Potential of camel-derived haemoglobin oxygen carriers as a blood substitute

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Abstract

Camel-derived haemoglobin O2 carriers (CHBOC) are being developed as a blood substitutes for allogeneic RBCs or to improve tissue oxygenation. The tolerability of the developed product was evaluated in 10 moderately exsanguinated (40%) experimental mongrel dogs that were randomized to receive either 40 g/l CHBOC dissolved in lactated Ringer's solution at a rate of 5 ml/kg (Test group-14 trials) or 6 g/l HES 200 solution - 6% hydroxyethyl starch (Fresenius Kabi-Germany) dissolved in saline at a rate of - 5ml/kg- (Control group-6 trials).

Overall, the clinical, haematological and biochemical responses were normal. The CHBOC was well tolerated and a promising product. However additional studies are needed to confirm the safety of the new CHBOC product in the realm of acellular blood transfusion.

Keywords: Camels, Blood substitute, Haemoglobin, Oxygen carrier, Prion

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Introduction

The quest for producing a blood substitute is the result of an incessant demand not only for routine surgery and accidents but also in cases of mass civilian casualties during natural disasters, terrorism and wars (Klein, 2008).The risks of allogeneic blood transfusion are multiple and include infection transmission (HIV and Hepatitis B and C), delayed postoperative healing, transfusion reactions, transfusion-related lung injury, immunodilution and potential risk of cancer recurrence (Lander et al.,1996; Beauchesne,2005; Brander et al.,2005; Webber et al.,2005).

Blood primarily functions to transport oxygen to tissues. This function is performed by haemoglobin (Hb), a protein encapsulated inside the red blood cells (RBCs) that is capable of binding and releasing oxygen (Smith et al., 2004).

Haemoglobin-based oxygen carriers (HBOCs) are being developed as substitutes to replace the oxygen-carrying functions of erythrocytes and thereby lessen the demand of donor blood during surgery and trauma situations (Chang, 2000). Artificial blood substitutes present several advantages over the use of donor blood for blood transfusions because the former have no antigenic blood groups on their surface, no possibility for transmitting infections, have a longer storage lifetime and are cost efficient (Standl,2004; Alayash,2004; Kiellstrom,2003; Chang, 2004).

An HBOC is a purified Hb compound stripped of its red cell stroma and reduced to the functional oxygen-carrying component. The HBOCs are designed to increase the

oxygen-carrying capacity while reducing the risks commonly associated with allogeneic RBC transfusion (Kim and Greenburg, 2006). Bovine and human Hb form the bases of many different types of HBOCs ranging from chemically modified Hbs, including cross-linked polymerized, polymerized conjugated to liposome-encapsulated to be clinically effective (Jahr et al., 2008; Smani and Hemospan, 2008; Chen et al., 2009; Taguchi et al., 2010).

Blood substitutes have important potential areas of clinical application including red cell replacement during surgery, emergency resuscitation of traumatic blood loss, oxygen therapeutic applications in radiography as oxygenation of tumor cells is beneficial to the effect of certain chemotherapeutic agents (Olaf and Michael, 2004). Other medical applications include organ preservation to meet the requirements of patients who cannot receive donor blood because of religious beliefs (Agrawal et al., 2005). Eventually, there is a possibility of athletes using such products to enhance performance (Varlet-Marie et al., 2004).

Bovine Hb is readily available and, potentially has a lower cost as a raw material than human Hb (Vlahakes et al., 1989 and Bradely et al., 1994) Currently, bovine-derived HBOC has been successfully used in South Africa to support a patient with autoimmune hemolytic anemia and has been approved by the FDA for routine use in canine anaemia (Jahr et al., 2002; Stowell, 2002). Infection agents present in animal blood, such as bovine spongiform encephalopathy prion are more difficult to detect than those in human blood (Baron, 1999). However, over the past decades, the field of blood substitutes of bovine origin has been exciting due to the potential risk of bovine spongiform encephalopathy (BSE), with its possible link to CJD (Creutzfeld-Jakob disease) that can resist sterilization procedures employed in the production of blood substitutes (O'Brien, 1996; Collinge, 1999). The form of spongiform encephalopathy that occurs in human is thought to result from the same protein (a prion) that causes BSE in cattle (Scott et al., 1996, Brown, 1997 and Hill, 1997). In order to eliminate the risks associated with prion protein infection, camel-derived HBOC (CHBOC) has attracted considerable attention as a potentially safe alternative to bovine-derived HBOC.

Camels (Camelus dromedarius) are found mostly in arid or semi-arid regions of Africa and Asia (Gauthier-Pilters and Dagge,1981) and their unique physiological system should motivate researchers to further exploit their potential (Field,1979). The camel is predominantly a browser, although it also grazes on tall succulent grass (Iqbal and Khan, 2001). Thus, its feeding on animal byproducts is ruled out. The camel's meat, milk, wool and leather are widely utilized. In some parts of east Africa, camels are regularly bled and their blood is consumed fresh or mixed with milk (Yagil, 1982). Also, camels have more RBCs and higher Hb and mean corpuscular Hb concentration (MCHC) (Aichouni et al., 2010) and express heavy-chain antibodies that can be used to clone nanobodies, which are antibody-derived therapeutic proteins. A major advantage of nanobodies is that they can be easily attached to other proteins and nanoparticles by a simple chemical procedure, with therapeutic applications in cancer (Deffar and Shi, 2008). In addition, camel blood is easily available from slaughter houses in countries where camel meat is consumed.

The objective of this study was to develop an effective and safe acellular CHBOC blood substitute. In addition, the clinical, haematological and biochemical evaluation

of CHBOC administration were investigated in a dog model with haemorrhagic hypovolemia.

Materials and Methods

The study was conducted in 2 stages:

Stage I: Preparation of CHBOC (Balassundaresan et al. 2004):

Fresh whole camel blood was collected in a container with EDTA as the anticoagulant. The RBCs were isolated from whole blood by centrifugation at 5000 rpm for 20 minutes, washed three times with two volumes of 0.9% saline, and haemolyzed by adding three volumes of triple distilled water. The haemolyzed solution was centrifuged at 15,000 rpm for 1 hour to yield a cell-debris free Hb solution. The Hb solution was mixed with stock solutions of polyethelene glycol (PEG) 4000 and K_2HPO_4/NaH_2PO_4 to obtain a solution containing 12.5% PEG 4000, 12.5% phosphate, and pH10 with the addition of 12.5% NaOH. Following a separation phase, the top phase was withdrawn and added to NaH_2P0_4 to a final composition of 12.5% PEG 4000, 12.5% phosphate, pH 7.0. After mixing and separation, the bottom phase containing the Hb solution was dialyzed with triple- distilled water and lyophilized. Samples of the developed product of 20 g were frozen at -20 ^{o}C and thawed before testing.

Stage II: Experimental model:

All animal experiments were undertaken in accordance with the guideline principles for the Care and Use of Laboratory Animals and Experiments were approved by the Ethical Committee of the Faculty of Veterinary Medicine, Cairo University. All experimental animals were kept under conventional housing conditions with food and water ad libitum.

Ten healthy dogs (7 males and 3 females) aged between 2 to 5 years and weighing between 20 and 25 kg, were used in this study. These animals were tested for two consecutive times with a 2-month interval.

Allergy testing (James, 2002)

All dogs were allergically assessed against the prepared CHBOC before administration. The allergy testing comprised:

- Percutaneous testing by scratching the skin surface of the forearm of each dog. The CHBOC product was diluted 1:10 and a small amount of the diluted product was distributed over the scratched skin.

- Intradermal testing by intradermal injection of 0.2 ml of the diluted product in the forearm. The other leg was used as a control. The test and control sites were examined for any enlarged flare reaction or wheel of erythema 3 mm greater than the control (positive reaction).

All experimental dogs were sedated with Xylazine HCl 2% (Xylaject, Adwia, Egypt) (0.5 mg/Kg) and subjected to moderate jugular exsanguinations to approximately 40% of estimated blood volume (70-110 ml/kg) approximately 500 ml blood) according to Wolfensohn and Lloyd (2003). Jugular exsanguinations were conducted using 18 gauge needle. The needle was attached to a rubber tube of suitable length, coated with paraffin wax to prevent clotting. The tube was left hanging freely into 1 l graduated glass vessel. In addition, an intravenous cannula was introduced into the cephalic vein of the forearm of each dog, ready for subsequent infusion.

The exsanguinated experimental dogs were randomized into two groups:

The test group (n= 7-14 trials) was infused with CHBOC (40 g/ l) dissolved in lactated Ringer's solution at a rate of 5 ml/kg.

The control group (n=3-6 trials) infused with HES 200 solution (6 g/l), 6% hydroxyethyl starch (Fresenius Kabi-Germany), devoid of oxygen-carrying capacity (Standl et al., 1996) dissolved in saline at a rate of (5 ml/kg).

Blood samples with and without the anticoagulant were collected from the dogs of both groups before exsanguinations (baseline-BE, after exsanguinations-AE, 1 hour after infusion-1 hA and 24 hour after infusion-24 hA) for analysis of the complete blood count, CBC (Hb, Haematocrit-HCT, RBCs, MCV, MCHC and WBCs) using spectrophotometry, microcentrifugation and haemocytometer) and for the blood chemistry(glucose, cholesterol, triglycerides, urea nitrogen, creatinine,bilirubin,albumin, aspartate aminotransferase, -AST and alanine aminotransferase-, ALT) using chemistry autoanalyzer (Roche/Hitachi 912, Switzerland).

Statistical Analysis

The results data and figures were presented as means ± S.E. Comparison between mean values were made for each time point using a two-tailed paired student's t test with $P<0.05$ considered statistically significant.

Results

Allergy testing

The CHBOC fluid showed a negative reaction for both percutaneous and intradermal testing. During transfusion, no signs of shortness of breath, itching, fever or chills were observed in both groups.

Survival

Survival was not altered in the experimental dogs of both groups. It was interesting to note that administration of CHBOC to the test group was associated with remarkable vitality, a high capacity for exercise and a great appetite for up to 2 days post-infusion. On the contrary, the control group infused with HES 200 solution exhibited weakness and reduced appetite for nearly 1 week post-infusion.

Clinical

The observed changes in certain physiological variables after exsanguinations (AE) and 1 hour after infusion (1 hA) included slight hypothermia and marked tachycardia in both groups (Table 1).

Haemodynamic

Moderate exsanguinations were followed by significant reduction ($P<0.05$) in the measured values of Hb, HCT and RBCs, while calculated values of MCV showed significant increase ($P<0.05$) at post-exsanguinations and infusion. The WBCs count showed mild changes in both groups at all-time points in both groups (Table 2; Figures 1, 2 & 3).

Blood biochemistry parameters reflecting renal function (BUN and creatinine) and liver function (bilirubin, AST and ALT) showed minor changes at post-infusion from the values at base line. Other parameters reflecting metabolic activities (glucose, cholesterol, triglycerides and albumin) showed a significant elevation of the glucose values ($P<0.05$) in the test group received CHBOC 1 h post-infusion (Table 3).

Discussion

In the present study, the dog model of moderate exsanguinations has been designed to clinically evaluate the newly prepared CHBOC as a blood substitute and comparing its effects on various haemodynamic measurements with colloid HES 200, devoid of oxygen-carrying capacity (hydroxyethyl starch 6%). The results showed that CHBOC improved early survival and stabilized physiological and haemodynamic functions.

Dogs resuscitated with CHBOC exhibited marked vitality, high exercise capacity and increased appetite that continued for 2 days post-infusion compared with dogs resuscitated with HES 200. Increased exercise capacity had also been observed in human subjects administered HBOC of bovine origin which resulted in greater O2 uptake and CO2 production and lower lactate levels (Hughes et al., 1995). Additionally, no adverse effects were recorded in terms of the clinical systemic response, CBC and blood biochemistry analysis. Nearly identical results were reported when HBOC of bovine origin was used for restoring muscular tissue oxygenation after profound isovolaemic haemodilution in dogs and for resuscitation following haemorrhagic shock in a swine model (Standl, 1998; Arnaud et al., 2005). Bovine HBOC- 201 (Hemopure) has been used to manage autoimmune haemolytic anaemia in critically ill Jehovah's Witness patients and for treatment of acutely anaemic surgical patients in South Africa (Jahr et al., 2002; Stowell, 2002; Argawal et al., 2005). The beneficial effects of cell-free Hb were also demonstrated in the presence of the animal's red blood cells in maintaining physiologicael viscosity and limiting vasoconstriction a result of the pharmacological properties of cell free Hb (Rochon et al., 2004). Although HBOC can function as a bridge to spontaneous haematopoiesis, it may also accelerate the haematopoiesis process as the serum erythropoietin level- increased by two-fold to six-fold over baseline at 24 hours after HBOC infusions (Hughes et al., 1995). Therefore, if further infusions of HBOC are administered as HBOC is metabolized, all banked red cell transfusion could be eliminated (Sprung et al., 2001).

The low HCT values 24 h post-CHBOC infusion suggests a repeat infusion of CHBOC to maintain tissue oxygenation. Similarly, the HBOCs agents persist for a relatively short time in the circulation with a half-life of 24-48 h while, the RBCs have a half-life of 28-36 days. Therefore, frequent monitoring of CBC is necessary to decide whether the patient needs redosing of HBOC to replenish the CBC defect, especially in chronic transfusion setting (Klein, 2008).

In the present study, the blood biochemical parameters reflecting the liver function (AST & ALT) showed no significant changes in both groups. Nevertheless, a marked elevation of bilirubin was observed 1 hour post-infusion of CHBOC. This is consistent with the results obtained in monkeys that received vesicular Hb (HbV) (Taguchi et al., 2012). Such elevation is related to the liver, which is the main organ for Hb metabolism, thus Hb particles captured by the mononuclear phagocytes system such as kupffer cells, results in an excess load on the liver during the metabolism of massive amounts of Hb (Sakai et al., 2001). The Hb derived from haemolysis can cause renal toxicity by the dissociation of tetramic Hb subunits into two dimmers, by extravasation and precipitation in the renal tubules (Parry, 1988). However, in the present study blood biochemical parameters reflecting renal function (BUN and creatinine) showed slight changes in both groups that were within the normal range. Additionally, other blood metabolic biochemical parameters (cholesterol, triglycerides and albumin) were unaffected, but the glucose levels increased significantly at 1 h post-CHBOC infusion and regained normality at 24 h post-CHBOC infusion.

Despite previous benefits of HBOC solutions, most of them have been reported to increase systemic and pulmonary vascular resistance in clinical and preclinical settings, thus limiting the range of the therapeutic

applications for these solutions (Doherty et al., 1998; Alayash, 1999). Furthermore, acellular-type HBOCs are associated with a significantly increased risk of death and myocardial infarction which could be a result of nitric oxide (NO) scavenging by cell-free Hb (Burhop et al., 1999). The reduction in NO levels in myocardial lesions is an important factor in inducing histological damage in cases of myocardial lesions (Natanson, 2008). To overcome the risks evolved from HBOCs agents, many safe HBOC products have been generated via either chemical or genetic modifications of Hb, using liposomes entrapping of Hb or nanocapsules adsorbing Hb or by generating polyethylene glycol (PEG)- conjugated liposomes (Mozzarelli et al., 2010).

The limitations of the current study model include the lack of means for assessing the blood gases and cardiac output, oncotic pressure and toxicity studies.

Conclusion

The promising effects of CHBOC as a safe acellular blood substitute was efficacious and well tolerated in animal experimentation. Nevertheless, further studies on the developed product aimed at improving its properties by ultra- purification and liposomal encapsulation may enforce its safety, minimize any possible side effects and maximize the benefits.

Table 1. Mean values ± SE of some physiological parameters before and after exsanguination and 1 hour and 24 hour after administration of CHBOC (test) and HES (control).

Physiological variable	Before exsanguination		After exsanguination		1 h after administration		24 h after administration	
	Test (n=14) BET	Control (n=6) BEC	Test (n=14) AET	Control (n=6) AEC	Test (n=14) AAT	Control (n=6) AAC	Test (n=14) AAT	Control (n=6) AAC
Body temp. (°C)	39.5 ± 0.1	39.5 ± 0.2	39.5 ± 0.1	38.6 ± 0.4	39.2 ± 0.1	38.3 ± 0.2	39.5 ± 0.2	38.8 ± 0.1
Heart rate/min.	99.4 ± 3.6	93.3 ± 2.8	121.0 ± 4.4	120.0 ± 4.9*	115.0 ± 2.8	111.0 ± 0.8*	100.0 ± 1.4	103.0 ± 1.8
Respiration rate/min.	15.1 ± 0.8	9.3 ± 0.4	18.6 ± 1.7	14.7 ± 1.1	18.0 ± 1.1	14. 0 ± 0.0	13.7 ± 0.7	12.0 ± 0.7

*significant at $P<0.05$

CHBOC: camel-derived haemoglobin O_2 carrier; HES 200: 6% Hydroxyethyl starch; BET: before exsanguination test; BEC: before exsanguination control; AET: after exsanguination test; AEC: after exsanguination control; AAT: after administration test; AAC: after administration control.

Table 2. Mean values ±SE of some haematological variables before and after exsanguination and 1 hour and 24 hour after administration of CHBOC (test) and HES 200 (control).

Physiological variable	Before exsanguination		After exsanguination		1 h after exsanguination		24 h after exsanguination	
	Test (n=14) BET	Control (n=6) BEC	Test (n=14) AET	Control (n=6) AEC	Test (n=14) AAT	Control (n=6) AAC	Test (n=14) AAT	Control (n=6) AAC
Hb (g/dl)	12.3 ± 0.3	13.2 ± 0.7	9.4 ± 0.2*	9.6 ± 0.3*	10.5 ± 0.3	9.5 ± 0.3	10.8 ± 0.3	9.6 ± 0.3
HCT (%)	38.7 ± 1.2	43.0 ± 0.6	30.7 ± 0.7*	33.6 ± 0.2*	32.3 ± 0.9	32.6 ± 0.2	33.6 ± 0.2	33.6 ± 0.2
RBCs (10^6/ml)	4.4 ± 0.2	4.9 ± 0.6	3.3 ± 0.1*	3.6 ± 0.1*	3.4 ± 0.1	3.5 ± 0.1	3.4 ± 0.1	3.4 ± 0.1
MCV (ft)	88.1 ± 1.4	87.1 ± 0.5	89.4 ± 1.0	92.7 ± 2.0*	93.6 ± 1.2*	95.6 ± 3.5*	94.5 ± 1.7*	102.3 ± 2.8*
MCHC (%)	31.4 ± 0.5	30.7 ± 0.4	30.7 ± 0.4	28.7 ± 1.2	33.0 ± 0.4	29.3 ±1.1	32.9 ± 0.3	28.7 ± 0.8
WBCs (10^3/ml)	8.4 ± 0.3	6.4 ± 0.3	5.8 ± 0.3	7.3 ± 1.2	7.1 ± 0.5	8.3 ± 0.8	8.8 ± 0.6	8.4 ± 0.5

*significant at $P<0.05$

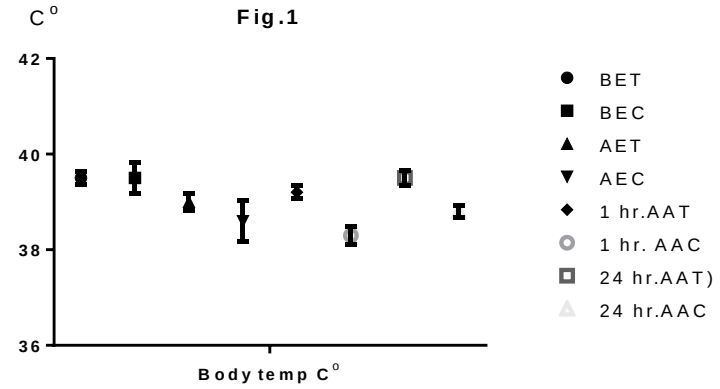
CHBOC: camel-derived haemoglobin O_2 carrier; HES 200: 6% Hydroxyethyl starch; BET: before exsanguination test; BEC: before exsanguination control; AET: after exsanguination test; AEC: after exsanguination control; AAT: after administration test; AAC: after administration control.

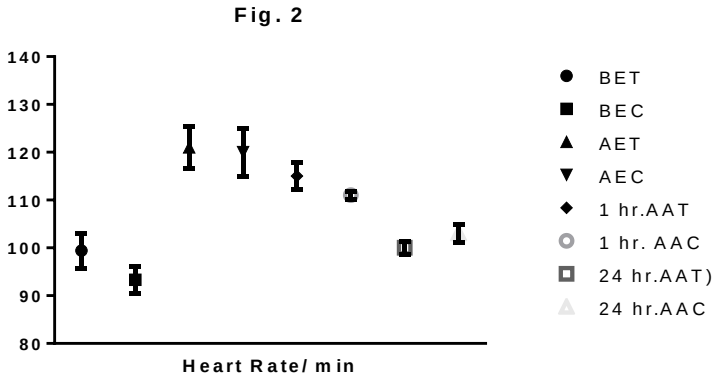
Table 3. Mean values ±SE of some blood variable before and after exsanguination and 1 hour and 24 hour after administration of camel derived haemoglobin O_2 carrier (CHBOC, test) and 6% of hydroxyethyl starch 200 (HES 200, control).

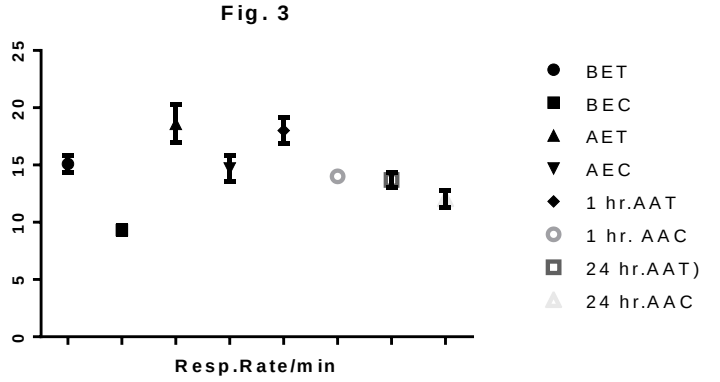
Blood variables	Before exsanguination		After exsanguination		1 h after administration		24 h after administration	
	Test (n=14) BET	Control (n=6) BEC	Test (n=14) AET	Control (n=6) AEC	Test (n=14) AAT	Control (n=6) AAC	Test (n=14) AAT	Control (n=6) AAC
Glucose (mg/dl)	88.0 ± 7.0	91.6 ± 5.1	97.7 ± 4.5	103.3 ± 2.8	176.0 ± 13.6*	98.6 ± 2.3	94.1 ± 5.9	90.6 ± 4.0
Cholesterol (mg/dl)	221.4 ± 14.1	158.7 ± 21.9	219.1 ± 14.1	162.7 ± 9.2	177.7 ± 11.5	151.0 ± 8.4	179.9 ± 8.3	141.7 ± 12.3
Triglycerides (mg/dl)	55.7 ± 5.9	44.6 ± 8.8	61.8 ± 4.9	46.6 ± 13.5	65.0 ± 4.0	47.3 ± 3.3	53.4 ± 4.2	45.0 ± 4.5
Urea-N (mg/dl)	28.2 ± 2.1	23.3 ± 0.9	32.0 ± 2.8	23.6 ± 1.7	33.8 ± 2.5	23.0 ± 1.3	29.5 ± 1.2	22.6 ± 2.0
Creatinine (mg/dl)	0.8 ± 0.1	1.0 ± 0.0	1.0 ± 0.1	1.6 ± 0.4	1.3 ± 0.2	1.3 ± 0.2	1.1 ± 0.1	1.4 ± 0.2
Bilirubin (mg/dl)	0.5 ± 0.4	0.7 ± 0.1	0.8 ± 0.5	0.9 ± 0.7	0.8 ± 0.1	0.7 ± 0.6	0.7 ± 0.0	0.7 ± 0.1
Albumin (g/dl)	3.5 ± 0.0	3.5 ± 0.1	2.8 ± 0.1	2.5 ± 0.1	2.8 ± 0.1	2.3 ± 0.1	3.1 ± 0.1	2.3 ± 0.1
ALT (U/L)	20.3 ± 3.1	16.7 ± 0.4	20.6 ± 3.6	14.7 ± 0.9	23.6 ± 2.5	16.3 ± 1.1	19.1 ± 1.9	15.0 ±1.0
AST (U/L)	25.6 ± 3.2	15.0 ± 0.6	25.3 ± 3.6	14.6 ± 1.3	21.6 ± 2.5	13.7 ± 0.8	20.9 ± 2.2	14.7 ± 0.4

*significant at $P<0.05$

CHBOC: camel-derived haemoglobin O_2 carrier; HES 200: 6% Hydroxyethyl starch; BET: before exsanguination test; BEC: before exsanguination control; AET: after exsanguination test; AEC: after exsanguination control; AAT: after administration test; AAC: after administration control.







Figs. 1, 2 &3. Some physiological parameters; BET: before exsanguination test; BEC: before exsanguination control; AET: after exsanguination test; AEC**:** after exsanguination control; AAT: after administration test; AAC: after administration control.

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