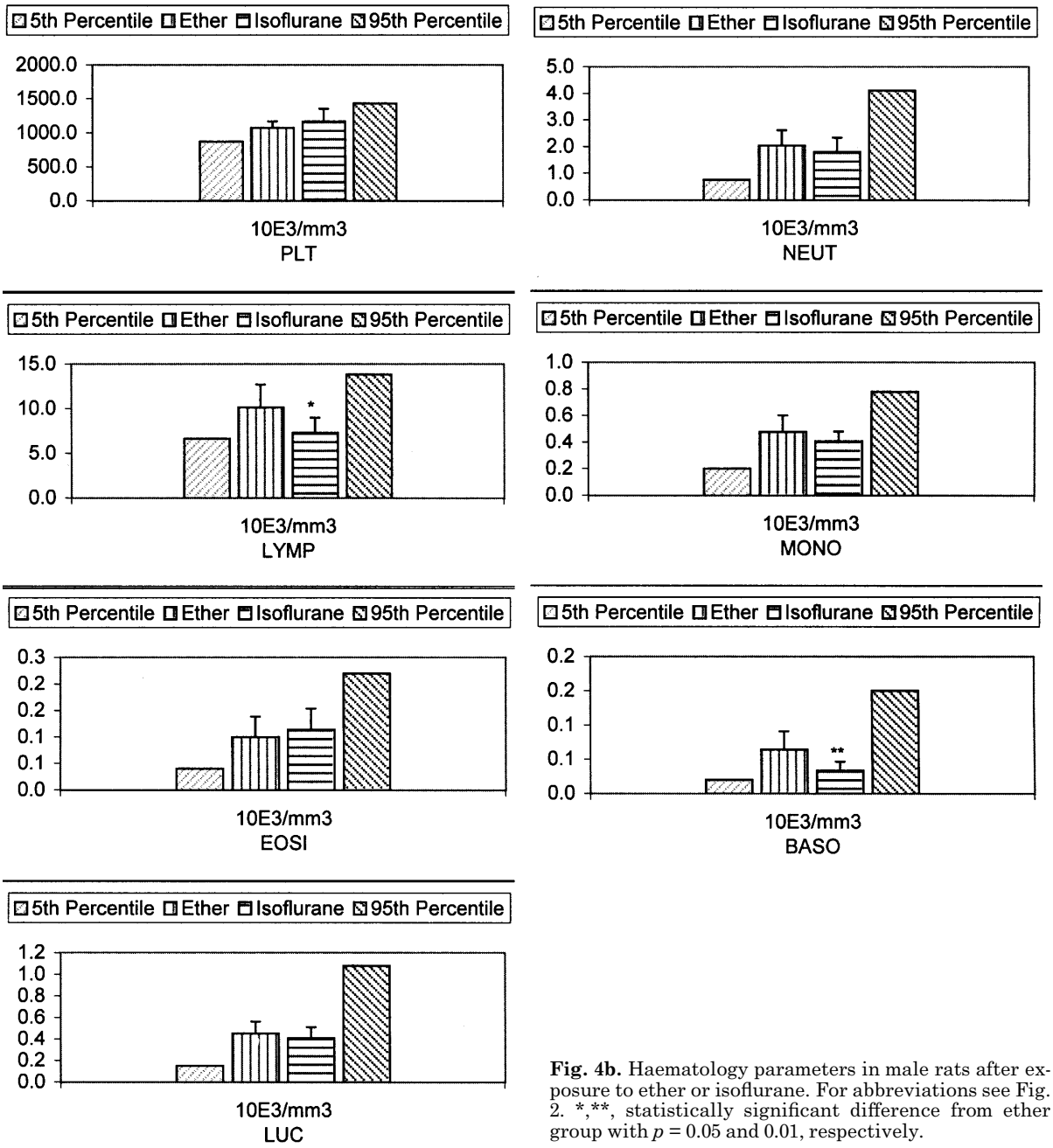

Fig. 4b. Haematology parameters in male rats after exposure to ether or isoflurane. For abbreviations see Fig. 2. *,**, statistically significant difference from ether group with $p = 0.05$ and 0.01, respectively.

tives were 2l flow rate, 4% isoflurane and 2.5 min exposure. When using the sublingual vein with exposure for 2 min the depth of anaesthesia was enough to allow blood sampling.

2. *Effects of isoflurane in rats submitted to repeated anaesthesia.* In rats repeatedly anaesthetised (4 times over 24 h) by exposure to isoflurane for 2 min there was no mortality.

3. *Effects of single exposure of rats to isoflurane on plasma chemistry and haematology.* Under optimal conditions the anaesthesia of male rats with isoflurane (2 l/min, 4% for 2.5 min) produced no clear changes in the clinical parameters examined when compared with ether anaesthesia (Fig. 3a,b, 4a,b).

In plasma chemistry, the only effect possibly related to duration of anaesthesia was a trend toward decreased potassium values and increased glucose values (taking 2 min exposure as the reference). The amplitude of changes was proportional to the duration of exposure (Fig. 5a,b) and was up to 3% and 15% for potassium and glucose respectively.

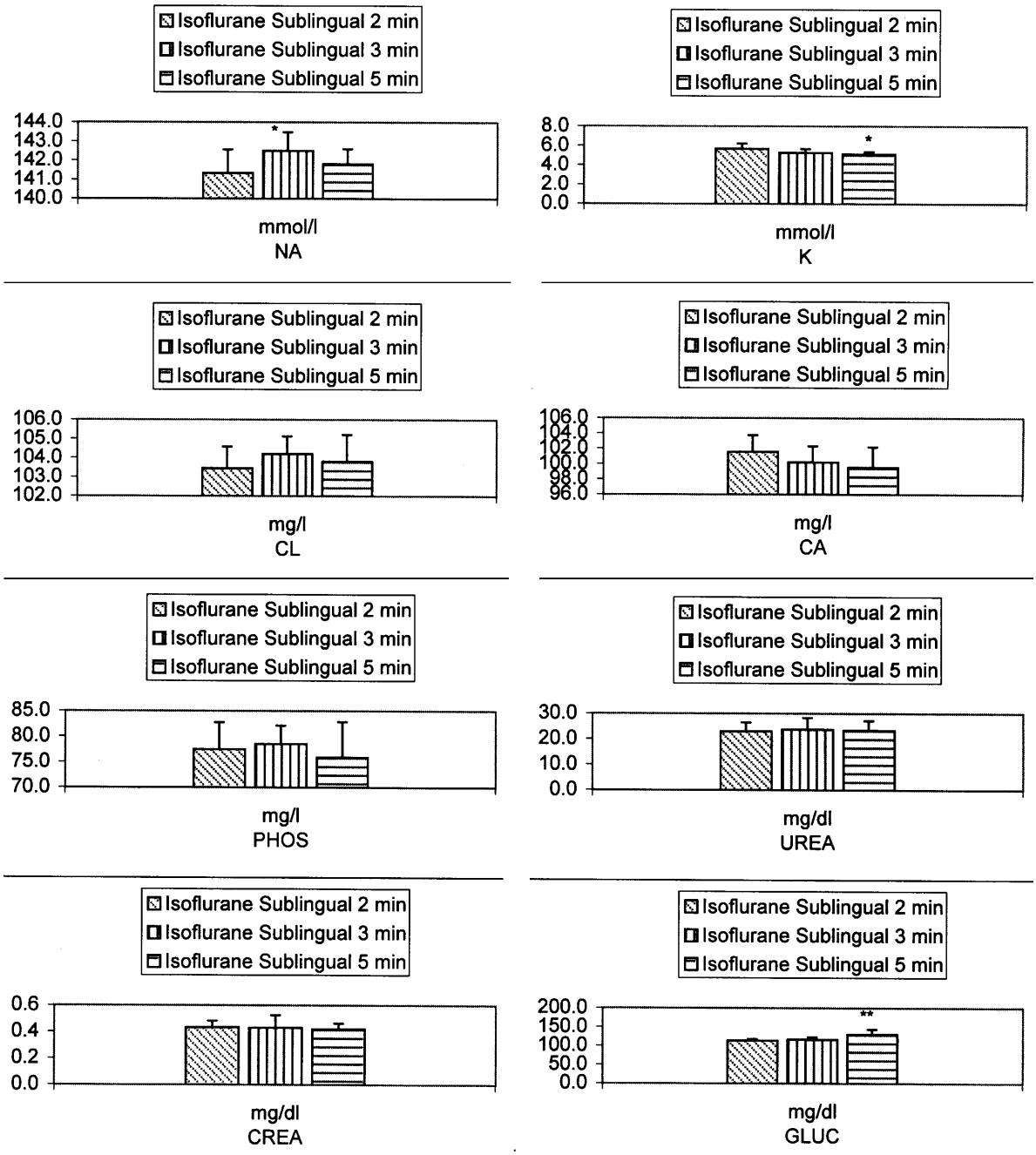

Fig. 5a. Effects of duration of exposure to isoflurane on chemistry parameters. For abbreviations see Fig. 1. *,**, statistically significant difference from 2 min group with $p = 0.05$ and 0.01, respectively.

Haematologically, in rats bled at the sublingual site, the red blood cell parameters tended to decrease with time of exposure as shown in Fig. 6a,b (taking 2 min exposure as the reference). However, these decreases remained of small amplitude (below 10%). Other significant differences were considered to result from random variations.

Discussion. The only consistent findings were a downward trend in the red blood cell parameters and potassium, and an increase in glucose. Although these effects remained of slight amplitude and of no toxicological relevance, they probably represent an effect of prolonged exposure to isoflurane. Splenic sequestration of RBC was demonstrated in

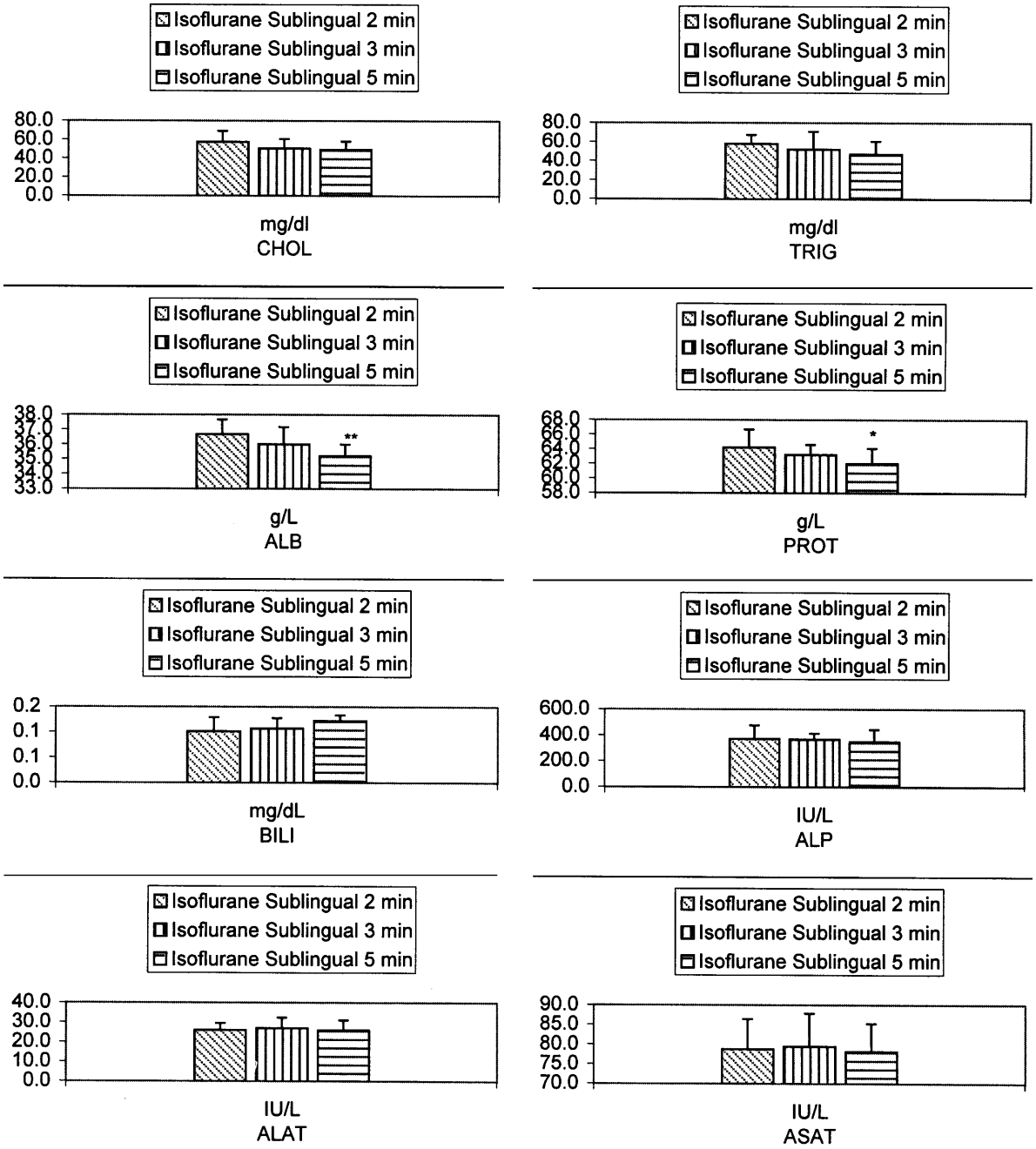

Fig. 5b. Effects of duration of exposure to isoflurane on chemistry parameters. For abbreviations see Fig. 1. *,**, statistically significant difference from 2 min group with $p = 0.05$ and 0.01, respectively.

ferrets exposed to isoflurane for 15 min leading to a decreased circulating red cell mass (Marini et al. 1997). In a comparative study with different volatile anaesthetics (Kofke et al. 1987), anaesthesia with isoflurane was shown to increase plasma glucose relative to halothane and enflurane. Therefore, to avoid variations in these parameters, the duration of exposure should be limited to 3 min.

SITES OF BLOOD SAMPLING

Sampling sites have been described in 1993 in the report of the BVA/FRAME/RSPCA/UFAW (Morton et al. 1993) and by Diehl et al. (2001) who recommended the saphenous, lateral and sublingual veins. The technique for the saphenous veins has been published by Hem et al. (1998). In rats, sites for blood sampling are: orbital sinus; sublingual vein;

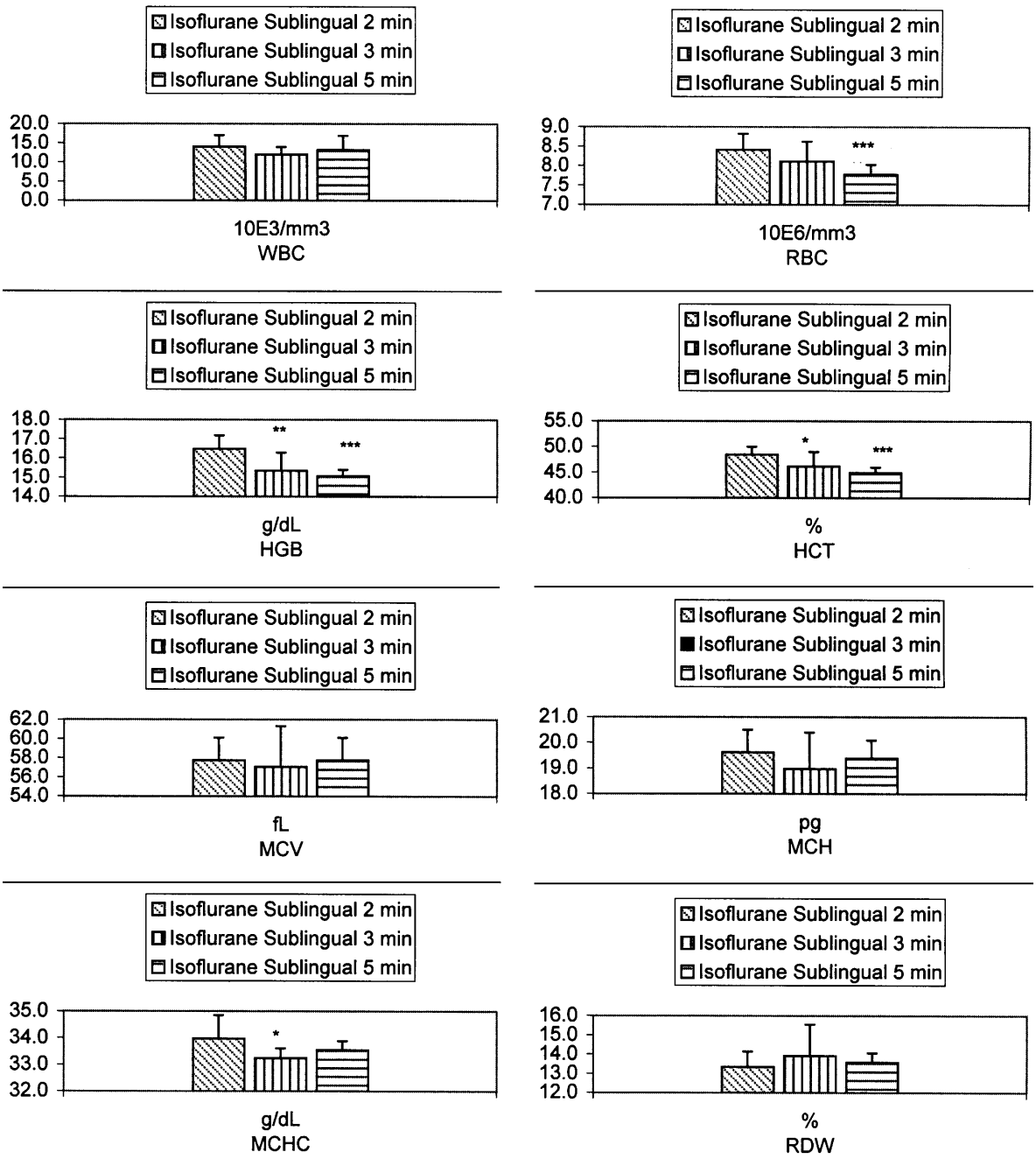

Fig. 6a. Effects of duration of exposure to isoflurane on haematology parameters. For abbreviations see Fig. 2. *,**,***, statistically significant difference from 2 min group with $p = 0.05$ and 0.01, respectively.

cardiac puncture; jugular puncture; jugular/femoral cannulation; saphenous vein; abdominal vena cava/aorta; lateral tail veins; tail tip amputation. The choice of sampling site is dependent on study design, skills of the technical staff, the volume of blood needed, the parameters to be analysed, the frequency of sampling and on ethical/animal welfare considerations.

Methodology for each sampling site will not be described in this paper for a detailed description, see Diehl et al. (2001), and Waynforth and Flecknell (1992). The advantages and disadvantages of each sampling site are given in Table 2. The sampling site may interfere with haematology and plasma chemistry parameters. Archer and Riley (1981) reported substantial differences in leucocyte counts

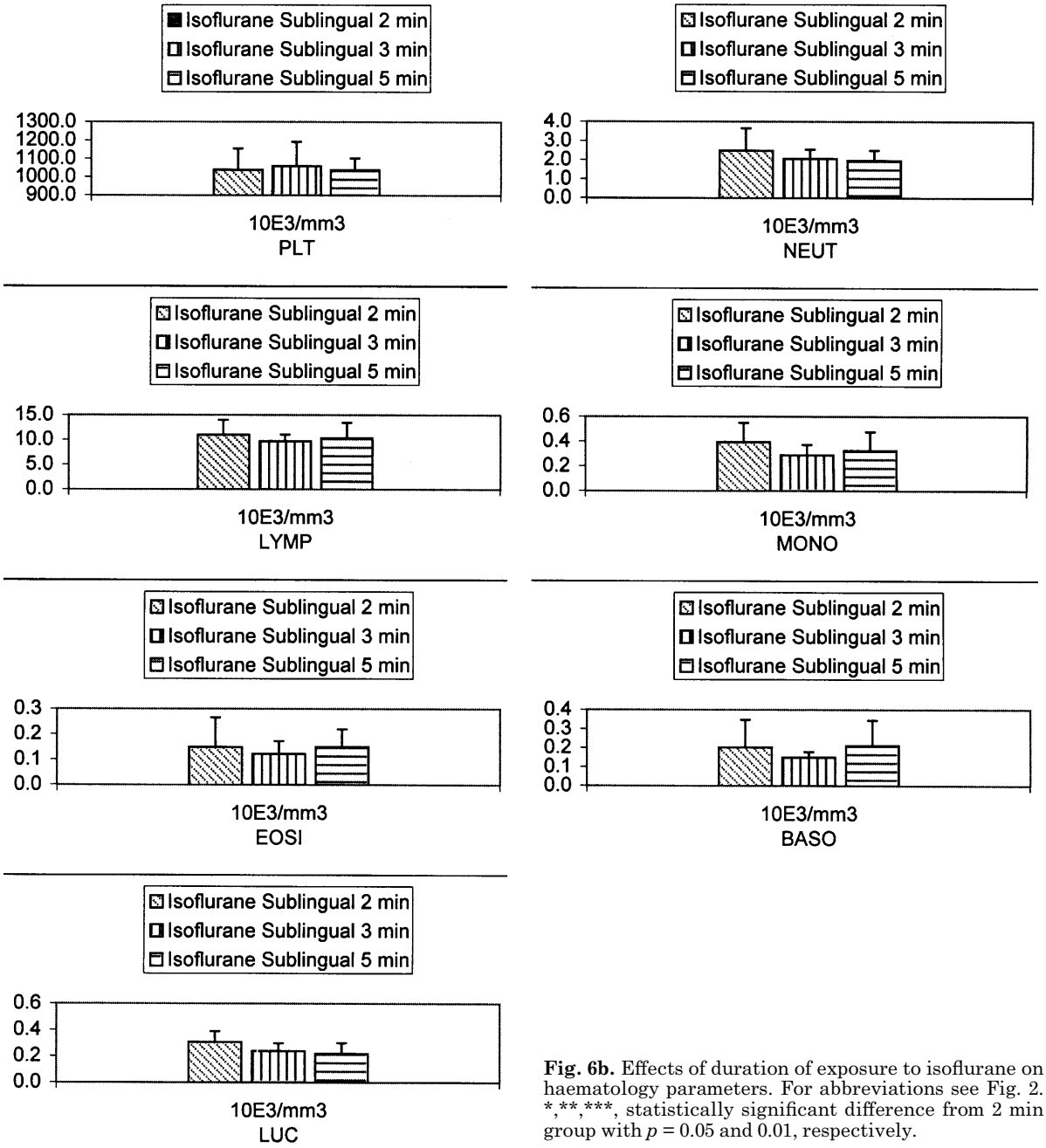

Fig. 6b. Effects of duration of exposure to isoflurane on haematology parameters. For abbreviations see Fig. 2. *,**,***, statistically significant difference from 2 min group with $p = 0.05$ and 0.01, respectively.

between samples taken from the heart and the tail or jugular veins. In our hands, when comparing sublingual to retro-orbital blood sampling following 2 min exposure to 4% isoflurane, no major differences were found in the plasma chemistry parameters examined (Fig. 7a,b and 8a,b). The small increase in potassium (9%) and the small decrease in aspartate aminotransferase (12%), although statistically significant were considered to have no biological significance. However, interference with plasma amylase levels following sampling from the sublingual vein has been suspected in one toxicity study (data not published).

EFFECTS OF BLOOD REMOVAL

The objectives of this experiment were to assess the clinical and haematological effects of acute blood removal, over 24 h, of 7.5, 10, 15 and 20% of circulating blood volume in rats, and to determine the time required for return to baseline values, in order to help establish guidelines for

Table 2. Advantages and disadvantages of sites for blood sampling

Sampling site	Advantages	Disadvantages
Vena cava Abdominal aorta	• Large volume	• Only terminal bleeding is possible • If histopathology is performed, exsanguination may not be complete leading to artefacts • Deep anaesthesia is needed, using injectable anaesthetics; increased time to induction • May not be possible in infusion studies
Cardiac puncture	• Large volume	• Skilled technicians • High rate of mortality – cardiac arrest
Jugular or femoral cannulation	• Large volumes • Repeated, frequent sampling • Do not need anaesthesia	• Initial surgery is needed • Not suitable for clinical pathology since catheters should be flushed – interference with flushing solutions and dilution
Lateral tail vein	• Do not need anaesthesia • Frequent sampling	• The tails need to be heated which may affect the physiology of the animal • Small volumes • Skilled technicians • Limitation in IV studies
Sublingual vein	• Large volume • Frequent sampling • Low tissue damage • Not painful	• Interference with amylase • Possible contamination if compound excreted in saliva
Jugular vein	• Do not need anaesthesia	• Skilled technicians • Limited frequency of sampling
Saphenous vein	• Do not need anaesthesia	• Small volumes • Limited to small size or young rats
Tip tail amputation		• Amputation is ethically not acceptable • Tissue damage • Limited number and frequency of sampling
Orbital sinus puncture	• Large volumes • Repeated and frequent sampling	• Optic nerve damage – Discomfort • Haematoma • Skilful technicians • More and more controversial

limiting volumes of blood removed and for recovery periods.

Materials and Methods

Animals. Male and female albino rats (Crl:COBS-VAF-CD(IGS)BR (France)) were obtained from Charles River (Saint-Aubin-Les-Elbeuf, France) and were acclimatised to laboratory conditions for one week. At the start of the experiments rats were aged 9 weeks and the mean body weight was 331.5 and 241.1 g for males and females, respectively.

Housing Conditions and Feeding. See description above.

Anaesthesia and Blood Collection. Isoflurane was delivered at a concentration of 4% using an oxygen flow rate of 2 l/min. Rats were exposed to the anaesthetic for 2 min then removed from the induction chamber. The tongue was then exteriorised to visualise the sublingual vein. The vein was punctured with a hypodermic needle and blood was allowed to drop freely into tubes.

Experimental Design. Animals were allocated to five groups of seven rats/sex. Initially we intended to remove 7.5, 10, 15 and 20% of total blood volume based on rats weighing 250 g and total circulating blood volume of 64 ml/kg (EFPIA/ECVAM- Diehl et al. 2001). Although the mean body weight of the animals of each sex on day 1 was different in our study (331.5 g for males and 241.1 g for females), for practical reasons we did not change the amount of blood removed. Consequently, the volume removed, the percentage of blood removed over 24 h in the different groups and the frequency of sampling are described in Table 3.

For groups 2 to 5 blood samples were taken on the first day at 8:30, 10:30 and 12:30 then on day 2 at 8:30 (24 hours after the first sample).

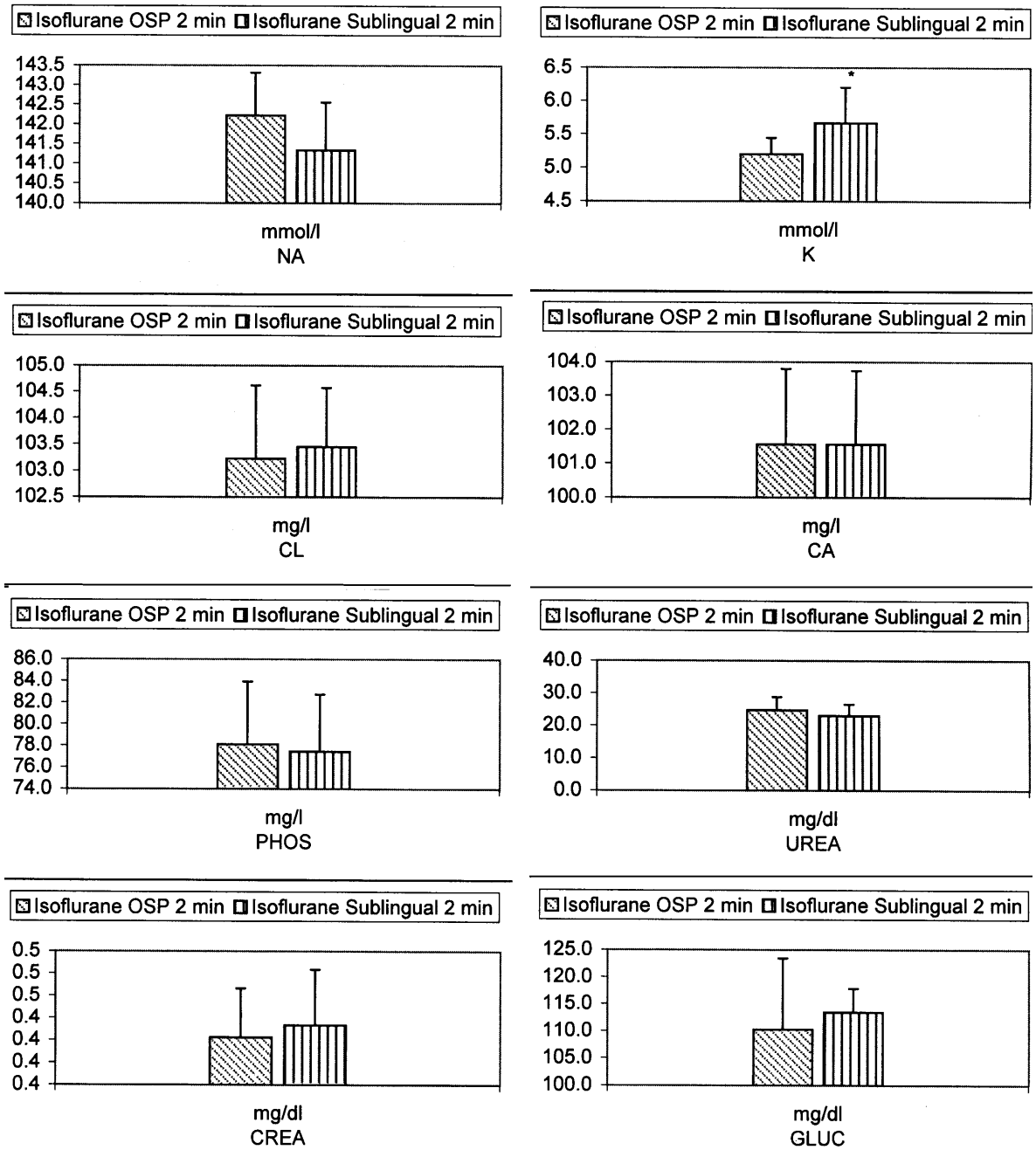

Fig. 7a. Effects of site of blood sampling on plasma chemistry parameters in male rats. For abbreviations see Fig. 1. *,**, statistically significant difference from 2 min group with $p = 0.05$ and 0.01, respectively.

Group 1, considered as a minimal regime group, served for comparing the results, with only 0.3 ml of blood being taken on one occasion on day one at each sampling period. Animals from this group were anaesthetised but not sampled at 10:30, 12:30 and the following day at 8:30.

Clinical Observations and Measurements. For all rats, clinical signs, body weight and food consumption were determined at scheduled intervals throughout the study. All animals were observed daily for survival.

Haematological Determinations. Blood samples were collected in tubes containing complexone (EDTA, K3). Selected haematological parameters: red blood cell count (RBC), haemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV) and mean

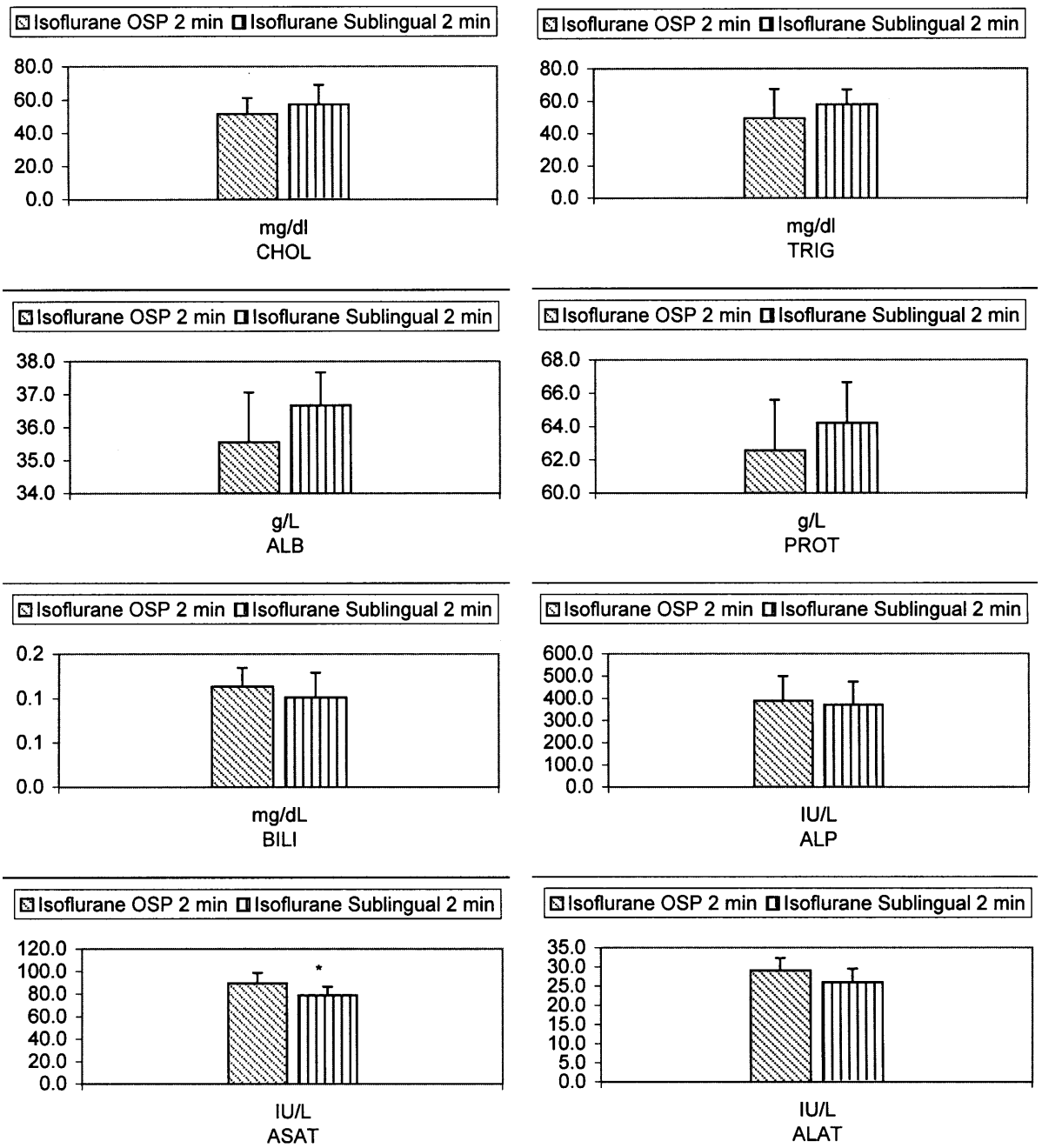

Fig. 7b. Effects of site of blood sampling on plasma chemistry parameters in male rats. For abbreviations see Fig. 1. *,**, statistically significant difference from 2 min group with $p = 0.05$ and 0.01, respectively.

red cell distribution width (RDW, the coefficient of variation of MCV) were measured using a Technicon H*1 (Bayer, France) haematology analyser.

Statistical Analysis. For each sex, group and parameter, two-tailed Student paired t-tests were used to compare values of the parameters measured at the first withdrawal (day 1) with each subsequent withdrawal. The 'MEANS' procedure from SAS was used (SAS STAT).

Results

Mortality, Clinical Signs, Body Weight and Food Consumption. There were no deaths and no adverse clinical signs. There were no statistically significant differences in the

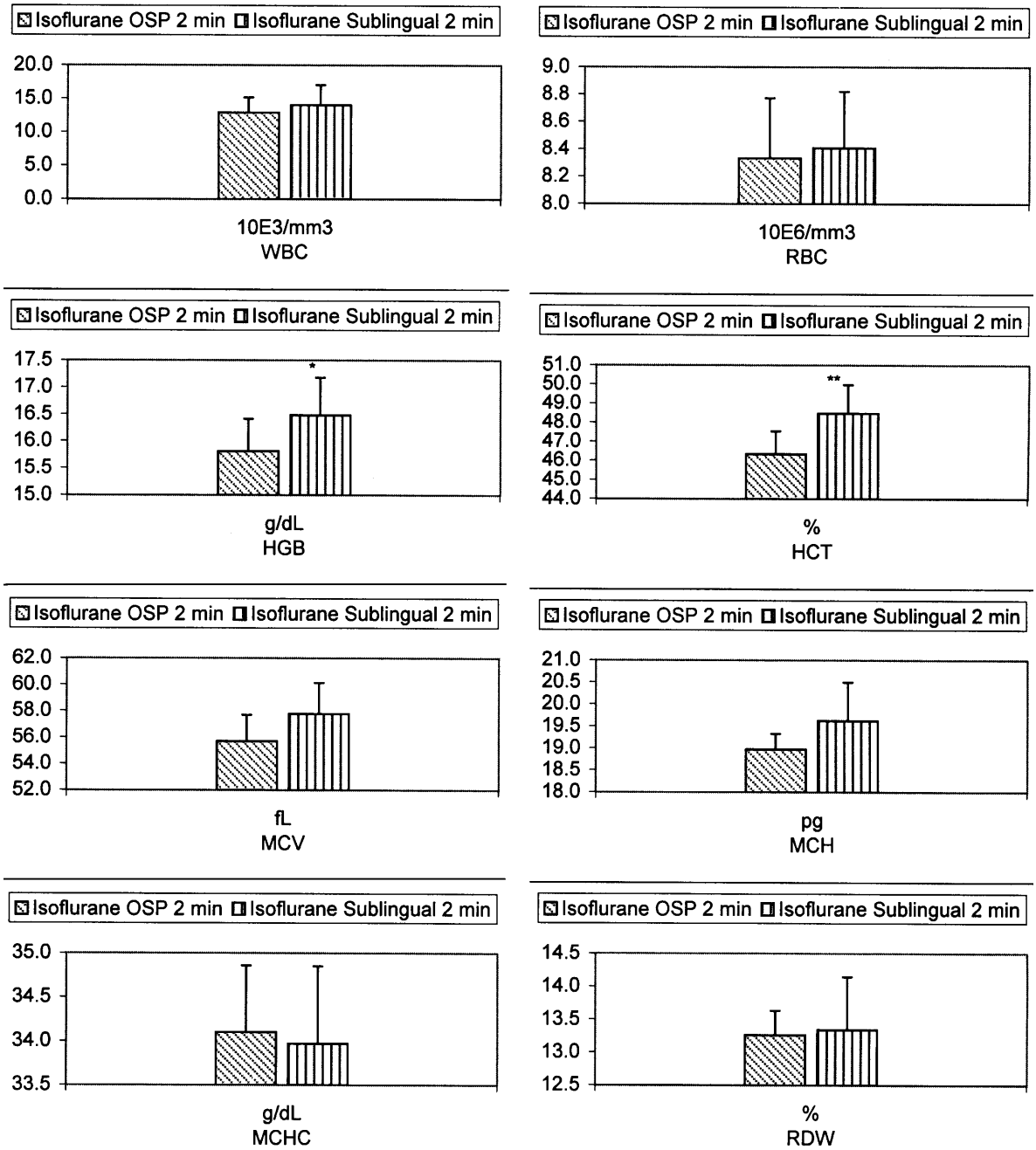

Fig. 8a. Effects of site of blood sampling on haematology parameters in male rats. For abbreviations see Fig. 2. *,**, statistically significant difference from 2 min group with $p = 0.05$ and 0.01, respectively.

mean body weight and mean body weight gain of the different groups in both sexes. On day 8, there was a body weight loss in a few rats in groups 1, 3 and 5. The amplitude of weight loss was 0.3–2.9% and the number of animals affected (two or three per group) were not related to the volume of blood collected. The body weight of these rats was comparable with other animals in the study on day 15 or 22.

There were no statistically significant differences in the mean food consumption of the different groups in both sexes.

Haematological parameters. Base lines for haematological parameters are shown in Table 4. The acute effects of removal of various amounts of blood over the 24 h period are shown in Table 5. As expected, a downward trend for core parameters RBC,

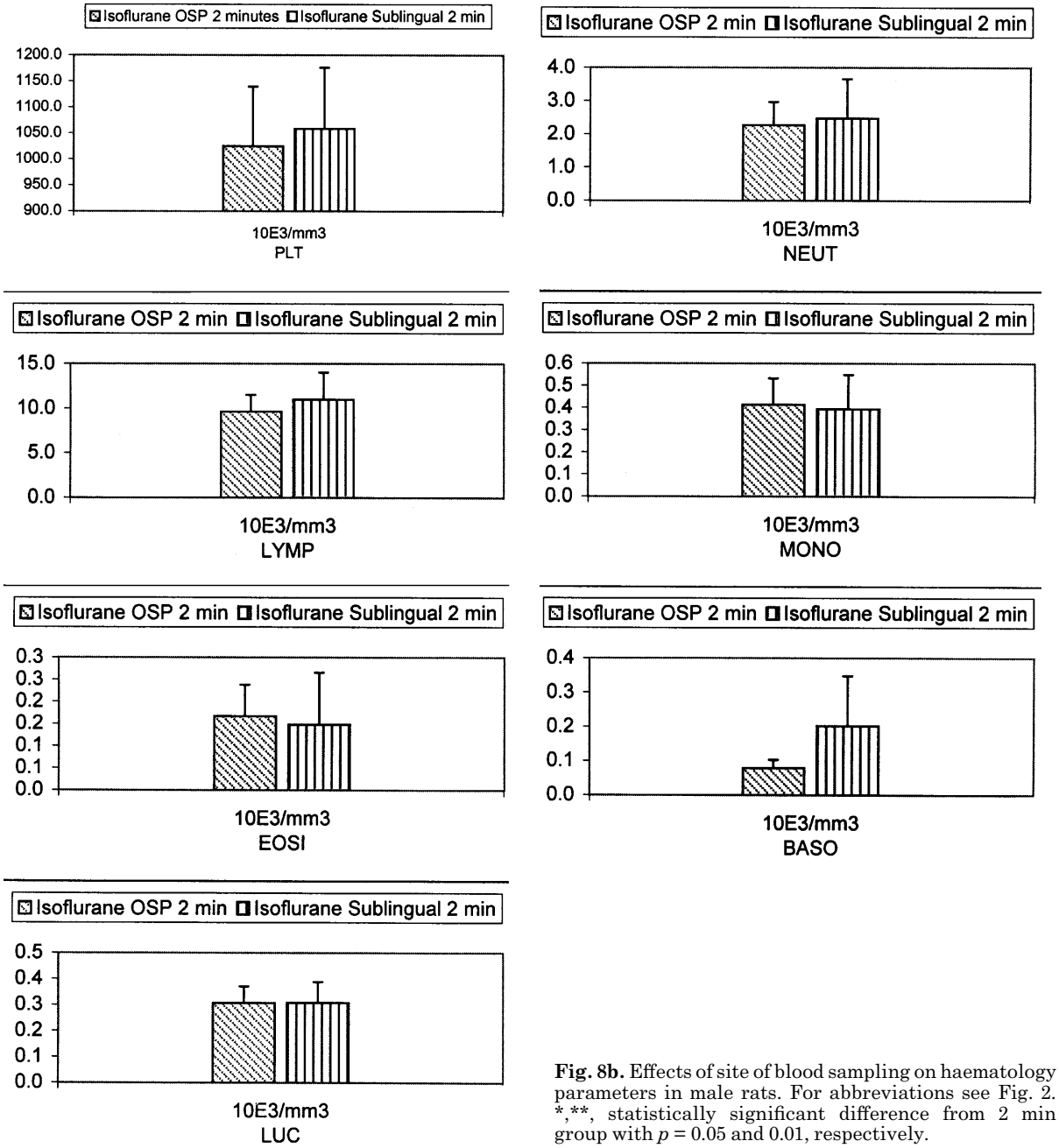

Fig. 8b. Effects of site of blood sampling on haematology parameters in male rats. For abbreviations see Fig. 2. *,**, statistically significant difference from 2 min group with $p = 0.05$ and 0.01, respectively.

HGB and HCT was observed in all groups except in group 1 males (volume removed 1.4 %). When compared to day 1, the amplitude of decreases in these parameters ranged from approximately 5% in group 1 females (volume removed 1.9%) to 30% in group 5 females (volume removed 20.8%). These reductions were similar for blood removal exceeding 7.5% but below 15% of circulating blood volume and were positively related to the amount of blood loss beyond 15%.

There were no clear changes in MCV whatever the amount of blood taken. The RDW was slightly but significantly increased from 7.8% blood volume removal, and the change was more marked after removal of 20.8%.

Haematological parameters from day 2 to 29 are shown in Table 6.

Group 1: In males and females from group 1 (volume removed 1.4% and 1.9%, respectively), there was no clear-cut effect on the

Table 3. Total blood removed and days of blood sampling

	Targeted[a]	Achieved[b]		Volume of blood removed										
		Males	Females	Day 1	Day 2	Day 3	Day 5	Day 8	Day 12	Day 15	Day 19	Day 22	Day 26	Day 29
Group1	–	1.4%	1.9%	0.3 ml		0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Group2	7.5%	5.7%	7.8%	0.9 (3 × 0.3 ml)	0.3	0.3	0.3	0.3						
Group3	10%	7.5%	10.4%	1.2 (3 × 0.4 ml)	0.4	0.3		0.3	0.3	0.3				
Group4	15%	11.3%	15.6%	1.8 (3 × 0.6 ml)	0.6	0.3		0.3	0.3	0.3	0.3	0.3	0.3	0.3
Group5	20%	15.1%	20.8%	2.4 (3 × 0.8 ml)	0.8	0.3		0.3	0.3	0.3	0.3	0.3	0.3	0.3

[a]Based on a circulating blood volume of 64 ml/kg for a 250 g rat.
[b]Based on mean body weight on day 1 of 331.5g for males and 241.1 g for females.

Table 4. Base line for haematological parameters on day 1, measured at the first blood withdrawal (mean ± SD)

	RBC (10^6/mm^3)	HB (g/dl)	HCT (%)	MCV (fl)
Males				
Group 1	6.907 ± 0.360	14.07 ± 0.51	43.25 ± 1.30	62.68 ± 2.01
Group 2	7.110 ± 0.336	14.30 ± 0.52	44.07 ± 1.85	62.06 ± 2.21
Group 3	6.873 ± 0.350	14.05 ± 0.50	42.78 ± 2.07	62.28 ± 1.61
Group 4	6.980 ± 0.152	14.37 ± 0.34	44.45 ± 1.18	63.72 ± 1.10
Group 5	7.141 ± 0.202	14.57 ± 0.50	45.09 ± 1.72	63.13 ± 1.75
Females				
Group 1	7.460 ± 0.216	14.45 ± 0.29	42.72 ± 0.73	57.25 ± 1.39
Group 2	7.611 ± 0.281	14.19 ± 0.47	41.97 ± 1.22	55.17 ± 1.76
Group 3	7.558 ± 0.462	14.52 ± 0.52	42.40 ± 1.90	56.16 ± 1.54
Group 4	7.336 ± 0.301	14.41 ± 0.56	42.73 ± 1.71	58.30 ± 1.79
Group 5	7.630 ± 0.347	14.73 ± 0.58	43.87 ± 1.39	57.53 ± 1.72

Table 5. Acute effect of increasing volumes of blood taken over a 24 h period on red blood cell parameters on day 3 (% change relative to day 1)

	Amount of blood removed (% circulating blood volume)									
	1.4 M	1.9 F	5.7 M	7.5 M	7.8 F	10.4 F	11.3 M	15.1 M	15.6 F	20.8 F
RBC	1.3	–4.3 ***	–8 **	–6.8 **	–19.4 **	–17.6 ***	–14.4 **	–18.9 ***	–24 ***	–31.2 ***
HGB	0.9	–3.3 **	–7.9 **	–8.3 ***	–19.2 **	–16.7 ***	–16.5 **	–19.7 ***	–24.5 ***	–31 ***
HCT	–0.1	–4.7 ***	–7.5 **	–6.5 **	–18.4 **	–16.1 ***	–14.3 **	–17.3 ***	–23.2 ***	–29.6 ***
MCV	–1.3	–0.3	0.4	0.3 **	1.4	1.7	0.1 *	1.9 ***	1 *	2.5 **
RDW	–0.7	2.4	3.7 **	4.8	17.5	11.5 **	14	14.1 ***	19.3 ***	49.9 **

M, Male; F, Female
*,**,***: Statistically significant with $p = 0.05$, 0.01 and 0.001, respectively

red blood cell values during the whole time course of the study. A statistically significant increase in RBC was observed in males from day 19 onwards together with slightly reduced MCV values at all bleeding times with a maximum of approximately 10% recorded at the end of the study. In females, a fairly constant increase (of below 30%) in RDW was present from day 5 onwards. There were no noticeable changes in MCV which was even slightly decreased on days 26 and 29.

Group 2: In males (blood removal 5.7%), the only noteworthy change was a minor increase in RDW on days 5 and 8. In females (blood

Table 6. Follow-up of haematological values with time (% change relative to day 1)

Blood loss %		Day 2	Day 3	Day 5	Day 8	Day 12	Day 15	Day 19	Day 22	Day 26	Day 29
1.4	RBC		1.3	2.8*	2.8*	4.9*	4.4	10.7**	8.5**	10.8	12.5**
Males	HGB		0.9	0.9	–0.9	1.8	1.4	6.5*	2.3	3.8	4.1
Group 1	HCT		–0.1	–1.5	0.1	1.3	–0.7	4.5*	0.7	0.1	0.2
	MCV		–1.3***	–4.2***	–2.6***	–3.5**	–5**	–5.6**	–7.2***	–9.9**	–11.1**
	RDW		–0.7	8.2**	10.4**	7.8**	6.6**	7.2**	5.4*	8.7**	10.3*
1.9	RBC		–4.3	–5.9***	–8.4***	–5.4***	–7***	–5.4***	–5.2**	–2.4	–2.9*
Females	HGB		–3.3	–6.9***	–8.9***	–4.9***	–6.2**	–4**	–4.8***	–2	–3*
Group 1	HCT		–4.7	–8.1***	–7.7***	–3.9***	–5.9***	–3.3***	–3.2**	–3.7*	–3.8*
	MCV		–0.3	–2.2***	0.8**	1.6***	1.3	2.2**	2.2*	–1.4	–0.9
	RDW		2.4	15.6***	28.3***	28.4***	26.3**	24.9***	24.5**	20.7**	18.3**
5.7	RBC	–6.5	–8	–4.9*	–0.9						
Males	HGB	–7.5	–7.9	–5.4**	–2.6						
Group 2	HCT	–6.5	–7.5	–5.6**	0.1						
	MCV	–0.1	0.4	–0.9	0.9						
	RDW	–0.6	3.7	24***	21.8***						
7.5	RBC	–5.9	–6.8		4.1*	9**	11.6				
Males	HGB	–9.8	–8.3		–0.3	4.3**	6.6				
Group 3	HCT	–7.5	–6.5		3.1*	5.9**	6.7				
	MCV	–1.7	0.3		–0.9	–3*	–4.5				
	RDW	–1.5	4.8		14.9**	11**	8.8				
7.8	RBC	–14.9	–19.4	–20.6**	–14.1***						
Females	HGB	–15.7	–19.2	–19.1**	–11.7***						
Group 2	HCT	–14.6	–18.4	–17.9***	–6.9***						
	MCV	0.3	1.4	4	8.6*						
	RDW	0.6	17.5	66.3*	59.1***						
10.4	RBC	–10.7	–17.6		–9.9**	–6.4*	–4.9				
Females	HGB	–10.6	–16.7		–8.1***	–2.7	–0.8				
Group 3	HCT	–10.6	–16.1		–4.8**	0.1	0.4				
	MCV	0	1.7		5.7*	6.8**	5.5**				
	RDW	0.1	11.5		46.1**	40***	35.7***				
11.3	RBC	–10.8	–14.4		–1	7.7**	9.3***	9.9***	10.9***	10.6***	12.7***
Males	HGB	–12.8	–16.5		–1.9	6.5**	7.5**	4.8**	6.2***	4.2**	3.8*
Group 4	HCT	–11.5	–14.3		1.2	7.4**	6.9**	3.9***	4.3**	0.1	0.4
	MCV	–0.9	0.1		2.2**	–0.3	–2.2**	–5.5**	–6***	–9.6***	–11***
	RDW	0.8	14		27.6***	20.1**	17.5***	15.4**	14.3**	12.8***	12.2**
15.1	RBC	–11.6	–18.9		–2.4*	3.9**	1.6	6.1***	2.8***	6.3***	9**
Males	HGB	–13.7	–19.7		–2.6*	2.2*	–0.5	2.5	–0.1	0.4	3.1
Group 5	HCT	–12	–17.3		1.3	3.5**	0.6	1.5	–1.5	–2.8*	–0.5
	MCV	–0.4	1.9		3.9	–0.3	–0.8	–4.2**	–4.1**	–8.5***	–8.5***
	RDW	–0.2	14.1		29.6***	20.5***	18.2***	13.7***	13***	12.3***	11.9***
15.6	RBC	–15.4	–24		–12.2***	–6.3**	–7.3**	–3.8*	–6.1***	–4.4**	–2.2*
Females	HGB	–17.3	–24.5		–10.3***	–3.1*	–4.4*	–2.2	–3.3**	–2.2	–2.4
Group 4	HCT	–15.7	–23.2		–6.2**	0.5	–1.9	–0.9	–2.8**	–3.4**	–2.2*
	MCV	–0.4	1		6.8***	7.3***	5.7***	3.1*	3.5***	0.9	–0.1
	RDW	1.7	19.3		59.6***	42***	37***	29.7***	27.1***	22.9***	18.9**
20.8	RBC	–24.8	–31.2		–17.9***	–12.5**	–13.3***	–7.8**	–9.6***	–6.2*	–5.2**
Females	HGB	–26	–31		–12.5***	–8.1**	–7.8*	–3.4	–4.9**	–3.4	–4.3*
Group 5	HCT	–25.8	–29.6		–7.1**	–5.3	–5.2	–2.6	–4.8**	–4.6*	–3.9**
	MCV	–1.2	2.5		13.3***	8.4**	9.5**	5.7**	5.3**	1.9	1.6
	RDW	4.3	49.9		83.9***	58.9***	53***	42***	36.1***	31.7***	27.5***

*,**,***: Statistically significant with $p = 0.05$, 0.01 and 0.001, respectively.

removal 7.8%), the decreases of RBC, HGB, HCT, present on day 5 were approximately of the same amplitude as those recorded on day 3. On day 8, a decrease was still present but with clearly reduced amplitude as compared to day 5 except for an increase of approximately 60% in RDW on both days 5 and 8.

Groups 3 to 5: In males and females a return to normality of RBC, HGB, HCT was present from day 8 onwards. Increases in MCV and RDW peaked on day 8 with an amplitude proportional to the amount of blood removed and then decreased with time. In male groups (11.3 and 15.1%), from day 12 onwards, the MCV even slightly decreased with a maximum amplitude observed on days 26 and 29.

Discussion

Laboratory diagnosis of anaemia is chiefly based on a reduction of the main red blood cell parameters RBC, HGB and HCT (McGrath 1993), and these are also core haematology measurements in toxicological studies (Andrews 1998). In our view, when considering variations of these three parameters, only a decrease greater than 10% relative to basal level should be considered as having a biological significance. Based on this principle, removal of up to 7.5% of blood (corresponding to 1.6 ml in the male rats) over 24 h did not have any biological effects on red blood cell values, and a complete recovery was obtained 48 h after sampling (day 3). At this time point, removal of higher quantities produced a significant effect on haematological parameters. The group 2 females showed a partial recovery on day 8 with haematocrit values approximately 7% below the day 1 value.

In groups losing from 10.4% to 15.1% of blood, a total recovery of HCT values was obtained on day 8, whereas in groups losing 15.6% and 20.8 % (2.4 ml and 3.2 ml in the female rats), recovery was not reached until days 12 and 19, respectively.

The nature and extent of effects on core haematological parameters were similar to those reported by other authors and showed that, after an initial blood loss, recovery periods lasted from 24 h to 4 weeks according to the sampling regime (single or multiple samplings over 24 h) and the amount of blood removed. Generally, it is recognised that mammals can tolerate roughly a 10–15% reduction in blood volume before marked and irreversible haemodynamic changes take place (Redondo et al. 1995). Scipioni et al. (1997) came to the conclusion that removal of up to 40% of a rat's total blood volume over a 24-hour period, and repeated 2 weeks later, caused no gross ill effects.

The heterogeneity of erythrocyte size as measured by RDW peaked on day 8 and corresponded to an increase of MCV in groups where higher blood volumes were taken. This may be explained by the simultaneous presence of a small number of young cells with a majority of mature erythrocytes.

It is noteworthy that in females the expected trend toward increased MCV values (spleen or bone marrow production of young and large erythrocytes) was widely present whereas in most male groups there was a decrease in MCV over time. The mechanism underlying this sex-dependent finding is unknown and was not reported in the aforementioned publications. A progressive, non-compensated loss of iron may have occurred as the animals were bled many times during the course of the study (up to 12 times) giving rise to microcytic red cells (Bertram et al. 1991). Further studies seem necessary to confirm this hypothesis. However, in the present study, on the basis of clinical observations and the core haematological data, the health of the animals was not impaired.

Thus the acute effect of blood removal over 24 h on the main haematological parameters consisted of decreases in RBC, HGB and HCT which were similar for blood removal exceeding 7.5% but below 15% of circulating blood volume and were positively related to the amount of blood loss beyond 15%. There were no biologically significant effects on these parameters below 7.5%. Time to recover was also proportional to the volume taken at the initial bleed, and was estimated to range from 48 h for amounts between 5% and 7.5%, 12 days for amounts between 7.5% and 20%, and 19 days for amounts above 20%.

Therefore, the collection of up to 20% of the circulating blood volume over 24 h did not affect the welfare of rats as indicated by the absence of mortality and clinical signs and the lack of effects on body weight and food consumption.

CONCLUSION

Anaesthesia should be deep enough to prevent pain to the animals and the anaesthetic should not interfere with the parameters evaluated. The duration of exposure should be limited to avoid effects on clinical pathology parameters due to hypoventilation. The choice of the site of blood sampling is dependent on the volume of blood needed, the frequency of sampling, the parameters measured and the skill of the technical staff. The volume of blood removed is dependent on the frequency of sampling and should not exceed 20% of total blood volume.

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