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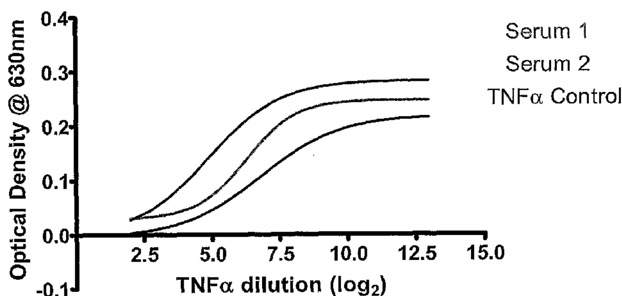

(57) Abstract: The invention provides a method for the treatment of inflammatory diseases and/or conditions in animals that involves the parenteral administration of hyperimmune plasma with anti-TNFα activity raised by vaccination of canines with *Escherichia coli* J5. Inflammatory diseases and/or conditions treatable by parenteral administration of hyperimmune plasma with anti TNFα activity include inflammatory diseases or conditions including, but not limited to, severe infections caused by endotoxins of any gram negative bacteria and gram positive such as *S. aureus*, shock, infection, ischaemia-reperfusion injury, pancreatitis, volvulus, colic, typhoid, cholera and other diarrhoeas, peritonitis, perforated bowel, bowel obstruction when ischaemia is also occurring due to the size of the obstructing foreign body, bowel impaction & constipation, prostatitis & prostatic abscess and colitis. The anti-TNFα activity may be attributable to soluble TNFα receptors present in the hyperimmune canine plasma, which receptors may be purified for subsequent therapeutic use.

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TITLE

ANTI-INFLAMMATORY BLOOD PRODUCT AND METHOD OF USE

FIELD OF THE INVENTION

THIS INVENTION relates to treatment of inflammatory diseases or conditions in
5 mammals. More particularly, this invention relates to treatment of inflammatory diseases or conditions in non-human mammals, particularly canines, using isolated hyperimmune canine plasma having anti- tumour necrosis factor alpha activity.

BACKGROUND OF THE INVENTION

Plasma therapy is still a major mode of treatment of human diseases and
10 conditions. Some of the more common plasma therapy products include Immune Globulin for the treatment of immune deficiency diseases, albumin for heart surgery patients and burn victims, anti-Thrombin III for the treatment of patients with bleeding disorders, Factor VIII for the treatment of hemophilia, Alpha 1 Proteinase Inhibitor for the treatment of Alpha-1 Antitrypsin Deficiency and Fibrin Sealant
15 which replicates the natural blood-clotting process to stop bleeding during surgical procedures.

Plasma therapy is also a well accepted veterinary treatment of equines, cattle and other large animals including camelids, such as Alpacas and Llamas.

Although plasma is more readily obtained from larger animals such as those
20 described above, International Publication WO 2005/007299 describes production of a canine hyperimmune plasma by vaccination with *Escherichia coli* J5, which plasma is safe and efficacious for use in the treatment of certain canine diseases and conditions.

SUMMARY OF THE INVENTION

25 Surprisingly, hyperimmune canine plasma produced by vaccination with *Escherichia coli* J5 is useful in the treatment of diseases and conditions not previously contemplated. More particularly, canine plasma has significant and therapeutically useful anti-inflammatory and/or anti-TNFα activity.

Thus the invention is broadly directed to use of plasma obtained from a
30 canine donor for prophylactically or therapeutically treating an inflammatory disease or condition in a mammalian recipient.

In one broad embodiment, the inflammatory disease or condition is responsive to inhibition, suppression or reduction in TNFα activity.

In one aspect, the invention provides a method of treating an inflammatory disease or condition in a mammalian recipient in need of such treatment, including the step of administering isolated plasma obtained from a canine donor to a mammalian recipient to thereby suppress, inhibit, ameliorate or otherwise treat said inflammatory disease or condition.

In another aspect, the invention provides a method of treating a disease or condition that is mediated by TNFα in a mammalian recipient in need of such treatment, said method including the step of administering isolated plasma or an isolated TNFα receptor obtained from a canine donor to said mammalian recipient to thereby suppress, inhibit, ameliorate or otherwise treat said disease or condition.

In yet another aspect the invention provides a method of producing a canine TNFα receptor including the step of obtaining a blood or a component thereof from said canine and isolating, enriching or purifying said TNFα receptor from said blood or component thereof.

Preferably, said blood component is hyperimmune plasma.

In still yet another aspect, the invention provides use of isolated canine plasma or an isolated canine TNFα receptor fragment in the manufacture of a medicament for treating an inflammatory disease or condition in a mammal.

In still yet another aspect, the invention provides use of an isolated canine TNFα receptor, or fragment thereof, in the manufacture of a medicament for treating an disease or condition that is mediated by TNFα in a mammal..

In a further aspect, the invention provides a TNFα receptor or fragment thereof, obtained from a blood component of a canine.

According to the aforementioned aspects, preferably said TNFα receptor is a soluble TNFα receptor or a TNFα-binding fragment thereof.

In another further aspect, the invention provides a composition comprising a TNFα receptor or fragment thereof, obtained from a blood component of a canine, together with a pharmaceutically or veterinarily acceptable carrier, diluent or excipient.

Suitably, the isolated canine plasma is "*hyperimmune canine plasma*" prepared by vaccination of said canine donor with *Escherichia coli* J5.

In a preferred embodiment, the isolated canine plasma or TNFα receptor is/are administered parenterally to the mammalian recipient to therapeutically or 5 prophylactically treat one or more diseases or conditions associated with the presence and/or activity of TNFα.

Throughout this specification unless the context requires otherwise, the word "comprise", and variations such as "comprises" or "comprising", will be understood to imply the inclusion of the stated integers or group of integers or steps but not the 10 exclusion of any other integer or group of integers.

BRIEF DESCRIPTION OF THE FIGURES

In order that the invention may be readily understood and put into practical effect, preferred embodiments will now be described by way of example with reference to the accompanying figures.

15 FIG. 1. Canine hyperimmune plasma anti-TNFα activity. Blind test of 2 canine sera for anti-TNFα activity in an L929 cell bioassay. Serum 1 was later confirmed as the negative control serum.

FIG. 2. Canine hyperimmune plasma anti-TNFα activity. Blind test of 4 canine sera for antiTNFα activity in an L929 cell bioassay. Serum 4 was later 20 confirmed as the negative control serum.

FIG. 3. Mean arterial blood pressure (MAP). Canine hyperimmune plasma ("Caniplas") pre-treatment (200ml administered IV over 1 hour) had no detectable effect on MAP in dogs injected with *E. coli* LPS (1.5mg/kg IV). Normal dog plasma appeared to improve MAP from 30 minutes after LPS infusion. The 25 pressures are expressed as a percentage of the pressure at time 0.

FIG. 4. Systolic blood pressure in a canine model of endotoxic shock. Pretreatment with canine hyperimmune plasma (200ml administered IV over 1 hour) in dogs that were challenged with *E. coli* LPS (1.5mg/kg IV). Canine hyperimmune plasma pre-treatment and pre-treatment with plasma from normal dogs at the same 30 dose rate had no detectable effect on mean SBP. The pressures are expressed as a percentage of the pressure at time 0.

FIG. 5. Diastolic blood pressure in a canine model of endotoxic shock. Canine hyperimmune plasma appeared to have minimal effects on diastolic blood pressure, although infusion with normal canine plasma increased diastolic blood pressure for most of the 180 minutes after LPS (1.5mg/kg) infusion. The pressures
5 are expressed as a percentage of the pressure at time 0.

FIG. 6. Pulse pressure in a canine model of endotoxic shock. An infusion of 200ml of canine hyperimmune plasma IV before challenge with LPS (1.5mg/kg IV) improved cardiac output. A greater pulse pressure was achieved than with saline pre-treatment. Dogs pre-treated with normal canine plasma did not show
10 an improvement in pulse pressure.

FIG. 7. Heart rate in a canine model of endotoxic shock. Heart rate was not significantly affected by LPS challenge although at 180 minutes the heart rate increased in dogs receiving saline. In this group this change was interpreted as a compensation for worsening cardiovascular function. The data are expressed as a
15 percentage of heart rate at time 0.

FIG. 8. Plasma TNFα concentrations in a canine model of endotoxic shock. LPS (1.5mg/kg) caused a marked rise in plasma TNFα concentration which was maximal at 60 – 120 minutes after challenge. An infusion of 200ml Canine hyperimmune plasma before challenge reduced the plasma concentration of TNFα as
20 measured by ELISA, but the variability in the data did not allow statistic significance.

FIG. 9. Activated clotting time (ACT) in a canine model of endotoxic shock. Blood clotting, as measure by ACT, was not affected by plasma treatments in this model.

FIG. 10. Total leukocyte counts in a canine model of endotoxic shock.
25 LPS infusion (1.5mg/kg *E. coli* LPS) caused a significant fall in the number of circulation leukocytes. Canine hyperimmune plasma and normal canine plasma did not moderate this change. Infusion of both canine hyperimmune plasma and normal canine plasma cause a small drop in total white blood cell count (WBCC) although this could be a dilution effect of the rapid infusion of plasma (200ml over 1 hour).
30 The saline would be less likely to cause dilution because it would more easily move out of the vascular compartment into the extracellular space. Treatments had no

effect on total leukocyte count. The upper and lower limits of normal WBCC are indicated on the graph.

FIG. 11. Circulating neutrophil counts in a canine model of endotoxaemia. The leukopaenia was due to the loss of all white cell types although 5 loss of neutrophils from the circulation contributed to the overall loss. Pre-treatment with canine hyperimmune plasma or normal dog plasma had no effect on the extent of neutropaenia. The normal range of neutrophils is indicated on the graph.

FIG. 12. Blood monocyte levels after LPS challenge. The numbers of monocytes are illustrated to show that all leukocyte cell types were affected by LPS 10 challenge. The normal range of monocyte numbers is indicated on the graph.

FIG. 13. Packed cell volume (PCV) in a canine model of endotoxaemia. Packed cell volume was used as a measure of vascular leakage and haemo-concentration in this model. PCV increased over the duration of the experiment indicating either a loss of fluid from the vascular compartment or an injection of 15 erythrocytes from stores in the spleen and bone marrow, or both. The concurrent leukopaenia (Figure 10) suggests that some loss of plasma is through "leaky" capillaries.

FIG. 14. Erythrocyte count in an LPS-induced sepsis model in dogs. The increase in RBCC correlates with an increase in PCV (Figure 13). Without a 20 marker of vascular leakage it cannot be ascertained if the increase in RBCC is due to haemo-concentration or mobilization of RBC reserves.

FIG. 15. Mean arterial pressure after high volume infusion of plasma or saline. Canine hyperimmune plasma blocked the hypotension induced by LPS injection (*E. coli* LPS 1.5mg/kg) from 90 minutes after LPS infusion. Infusion of 25 normal canine plasma also had an effect on MAP.

FIG. 16. Effects of high volume infusion on systolic blood pressure. Canine hyperimmune plasma and normal canine plasma treatments improved systolic blood pressure from about 90 minutes after LPS infusion.

FIG. 17. Diastolic blood pressure in a canine model of endotoxic shock. 30 High volume infusion of either canine hyperimmune plasma or normal dog plasma had no effect on diastolic blood pressure.

FIG. 18. Effects of plasma treatment on pulse pressure in a canine model of endotoxaemia. From 90 minutes after LPS challenge canine hyperimmune plasma at high volume, but not normal plasma, moderated the fall in pulse pressure.

FIG. 19. Heart rate before and after LPS challenge and the effects of 5 plasma treatments. Heart rate was not affected by LPS infusion (1.5mg/kg IV).

FIG. 20. TNFα in a canine model of endotoxic shock. Using an ELISA to determine plasma levels of the cytokine showed no effect of either canine hyperimmune plasma or normal plasma pre-treatments. This assay measures the presence of TNFα, not its activity. There was a small difference in activity between 10 low dose bolus (200ml) and high dose infusion (800ml), but the small numbers of dogs in the groups did not confirm the significance of this observation.

FIG. 21. Activated clotting time after LPS infusion. LPS infusion caused a small increase in ACT, but this was not significant. The plasma treatments did not affect clotting as measure by ACT.

15 FIG. 22. Leukocyte numbers in a canine model of endotoxic shock. LPS infusion caused a marked leukopaenia. The effects on neutrophil and monocyte numbers are displayed. Pre-treatment with either canine hyperimmune plasma or normal canine plasma had no effects on the severity of leukopaenia. Normal levels are marked on the graphs.

20 FIG. 23. Effects of LPS infusion on haematocrit. In contrast to the bolus infusion where there was a marked haemoconcentration (Figure 13), high volume infusion of fluids maintained the haematocrit in the normal range. This was seen with infusions of saline, canine hyperimmune plasma and normal canine plasma.

25 DETAILED DESCRIPTION OF THE INVENTION

A number of acute disease states and injuries lead to an exaggerated response by a patient's immuno-inflammatory system that may eventually lead to death. Activation of this cascade of events may be triggered by bacterial infections, injuries and cancer. The clinical presentation is usually shock. Systemic inflammatory 30 response syndrome (SIRS) is the clinical manifestation of this pathophysiology. If this inflammation is not adequately regulated, either naturally or pharmacologically,

the patient may undergo organ damage and loss of function leading to multiple organ dysfunction syndrome (MODS). Both SIRS and MODS have high mortality and at this time there are few effective drug treatments to control excessive activation of the inflammatory cascade.

5 One pro-inflammatory mediator that occurs in sepsis and which has been targeted for drug treatment is tumour necrosis factor alpha (TNFα). In particular, a number of specific anti-TNFα therapeutic agents have been developed for human applications and are registered for use in the treatment of inflammatory bowel disease and rheumatoid arthritis in man. They have been evaluated in the treatment of SIRS

10 and sepsis and provide a modest improvement in severely ill human patients. In both medical and veterinary practice, it would be more desirable to target a number of pro-inflammatory mediators, but identification of key cytokines in a particular patient at a particular stage of the disease process is difficult. Human and non-human patients presented with sepsis and SIRS need urgent treatment to avoid immediate death.

15 The present invention has arisen from the discovery that canine plasma produced by vaccination with *Escherichia coli* J5 has significant and therapeutically useful anti-inflammatory activity, at least partly due to the presence of an anti-TNFα activity.

While not wishing to be bound by any particular theory, it is proposed that

20 canine plasma produced by vaccination with *Escherichia coli* J5 comprises soluble TNFα receptors that are capable of inhibiting, suppressing or otherwise reducing TNFα activity.

However, it may also be the case that the canine plasma may have anti-inflammatory activity also or alternatively resulting from inhibition, suppression or

25 reduction in activity of other pro-inflammatory cytokines such as IL-1 and/or IL-6.

Unless defined otherwise, all technical and scientific terms used herein have a meaning as commonly understood by those of ordinary skill in the art to which the invention belongs. Although any method and material similar or equivalent to those described herein can be used in the practice or testing of the present invention,

30 preferred methods and materials are described. For the purpose of the present invention, the following terms are defined below.

"*Plasma*" refers to a liquid uncoagulated component of blood comprising proteins, including antibodies and clotting factors.

"*Plasma Therapy*" means a process of administering plasma or plasma-based product to an individual to increase an amount of one or more serum protein(s) in the
5 blood of a recipient. The serum protein(s) may include antibodies.

"*Plasmapheresis*" is a process in which plasma is separated and removed from blood cells of an individual using, for example, a cell separator. The separator may comprise a centrifuge and/or membrane with a pore size suitable to separate blood cells from plasma. The blood cells are returned to the individual and the
10 plasma is collected and replaced within the donor's body with other fluids, by the normal compensatory mechanisms in such situations. Medication to keep the blood from clotting (an anticoagulant) may be mixed with the donor blood at the venipuncture site during the plasmapheresis. Plasma collected according to the method of the present invention is sterile (sterility is tested for each batch of plasma
15 produced).

"*Hyperimmune plasma*" refers to plasma comprising an increased concentration of gamma globulins, preferably all gamma globulins and in particular antibodies capable of binding to a specific antigen, when compared to a non-immunised animal. Hyperimmune plasma is obtainable from a donor that has been
20 subject to a hyperimmunisation regimen comprising administration of an antigen, including commercial and custom-made vaccines, to thereby elicit antibodies that target specific antigens in the recipient. In response to hyperimmunisation, donors may accumulate an antibody concentration in their bloodstream 2-3 times higher than those of normal animals.

25 The invention provides use of hyperimmune canine plasma with anti TNFα and/or anti-inflammatory activity to treat diseases or conditions including (but not limited to) shock, infection, ischaemia reperfusion injury, pancreatitis, volvulus and colic.

While the invention in its broadest form relates to treatment of any human or
30 non-human mammal, in a preferred form the invention provides a method of treating an inflammatory disease or condition that is mediated by TNF-α, in a canine

recipient, including the step of administering isolated plasma obtained from a canine donor to said canine recipient to thereby suppress, inhibit, ameliorate or otherwise treat said inflammatory disease or condition.

The term "*non-human mammal*" includes domestic mammals (*e.g.* canines
5 and felines) livestock (*e.g.* horses, cattle, sheep, goats), performance mammals (e.g. racehorses, greyhounds and racing camels) and other large mammals such as camelids (*e.g* Alpacas and Llamas).

As used herein "*canine*" includes and encompasses any member of the family *Canidae*, including, dogs (domestic or wild), wolves, jackals, hyenas, coyotes and
10 foxes.

In one particular embodiment, the invention provides a method of treating an inflammatory disease or condition that is preferably mediated by TNF-α in a mammalian recipient, including the steps of:

(I) selecting a canine donor having a blood group compatible
15 with said mammalian recipient;

(II) collecting blood from the canine donor;

(III) isolating plasma from blood collected in step (II); and

(IV) administering the plasma to the mammalian recipient to thereby suppress, inhibit, ameliorate or otherwise treat said
20 inflammatory disease or condition.

Preferably, the canine donor is treated with *E. coli* J5 prior to step (II).

Preferably, the *E. coli* J5 is inactivated or attenuated such as by chemical (e.g. formaldehyde) or heat treatment.

More preferably, the *E. coli* J5 is inactivated by heat treatment.

25 In embodiments relating to administration to canine recipients, a detailed description of canine donor selection according to blood group compatibility and selection of universal donors (*e.g.* according to Dog Erythrocyte Antigens) is provided in International Publication WO 2005/007299.

A detailed description of obtaining blood from canine donors, subsequent
30 plasmapheresis and therapeutic administration of hyperimmune plasma to mammalian recipients is provided in International Publication WO 2005/007299.

The invention also contemplates isolation, enrichment or purification of soluble TNFα receptors, or fragments thereof, from a canine blood component such as hyperimmune plasma.

Suitably, soluble TNFα receptor fragments bind TNFα sufficiently to at least
5 partly neutralize, block or inhibit TNFα activity.

Isolation, enrichment or purification of soluble TNFα receptors may be performed using protein purification techniques as are well known in the art. These include affinity chromatography (*e.g* using TNFα or anti- TNFα receptor antibody coupled to a solid phase matrix), high performance liquid chromatography (HPLC or
10 RP-HPLC), ion exchange chromatography (*e.g* cation or anion exchange chromatography), although without limitation thereto.

Purified, soluble TNFα receptors or receptor fragments may be administered together with a pharmaceutically- or veterinarily-acceptable carrier diluent or excipient.

15 By "*pharmaceutically or veterinarily-acceptable carrier, diluent or excipient*" is meant a solid or liquid filler, diluent or encapsulating substance that may be safely used in systemic administration. Depending upon the particular route of administration, a variety of carriers, well known in the art may be used. These carriers may be selected from a group including sugars, starches, cellulose and its
20 derivatives, malt, gelatine, talc, calcium sulfate, vegetable oils, synthetic oils, polyols, alginic acid, phosphate buffered solutions, emulsifiers, isotonic saline and salts such as mineral acid salts including hydrochlorides, bromides and sulfates, organic acids such as acetates.

Any safe route of administration may be employed for providing a patient
25 with the composition of the invention. For example, oral, rectal, parenteral, sublingual, buccal, intravenous, intra-articular, intra-muscular, intra-dermal, subcutaneous, inhalational, intraocular, intraperitoneal, intracerebroventricular, transdermal and the like may be employed.

Preferably, administration is parenteral.

30 Dosage forms include tablets, dispersions, suspensions, injections, solutions, syrups, troches, capsules, suppositories, aerosols, transdermal patches and the like.

These dosage forms may also include injecting or implanting controlled releasing devices designed specifically for this purpose or other forms of implants modified to act additionally in this fashion. Controlled release of soluble TNFα receptors may be effected by coating the same, for example, with hydrophobic polymers including 5 acrylic resins, waxes, higher aliphatic alcohols, polylactic and polyglycolic acids and certain cellulose derivatives such as hydroxypropylmethyl cellulose. In addition, the controlled release may be effected by using other polymer matrices, liposomes and/or microspheres.

Compositions of the present invention suitable for oral or parenteral 10 administration may be presented as discrete units such as capsules, sachets or tablets, as a powder or granules or as a solution or a suspension in an aqueous liquid, a non-aqueous liquid, an oil-in-water emulsion or a water-in-oil liquid emulsion. Such compositions may be prepared by any of the methods of medical and/or veterinary pharmacy.

15 While in principle the invention is applicable to treating any mammalian recipient using isolated plasma obtained from a canine donor, the invention is preferably directed to treatment of canines using isolated plasma obtained from canines.

Generally, whether in canines or other mammals, inflammatory diseases or 20 conditions include, but are not limited to, severe infections caused by endotoxins of any gram negative bacteria and gram positive such as *S. aureus*, shock, infection, ischaemia-reperfusion injury, pancreatitis, volvulus, colic, typhoid, cholera and other diarrhoeas, peritonitis, perforated bowel, bowel obstruction when ischaemia is also occurring due to the size of the obstructing foreign body, bowel impaction & 25 constipation, prostatitis & prostatic abscess and colitis.

Particular canine diseases or conditions contemplated by the present invention include auto immune diseases (*e.g.* arthritis, haemolytic anaemia, Lupus-type disease, Pemphigas-type disease), canine distemper, infectious canine hepatitis, canine herpes virus infection, diarrhoea, haemorrhagic gastro-enteritis, gastric 30 dilatation with or without volvulus, intestinal catastrophes (*e.g.* obstruction, intussusception, torsion, volvulus, mesenteric entrapment), cachexia induced by

metastatic tumours, fading puppy complex or fading puppy syndrome, mastitis, vomiting, gastritis, tick paralysis, megoesophagus, pleuritis and pleurisy, tonsillitis, pharyngitis, sinusitis, encephalitis, meningitis, myositis, neuritis, osteitis, chondritis, hepatitis, proctitis, colitis, uveitis, rhinitis, glossitis, gingivitis, dental infections, otitis, gall bladder diseases (*e.g* cholecystitis, cholangitis), biliary obstructive disease, Cushings syndrome, Addisons disease, tendonitis, tendosynovitis, vaginitis, cystitis, endometritis, salivary gland inflammation/infection, helminthiasis and dermatitis.

It will be appreciated that administration of hyperimmune canine plasma and/or soluble TNFα receptors to a mammalian recipient may be as a "bolus" (singly or repeatedly) or by continuous infusion.

Preferably, the canine hyperimmune plasma plasma is administered in a range of 2-25 mL/kg weight of the recipient mammal per hour.

In embodiments relating to treatment of a canine recipient, isolated canine plasma is administered preferably in a range of 5-20 mL/kg weight of the recipient canine per hour.

More preferably, the canine hyperimmune plasma is administered in a range of 5-10 mL/kg weight of the recipient canine per hour.

In one particular embodiment, the hyperimmune canine plasma is administered to a canine recipient as a "standard" ~200 mL bolus (5-20 ml/kg).

In another particular embodiment, the hyperimmune canine plasma is administered to a canine recipient by continuous infusion.

For example, the invention contemplates continuous infusion over 2-8 hours, but preferably for about four (4) hours.

In one particular example, the invention contemplates continuous infusion of 200 mL per hour over about four (4) hours.

In order that the invention may be readily understood and put into practical effect, particular preferred embodiments will now be described by way of the following non-limiting examples.

EXAMPLES

EXAMPLE 1

Preparation of canine hyperimmune plasma

Canine hyperimmune plasma was produced exactly as described in International Publication WO 2005/007299.

anti-TNFα bioassay

5 This assay is a modification of that described by Wadhwa *et al.*, Quantitative biological assays for individual cytokines. *In* Cytokines: a practical approach. 2nd edit, Editor: Balkwill FR. IRL Press, Oxford UK 1997 pp 357-391 and Burke *et al.*, Measurement of proliferative cytolytic and cytostatic activity of cytokines. *In* Cytokines: a practical approach. 2nd edit, Editor: Balkwill FR. IRL Press, Oxford UK 10 1997 pp 279-296.

Briefly, 100μl of 2 x 10^5 L929 cells/ml suspension in DMEM containing 10% FCS were dispensed into each well of a 96 well microtitre plate and incubated ON @ 37°C in 5% CO_2.

In a separate microtitre plate serial double dilutions of recombinant 15 murineTNFα (commencing at 50ng/ml in well 1) were prepared in the test serum that had been diluted 1:4 in DMEM containing 10% FCS and 2μg/ml actinomycin D. The titration was incubated for 60 min @ 4°C, allowed briefly to equilibrate to RT and 100μl of each dilution was added to a well of the now confluent L929 cells. The cells were incubated for 20 hrs @ 37°C in 5% CO_2 following which the media was flicked 20 out and cells fixed by the addition of methanol for 30 sec. The methanol was removed and 100μl of aq crystal violet (0.5% in 20% MeOH) was added to each well and left to stain for 15 min @ RT. The stain was flicked out and the wells were gently washed in distilled water and allowed to dry on paper towelling. The OD was measured @ 630 nm in a multiscan plate reader following the manufacturer's 25 instructions.

Positive control was a serial dilution of recombinant murine TNFα diluted in DMEM containing 10% FCS and 2μg/ml actinomycin D. The recombinant TNFα concentration varied and was initially tested from 500ng/ml to 50 ng/ml in the first test well and thereafter double diluted.

30 The negative control was DMEM containing 10% FCS and 2μg/ml actinomycin D.

Results

Initially, canine sera were assayed for anti-TNFα activity using the abovementioned assay. One specimen was hyperimmune serum and the second a control serum (Figure 1). In a blind experiment, the hyperimmune canine plasma was distinguished from the control serum using the anti-TNFα bioassay, the hyperimmune serum having anti-TNFα activity.

In a more statistically significant blind assessment of canine hyperimmune plasma, the results for four canine sera (labelled 1-4 in Figure 2; the samples contained 3 hyperimmune plasma and 1 control serum) compared to negative control sera using the antiTNFα bioassay, are shown in Figure 2.

EXAMPLE 2

Methods

Animals

Mongrel dogs or greyhounds of both sexes and various ages (n = 21, estimated ages 1 – 7 years) were obtained from the pound or were donated by trainers (Table 1). They were housed in kennels for between 1 and 7 days before experiments. All dogs appeared healthy at the time of experiment.

Dogs were anaesthetized with intravenous thiopentone, incubated, and maintained on halothane/oxygen for the duration of the experiment. During periods of apnea, ventilation was maintained by compression of the rebreathing bag until spontaneous respiration returned. Dogs remained unconscious for the duration of the experiment when euthanasia was performed. An incision was made in the skin on the medial aspect of the thigh so catheters filled with heparin-saline could be placed into the aorta via the femoral artery to record arterial blood pressure and into the right atrium via the femoral vein to record central venous pressure (CVP). Catheters were connected to Grass pressure transducers and the signal was captured by a Grass pen recorder. Heart rate was also recorded by the Grass recorder. Respiratory rate was observed on the central venous pressure trace. Electrocardiographs (ECG) were recorded at intervals throughout the procedure on a portable machine. Blood samples were collected from the jugular veins by needle and syringe at set times during the experiment (-60, 0, +5, +15, +30, +60, +120, +180 minutes relative to LPS injection).

Blood samples were collected for hematology, clinical biochemistry and cytokine analysis. An indwelling catheter was placed in one cephalic vein to facilitate infusion of either canine hyperimmune plasma, normal dog plasma or saline. The researchers were blinded to the source of the plasma.

5 Infusion of plasma or saline was commenced 60 minutes prior to lipopolysaccharide infusion (time 0). The infusion rate was approximately 200ml/hour. In the first 11 dogs treated, the volumes of plasma and saline were 200ml (a "standard" pack of canine hyperimmune plasma) and the final 10 dogs were infused with up to 800ml of saline or plasma over 4 hours. In the latter group 10 the infusion continued for the duration of the experiment (4 hours). This was done to compare continuous infusion with "bolus" treatment.

At time 0, lipopolysaccharide (1.5mg/kg *Escherichia coli* 055:B5, Sigma) was injected into the cephalic vein catheter over 60 seconds. Dogs were then maintained under anesthesia for a further 3 hours before being euthanised without 15 regaining consciousness. Arterial blood pressure was recorded continually and respiration was monitored via the central venous pressure trace. At 180 minutes after LPS infusion dogs were given a lethal injection of pentobarbitone IV. A selection of tissues were then collected into 10% formalin and fresh tissue was stored at -70°C.

20 **Results**

LPS injection caused a drop in mean arterial blood pressure within 5 minutes. Some dogs in the saline treatment group had marked hypotension, whilst in other individuals in this group the mean arterial pressure was minimally affected indicating the population of dogs used in this study had individuals that responded weakly to 25 LPS challenge. Previous studies had shown that the blood pressure response in dogs could vary, but the dose of endotoxin chosen caused death is some of these dogs, so increasing the dose of LPS to achieve responses in all dogs was not attempted. It appears some dogs have innate resistance to challenge with endotoxin, even at high doses.

30 Dog #5 (canine hyperimmune plasma bolus) passed bloody diarrhoea at 150 minutes and at autopsy showed haemorrhagic enteritis. The data from this dog have

been included.

The results are presented as experiments in which the plasma treatments were given as a bolus of 200ml per dog (approximately 5.6 – 20 ml/kg) and as a continuous high volume infusion.

5 All data are presented as mean ± SEM. The physiological data are presented as a comparison between saline (control), canine hyperimmune plasma and normal dog plasma on one graph. The data are then presented as a comparison between saline and one plasma treatment with one-sided error bars to make it easier to see the relative differences between treatment and control and the degree of variability in the

10 data. Mean arterial pressure (MAP), systolic blood pressure (SBP), diastolic blood pressure (DBP), pulse pressure and heart rate (HR) are presented in this manner.

Central venous pressure was recorded qualitatively, but provided no useful data. The respiratory rates were too variable to be of use. No identifiable changes were recorded in the ECG's.

15

Treatment by bolus infusion

Canine hyperimmune plasma (200ml bolus) reduced the fall in pulse pressure for the duration of the experiments (3 hours post-LPS infusion) (Figure 6). It had no detectable effects on the other physiological parameters; MAP (Figure 3), SBP

20 (Figure 4), DBP (Figure 5) or HR (Figure 7). Similarly, normal plasma (200ml infusion) did not have detectable effects except for reducing the fall in mean arterial pressure (MAP) after 30 minutes (Figure 3). The effects of normal plasma on MAP appeared to be mainly due to effects on diastolic blood pressure (DBP) (Figure 5).

Canine hyperimmune plasma infusion appeared to reduce the expression of

25 TNFα as measured by commercial ELISA (Figure 8). There was considerable variation in the degree of response in different dogs so the small number of dogs in each group did not allow statistical significance. The ELISA did not measure TNFα activity, only the presence of the cytokine.

Neither canine hyperimmune plasma nor normal plasma treatment had a

30 significant effect on activated clotting time, haematocrit (Figure 13) or haematology (Figures 10, 11, 12 and 14).

The plasma samples collected at time 0 and +180 minutes were assayed for standard biochemical parameters. No significant changes were identified (data not shown).

5 **Canine hyperimmune plasma infusion over 4 hours.**

Continuous infusion of saline, canine hyperimmune plasma or normal canine plasma for 4 hours resulted in nearly 4 times the volume of fluid being infused into the dogs than in the bolus experiments.

Results of measurements of systolic pressure, diastolic pressure, pulse
10 pressure, heart rate and leukocyte numbers are shown in Figures 16-19 and 22.

In the infusion experiments, cardiac output and mean arterial pressure were maintained to a greater degree than when the lesser bolus volume (200ml) was infused. This finding was likely the result of adequate volume expansion in these severely ill dogs. The adequacy of volume expansion can be gauged from the
15 haematocrit which was maintained in the normal range in these experiments (Figure 23). In these experiments canine hyperimmune plasma infusion blocked the late development of hypotension whilst normal plasma was less effective (Figure 15).

In these experiments the concentrations of TNFα in the plasma were not altered by either canine hyperimmune plasma or normal canine plasma infusion when
20 they were given at high volume (Figure 20). However, it is noted that the ELISA only measures the presence of the cytokine and not the cytokine activity.

Activated clotting time (Figure 21) was not significantly affected by LPS infusion. The values of ACT varied widely in the dogs after LPS challenge and plasma treatments did not alter ACT in this model.

25 The plasma samples collected at time 0 and +180 minutes were assayed for standard biochemical parameters. No significant changes were identified (data not shown).

All dogs survived for 3 hours after LPS challenge and no dog appeared to be at risk of dying during the experiments. This was in contrast to earlier studies where
30 the same dose of LPS caused severe respiratory distress in dogs approximately 1 hour after infusion. The cyanosis was accompanied by a cardiac arrhythmia. The LPS was

from different batches, but were all sourced from Sigma.

When canine hyperimmune plasma was administered as a 200ml bolus per dog (approximately 5.6 – 20 ml/kg), there was a degree of protection against some of the pathophysiological changes induced by infusion of LPS. There was an
5 improvement in pulse pressure and a reduction in the expression of TNFα in plasma. The data from the high volume infusion experiments were analysed separately because of the differences high volume infusion made to the physiological data.

Canine hyperimmune plasma treatment improved pulse pressure, particularly later in the experiment when saline and normal plasma treated dogs were displaying
10 a fall in pulse pressure.

Canine hyperimmune plasma has specific anti-TNFα activity *in vitro*. By using an ELISA to measure the concentrations of this cytokine in the plasma it was not possible to conclusively determine if this finding applied *in vivo*. It is hypothesized that canine hyperimmune plasma contains soluble TNFα receptors and
15 these may bind to TNFα in the plasma without affecting detection in the ELISA.

The bolus dosage appeared to be as effective as the 4X dose delivered as a constant infusion over the duration of the experiment. The rationale behind changing the protocol was to observe if continuous infusion was more effective at "mopping up" TNFα as it was formed during the endotoxic shock episode. The higher volume
20 infusion did improve the hydration and haemodynamic status of the animals as measured by packed cell volume. The bolus treatment did not prevent haemoconcentration occurring, but the high volume infusion did. The high volume infusion of saline, canine hyperimmune plasma and normal canine plasma prevented haemo-concentration, a useful property in the treatment of sepsis.

25 The invention provides a method for the treatment of animals that involves the parenteral administration of hyperimmune plasma with anti TNFα activity raised by vaccination of dogs with *Escherichia coli* J5.

TNFα is a potent pro-inflammatory mediator associated with a range of systemic and localised diseases in humans and animals ranging from septic shock,
30 inflammatory bowel disease to rheumatic syndromes. The cytokine is particularly associated with endotoxic shock that is induced by the presence of bacterial

lipopolysaccharide (LPS; more specifically lipid A moieties) in the systemic circulation. It is postulated that the a compromised gut mucosa (displaying a loss of gut mucosal integrity) forms the basis of the gut sepsis model. The current understanding of the gut origin of sepsis is that LPS leaking into the blood stream complexes to an epithelial-cell secreted LPS binding protein (LBP). The LPB, although not essential to the process, appears to accelerate the binding of LPS to a variety of host cell receptors, either near the site of the insult or in remote organs such as the lungs and liver. There is also some evidence that LPB may play a role in reducing the bioavailability of LPS in serum and tissues, although the mechanism for process requires further elucidation. The best understood receptor mechanism is the association of LPS with CD14 (a 55-KD protein found on the surface of monocytes, macrophages and neutrophils or as a serum soluble protein). LPS binding to CD14 receptors activates a signalling complex which may include Toll like receptors (TLRs), heat shock proteins (HSPs) 70 and 90, chemokine receptor 4 (CXCR4) and growth differentiation factor 5 (GDF5). The outcome, in a process not yet fully understood, results in the secretion of hyperinflammatory cytokines such as, but not exclusively TNFα, IL1 and IL6, which if remain unchecked, lead to the systemic pathophysiology associated with fulminant endotoxic shock.

Consequently, it appears that the control of the hyper-inflammatory cascade is a paramount ideal in the control of septic shock. TNFα is a prime target for such control measures and in fact antibodies against human forms of TNFα have shown promising results (Infliximab or Remicade) in human applications. It appears that the normal vertebrate host mechanism for controlling hyper synthesis of TNFα is the secretion of two types of soluble receptors (a p55 (TNFR1) and a p75 (TNFR2).

These receptors are expressed on most cell surfaces, with the majority of the effects of TNFα being mediated by activation of TNFR1. It is thought that soluble forms of TNFR1 and TNFR2 are released into the circulation for the purpose of antagonizing the activity of free TNFα. In syndromes such as gut induced endotoxic shock it would appear that the concentration of available intrinsic soluble receptors is overwhelmed by the amount of TNFα and that the pro-inflammatory mediate initiates a pro-inflammatory cytokine cascade (*e.g.* IL1 and IL6 amongst others).

According to the present invention, it is postulated that repeated vaccinations of dogs with *Escherichia coli* J5 stimulates the host (most likely through macrophage activation) to produce sub-lethal levels of TNFα which induces an increased titre of TNFR1 and TNFR2.

5 In experimental testing of anti-TNFα activity of hyperimmune plasma in the L929 TNFα bioassay (which is an *in vitro* test for TNFα activity rather than simple binding), the present invention demonstrated specific activity of hyperimmune plasma compared to non-vaccinated sera and compared to TNFα controls.

Furthermore, in a canine model of endotoxic shock, both bolus administration 10 and continual infusion of canine hyperimmune plasma demonstrated anti-inflammatory activity, probably at least partly mediated by inhibition of TNF-α activity.

Thus the invention provides a blood plasma product that provides a relatively inexpensive, safe, reproducible and highly efficacious treatment of inflammatory 15 diseases or conditions, particularly those that are at least partly responsive to inhibition of TNFα activity.

Throughout the specification the aim has been to describe the preferred embodiments of the invention without limiting the invention to any one embodiment or specific collection of features. It will therefore be appreciated by those of skill in 20 the art that, in light of the instant disclosure, various modifications and changes can be made in the particular embodiments exemplified without departing from the scope of the present invention.

All computer programs, algorithms, patent and scientific literature referred to herein is incorporated herein by reference.

TABLE 1

5

Dog	Weight dog kg	Treatment infusion	Treatment dose mLs/kg	Treatment tally wrt amt admin
Dog 1	12.4	200mLs/hr	16 /hr	S 1 CRI
Dog 2	10	200mLs/hr	20 /hr	C 1 CRI
Dog 3	18.7	200mLs total 1st hr	10.7	S 1 BOLUS
Dog 4	26	200mLs total 1st hr	7.7	C 1 BOLUS
Dog 5	26	200mLs total 1st hr	7.7	C 2 BOLUS
Dog 6	36	200mLs total 1st hr	5.6	P 1 BOLUS
Dog 7	34	200mLs total 1st hr	5.9	C 3 BOLUS
Dog 8	27	200mLs total 1st hr	7.4	S 2 BOLUS
Dog 9	32	200mLs total 1st hr	6.3	S 3 BOLUS
Dog 10	26.5	200mLs total 1st hr	7.5	P 2 BOLUS
Dog 11	24.7	200mLs total 1st hr	8.1	S 4 BOLUS
Dog 12	26	200mLs total 1st hr	7.7	P 3 BOLUS
Dog 13	32	200mLs total 1st hr	6.3	C 4 BOLUS
Dog 14	16.7	200mLs total 1st hr	12.0	S 5 BOLUS
Dog 15	15	200mLs/hr	13.3 /hr	C 2 CRI
Dog 16	15.7	200mLs/hr		S 2 CRI
Dog 17	22	200mLs/hr	10.22/hr	P 1 CRI
Dog 18	18	200mLs/hr	13.8/hr	P 2 CRI
Dog 19	18	200mLs/hr	12.5/hr	P 3 CRI
Dog 20	15.3	200mLs/hr		C 3 CRI
Dog 21		200mLs/hr		P 4 CRI
Dog 22	23	200mLs/hr		S 3 CRI

CLAIMS

1. A method of treating an inflammatory disease or condition in a mammalian recipient in need of such treatment, including the step of administering isolated hyperimmune plasma obtained from a canine donor to a mammalian recipient to thereby suppress, inhibit, ameliorate or otherwise treat said inflammatory disease or condition.

2. The method of Claim 1, wherein the mammalian recipient is a non-human mammal.

3. The method of Claim 2, wherein the non-human mammal is a canine.

4. The method of Claim 3, wherein the one or more diseases or conditions are selected from the group consisting of an auto immune disease, canine distemper, infectious canine hepatitis, canine herpes virus infection, diarrhoea, haemorrhagic gastro-enteritis, gastric dilatation with or without volvulus, an intestinal catastrophe, cachexia induced by metatstatic tumours, fading puppy complex syndrome, mastitis, vomiting, gastritis, tick paralysis, megoesophagus, pleuritis and pleurisy, tonsillitis, pharyngitis, sinusitis, encephalitis, meningitis, myositis, neuritis, osteitis, chondritis, hepatitis, proctitis, colitis, uveitis, rhinitis, glossitis, gingivitis, dental infections, otitis, gall bladder diseases, biliary obstructive disease, Cushings syndrome, Addisons disease, tendonitis, tendosynovitis, vaginitis, cystitis, endometritis, salivary gland inflammation/infection, helminthiasis and dermatitis.

5. The method of Claim 3, wherein the isolated hyperimmune plasma is administered to a canine recipient as a 200 mL bolus.

6. The method of Claim 3, wherein the isolated hyperimmune plasma is administered to a canine recipient by continuous infusion over four (4) hours.

7. A method of treating a disease or condition that is mediated by TNFα in a mammalian recipient in need of such treatment, said method including the step of administering isolated plasma or an isolated TNFα receptor obtained from a canine donor to said mammalian recipient to thereby suppress, inhibit, ameliorate or otherwise treat said disease or condition.

8. The method of Claim 7, wherein the mammalian recipient is a non-human

mammal.

9. The method of Claim 8, wherein the non-human mammal is a canine.

10. The method of Claim 9, wherein the one or more diseases or conditions are selected from the group consisting of an auto immune disease, canine distemper, infectious canine hepatitis, canine herpes virus infection, diarrhoea, haemorrhagic gastro-enteritis, gastric dilatation with or without volvulus, an intestinal catastrophe, cachexia induced by metatstatic tumours, fading puppy complex syndrome, mastitis, vomiting, gastritis, tick paralysis, megoesophagus, pleuritis and pleurisy, tonsillitis, pharyngitis, sinusitis, encephalitis, meningitis, myositis, neuritis, osteitis, chondritis, hepatitis, proctitis, colitis, uveitis, rhinitis, glossitis, gingivitis, dental infections, otitis, gall bladder diseases, biliary obstructive disease, Cushings syndrome, Addisons disease, tendonitis, tendosynovitis, vaginitis, cystitis, endometritis, salivary gland inflammation/infection, helminthiasis and dermatitis.

11. The method of Claim 9, wherein the isolated hyperimmune plasma is administered to a canine recipient as a 200 mL bolus.

12. The method of Claim 9, wherein the isolated hyperimmune plasma is administered to a canine recipient by continuous infusion over four (4) hours.

13. A method of obtaining a canine TNFα receptor or fragment thereof including the step of obtaining a blood or a component thereof from said canine and isolating, enriching or purifying said TNFα receptor or fragment from said blood or component thereof.

14. The method of Claim 13, wherein, said blood component is hyperimmune plasma.

15. An isolated canine TNFα receptor or fragment thereof obtainable from canine blood or a component thereof.

16. The isolated canine TNFα receptor of fragment thereof of Claim 15, wherein the blood component is hyperimmune plasma.

17. A composition comprising the isolated canine TNFα receptor or fragment thereof of Claim 15 and a pharmaceutically or veterinarily acceptable carrier, diluent or excipient.

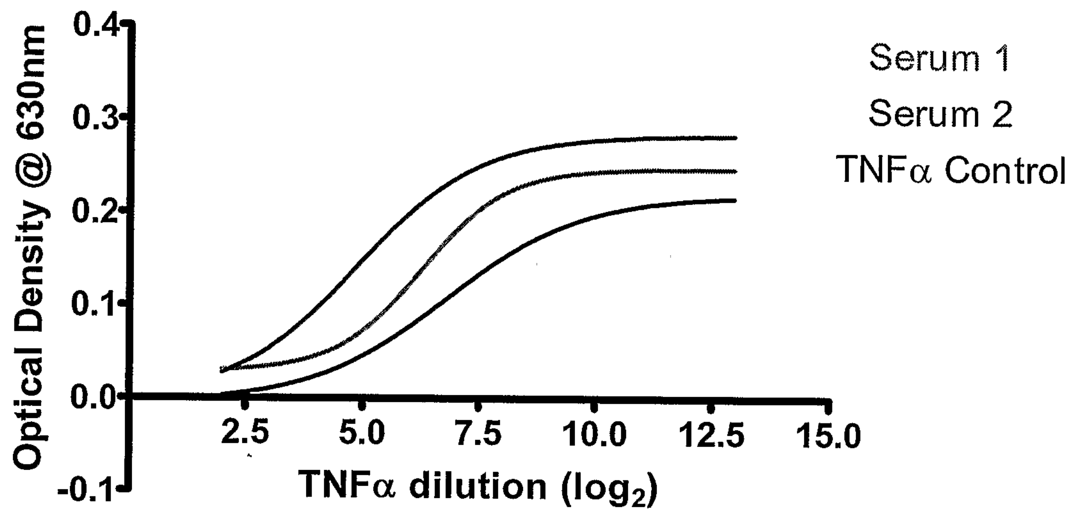


FIG. 1

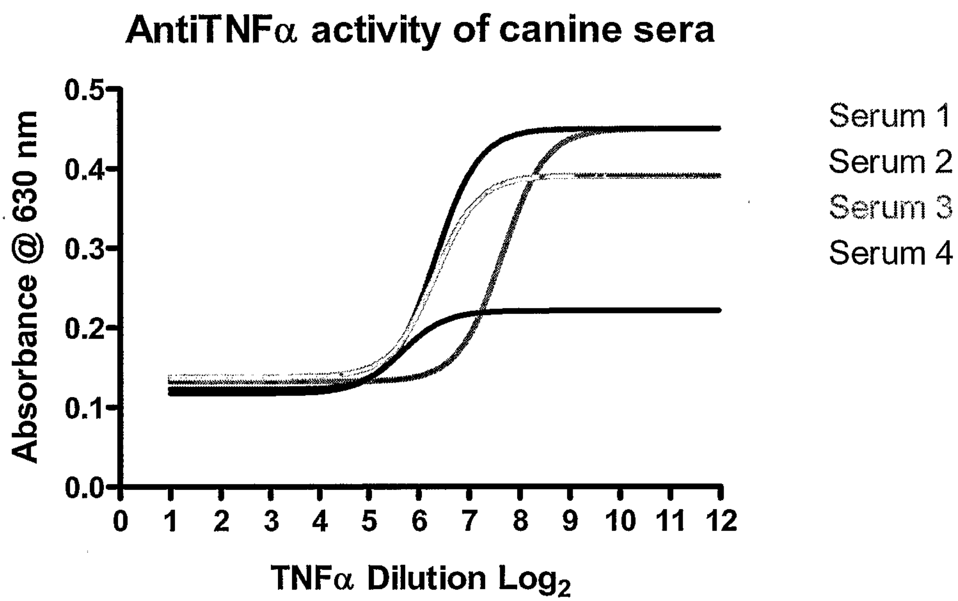
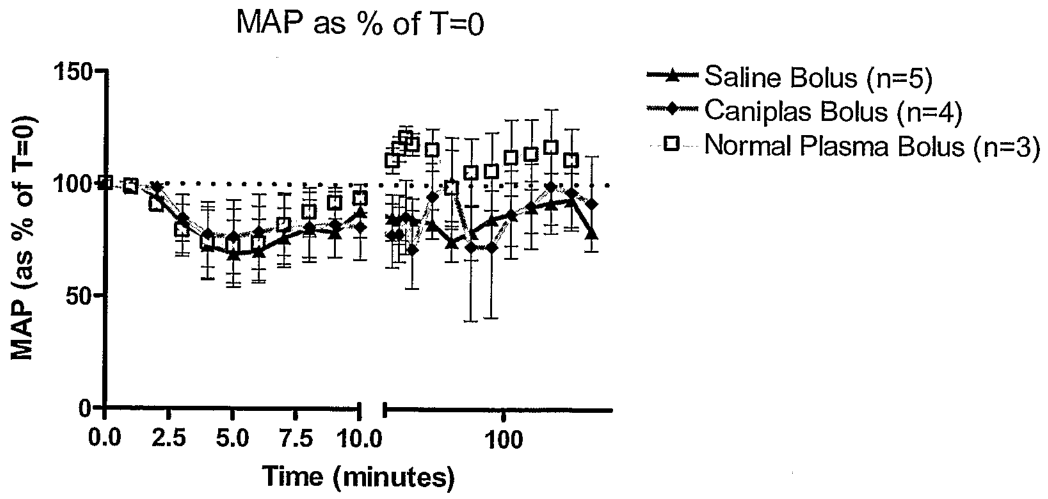
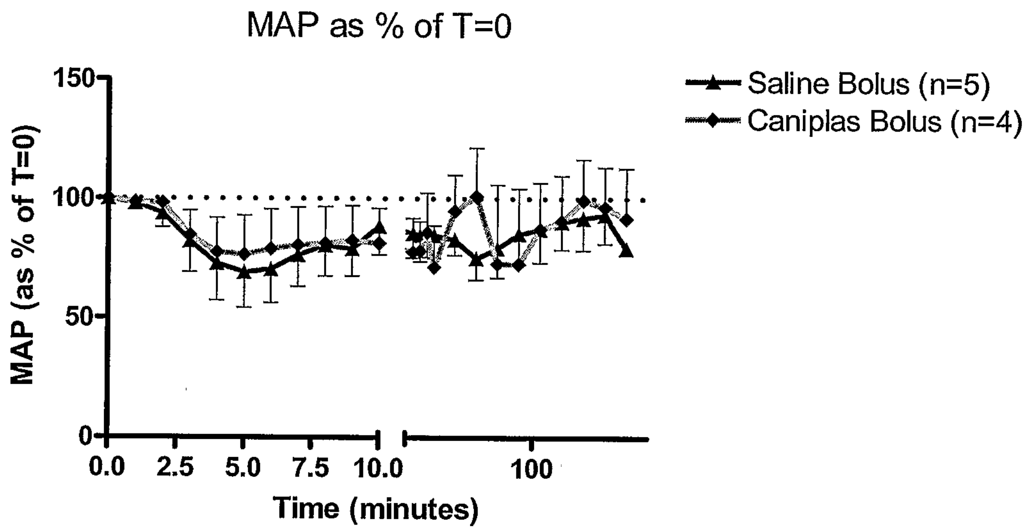


FIG. 2





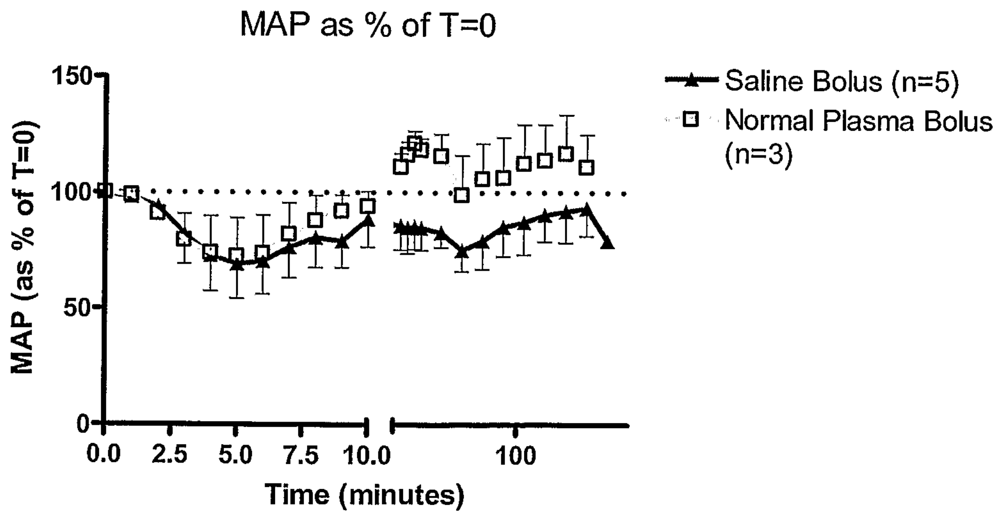
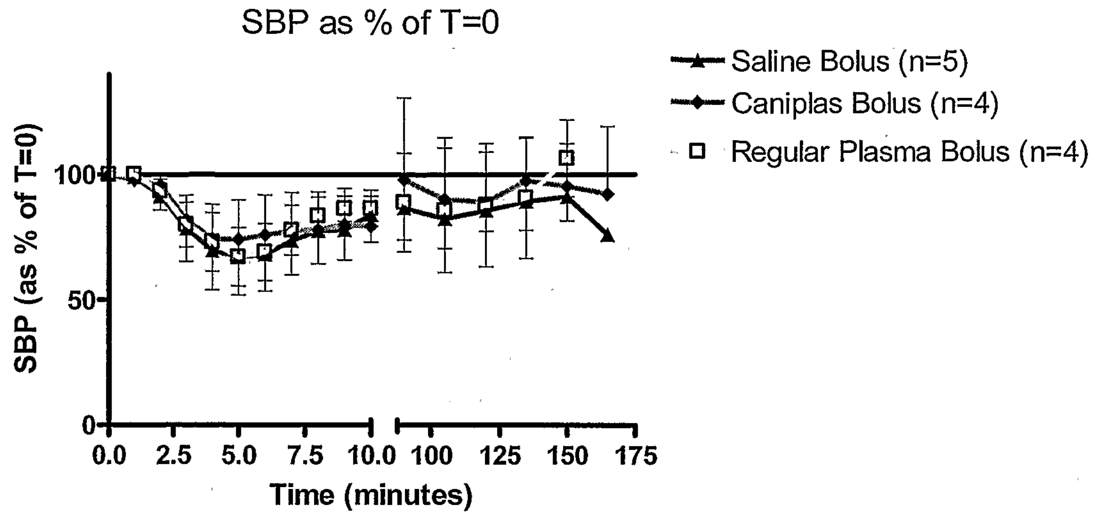
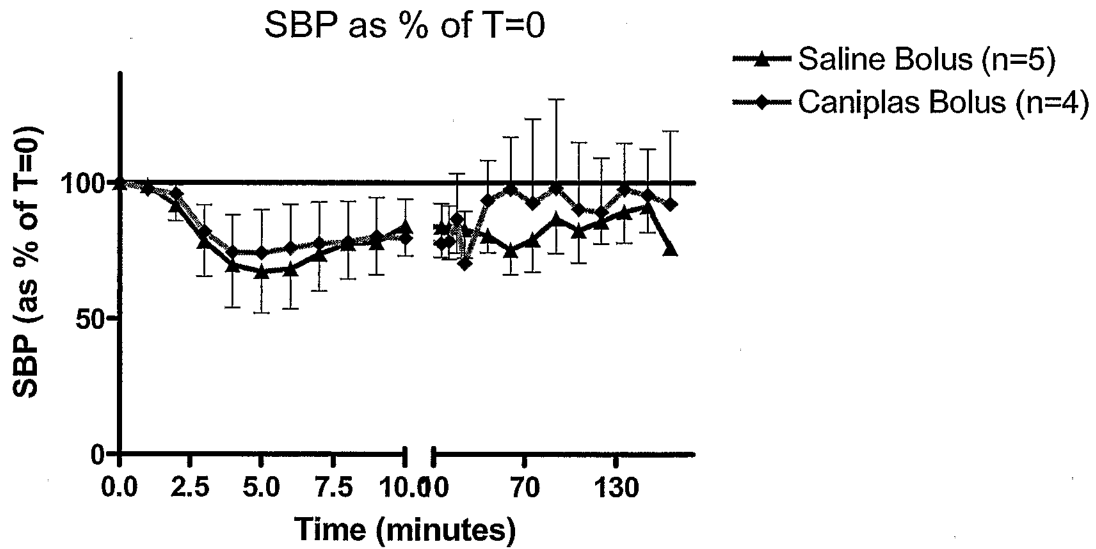


FIG. 3





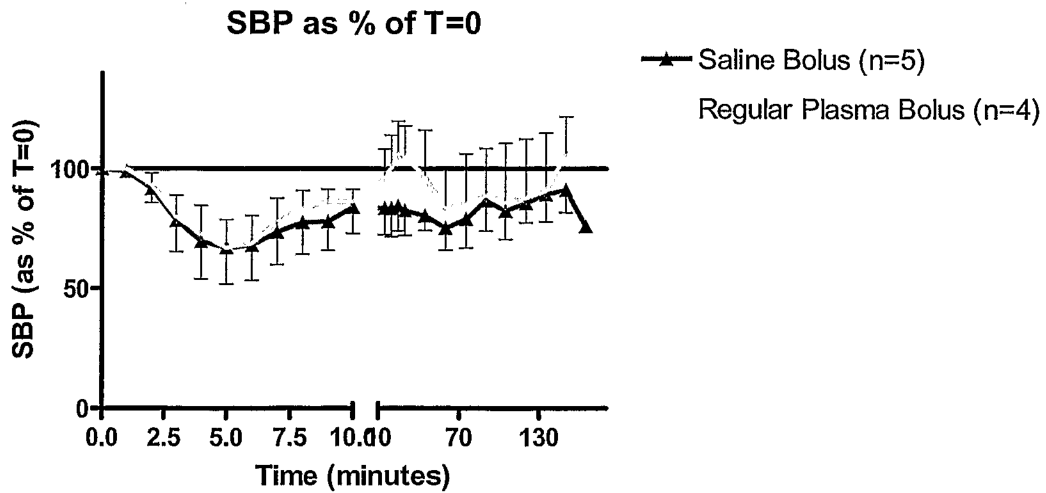


FIG. 4

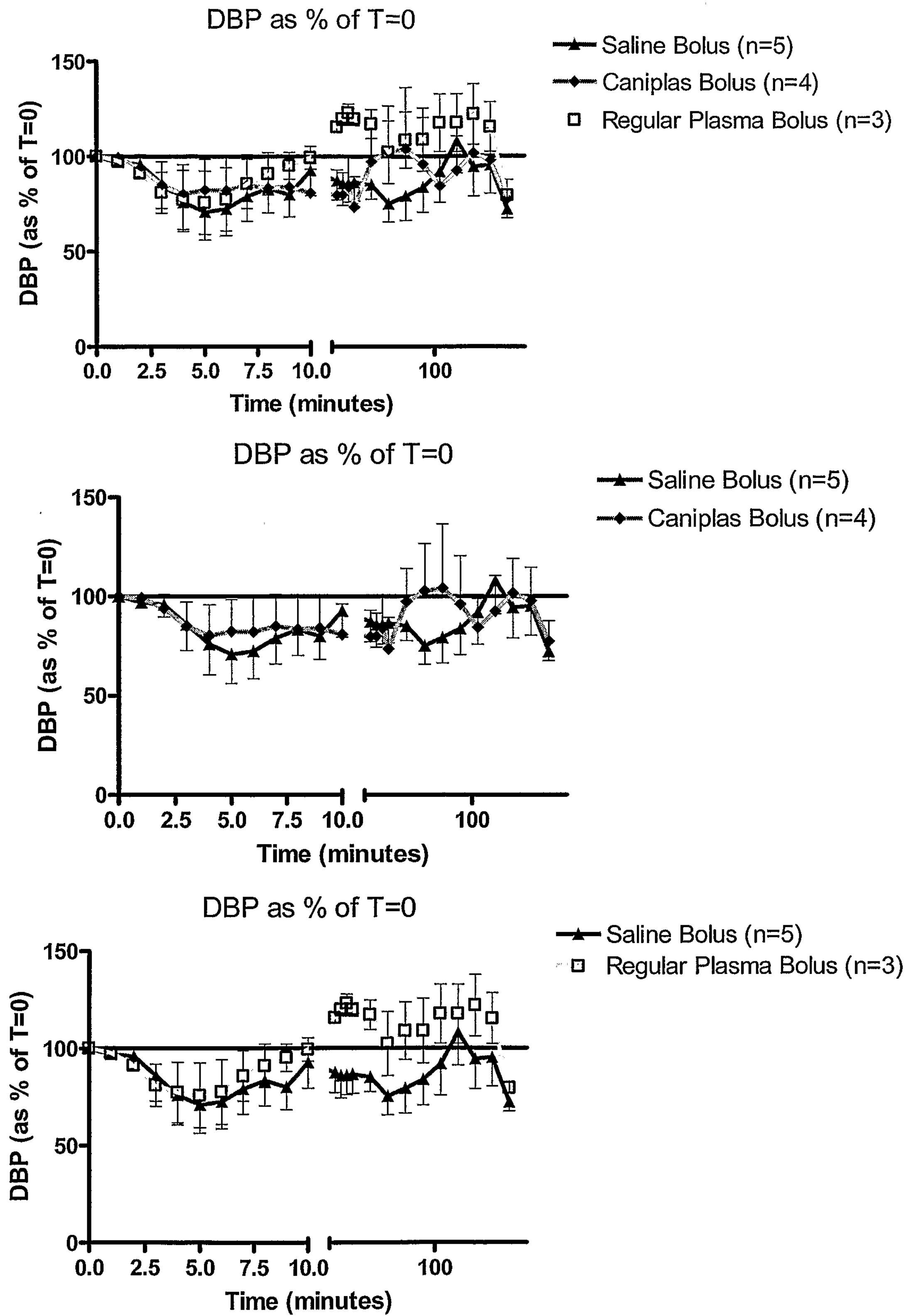


FIG. 5

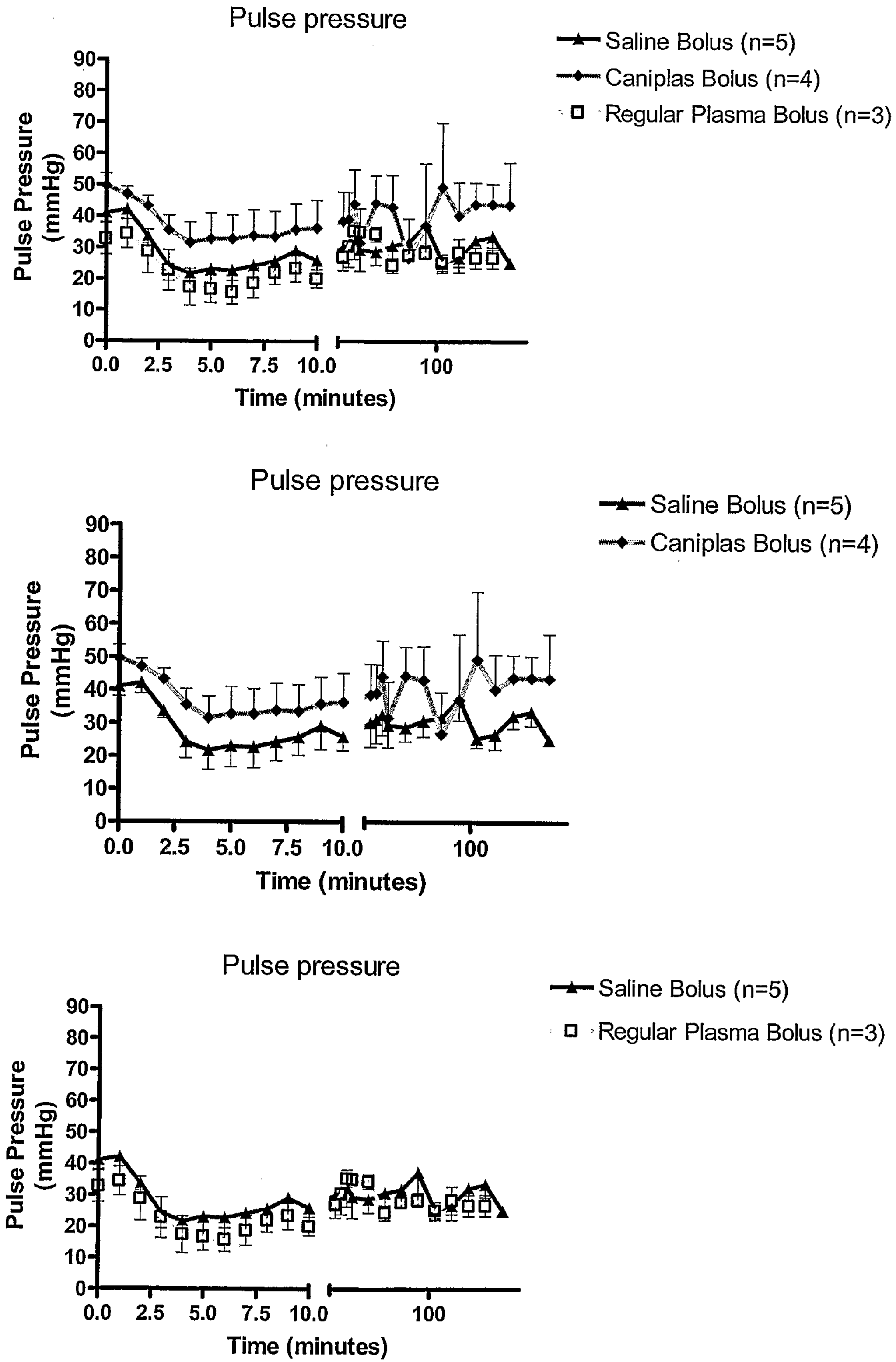


FIG. 6

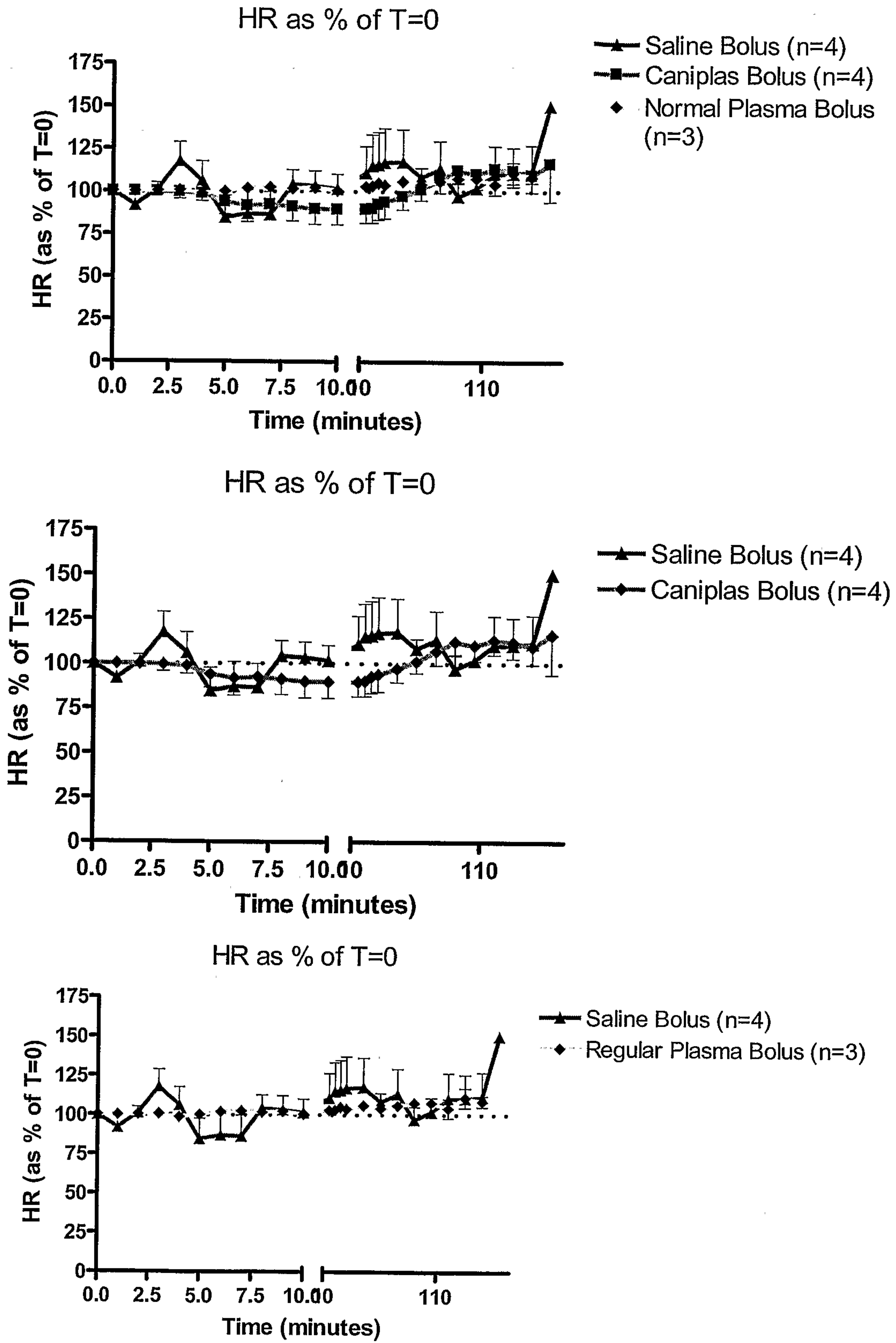


FIG. 7

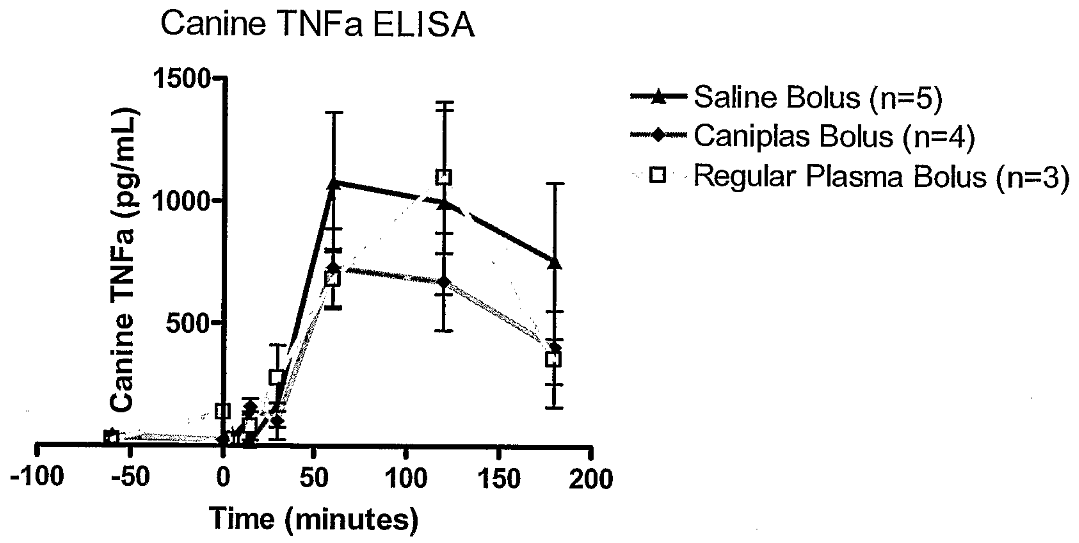


FIG. 8

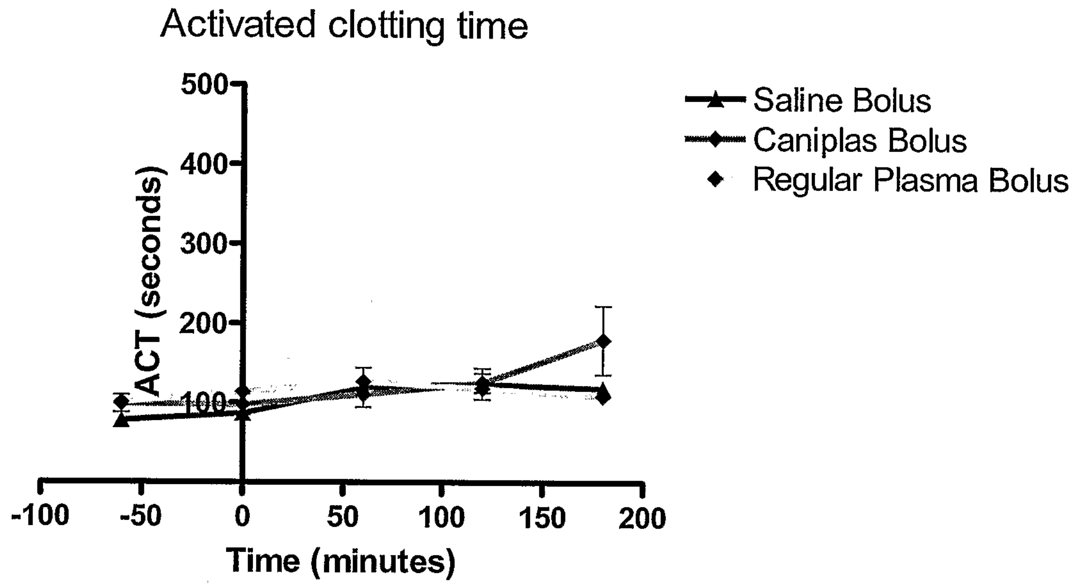


FIG. 9

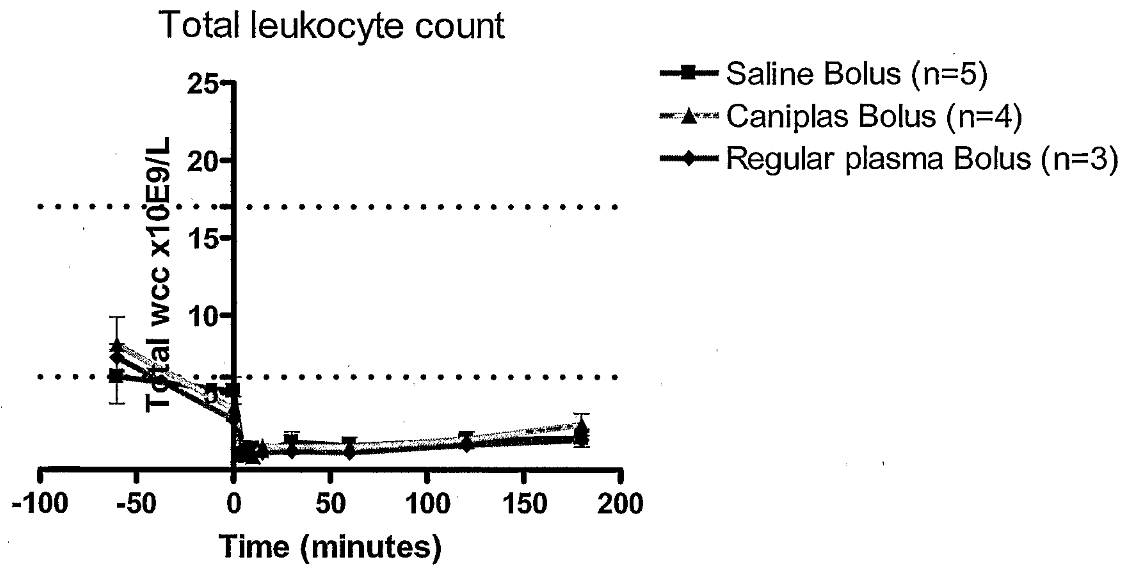


FIG. 10

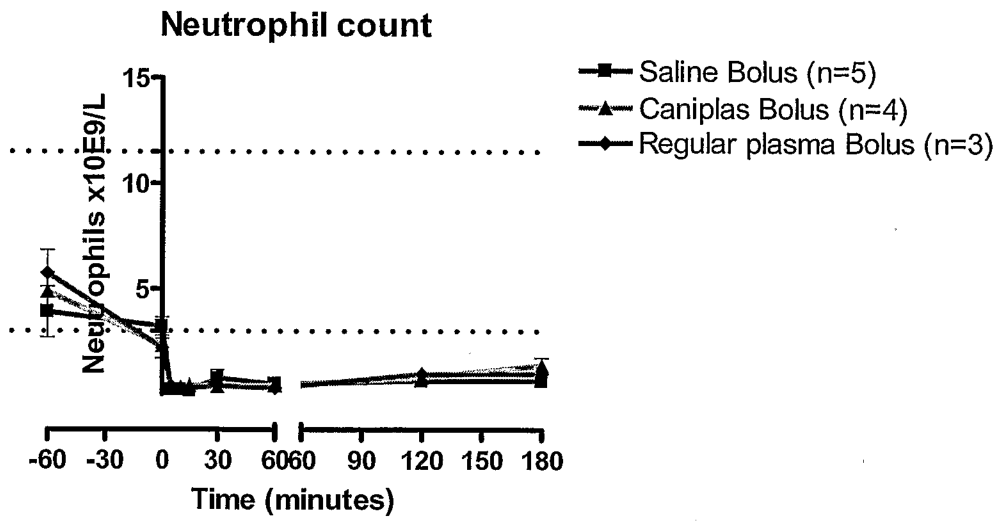


FIG. 11

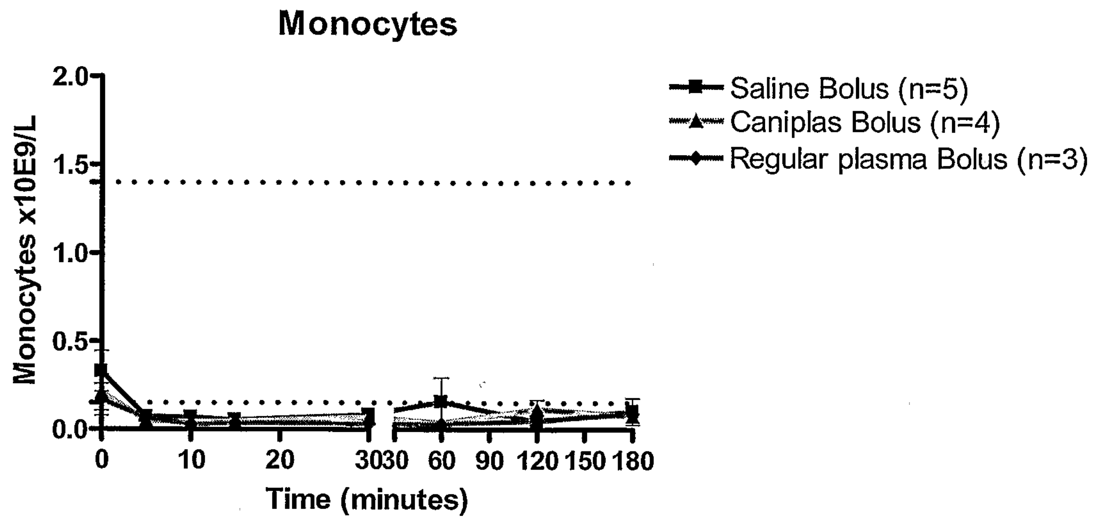


FIG. 12

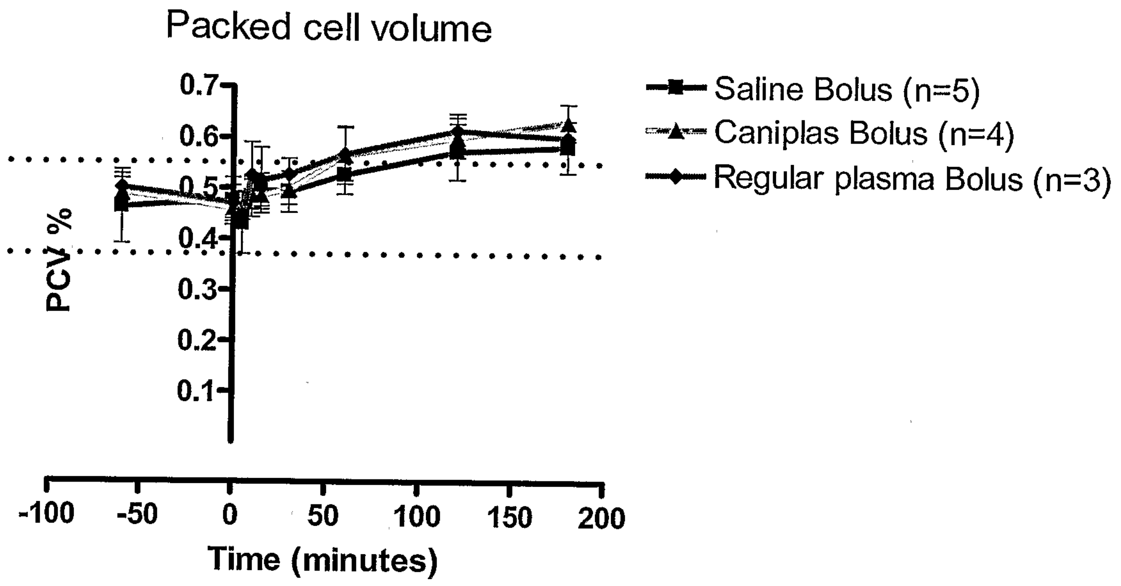


FIG. 13

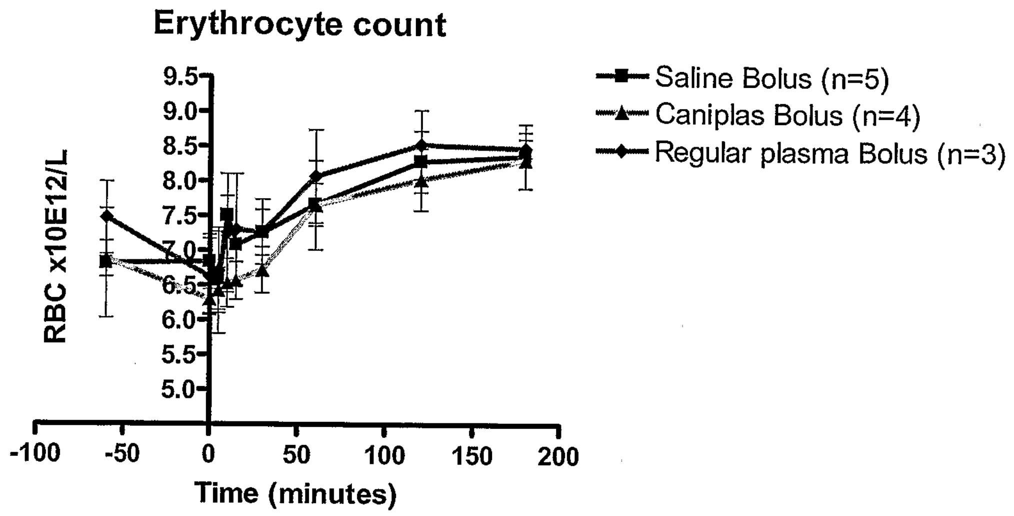
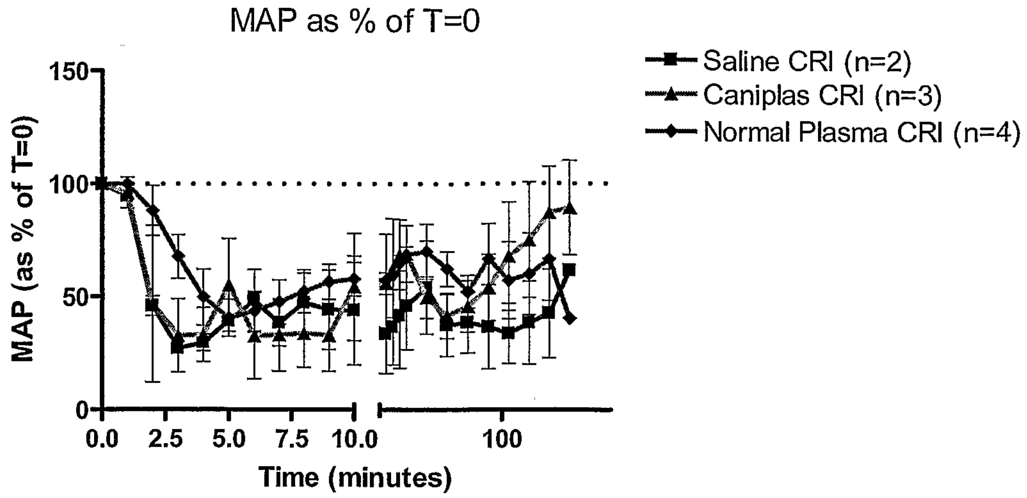
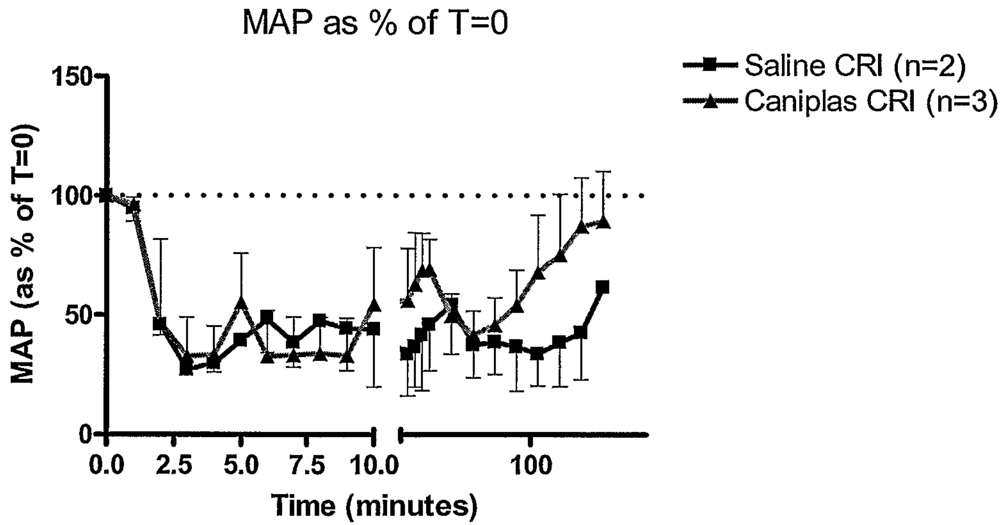


FIG. 14





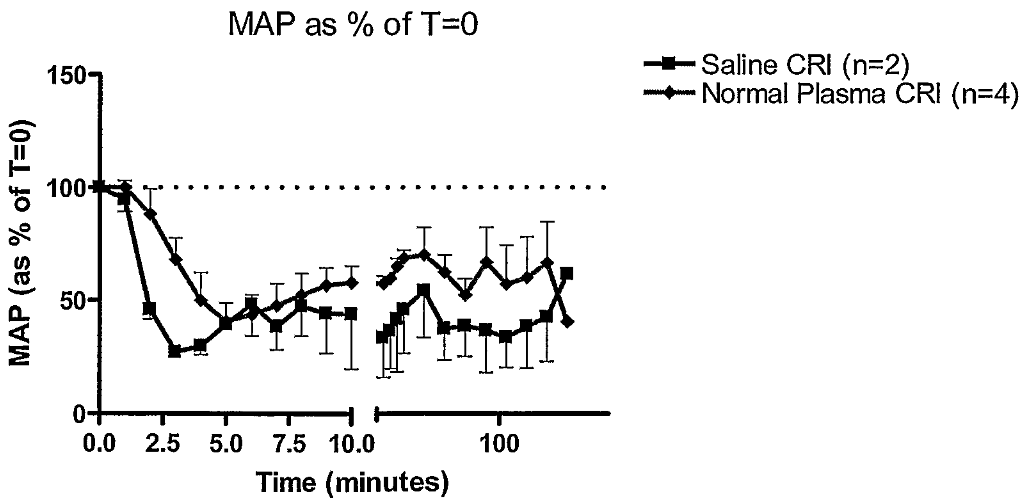


FIG. 15

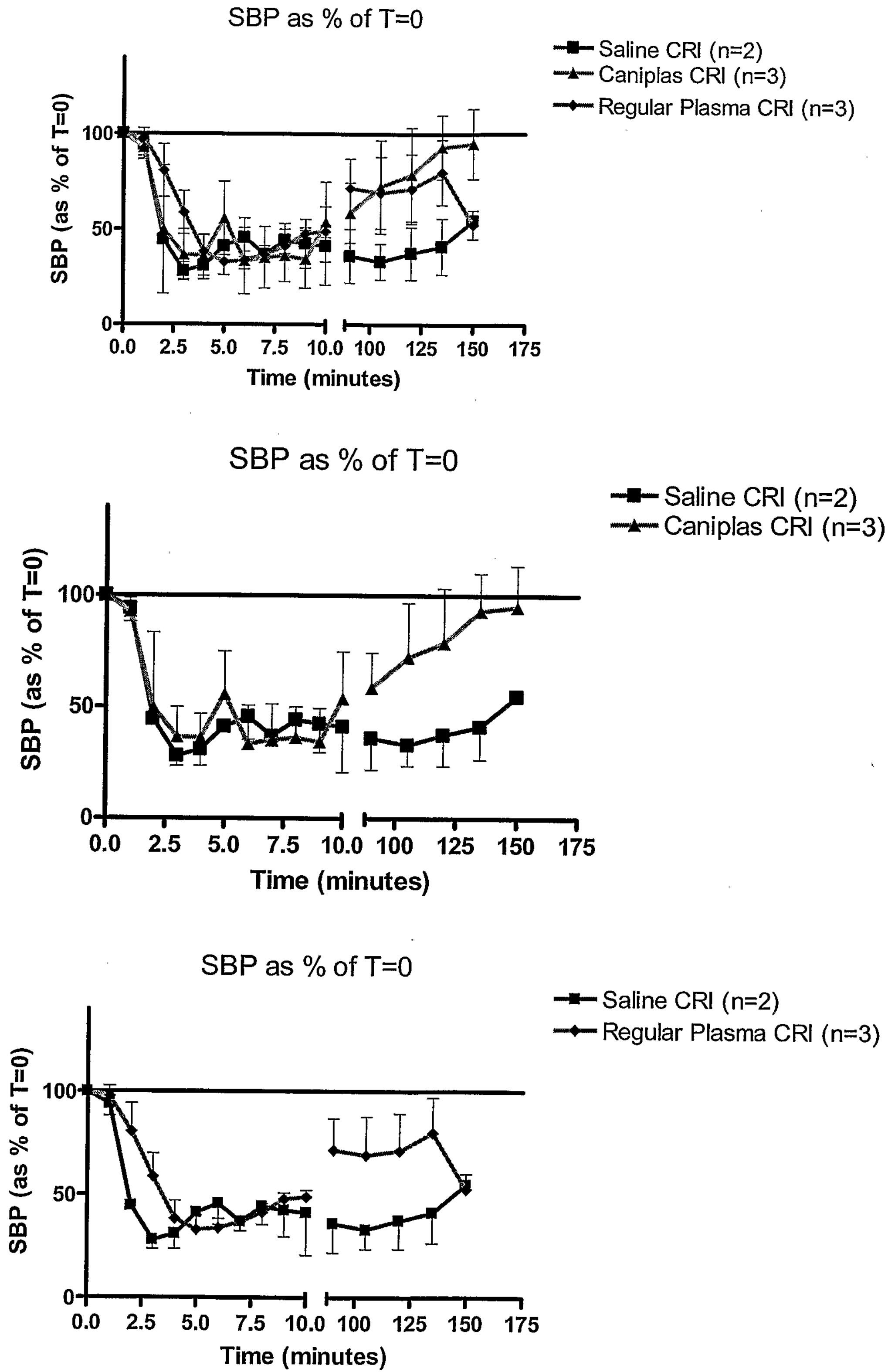
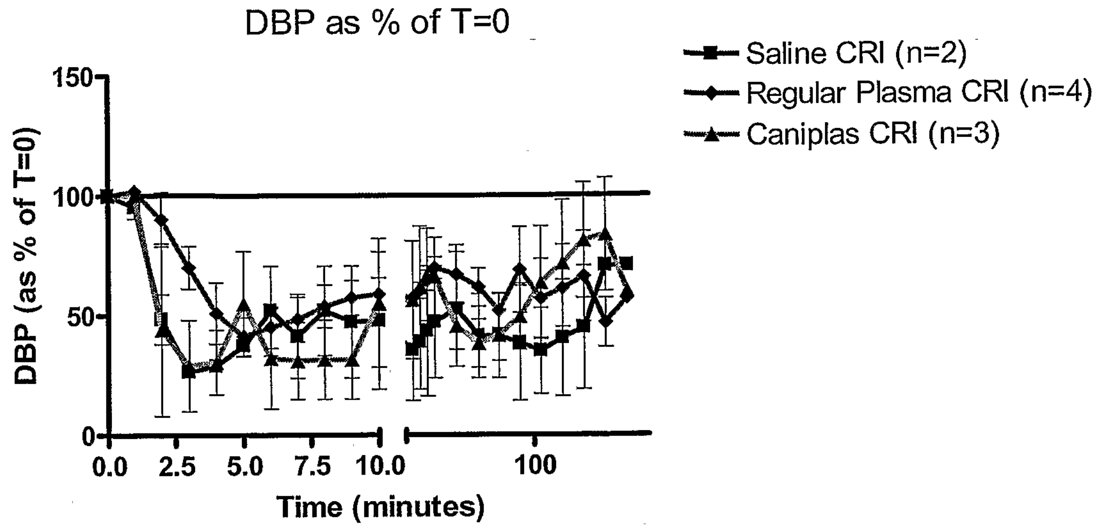
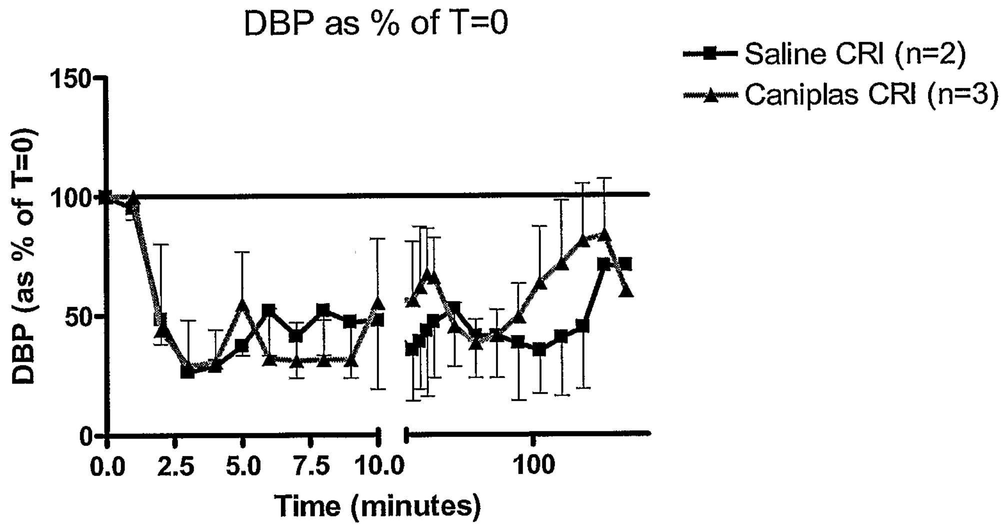


FIG. 16





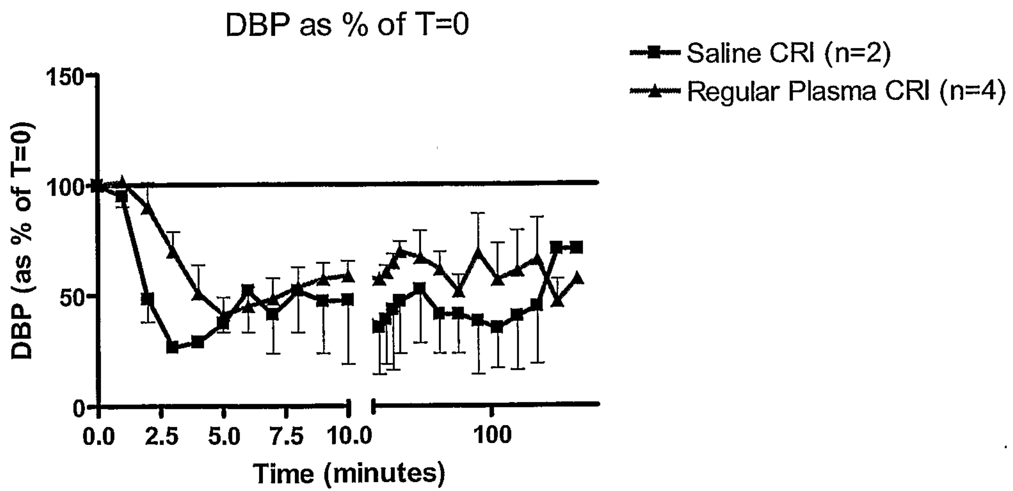
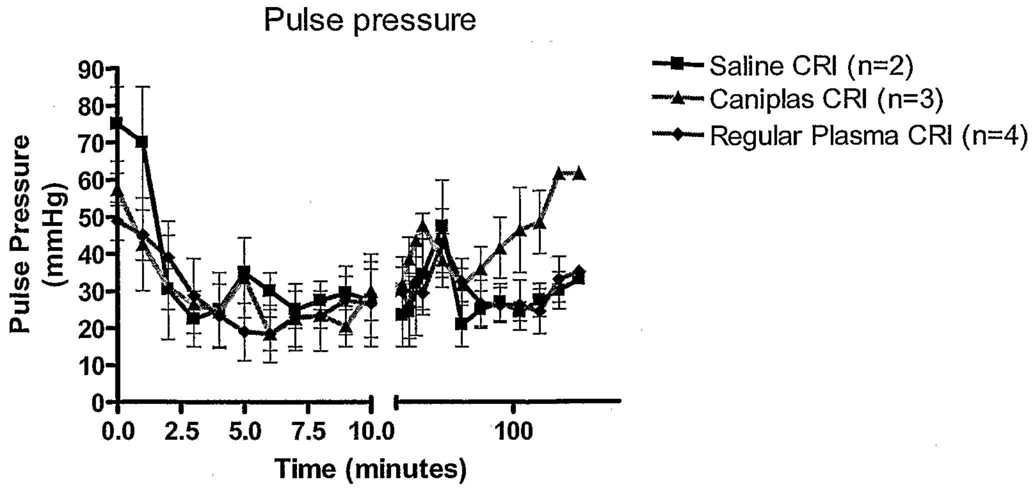
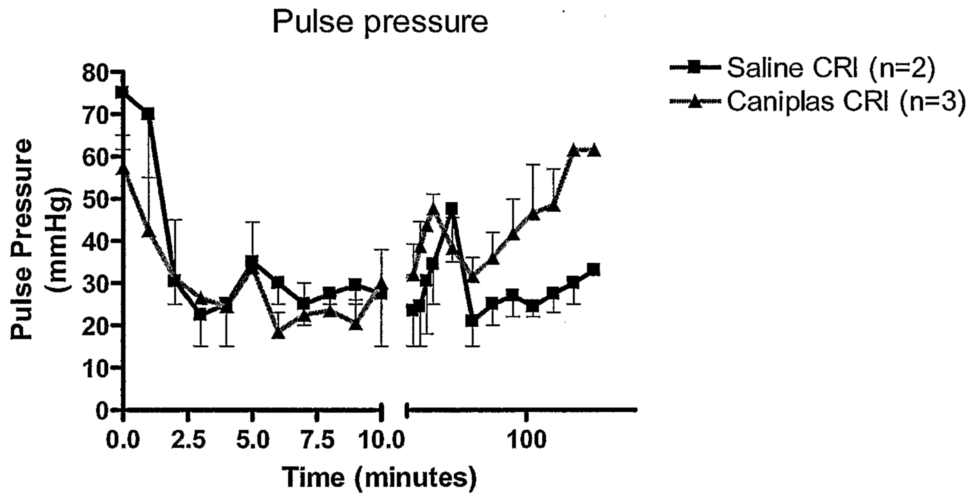


FIG. 17





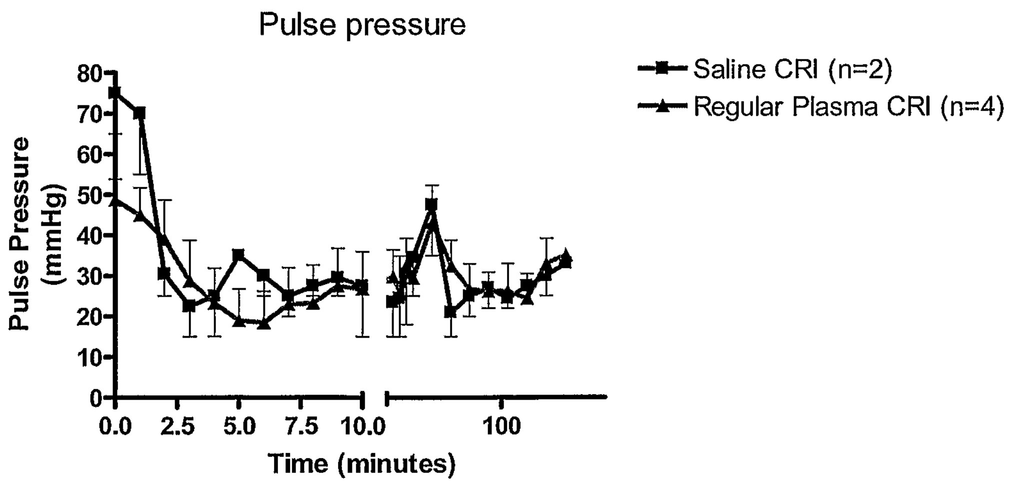


FIG. 18

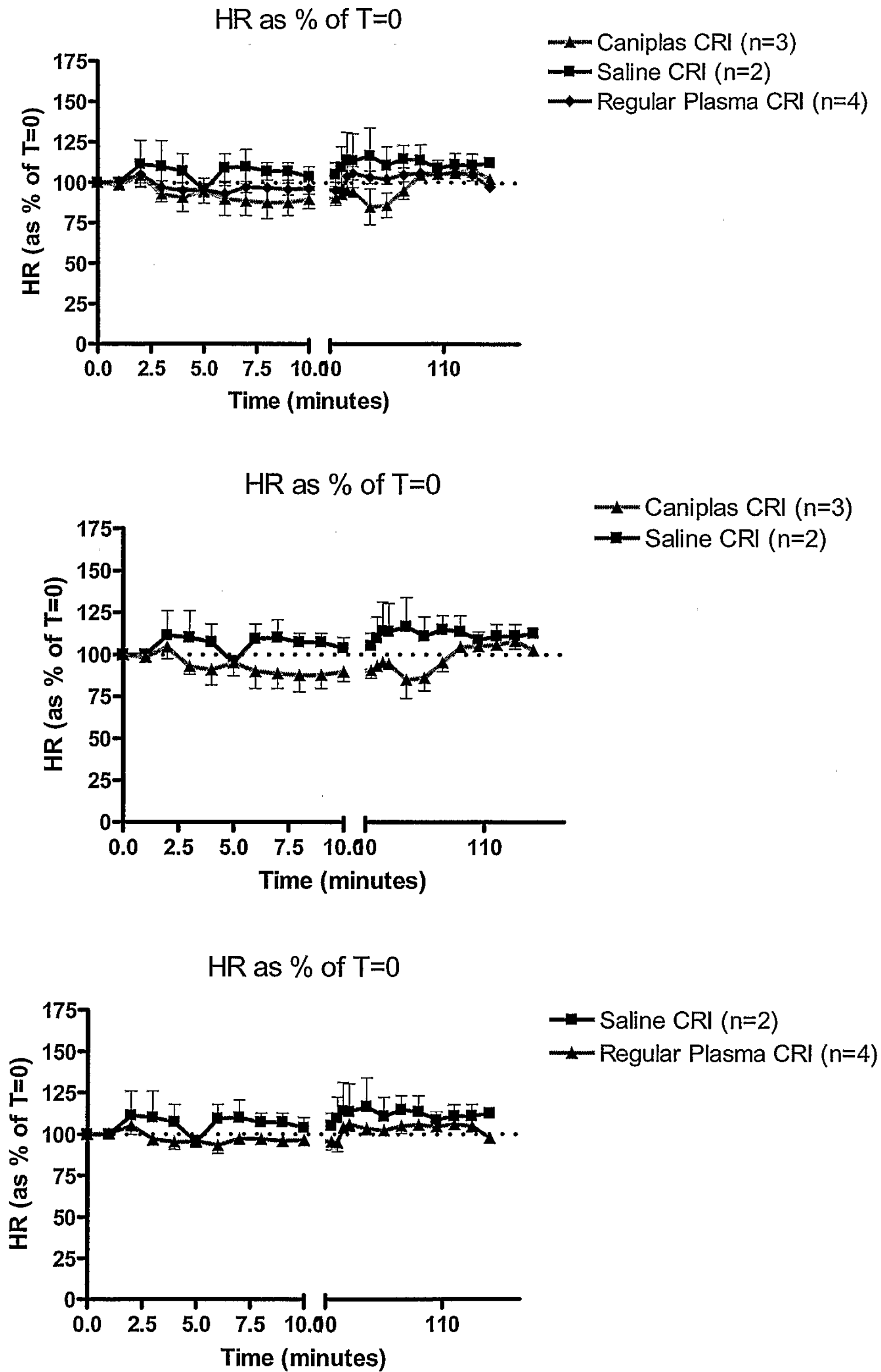
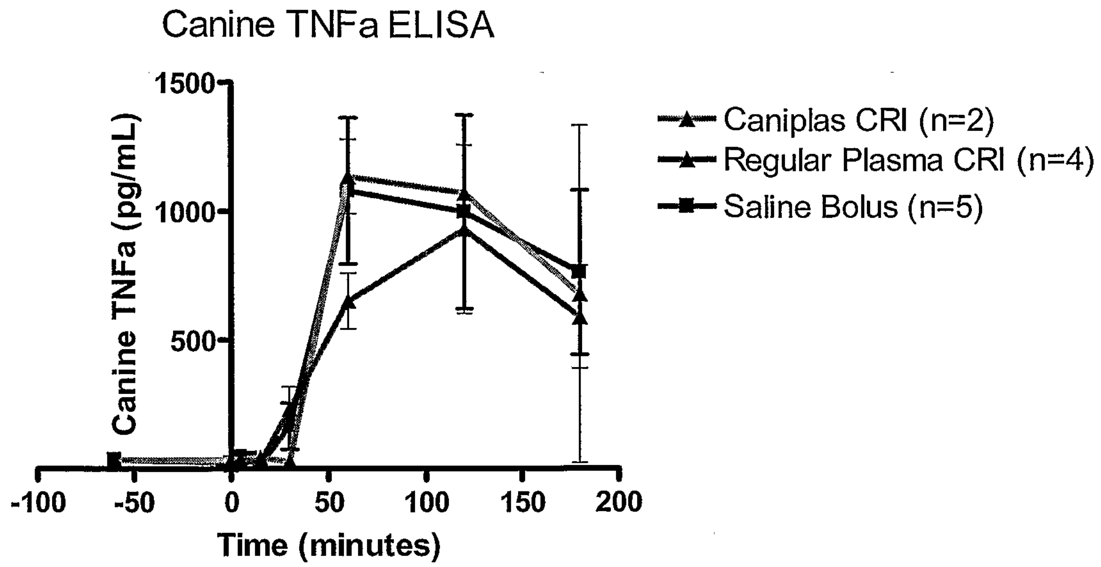


FIG. 19



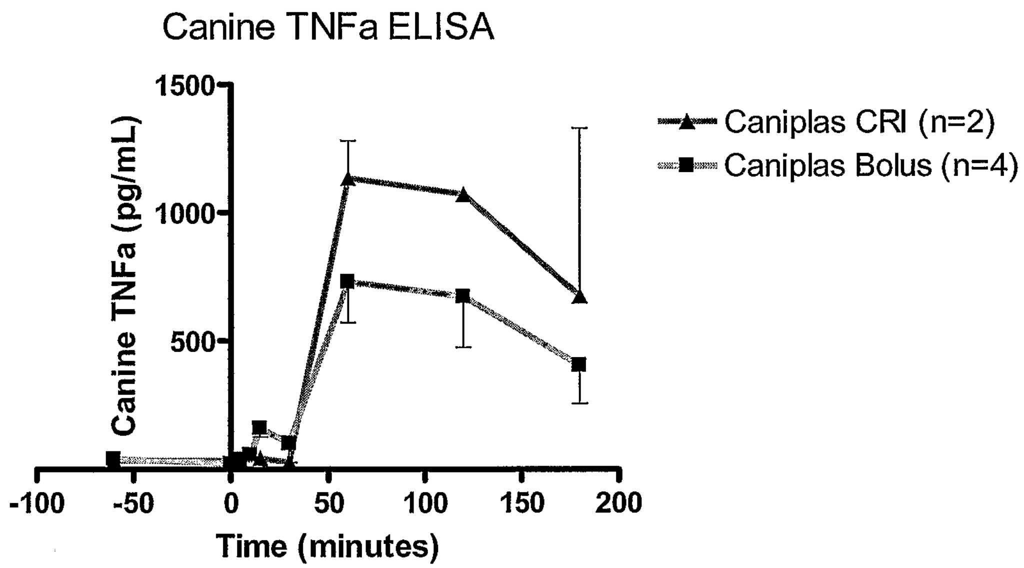


FIG. 20

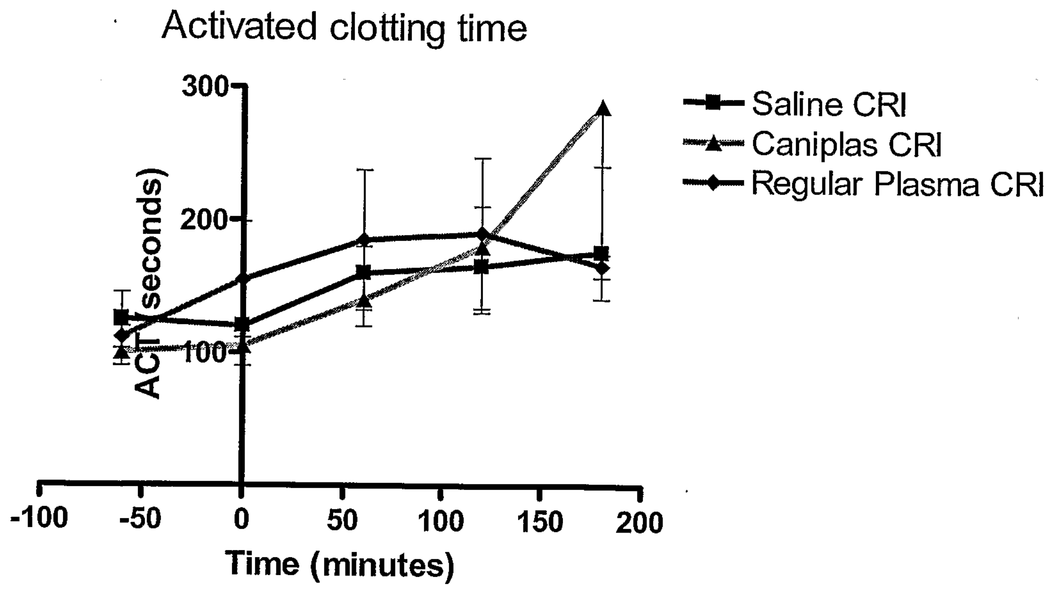


FIG. 21

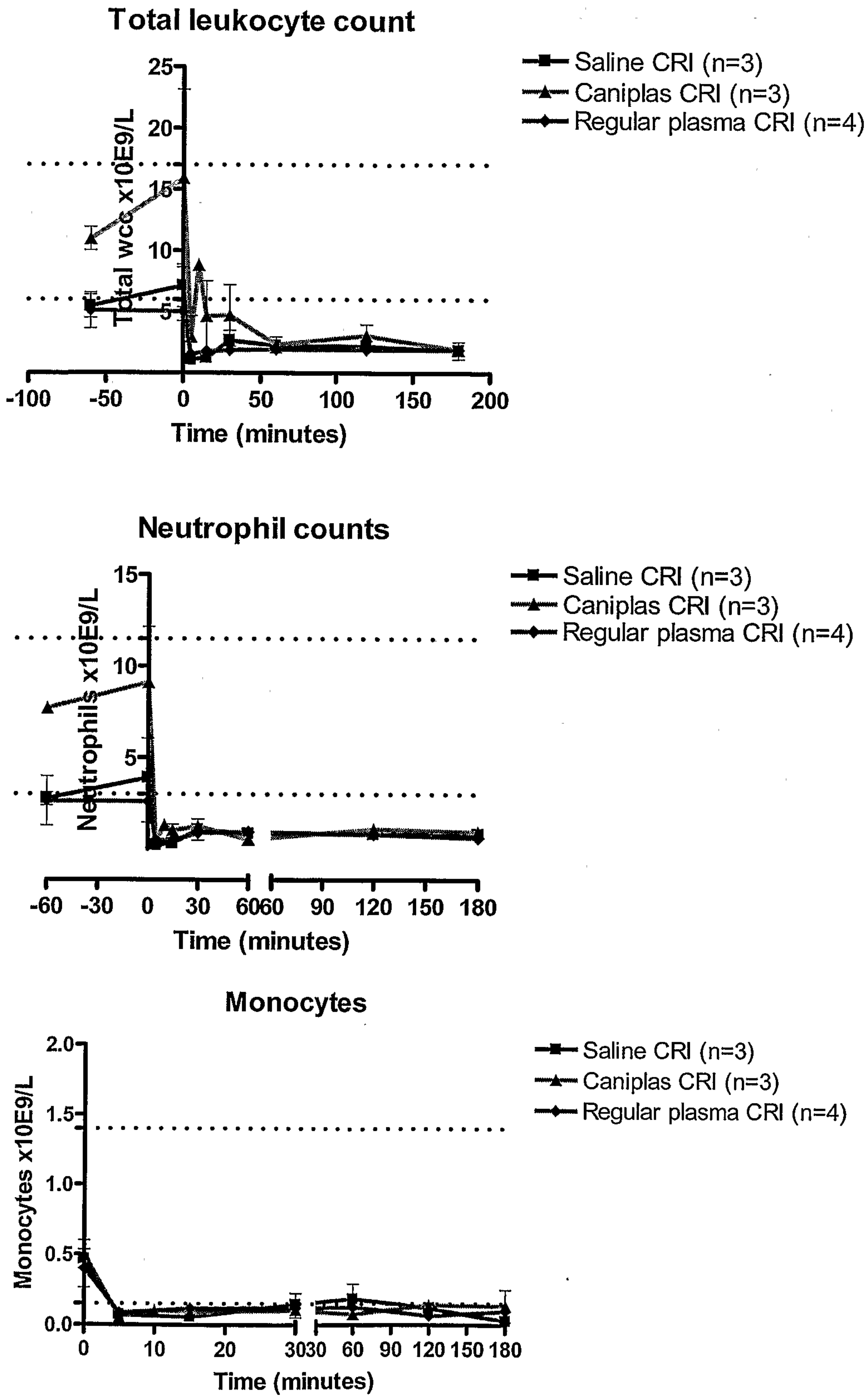


FIG. 22

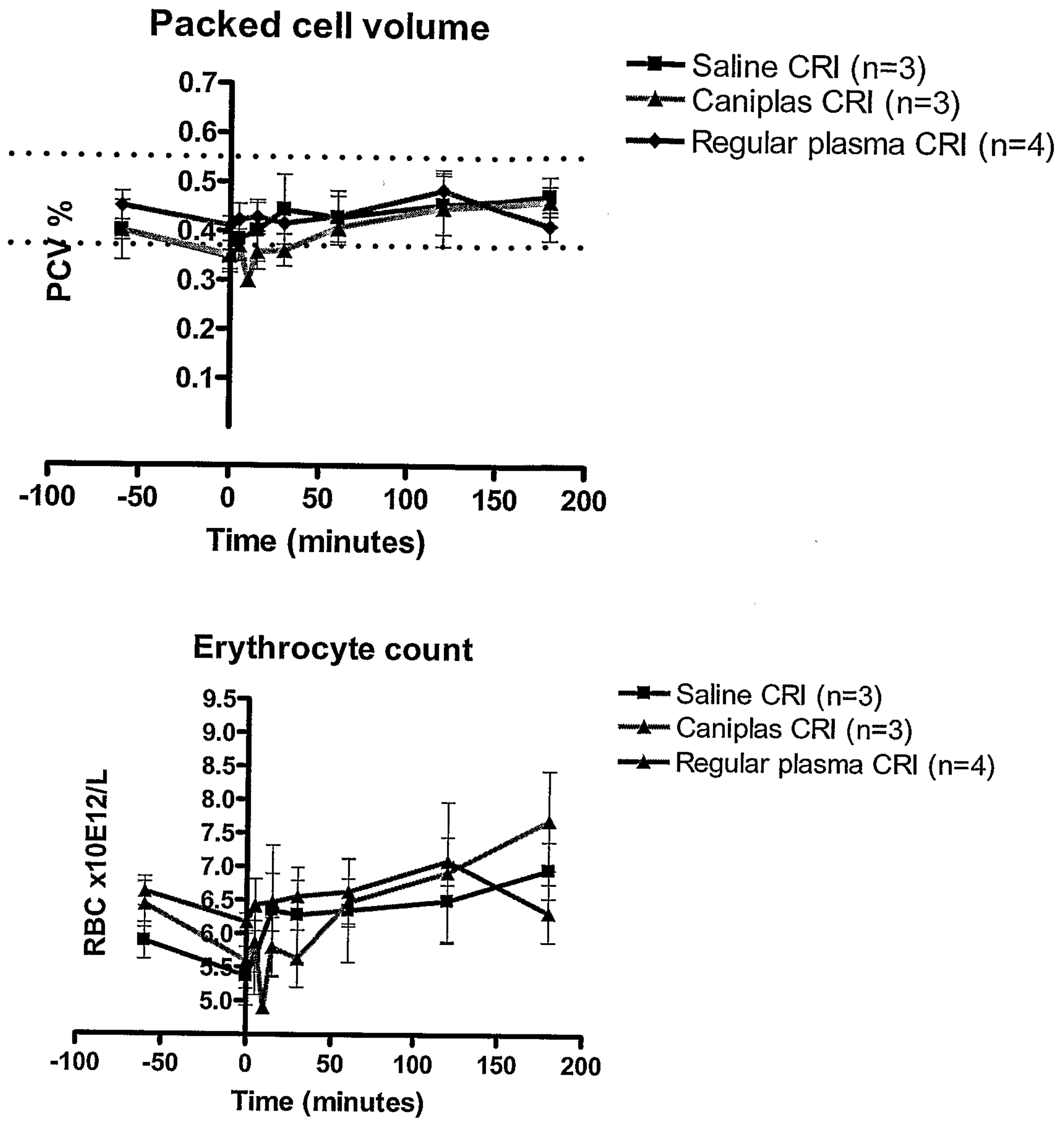


FIG. 23

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2007/000545

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

A61K 35/16 (2006.01) *A61P 37/00* (2006.01)
A61K 38/19 (2006.01) *A61P 37/02* (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPIDS, HCA, MEDLINE, BIOSIS: plasma, antisera, hyperimmune, inflammation, immune, canine, dog, TNF alpha, TNF receptor, Escherichia coli J5.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO2005077299 A (PLASMA VENTURES PTY LTD) 25 August 2005. (See abstract, page 11, lines 9-15)	1-6
X	Spier, S.J. et al. 1989. Protection against clinical endotoxemia in horses by using plasma containing antibody to an Rc mutant *E.coli* (J5). Circulatory Shock. 28:235-248. (See abstract, page 238)	1-6
X	Sekut, L. and Connolly, K. 1998. AntiTNF-α agents in the treatment of inflammation. Exp. Opin. Invest. Drugs. 7(11):1825-1839. (See abstract, page 1829-1830)	7-12, 15-17

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
31 May 2007	02 JUN 2007
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer **RAMILA DEWALAGAMA** AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No : (02) 6222 3659

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO2000062790 A (IMMUNEX CORPORATION) 26 October 2000. (See page 6, lines 19-25, page 7, lines 29-35)	7-12, 15-17
X	US6939950 B2 (Taylor et al.) 6 September 2005. (See abstract, page 8, lines 25-40)	7-12, 15-17
X	WO2001062272 A (IMMUNEX CORPORATION) 30 August 2001. (See page 9, lines 5-25)	7-12, 15-17
X	Gatanaga, T. et al. 1990. Purification and characterization of an inhibitor (soluble tumor necrosis factor receptor) for tumor necrosis factor and lymphotoxin obtained from the serum ultrafiltrates of human cancer patients. PNAS. 87(22):8781-4. (See whole document)	13-14

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/AU2007/000545**

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	2005077299	AU	2004315533	CA	2563904	EP	1718238
WO	2000062790	AU	43632/00	AU	45336/01	CA	2366785
		CA	2416250	EP	1171148	EP	1259252
		US	2001021380	US	2003148955	US	2004220103
		WO	0162272				
US	6939950	US	6406907	US	2002076765	AU	43632/00
		AU	45336/01	CA	2366785	CA	2416250
		EP	1171148	EP	1259252	US	2001021380
		US	2003148955	US	2004220103	WO	0062790
		WO	0162272				

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX