

Effect of Administration of Copper plasma Activated water and Anesthetic on Hematological and Pancreatic tissues of Wister rats

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Abstract

In biomedicine, applications of PAW span from biofilm removal, wound healing, deactivation of bacteria and viruses, dentistry (for teeth disinfection and whitening), and cancer therapy. The biochemical activity of PAW is derived from synergistic effects of the highly reactive species, specifically reactive oxygen and nitrogen species (RONS). PAW is considered a biofriendly and prospective solution for biotechnology applications due to the time dependent nature of its biochemical activity because of the active species, and its economic and environmental benefits of using air rather than toxic chemicals as the raw material. Thus, the study on Effect of Administration of Copper plasma Activated water and Anaesthetic on Haematological and Pancreatic tissues of Wister rats. Material and Methods: Preparation of plasma-activated water a non-thermal micro-hollow cathode discharge (MHCD) was used to generate plasma-activated waters (CU-PAWs). Results: The results showed the haematological profile of the animals treated with 400mgkg⁻¹ had a decrease in Haemoglobin, Mean capsular Haemoglobin (11.21 ± 1.23 and 22.93 ± 21.11) compared to the controlled group (15.03 ± 1.72 and 29.93 ±

7.27) and in animals treated with 200mgkg⁻¹ and 400mgkg⁻¹ there is a significant decrease in the platelets (21.96 ± 23.16 and 20.89 ± 24.03) compared to animals on control group (32.08 ± 16.70) (table 2). Animals treated with 200mgkg⁻¹ and 400mgkg⁻¹ have a decrease in platelet (22.36 ± 4.88 and 20.89 ± 24.03) compared to animals on control (32.08 ± 16.70) and also animals treated with 400mgkg⁻¹ have an elevated white blood cell count ($173-56 \pm 4.50$) compared to the control group (3.66 ± 3.06). Conclusion: Copper plasma water is safe to be used since it do not have much effect on the blood biochemistry and haematological parameters unlike the aesthetic agents cause changes on the haematological and blood biochemistry parameters. Hence, it is important to be aware of the effects of these agents before using them in experiments.

Keywords: Effect, Administration, Copper, plasma, Activated, water, Anaesthetic, Haematological, Pancreatic, tissues, Wister rats

INTRODUCTION

Plasma activated water (PAW) produced by interaction of non-thermal plasma and water possess outstanding biological applications in agricultural and biomedical applications. PAW possess highly potent biological applications in fields of agriculture and biomedicine. In the field of agricultural applications, owing to its biochemical activity, PAW is used to increase the rate of germination of seeds and subsequent growth of the seedlings and plants, inactivate plant-based pathogenic organisms and cure fungus-infected plants. In biomedicine, applications of PAW span from biofilm removal, wound healing, deactivation of bacteria and viruses, dentistry (for teeth disinfection and whitening), and cancer therapy. The biochemical activity of PAW is derived from synergistic effects of the highly reactive species, specifically reactive oxygen and nitrogen species (RONS). The RONS in PAW include long-lived species as nitrates (NO_3^-), nitrites (NO_2^-), hydrogen peroxide (H_2O_2) and ozone (O_3), with

corresponding half-lifetime of years, several days, $\sim 10^4$ s, and several to dozens of minutes, and short-lived ones: hydroxyl radicals ($\text{OH}^\bullet$), nitric oxide ($\text{NO}^\bullet$), superoxide (O_2^-), peroxynitrate (OONO_2^-) and peroxynitrites (ONOO^-), the half-lifetimes of which have been proved as 1 ns, few seconds, 1.5 s and less than 1s, respectively.

Furthermore, PAW is considered a biofriendly and prospective solution for biotechnology applications due to the time dependent nature of its biochemical activity because of the active species, and its economic and environmental benefits of using air rather than toxic chemicals as the raw material [1].

It has been confirmed that the biological activity of PAW is directly related to the chemistry and relative concentration of RONS, which are produced in the aqueous medium or at the interface between the gas and liquid phases. The production of plasma induced RONS depends on different typical parameters such as types of plasma power supply, type of carrier gas, electrode configuration, applied voltage, voltage polarity, treatment time, gas flow, the volume of solution, the distance between electrode and liquid surface [1].

Plasma activated water (PAW) is a relatively new and innovative technology that has gained attention in various industries, including the poultry industry. PAW is created by subjecting water to a plasma discharge, that generates chemically reactive species such as reactive oxygen species and reactive nitrogen species, which makes PAW acidic [2-5]. These species can have various antimicrobial and disinfectant properties, making PAW potentially useful in poultry farming and processing [6,7,8].

The Plasma activated water (PAW) antibacterial modes of action were reviewed by Zhao et al. [16] and subdivided into 4 main modes of action as follow: First, primary and secondary reactive oxygen and nitrogen species (RONS) are generated in PAW, leading to reduced pH, increased ORP, and electrical conductivity, which cause physical stress on microbial cells. Second, these RONS induce oxidative stress, damaging the peptidoglycan structure of the cell wall and initiating lipid and protein peroxidation on the cell membrane.

Several studies have shown that PAW is non-toxic and safe for use in animals including person [9]. Plasma activated water (PAW) is a promising technology for pharmaceutical industry, but its safety has not been assessed before in human.

It was also reported that plasma is the fourth state of matter (after solid, liquid and gas). The macroscopic temperature of non-thermal equilibrium in plasma can be as low as 300 K, which is close to room temperature (non-thermal equilibrium plasma is also called cold plasma). As the generation of cold plasma does not require a special vacuum environment and can occur under atmospheric pressure, it is also referred to as cold atmospheric plasma (CAP). The main chemically active groups in CAP are ions, free electrons, neutral particles,

free radicals, and electromagnetic waves (UV, electric field, and visible light) [11,12]. The active species produced by CAP mainly include reactive oxygen species (ROS) and reactive nitrogen species (RNS), including H_2O_2 , OH , O_2 , O_3 , NO_2 , NO_3 , NO , and ONOO [13-18].

The objectives of this study is to evaluate the Effect of Administration of Copper plasma Activated water and anesthesia on Hematological and Pancreatic tissues of Wister rats. Using two working gases (air and oxygen) to generate plasma and treated Deionized (D.I) water for specified times to create PAWs,

MATERIAL AND METHODS

Preparation of Copper Plasma Activated Water (CU-PAW)

Preparation of plasma-activated water a non-thermal micro-hollow cathode discharge (MHCD) was used to generate plasma-activated waters (CU-PAWs) and the experimental set-up was similar to that reported previously [19]. In this study, the MHCD was operated at atmospheric pressure by using two different gases (air and oxygen) as working gases. The gas flow and discharge power were controlled at 4 l per minute and 100 W, respectively.

During this study, deionized water (D. I. water) was used to generate PAW. Discharge plasma was generated by fixing the discharge probe 5.0 mm away from the surfaces of D. I. water in 50-mL conical tubes. After exposure to plasma for specified treatment time (10, 15, and 30 min), those plasma-activated waters were immediately used or collected in 15-mL conical tubes and stored in the refrigerator.

Detection of Copper

Detection of copper concentration within plasma-activated water, the concentration of copper and zinc within PAWs was detected by inductively coupled plasma optical emission spectrometry (ICP-OES). ICP-OES measure light emitted at element-specific wavelength from thermally excited analyte ions and atoms. The intensity of this emitted electromagnetic radiation can be converted into an elemental concentration by comparison to calibrated reference standards.

Delay Time Experiments

Delay time is the time that starts from when PAW was created by plasma until PAW was exposed to agent suspensions. To evaluate the effect of delay time, three different time

points were selected (0 min, 1 day, and 14 days after plasma treatment). For 0-min delay time, PAW was exposed to bacterial suspensions immediately after plasma treatment. For 1- and 14-day delay time points, PAW was stored at 4 °C (in the refrigerator) before antibacterial activity assay.

Experimental Animals grouping

20 Albino Swiss rats of weight 120 -225g of the same sex were obtained from HAUEMM Animal House Federal Housing estate Bajabure Gerie Adamawa State. The animals were kept in constant 12 hours light and dark cycle and maintained at a temperature of 35°C ± 2°C for 14 days for acclimatization. They were maintained on standard animal pellets with enough water. The animals were randomly grouped into four groups of 5 rats each identified as group 1, 2, 3 and 4.

Experimental procedure

The rats were weighed just before the administration of CU-PAW and just before they were killed. The administration of the plasma water was performed via oral route of administration as follows:

Group 1 was the control group and was administer distilled water,

Group 2 was administered the CU-PAW of 100mgkg⁻¹, body weight

Group 3 was administered the CU-PAW of 200mgkg⁻¹ body weight

Group 4 was administered the CU-PAW of 400mgkg⁻¹ body weight

Over a period of 28days and at the end of the 29th day the rats were fasted overnight. The animals were subject to Deep anaesthesia with chloroform on the 30th day and the organs liver, spleen lung and kidney were obtained and immediately fixed in formaldehyde solution.

Blood Biochemistry and Haematology

Separate portions of blood were transferred to tubes containing either lithium heparin or trisodium EDTA as anticoagulants. Heparinized samples were centrifuged 1,900 × g at 4°C for 10 min and resulting plasma supernatants were transferred to fresh tubes for blood biochemistry analysis using a TBA-120FR automatic chemical analyser (Toshiba Medical Systems, Co., Ltd., Tochigi, Japan). EDTA blood was analysed for haematology using XT-2000iV (SYSMEX, Co., Ltd., Kobe, Japan).

Histopathology

Haematoxylin and eosin (HE) staining was used to assess pathological pulmonary changes after CU-PAW administration. Pulmonary tissue samples were fixed in 10% neutral buffered formalin. Preserved pulmonary samples then were trimmed, embedded in paraffin, sectioned at 3- μ m thicknesses, mounted on glass slides, stained with HE, and cover-slipped using standard methodologies. Confirmation of the effect of CU-PAW administration

Changes in haematological and biochemical parameters in the Control (N=5), CU-PAW Low (N=5), CU-PAW Mid (N=5) and CU-PAW High groups (N=5) were compared to those in the Ati group (N=5). Depth of anaesthesia was evaluated 10, 30, 45 and 60 min after injection to confirm anaesthesia induction time and sustained time. All groups, excluding the Control group, were administered atipamezole (Ati) 1 hr after the start of the experiment to provide recovery from anaesthesia. Blood samples (0.3 ml) were collected from the jugular vein at -24, 1, 4 and 24 hr after the start of the experiment. Following the final blood sample collection, haematological and blood biochemistry parameters were compared among groups. Clinical observations of body weight and food intake were measured 0, 2, 4, 6 and 24 hr after the start of the experiment. Food intake was defined as a decrease in food weight for 3 rats per cage (Table 5).

RESULTS AND DISCUSSION

Haematological Parameters

The results showed the haematological profile of the animals treated with 400mgkg⁻¹ had a decrease in Haemoglobin, Mean capsular Haemoglobin (11.21 ± 1.23 and 22.93 ± 21.11) compared to the controlled group (15.03 ± 1.72 and 29.93 ± 7.27) and in animals treated with 200mgkg⁻¹ and 400mgkg⁻¹ there is a significant decrease in the platelets (21.96 ± 23.16 and 20.89 ± 24.03) compared to animals on control group (32.08 ± 16.70) (table 2). Animals treated with 200mgkg⁻¹ and 400mgkg⁻¹ have a decrease in platelet (22.36 ± 4.88 and 20.89 ± 24.03) compared to animals on control (32.08 ± 16.70) and also animals treated with 400mgkg⁻¹ have an elevated white blood cell count 173.56 ± 4.50 compared to the control group (3.66 ± 3.06) (table 2), (21.96 ± 23.16 and 20.89 ± 24.03) compared to animals on control group (32.08 ± 16.56).

Table 2: The effect of CU-PAW on the haematological parameters of the rats after 29 days of treatment.

Parameters	Control (std)	100mg/kg-1	200mg/kg-1	400mg/kg-1
WBC (103 U1-1)	3.66 ± 3.06	4.31 ± 4.31	4.03 ± 0.50	173.56 ± 4.50
RBC (!) U1)	4.25 ± 0.14	14.70 ± 14.70	3.70 ± 0.63	3.97 ± 2.13
Haemoglobin (Gdl)	15.03 ± 1.72	14.70 ± 14.70	11.56 ± 0.84	11.21 ± 1.23
Heamatocrit (%)	42.5 ± 4.57	100.70 ± 1.70	35.6 ± 1.02	36.02 ± 2.23
MCV (FL)	80.86 ± 1.99	34.10 ± 34.10	97.33 ± 19.40	79.40 ± 24.02
MCH (pg)	29.93 ± 7.27	33.90 ± 33.90	31.96 ± 8.75	21.96 ± 23.16
MCHC (%)	33.73 ± 2.19	17.10 ± 17	32.40 ± 1.90	28.82 ± 7.19
Platletes	32.08 ± 16.70	3.80 ± 3.80	22.36 ± 4.88	20.89 ± 24.03

Table 3: The mean body weight of the treated and control group after 29days of treatment with CU-PAW.

Treatment	Day 0	Day 14	Day 28	% increase body weight
Control (std)	95.36±52.58	150.35± 3.8	189.33 ± 34.94	74.57
100mg/kg	148.41v 19.56	161.10± 18.16	217.8 ± 217.80	27.65
200mg/kg	180.02± 14.75	190.86±111.70	221.2 ± 44.98	14.45
400mg/kg	206.12v 25.06	209.14 ± 60.85	208.6 ± 87.19	1.38

The result is in mean ± sem

Table 4: The mean relative organ weight of liver and kidney after 29 days of treatment with CU-PAW

Organs (Ref Val)	Control (std)	100mg/kg-1	200mg/kg-1	400mg/kg-1
Liver (0.82)	0.39± 0.10	0.37±0.37	0.38±0.05	0.49±0.21
Kidney (0.76)	0.46 ±0.36	0.51±0.51	0.38±0.24	0.81±0.35

The effect of the U-PAW on the percentage average weight of the treated and control group after 29days of treatment. The results showed progressive increase in the average body weight of the animals in the control groups up to 74.57%. While the treated groups showed inverse proportion in the average body weight between 27.65% for group treated with 100 mg/kg to 1.3% for group treated with 400mg/kg (Table 3).

Mean relative organ weight (MROW) The mean relative organ weight of the control for both kidney and liver were not significantly different except for the group treated with 400 mg/kg which are both significantly higher than the normal group (table 4)

Table 5. Influence of I.P. and S.C. CU-PAW Administration.

Administration route	Group	No	Under Anaesthesia (mm)		Clinical Observation	Necropsy Observation
			Introduction Point (Heads/Groups)	End points (Heads/Groups)		
I.P	CU-PAW Low	1	10	60	Reacted at Atipamezole administration	Hydronephrosis of the left kidney
		2	No Introduction		No particular	No particular
		3	10	60	No particular	No particular
	CU-PAW Mid	4	5	60	No particular	No particular
		5	10	60	Rapid breathing	No particular
		6	10	60	No particular	No particular
	CU-PAW High	7	5	60	Rapid breathing Irregular respiration Dark skin	No particular
		8	10	60	Dark skin	No particular
		9	5	60	Piloerection Irregular respiration Dark skin	No particular
S.C	CU-PAW Low	1	10	60	No particular	No particular
		2	10	60	No particular	No particular
		3	5	60	No particular	No particular
	CU-PAW Mid	4	5	60	No particular	No particular
		5	5	60	No particular	No particular
		6	5	60	No particular	No particular
	CU-PAW High	7	5	60	Dark skin and white ear	No particular
		8	5	60	Rapid breathing Dark skin	Dead body discovery in 24 hr
		9	5	60	Rapid breathing Dark skin	A dark red point lay scattered in the pulmonary whole

Verification of the route of administration, Influence of CU-PAW route of administration on anaesthesia induction and endpoint, and clinical observations. Anaesthesia induction and endpoint were compared for i.p. and s.c. administration routes. After i.p. CU-PAW injection, anaesthesia was induced in all rats (except for 1 rat from the i.p. CU-PAW Low group), and the induction time ranged from 5 to 10 min, regardless of dose, and the end point of anaesthesia was 60 min (Table 5). In the s.c. CU-PAW Low group, anaesthesia induction time ranged from 5 to 10 min, whereas induction occurred at 5 min in all rats in the s.c. CU-PAW Mid and High groups. The endpoint for all s.c. groups was 60 min (Table 5).

All rats, regardless of the administration route, exhibited sustained urination while under anaesthesia. Clinical observations of animals under anaesthesia revealed rapid breathing, irregular respiration, and darkening of the skin in the CU-PAW High group (Table 5). Rapid breathing was observed in the i.p. CU-PAW Mid group. Additionally, 1 rat from the s.c. CU-PAW High group was found dead 24 hr after CU-PAW administration, and at necropsy dark red spots were observed across both lungs (Table 5). One rat from the i.p. CU-PAW Low group showed congenital hydronephrosis of the left kidney at necropsy (Table 5).

Table 6: Clinical Observations of Health Condition at Each Time Point After CU-PAW Administration

	Control (N=5)	CU-PAW (N=5)	CU-PAW Mid (N=5)	CU-PAW High (N=5)	Ati (N=5)
0–1 hr (Under anaesthesia)					
No abnormality	8	0	0	0	8
Rapid breathing	0	0	0	5	0
Irregular respiration	0	0	0	3	0
Deep respiration	0	0	0	2	0
Dark skin	0	0	0	2	0
Urination	0	8	8	8	0
2 hr					
No abnormality	8	8	0	0	8
Lying down but normal	0	0	4	7	0
Low activity	0	0	4	0	0
Lying down and immobility	0	0	0	1	0
4 hr					
No abnormality	8	8	0	0	8
Lying down but normal	0	0	8	1	0
Low activity	0	0	0	3	0
Lying down and immobility	0	0	0	4	0

Abnormal vocalization	0	0	0	1	0
6 hr					
No abnormality	8	8	3	0	8
Low activity	0	0	2	6	0
Abnormal vocalization	0	0	0	2	0
Low temperature	0	0	4	4	0
24 hr					
No abnormality		8	8	8	8
Necropsy					
No particular	8	8	8	6	8
A dark red point lies scattered	0	0	0	2	0

Blood biochemistry analysis 24 hr after CU-PAW administration revealed increased aspartate aminotransferase (AST) levels in the s.c. CU-PAW Mid group (74.0 ± 5.3 U/l) compared to those of the i.p. CU-PAW Mid group (57.0 ± 6.1 U/l, $P=0.0225$). Increased AST levels were also observed in the s.c. CU-PAW high group (100.0 ± 1.4 U/l) compared to those of the i.p. CU-PAW High group (61.7 ± 6.4 U/l, $P=0.0061$). Alanine aminotransferase (ALT) levels in the s.c. CU-PAW High group (42.5 ± 0.7 U/l) were higher than those of the i.p. CU-PAW High group (29.0 ± 2.0 U/l, $P=0.0030$) (Table 8). In contrast, no significant differences between groups were observed in haematological parameters (Table 8). We found statistically significant changes in other parameters including triglyceride (TG), total bilirubin (TBIL), GLUC, potassium (K) and reticulocyte (RET); however, these changes were not considered relevant because they were not dependent on anaesthesia dose.

Changes in clinical observation, body weight and food intake at 0, 2, 4, 6 and 24 hr after CU-PAW administration. The s.c. route of administration was chosen for all subsequent experiments because of the rapid breathing observed in the i.p. CU-PAW Mid group and the unstable anaesthesia induction in the i.p. CU-PAW Low group. Adequate depth of surgical anaesthesia was observed at all relevant time points after s.c. administration. Rapid breathing, irregular respiration, and darkening of the skin was observed in all rats in the CU-PAW High group (Table 6). No abnormalities were observed in the CU-PAW Low group 2 hr after CU-PAW administration. Conversely, low activity was observed 6 hr after CU-PAW administration, in 2 rats from the CU-PAW Mid group rats and 6 from the CU-PAW High group (Table 7). CU-PAW dosing resulted in dose-dependent effects on body weight (Supplementary Table 8), with significant decreases at 24 hr in the CU-PAW Low, Mid and High groups ($P=0.0254$, $P<0.0001$ and $P<0.0001$, respectively), Necropsy

observations revealed dark red spots across both lungs in 2 of the 5 CU-PAW High dose animals (Table 7).

Changes in blood biochemistry at -24, 1, 4 and 24 hr after CU-PAW administration in the CU-PAW Low group, GLUC ($P<0.0001$) and blood urea nitrogen (BUN) ($P=0.0406$) values were elevated 1 hr after CU-PAW administration (Table 7, and Table 8). GLUC ($P<0.0001$) levels remained significantly elevated 4 hr after.

Table 7: Changes in Blood Biochemistry At -24, 1, 4 and 24 Hr After CU-PAW Administration

	Control (N-6)			CU-PAW Low (N 6)				CU-PAW Med (N-6)				
	-24hrs	1hrs	4hrs	-24	1hr	4hrs	24hrs	24hrs	-24 hrs	1 hr	4 hrs	24hrs
AST (U/l)	80.3±20.4	75.8±16.6	86.5±21.3	78.5±20.5	70.3±11.7	61.3±12.6	73.1±12.3	72.8±15.6	71.8±14.9	64.3±12.8	76.5±10.1	89.0±17.4b)
ALT (U/l)	34.3±2.7	31.0±3.7	35.5±8.7	34.8±4.9	31.5±2.1	28.5±3.7	29.6±5.2	30.8±3.4	32.0±4.8	27.3±4.9	29.3±4.3	34.8±4.7
BUN (mg/dl)	19.2±1.7	17.5±1.9	15.3±2.0	17.8±1.7	19.1±1.1	19.3±1.8a)	14.9±1.8	19.2±1.0	18.6±2.6	18.0±2.0	13.3±2.4	17.9±2.9
GLUC (mg/dl)	149.8±7.0	140.5±16.9	138.5±13.7	140.3±17.2	148.0±12.0	347.5±20.7b)	186.6±15.9b)	133.3±7.1	143.3±8.9	358.3±21.3b)	184.0±13.0b)	127.3±8.3
TP (mg/dl)	6.1±0.2	6.0±0.2	5.8±0.3	6.1±0.1	6.1±0.4	5.8±0.2	5.9±0.3	5.9±0.2	6.0±0.3	5.5±0.2b)	5.8±0.3	5.8±0.2
Cl (mmol/l)	99.8±1.3a)	99.3±2.1	98.5±3.0	98.8±2.1	100.5±0.9	98.5±1.8	97.0±4.4	100.8±1.5	101.5±1.8	94.8±2.8b)	100.0±1.9	100.8±1.8
WBC (10 ³ /μl)	7.3±0.9	7.2±1.5	7.7±1.4	7.5±2.0	8.6±0.9	6.8±0.7	5.3±1.3b)	8.3±1.2	7.9±1.8	6.5±1.8	4.8±1.6b)	8.0±1.5
HGB (g/dl)	14.4±0.9	14.2±0.4a)	13.8±0.5	13.4±0.4	14.0±0.8	13.8±0.4	14.3±0.4	13.1±0.3	14.4±1.1	14.4±0.3b)	14.4±0.6a)	13.5±0.3
HCT (%)	41.7±2.4	41.4±1.3	40.2±1.5	39.6±1.2	40.9±2.1	41.3±1.3	42.3±1.6a)	38.9±0.9	42.0±2.7	43.5±0.8b)	42.7±1.4b)	39.8±1.1
LYMP (10 ³ /μl)	5.89±0.98	5.95±1.55	6.15±1.40	5.99±2.02	6.84±0.74	5.41±0.47	3.69±0.45b)	6.56±0.88	6.54±1.70	5.13±1.14	3.00±0.73b)	6.27±1.09
Insuline(ng/ml)	1.7±0.4	1.8±0.7	1.6±0.3	2.0±0.9	1.6±0.6	0.8±0.3b)	2.5±0.9b)	1.8±0.7	1.6±0.7	0.7±0.2b)	1.7±0.3	1.3±0.4

Table 8: Changes in Blood Biochemistry At -24, 1, 4 And 24 Hr After CU-PAW High and Ati Administration.

	CU-PAW High (N-5)					Ati (N-5)			
	-24hrs	1hr	4hrs	24hrs	-24hrs	-24hrs	1hr	4hrs	24hrs
AST (U/l)	72.5 ± 17.1	62.3 ± 5.9	84.4 ± 14.3	112.0 ± 6.7b)	70.8 ± 7.3	67.8 ± 6.7	81.0 ± 8.5	65.0 ± 7.4	72.5 ± 17.1
ALT (U/l)	30.5 ± 3.7	28.0 ± 4.1	38.4 ± 22.9	39.5 ± 5.0b)	32.8 ± 4.9	30.3 ± 4.2	30.4 ± 3.8	29.5 ± 4.0	30.5 ± 3.7
BUN (mg/dl)	20.0 ± 1.5	20.9 ± 1.2b)	20.0 ± 4.5b)	17.8 ± 2.1	18.6 ± 2.1	16.6 ± 2.9	13.3 ± 2.0	18.4 ± 2.3	20.0 ± 1.5
GLUC (mg/dl)	142.5 ± 8.7	399.8 ± 13.8b)	178.4 ± 10.6b)	130.3 ± 7.5	138.8 ± 10.3	139.5 ± 5.8	126.0 ± 4.7	137.5 ± 5.8	142.5 ± 8.7
TP (mg/dl)	6.1 ± 0.3	5.5 ± 0.3b)	5.7 ± 0.1	5.7 ± 0.2	6.2 ± 0.3	5.9 ± 0.2	5.7 ± 0.2	5.9 ± 0.3	6.1 ± 0.3
Cl (mmol/l)	100.8 ± 1.8	95.8 ± 2.0b)	99.6 ± 1.7	101.5 ± 0.9	102.0 ± 1.9	100.8 ± 1.0	100.1 ± 1.4	100.0 ± 1.5	100.8 ± 1.8
WBC (103/μl)	7.4 ± 1.7	5.8 ± 1.1	4.6 ± 1.0b)	7.7 ± 1.4	8.4 ± 1.8	6.2 ± 1.3	7.7 ± 1.2	7.4 ± 1.4	7.4 ± 1.7
HGB (g/dl)	14.6 ± 0.6	14.8 ± 0.5b)	15.2 ± 0.6b)	13.2 ± 0.4	14.2 ± 0.6	13.7 ± 0.5	13.7 ± 0.3	13.2 ± 0.6	14.6 ± 0.6
HCT (%)	42.6 ± 1.6	45.0 ± 1.4b)	45.6 ± 2.1b)	39.2 ± 1.4	41.3 ± 1.6	40.1 ± 1.1	40.1 ± 0.8	39.0 ± 2.0	42.6 ± 1.6
LYMP (103/μl)	6.24 ± 1.59	4.98 ± 0.90	3.08 ± 1.09b)	5.84 ± 1.19	6.74 ± 1.45	4.89 ± 0.84	5.96 ± 0.82	5.85 ± 1.19	6.24 ± 1.59
Insulin (ng/ml)	1.4 ± 0.6	0.7 ± 0.2b)	1.8 ± 0.5	1.4 ± 0.2	1.3 ± 0.3	2.0 ± 0.6	1.5 ± 0.5	1.7 ± 0.7	1.4 ± 0.6

Ati (N=6) The value is mean ± standard deviation. The statistics processing uses the parametric Dunnett's multiple comparison tests. a) P<0.05 vs. Ati, b) P<0.01 vs. Ati. All point of items was basically N=6 because all group had 8 rats. But some of samples were no data because of the fault of sampling. So, insulin of CU-PAW Low, Mid, High and Ati group was N=5.

CU-PAW administration (Table 7 and Table 8). In the CU-PAW Mid group, GLUC (P<0.0001) levels were elevated, whereas the total protein (TP) (P=0.0035) level was decreased 1 hr after CU-PAW administration (Table 7 and Table 8). GLUC (P<0.0001)

levels remained elevated 4 hr after CU-PAW administration. Furthermore, AST ($P=0.0083$) levels were elevated 24 hr after CU-PAW administration (Table 3, Supplementary table 4). In the CU-PAW High group, GLUC ($P<0.0001$) and BUN ($P=0.0005$) levels were elevated and TP ($P=0.0035$) levels were decreased 1 hr after CU-PAW administration (Table 3, Supplementary Table 4). GLUC ($P<0.0001$) and BUN ($P<0.0001$) levels remained elevated 4 hr after CU-PAW administration (Table 3, Supplementary Table 4). Furthermore, AST ($P<0.0001$) and ALT ($P=0.0003$) levels were elevated 24 hr after CU-PAW administration (Table 7 and Table 8). We found statistically significant changes in other blood biochemistry parameters (TBIL, IP, Ca, TG and K). However, these changes were not considered relevant because these effects appeared to be unrelated to either the aesthetic dose or the passage of time.

Changes in Haematology –24, 1, 4 And 24 Hr After CU-PAW Administration

In the CU-PAW Low group, white blood cell (WBC) ($P=0.0022$) and lymphocyte (LYMP) ($P=0.0001$) counts decreased and haematocrit (HCT) ($P=0.0286$) values increased at 4 hr after CU-PAW administration (Table 7, and Table 8). In the CU-PAW Mid group, HCT values ($P<0.0001$) and haemoglobin (HGB) levels ($P=0.0039$) were elevated 1 hr after CU-PAW administration (Table 7 and Table 8). HCT ($P=0.0059$) and HGB ($P=0.0184$) values remained elevated 4 hr after CU-PAW administration, whereas WBC ($P=0.0002$) and LYMP ($P<0.0001$) counts decreased. In the CU-PAW High group, HCT ($P<0.0001$) and HGB ($P<0.0001$) values were elevated 1 hr after CU-PAW administration. HCT ($P<0.0001$) and HGB ($P<0.0001$) values remained elevated at 4 hr after CU-PAW administration, whereas WBC ($P=0.0001$) and LYMP ($P<0.0001$) counts decreased. Statistically significant changes observed in other haematological parameters (Eosinophil (EO), Monocyte (MONO) and Mean either the aesthetic dose or the passage of time.

Changes in Blood Insulin Levels At –24, 1, 4 And 24 Hr After CU-PAW Administration

Temporary decreases in blood insulin levels were observed in the CU-PAW Low ($P=0.0003$), Mid ($P<0.0001$) and High ($P<0.0001$) groups 1 hr after CU-PAW administration (Table 7). Insulin levels were only elevated in the CU-PAW Low group 4 hr after CU-PAW administration ($P=0.0089$). In all CU-PAW groups, 24 hr after CU-PAW administration, insulin levels were no longer statistically different from those of the Ati group (Table 7).

Thus, Comparison of two routes of administration revealed that induction of anaesthesia using an intermediate and high dose of CU-PAW occurred 5–10 min after i.p. administration and 5 min after s.c. administration (Table 1). However, in the i.p. MMB Low group, CU-PAW administration failed to induce anaesthesia in one rat, as confirmed by a pain reaction at the time of Ati administration (Table 5). Additionally, one rat in the s.c. CU-PAW High group was found dead 24 hr after CU-PAW administration. Based on these results, we determined that s.c. administration provided more stable anaesthesia induction than i.p. administration. Under anaesthesia, respiratory problems and skin darkening were observed after administration by either route in the CU-PAW High groups. In contrast, only one rat in the i.p. CU-PAW Mid group exhibited such clinical observations (rapid breathing). At necropsy, no abnormalities were observed in i.p. dosed animals, apart from hydronephrosis of the left kidney, an event that was presumably congenital. Necropsy of s.c.-dosed animals revealed one abnormal pulmonary case. Based on these results, the CU-PAW High dose appeared to be an overdose when administered by either route. Blood biochemistry and haematological parameters were compared 24 hr after CU-PAW administration among groups.

AST and ALT levels were elevated after s.c.-dosing (Table 8); however, these effects were not supported by abnormalities at necropsy. The s.c. route was selected to confirm other changes induced by CU-PAW administration, as it produced stable induction of anaesthesia that did not differ based on dose. After CU-PAW administration, dose-dependent decreases in body weight were observed in the CU-PAW Mid and High groups. These effects were presumed to reflect differences at the time of awakening and recovery of activity following anaesthesia, consistent with reduced food consumption. No clinical abnormalities were found in the CU-PAW Low group 2 hr after CU-PAW administration. However, low activity (lethargy) was observed in 2 of 5 CU-PAW Mid group rats and 3 of 5 CU-PAW High group rats 6 hr after CU-PAW administration. As the effect of medetomidine is antagonized by Ati, low activity after awakening may be attributed to the influence of midazolam. Low activity is associated with decreased food and water consumption [20-23].

The resulting food and water deprivation were presumed to influence blood biochemistry and haematological parameters. Consistent with previous studies, we found that fasting and dehydration resulting from decreased activity caused decreases in WBC and elevations in HCT and HGB values [24, 25, 26, 27, 28, 29, 30]. The WBC count decrease observed here

was observed in all CU-PAW administration groups, and values were restored to control values at similar time points, indicating that effects on WBC reflected the pharmacological action of CU-PAW. We also detected a decrease in the number of WBC and LYMP counts; however, the basis for these effects is unclear.

The observed decrease in TP and elevation in BUN levels were considered a result of fasting [31, 32, 33, 34]. The presence of dark red spots in the lungs was only observed in the CU-PAW High group. These focal lesions in the lungs were presumed to reflect injury to the pulmonary system caused by abnormal breathing during anaesthesia, rather than to the pharmacological action of CU-PAW. Clinical observations under anaesthesia indicated that CU-PAW influenced systemic circulation and respiration. MMB has been reported to decrease heart rate and blood oxygen saturation in rats [35].

A previous report indicated that surgical stress could induce hyperglycaemia [37]. Thus, the increase in GLUC levels observed in the present study may reflect administration stress. In all CU-PAW -dosed rats, GLUC values were elevated 1 and 4 hr after CU-PAW administration (compared to those of controls at the respective time points), potentially due to the pharmacological action of CU-PAW: specifically, due to inhibition of insulin secretion by pancreatic β -cells, and ATP-dependent potassium ion channel closing of the cell membrane by α 2-adrenoreceptor agonists [35-43] Medetomidine, an α 2-adrenoreceptor agonist, suppresses insulin secretion by stimulating α 2-adrenoreceptors [47]. We previously found a similar change in mice ([40] and data not shown). Additionally, Ati has been reported to have α 2-adrenoreceptor-blocking effects [46, 41]. Consistent with this, all CU-PAW dosed animals showed decreased blood insulin values 1 hr after CU-PAW administration. By 4 hr after CU-PAW administration, blood insulin levels were elevated in rats in the CU-PAW Low group, and were restored to levels equivalent to those of the control (Ati) group in the CU-PAW Mid and High groups. Notably, larger Ati doses have been reported to induce larger increases in blood insulin concentrations. Specifically, peak blood insulin levels increased to about 1.5-fold of the blood concentration prior to medetomidine administration [48]. Several hours later, blood insulin levels returned to levels indistinguishable from those before medetomidine administration, suggesting that there is no risk that the sudden insulin increase will cause hypoglycaemia [48].

In this study, administration of CU-PAW in rats caused a temporary change in the levels of blood biochemistry and haematological parameters. These changes were not observed for

more than 24 hr after CU-PAW administration. CU-PAW administration in rats also attenuated body weight gain. However, these changes did not appear to adversely affect the health of rats, given that the clinical and necropsy observations were normal. The CU-PAW High dose was considered an overdose based on clinical and necropsy observations.

CONCLUSION

Copper plasma water is safe to be used since it does not have much effect on the blood biochemistry and haematological parameters. However, our study also shows that the aesthetic agents usually used in animal experiments may cause changes in body weight, food intake, haematological and blood biochemistry parameters as well. Hence, it is important to be aware of the effects of these agents before using them in experiments.

Competing interests

The authors declare no competing interests.

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