Blood sampling from the retro-orbital plexus, the saphenous vein and the tail vein in rats: comparative effects on selected behavioural and blood variables

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Summary

We compared the behaviours of rats, and measured various blood parameters, after three blood sampling techniques: orbital puncture while they were under diethyl-ether anaesthesia, blood collection by tail vein puncture under O_2-N_2O-halothane anaesthesia and puncture of the saphenous vein without anaesthesia. Twelve rats were subjected to the three treatments according to a Latin square design. After each treatment, the behaviour of the rats was automatically monitored using the so-called LABORAS^{TM} method, which discriminates between grooming, locomotion and inactivity in rats. Based on excitation scores and urine production, it was found that induction of diethyl-ether anaesthesia combined with orbital puncture caused more distress than did the other two blood sampling techniques. The three techniques had no differential effects on the behaviours of grooming, locomotion and inactivity. Collecting 0.5 ml of blood by orbital puncture was ± 7 times faster than doing so by saphenous vein puncture and ± 15 times faster than collecting blood by tail vein puncture while the rats were under O_2-N_2O-halothane anaesthesia. The levels of some haematological and plasma variables differed significantly between the three blood collection techniques. These observations may help to select the most appropriate technique of blood sampling with respect to anticipated discomfort in the animals.

Keywords Rat; discomfort; blood collection; orbital puncture; retro-orbital puncture; vena saphena; tail vein; behaviour; LABORAS; haematology; clinical chemistry

Blood can be sampled from animals using different techniques with differing impacts on animal discomfort due to differences in handling, restraining, anaesthesia, invasiveness and the volume taken. The method of blood sampling can also affect the outcome of blood analysis. Orbital puncture is frequently used to obtain blood samples from rats.

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However, the technique is controversial because it possibly causes pain and distress to the animals and because of aversion amongst experimenters (van Herck *et al.* 1992a; van Herck *et al.* 1999). The BVA/FRAME/RSPCA/UFAW joint working group has stated that orbital puncture is acceptable only as a terminal procedure while the animal is under anaesthesia (Morton *et al.* 1993). For non-terminal blood sampling in rats, the working group advises collecting a maximum

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of 0.5 ml of blood by puncturing the tail vein while applying a short-acting anaesthetic to ease the rats. Hem *et al.* (1998) prefer to puncture the saphenous vein.

Orbital puncture causes tissue damage (van Herck *et al.* 1992b, Beynen *et al.* 1988), the severity of which depends on the technique and skill of the person performing the puncture (van Herck *et al.* 1998). In addition to tissue damage, behaviour may serve as an indicator of discomfort (Barclay *et al.* 1988). In a previous study, we found that orbital puncture, while the rats were under diethyl-ether anaesthesia decreased locomotor activity and diminished the frequency of inactivity periods during the dark period (12 h) as assessed with an automatic system for recording and analysing behaviour (van Herck *et al.* 2000). Although the impact of these behavioural changes on discomfort cannot be objectively determined in absolute terms, we felt that comparison of the effects of orbital puncture with those of tail vein puncture under O_2-N_2O-halothane anaesthesia and saphenous vein puncture without anaesthesia in a study with the same experimental design could provide us with clues as to the appropriate blood sampling techniques in rats. Apart from comparing the effects on behaviour, we also compared the outcomes of blood analyses selected to give an insight into the effect of these procedures on the animals.

Animals, materials and methods

The research project was approved by the Animal Ethics Committee of the Utrecht Faculty of Veterinary Medicine and the protocol of the present experiment by that of the Department of Laboratory Animal Science, Utrecht University.

Animals, housing and diet

Twelve male, virus-antibody-free U:WU(Cpb) rats, aged 8 weeks, were housed individually in Macrolon cages type III, provided with a layer of sawdust (3/4; BMI, Helmond, The Netherlands). The animals had free access to tap water and pelleted food (RMH-B®; Hope Farms BV, Woerden, The Netherlands). The cages were placed in a room with relative humidity of 40–70%, temperature of 20–24°C and a ventilation rate of 15–20 air changes per hour. The light period was between 23:15–11:15 h with a light intensity in the cage of 60–80 lux. The change from light to dark was gradual, between 11.15–11.45 h, and vice versa between 23:15–23:45 h. Daily care for the animals was provided between 09:00 and 11:00 h.

Experimental design

From the first day of the acclimatization period, which lasted 8 days, the animals received a clean cage daily. The experiment had a balanced Latin square design (Table 1) with three treatments, i.e. orbital puncture while the rats were under diethyl-ether anaesthesia, tail vein puncture while they were under halothane anaesthesia, and puncture of the lateral saphenous vein without anaesthesia. The treatments were performed at 48 h intervals. Three technicians, each experienced in an individual technique (van Herck *et al.* 1998) performed all the blood collections by orbital puncture, tail vein puncture and saphenous vein puncture. Technician 1 was experienced in technique A and only performed technique A, Technician 2 was experienced in technique B and only performed technique B, Technician 3 was experienced in technique C and only performed technique C. Treatment took place on Monday (Day 1), Wednesday (Day 3) and Friday (Day 5) between 10:45 and 11:15 h. During treatments, 0.5 ml of blood was collected in Eppendorf tubes coated with heparine (25 IU), as were the tips of the Pasteur's pipettes used for orbital puncture.

For treatment, the animals were transported one by one, while in their home cage, to an adjacent room with the same ambient temperature as the animal room. If appropriate, they were anaesthetized in a Perspex™ (Plexiglass™) anaesthesia box ($25 \times 15 \times 15$ cm, l × w × h) after the box was flushed for 1 min. For orbital puncture the box was flushed with room air that had been led through a bottle containing diethyl-ether and water. For tail vein puncture it was flushed with a mixture of O_2 (1 l/m), N_2O

Table 1 Treatments per animal

			Sensing platform*			
Week	Day	Period	A Rat 01	B Rat 02	C Rat 03	D Rat 04
0	Sunday	Day prior to first treatment				
1	Monday	Treatment	AP	A	SA	SA
	Tuesday	Day after treatment				
	Wednesday	Treatment	A	AP	A	AP
	Thursday	Day after treatment				
	Friday	Treatment	SA	SA	AP	A
	Saturday	Day after treatment				
			Rat 05	Rat 06	Rat 07	Rat 08
	Sunday	Day prior to first treatment				
2	Monday	Treatment	A	AP	AP	A
	Tuesday	Day after treatment				
	Wednesday	Treatment	SA	SA	A	AP
	Thursday	Day after treatment				
	Friday	Treatment	AP	A	SA	SA
	Saturday	Day after treatment				
			Rat 09	Rat 10	Rat 11	Rat 12
	Sunday	Day prior to first treatment				
3	Monday	Treatment	SA	SA	A	AP
	Tuesday	Day after treatment				
	Wednesday	Treatment	A	AP	SA	SA
	Thursday	Day after treatment				
	Friday	Treatment	AP	A	AP	A
	Saturday	Day after treatment				

*See `Behavioural observations after treatment' under `Animals, materials and methods' section. Overview of periods and treatments per animal: sham anaesthesia (SA), diethyl-ether anaesthesia (A) and anaesthesia plus orbital puncture (AP)

(1.5 l/m) and halothane set at the maximum concentration. The animal was taken out of the Perspex™ box as soon as the palpebral reflex had disappeared. Between treatments, the box was cleaned with warm water and dried with a paper tissue.

Orbital puncture was performed with a Pasteur's pipette (QTE 250, length 150 mm; Bilbate, Daventry, UK) as described by van Herck *et al.* (2000). Directly after sampling, blood was transferred from the pipette into the Eppendorf™ tube. For tail vein puncture, the rat was put on the edge of the table on a heating pad in a ventral position to enhance blood flow. Anaesthesia (O_2 1 l/m, N_2O 1.5 l/m, halothane vaporizer was set at 2) was continued throughout the procedure using a breathing mask made in-house. A 23 g × $1\frac{1}{4}$ needle was inserted into the vein at approximately 1/3 of the length of the tail from the tail base and blood was collected from the needle cone with the tail remaining on the heating pad. The saphenous vein was punctured without anaesthetizing the rats. Animals were restrained by putting the head and trunk in a tightly fitting, in-house-made, bag of towelling, leaving the hind legs free. One of the hind legs was immobilized in extended position by hand so that the vein was distended. The leg was shaved above the tarsal joint where the saphenous vein was visible underneath the skin. The vein was then punctured with a 21 g × $1\frac{1}{2}$ needle and the blood dripping from the puncture site was collected (Hem *et al.* 1998). As soon as 0.5 ml of blood was collected, a tissue was pressed on the punctured site of the saphenous vein and tail to prevent further

bleeding. If bleeding occurred after orbital puncture, it was stopped by gently pressing a gauze pad on the eyeball after closing the eyelids. During treatments, faeces and urine produced were collected on a paper tissue. Immediately after treatment, the animal was placed back in its home cage and transported to the test cage, which was placed on a sensing platform (see below).

Observations during treatment

Treatments were defined to start by placing the animal in the Perspex™ box for either orbital and tail vein puncture anaesthesia and by manually immobilizing the animal for saphenous vein puncture. Treatment was defined to end when bleeding stopped and the animal was placed back in its home cage. During treatment, the following variables were determined if appropriate: time needed to induce anaesthesia (palpebral reflex absent), duration of continued exposure to halothane outside Perspex box, time needed for recovery (righting reflex present), signs of excitation (0 = no excitation, 1 = slightly excited, 2 = clearly excited), the amount of faeces (no. of faecal boli and faecal weight) and urine produced (weight). Urine weight was determined as the weight increase of the paper tissue.

Analysis of blood samples

The blood in the heparinized Eppendorf™ tubes was stored at 0°C for haematological examination on the same day. Plasma was isolated by centrifugation (15 min, 3000 rpm) and stored at −20°C until chemical analysis. Haemoglobin concentration (Hb), haematocrit (Ht), red blood cell count (RBC), white blood cell count (WBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and number of platelets (PLT) were analysed with a blood cell counter (model K-1000, Sysmex IJsselstein, The Netherlands). This apparatus also calculated the mean corpuscular haemoglobin concentration (MCHC), K^+, Na^+, Ca^{2+}, pH (38°C), pO_2 (38°C), pCO_2 (38°C), and bicarbonate (HCO_3^-), which were analysed with an acid-base laboratory (ABL) type 505 Radiometer (Copenhagen, Denmark). This apparatus also calculated the standard bicarbonate (SBC) and actual base excess (ABE). Alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT) and alkaline phosphatase (ALP) were determined spectrophotometrically using a test kit (Roche Diagnostic Systems, Almere, The Netherlands) and a CobasBio automatic analyser (Roche Diagnostics, Basel, Switzerland).

Behavioural observations after treatment

Observations were performed from 11:30 h on Day 0 (Sunday) until 07:30 h on Day 6 (Saturday). The test cages were located in the animal room and were similar to the home cages, including bedding, food and water. They were placed on one of four sensing platforms of LABORAS™ (Laboratory Animal Behaviour Observation, Registration and Analysis System, Metris System Engineering, Hoofddorp, The Netherlands). LABORAS™ is a fully automated device for the recording and analysis of the behaviour of individually-housed mice or rats. The system consists of a triangular-shaped sensing platform, which is positioned on two orthogonally placed sensors and a third fixed point. A Macrolon type II cage (375 cm^2) or type III cage (840 cm^2) is placed on the sensing platform. Each sensor transforms the mechanical vibrations caused by the movements of the animal in the macrolon cage into electrical signals, which are amplified and filtered to eliminate noise and then stored on a computer. The software (Metris System Engineering, Hoofddorp, The Netherlands) contains an analysis and classification module, which processes the stored data and compares the signals with the predetermined characteristic vibration patterns and thus classifies the data into behavioural categories. LABORAS™ follows the movements continuously, but classifies these signals into behavioural categories every 10 s. Details of LABORAS™ are described by Bulthuis *et al.* (1997). Van de Weerd *et al.* (2001) have validated the LABORAS™ registrations by comparing them with data from observations of videotapes by human observers. Therefore, LABORAS™ is considered a reliable system for the automated registration of the behaviours 'grooming'

(shaking, scratching, wiping or licking fur, snout, ear or genitals), 'locomotion' (walking, running, jumping), and 'inactivity' (no movements while the animal is in a lying or sitting position; short movements, e.g. turning over while sleeping, are not considered an interruption) in rats. In this study, vibration patterns other than the validated ones were assigned to the category 'undefined' (results not shown).

Data processing and statistical analyses

The patterns of grooming, locomotion and inactivity on the day prior to the first treatment (Day 0) were comparable with previous results and the 20 h period was subdivided into the intervals 11:30–16:30 h, 16:30–21:30 h, 21:30–01:00 h and 01:00–07:30 h which had distinct activity patterns (van Herck *et al.* 2000). Behaviours were averaged per rat per treatment for either the whole 20 h period of observation (11:30–07:30 h), the dark period (11:30–23:15 h), part of the light period (23:15–07:30 h) or for the various intervals. For each behaviour, relative duration (s/h) and frequency (number/h) were calculated. Results in the tables are presented as means ± SD. For the behavioural measurements, treatment comparisons were done only within (but not between) the predetermined observational periods. The data for each measure, except for the excitation scores and the number of faecal boli, were subjected to MANOVA (repeated measurements) to identify treatment effects. For excitation score and number of faecal boli, the significance of the differences between treatments was calculated by Friedman two-way ANOVA, whereas the Student's one-sample *t*-test for paired data was used for the parameters 'time needed to induce anaesthesia' and 'time needed for recovery'. The pre-set P value was < 0.05. Day of observation, observation period and treatment were used as main within-subject factors. If treatment and/or day of observation after MANOVA (main effect and/or interaction) were found to have a significant effect ($P < 0.05$) on the behavioural measurements, the Student's one sample *t*-test for paired data was used to compare the three treatments and/or the days per treatment. To take into account the increased risk of a type I error due to multiple comparisons, the pre-set P value was reduced to $< 0.05/\text{number of meaningful comparisons}$ (Bonferroni correction) i.e. $< 0.05/3 = 0.0167$ for the 20 h period, to $< 0.05/4 = 0.0125$ for the dark/light periods and to $< 0.05/6 = 0.0083$ for the activity blocks. Pairwise comparisons of means for excitation score and number of faecal boli were performed with the Wilcoxon matched-pairs signed rank test if treatment after Friedman two-way ANOVA was found to have a significant effect on these parameters. The level of significance was then pre-set according to Bonferroni's adaptation at $P < 0.025$. If treatment after MANOVA was found to have a significant effect on faeces and urine production the Student's one-sample *t*-test for paired data ($P < 0.025$, Bonferroni correction) was used to compare the three treatments. All statistical analyses were carried out according to Steel and Torrie (1981), using a SPSS/PC + computer program (SPSS Inc. 1990).

Results

Observations during treatment (Table 2)

Total durations of treatments differed significantly, orbital puncture being the fastest and tail vein puncture being the slowest method. Preparation of the animal for blood collection was fastest for the saphenous vein puncture. An adequate level of anaesthesia was reached faster with diethyl-ether than with O_2-N_2O-halothane. The total time during which anaesthetics were administered was much shorter when collecting blood by orbital rather than by tail vein puncture. The time needed to collect the blood sample was shortest for orbital puncture and longest for tail vein puncture. The animals showed no signs of consciousness during orbital puncture. Righting reflexes returned well after completing the blood collection by orbital puncture.

Induction of diethyl-ether anaesthesia caused significantly more excitation and production of urine than either exposure to O_2-N_2O-halothane or manual restraining.

Table 2 Results of observations during treatment for orbital puncture, tail vein puncture and saphenous vein puncture (type of anaesthesia in parenthesis)[1]

	Orbita (diethyl-ether)	Tail vein (O_2-N_2O-halothane)	Saphenous vein (no anaesthesia)
Anaesthesia or immobilization			
Induction (s)	$64.25 \pm 13.22^{a,b}$	$95.00 \pm 18.10^{a,c}$	$40.92 \pm 8.28^{*\,b,c}$
Continued exposure (s)	–	327.33 ± 125.68	–
Exposure total (s)	64.25 ± 13.22^{a}	422.33 ± 139.32^{a}	–
Recovery (s)	37.58 ± 11.76	35.42 ± 12.96	–
Blood collection (s)	$18.42 \pm 7.24^{a,b}$	$283.42 \pm 120.57^{a,c}$	$122.92 \pm 71.62^{b,c}$
Duration of treatment (s)	$101.83 \pm 20.66^{a,b}$	$457.75 \pm 139.33^{a,c}$	$195.67 \pm 66.22^{b,c}$
Distress			
Excitation (score 0, 1, 2)	$1.75 \pm 0.05^{a,b}$	0.25 ± 0.45^{a}	0.58 ± 0.51^{b}
Faeces production			
(No. of boli)	2.08 ± 1.16	0.92 ± 1.16^{a}	2.33 ± 1.30^{b}
(g)	0.67 ± 0.40	0.26 ± 0.29^{a}	0.76 ± 0.43^{b}
Urine production			
(g)	$0.63 \pm 0.34^{a,b}$	0.12 ± 0.15^{a}	0.07 ± 0.14^{b}

* Time needed to prepare the animal for blood collection (immobilization and shaving); [1]Results are expressed as means ± SD ($n = 12$). Within rows, values with the same letter are significantly different

During anaesthesia, excitation and production of faeces and urine was limited to the first half minute of the induction phase, whereas it occurred throughout the whole treatment period of the saphenous vein puncture.

Blood parameters (Table 3)

Between treatments no significant differences were found for ASAT, Ca^{2+}, HCO_3^-, WBC, MCV, MCH and PLT. Blood collected by saphenous vein puncture had higher values for RBC, Hb and Ht than that collected by either orbital or tail vein puncture. Hb found in tail vein blood was lower than in orbital blood. pCO_2 and Na^+ were significantly higher and pH, K^+, ABE and SBC were significantly lower in blood collected by orbital puncture. K^+, ALP, ALAT, and MCHC values were significantly higher in blood of the saphenous vein than in that of the tail vein. The pO_2 was significantly higher in blood collected by tail vein puncture while the rats were under halothane anaesthesia.

Behavioural observations after treatment

The observations after orbital puncture in this study were similar to those found in a previous study (van Herck *et al.* 2000). The behaviours, grooming, locomotion and inactivity were not significantly influenced by the type of treatment (data not shown).

Discussion

In this study we compared three different techniques to collect a single blood sample of 0.5 ml in rats. Tail vein puncture took more time than the orbital puncture technique. The time needed to perform saphenous vein puncture was intermediate. The total time needed to collect blood can be subdivided into time needed to immobilize, fix and shave the animal and/or induce anaesthesia, and time needed for drawing the blood. Both the time needed to induce anaesthesia and/or produce immobilization and that needed to collect the blood differed significantly between the three techniques. Preparation for subsequent punctures of the saphenous vein took less time, because shaving was not needed again until the fur had regrown. O_2-N_2O-halothane anaesthesia was continued during collecting blood from the tail vein whereas diethyl-ether exposure was ended before collecting blood by orbital puncture. Thus, total exposure time for halothane was significantly longer than that for diethyl-ether. Despite this difference, the time to regain the righting reflex was similar after diethyl-ether and halothane anaesthesia.

Table 3 Results of analyses of blood samples obtained by orbital puncture, tail vein puncture and saphenous vein puncture (type of anaesthesia in parentheses)[1]

	Orbita (diethyl-ether)	Tail vein (O_2-N_2O-halothane)	Saphenous vein (no anaesthesia)
Haematology			
Hb (g/l)	$149.8 \pm 6.4^{a,b}$	$143.4 \pm 8.1^{a,c}$	$162.7 \pm 6.4^{b,c}$
Ht (l/l)	0.50 ± 0.05^{a}	0.49 ± 0.03^{b}	$0.55 \pm 0.02^{a,b}$
MCV (fl)	61.0 ± 2.0	60.9 ± 2.1	60.8 ± 2.1
MCH (pg)	18.3 ± 2.5	17.7 ± 0.6	17.9 ± 0.5
MCHC (g/l)	301.3 ± 41.9	291.6 ± 4.8^{a}	294.8 ± 4.8^{a}
WBC ($\times 10^9$/l)	13.0 ± 3.2	12.9 ± 2.1	10.5 ± 3.7
RBC ($\times 10^{12}$/l)	8.21 ± 0.78^{a}	8.06 ± 0.32^{b}	$9.08 \pm 0.45^{a,b}$
PLT ($\times 10^9$/l)	172 ± 202	168 ± 174	167 ± 180
Clinical chemistry (plasma)			
ALP (U/l)	149.94 ± 29.91	143.96 ± 25.95^{a}	161.05 ± 25.62^{a}
ALAT (U/l)	26.97 ± 3.28	25.95 ± 3.15^{a}	30.45 ± 5.31^{a}
ASAT (U/l)	58.82 ± 10.09	50.15 ± 11.34	60.87 ± 16.25
pH 38°C	$7.40 \pm 0.03^{a,b}$	7.50 ± 0.03^{a}	7.46 ± 0.04^{b}
pCO_2 38°C (kPa)	$6.4 \pm 0.5^{a,b}$	5.0 ± 0.6^{a}	5.4 ± 0.7^{b}
pO_2 38°C (kPa)	6.1 ± 1.5^{a}	$12.1 \pm 3.1^{a,c}$	7.8 ± 1.9^{c}
K^+ (mmol/l)	$4.4 \pm 0.4^{a,b}$	$5.0 \pm 0.4^{a,c}$	$6.3 \pm 0.7^{b,c}$
Na^+ (mmol/l)	$140 \pm 1^{a,b}$	138 ± 2^{a}	137 ± 2^{b}
Ca^{2+} (mmol/l)	1.14 ± 0.10	1.08 ± 0.07	1.07 ± 0.06
HCO_3^- (mmol/l)	27.6 ± 1.3	27.8 ± 2.1	28.2 ± 2.7
SBC (mmol/l)	$26.3 \pm 0.9^{a,b}$	28.8 ± 1.5^{a}	28.4 ± 2.3^{b}
ABE (mmol/l)	$2.6 \pm 1.3^{a,b}$	5.0 ± 1.6^{a}	4.7 ± 2.4^{b}

[1]Results are expressed as means ± SD ($n = 12$). Within rows, values with the same letter are significantly different ($P < 0.025$, paired two-tailed Student's *t*-test)

Orbital puncture combined with diethyl-ether anaesthesia produced the most severe signs of distress, as determined by excitation and production of urine. These signs however, were limited to the first half minute of the time needed to induce adequate diethyl-ether anaesthesia, whereas they occurred throughout the entire treatment procedure of puncturing the saphenous vein. The three blood sampling techniques caused no significant differences in the behaviours of grooming, locomotion and inactivity. In a previous study with an identical number of rats, we showed that orbital puncture, while the rats were under diethyl-ether anaesthesia versus sham anaesthesia, reduced the frequency and relative duration of locomotion and also the frequency of inactivity (van Herck *et al.* 2001). It thus appears that the three techniques produced similar behavioural changes, when compared with no treatment. When judged on the basis of behavioural changes, the degree of discomfort caused by the three techniques of blood sampling was similar.

The three blood collection techniques used differ with regard to the punctured blood vessels and surrounding tissues, and also with regard to the method of immobilization, the type of puncture needle and the technician performing the technique. Apart from the degree of anticipated discomfort in the animals, the technique of blood sampling may also be chosen on the basis of the blood volume needed and the requirements for blood analyses. It is important to consider any possible differences in these measured blood constituents caused by the technique of collection, to avoid increased experimental variation. This study was not designed primarily to measure these differences, but analyses carried out to assess the effect of the procedure on the animal will also highlight any differences for future study.

In rats, K^+ is mainly an intracellular and Na^+ mainly an extracellular ion. Lower K^+ and higher Na^+ levels in plasma point at better separation of the intra and extracellular compartments due to less erythrocyte damage during blood collection.

With the initial hypothesis that orbital puncture would more likely cause more tissue damage (McGee & Maronpot 1979, Krinke *et al.* 1988, van Herck *et al.* 1992b) than venepuncture, higher K^+ values and lower Na^+ values might be expected from orbital puncture using a pipette compared to venepucture via a needle. Our results of lower K^+ with orbital puncture corroborate those of Schwabenbauer (1991) who found higher levels in blood samples obtained by tail vein puncture compared with orbital puncture. The observed K^+ level in plasma from blood obtained by orbital puncture while the rats were under diethyl-ether anaesthesia coincides with the K^+ level of plasma from arterial blood (4.4 mmol/l) found by Braam *et al.* (1994) in rat's blood obtained from the cannulated A. femoralis. The Na^+ level was slightly, but significantly, higher in the blood samples obtained by orbital puncture, when compared with tail vein and saphenous vein blood. It would follow that orbital puncture affected the barrier between the intra and extracellular compartments least and saphenous vein puncture most.

One possible explanation for these results is that the contraction of skeletal muscle cells causes a rise in plasma K^+ (McKenna 1995). Both hyperventilation and isotonic as well as isometric muscle contractions could be expected to occur during the induction phase of O_2-N_2O-halothane anaesthesia. Furthermore, immobilizing conscious rodents is very stressful to these animals and is therefore often used to study stress responses. In rats, it may even be more distressing than footshock stress of the same duration (Okazaki *et al.* 1995). Given the stressfulness of immobilization, especially isometric, but also isotonic muscle contractions could be anticipated throughout the procedure of collecting blood samples from the saphenous vein.

Support for this hypothesis comes from examination of other data. Compared with orbital blood samples, the samples obtained by tail and saphenous vein puncture gave significantly higher pH, SBC and ABE values and significantly lower pCO_2 values. A literature search revealed no reference values for venous blood pH, pCO_2 and HCO_3^- in rats. If we take the normal values of human venous blood as a reference (pH: 7.32–7.43, pCO_2: 5.1–6.7 kPa, HCO_3^-: 23–30 mmol, Jacobs 1996; ABE: −3.3–2.7 mmol/l, extrapolated from the Siggaard-Andersen alignment nomogram), the data obtained with the saphenous and tail vein blood samples would show an alkalosis with a metabolic (higher SBC and ABE) and for the tail vein also respiratory (decreased pCO_2) origin. Hyperventilation is the most common cause for a decreased pCO_2 (Houpt 1993). As the time needed to collect the blood samples is far too short to excrete sufficient amounts of acid (H^+ ions) via the kidneys, the increase of SBC and ABE should be caused by a change in metabolism. Muscle contractions have a high energy expenditure, which can be generated by aerobic catabolism, provided enough oxygen is available. This catabolism can increase the SBC and ABE (Dr F. Boshouwers, personal communication). The extra O_2 administered during O_2-N_2O-halothane anaesthesia is the most likely reason for the higher pO_2 noted during tail vein collection. Although possibly not all are biologically relevant, the results of the examination of blood parameters indicate the need for further investigation.

In conclusion, the induction of diethyl-ether anaesthesia before orbital puncture caused significantly more visible distress than did either the induction of O_2-N_2O-halothane anaesthesia needed for tail vein puncture or manual fixation combined with saphenous vein puncture. The three blood sampling techniques had no differential effects on the behaviours of grooming, locomotion and inactivity. Of the three methods, orbital puncture appeared to be the fastest technique. It produced the lowest plasma K^+ and highest Na^+ levels, possibly indicating that it caused lesser erythrocyte damage. The acid-base equilibrium of the blood samples indicated that saphenous and tail vein puncture might have induced a slight alkalosis that might be stress related.

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