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(57) Abstract: The present invention provides methods for treating and preventing cardiac deficiencies and injuries (e.g., cardiac injury and ventricular dilation) in dystrophic subjects using poloxamer containing compositions. In some embodiments, the poloxamers are administered to dystrophic subjects prior to cardiac stress, such as that caused by anaesthesia. In other embodiments, the present invention provides systems comprising poloxamer compositions and at least one anaesthetic agent.



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METHODS AND SYSTEMS FOR TREATING AND PREVENTING CARDIAC INJURY IN DYSTROPHIC SUBJECTS

The present application claims priority to U.S. Provisional application serial number 61/264,560 filed November 25, 2009, which is herein incorporated by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with government support under Grant No. AG015434 awarded by the National Institutes of Health. The government has certain rights in the invention.

FIELD OF THE INVENTION

The present invention provides methods for treating and preventing cardiac deficiencies and injuries (e.g., cardiac injury and ventricular dilation) in dystrophic subjects using poloxamer containing compositions. In some embodiments, the poloxamers are administered to dystrophic subjects prior to cardiac stress, such as that caused by anaesthesia. In other embodiments, the present invention provides systems comprising poloxamer compositions and at least one anaesthetic agent.

BACKGROUND OF THE INVENTION

Duchenne Muscular Dystrophy (DMD) is an X-linked genetic disease caused by a mutation in the dystrophin gene. As a result, muscles from patients with DMD lack dystrophin, a 427 kDa protein located on the cytoplasmic surface of the plasma membrane, the sarcolemma, of muscle fibres (see, e.g., Blake DJ, et al., (2002) *Physiol Rev* 82, 291-329). Dystrophin is required for the assembly of the dystrophin-associated glycoprotein complex that is embedded in the sarcolemma (see, e.g., Ohlendieck K & Campbell KP (1991) *J Cell Biol* 115, 1685-1694). The dystrophin-glycoprotein complex links the actin cytoskeleton to the basement membrane and is thought to provide mechanical stability to the sarcolemma (see, e.g., Petrof BJ (2002) *Am J Phys Med Rehabil* 81, S162-S174). Duchenne muscular dystrophy (DMD) is a fatal disease of striated muscle deterioration resulting from the loss of the cytoskeletal protein dystrophin. In the absence of dystrophin small tears in the sarcolemma arise causing loss of membrane integrity, muscle wasting, and heart failure in DMD patients. There is no cure or effective treatment for progressive dystrophic

cardiomyopathy. As such, there exists a need for new compositions, methods, and systems for treating and preventing cardiac injury in subjects with DMD.

SUMMARY OF THE INVENTION

5 The present invention provides methods for treating and preventing cardiac deficiencies and injuries (e.g., cardiac injury and ventricular dilation) in dystrophic subjects using poloxamer containing compositions. In some embodiments, the poloxamers are administered to dystrophic subjects prior to cardiac stress, such as that caused by anaesthesia. In other embodiments, the present invention provides systems comprising poloxamer
10 compositions and at least one anesthetic agent.

 In some embodiments, the present invention provides methods of treating a dystrophic subject to prevent or reduce cardiac injury or ventricular dilation comprising: administering to the dystrophic subject a composition comprising a poloxamer under conditions such that the cardiac injury or the ventricular dilation is reduced or prevented.

15 In certain embodiments, the administering is conducted prior to cardiac stress. In further embodiments, the cardiac stress is caused by anesthesia or other medical procedure. In particular embodiments, the cardiac stress is caused by blood loss or hypotension. In particular embodiments, the administering is under conditions such that the cardiac injury is reduced or prevented. In other embodiments, the administering is under conditions such that
20 the ventricular dilation is reduced or prevented. In particular embodiments, the methods further comprise measuring cardiac damage in said subject before, or after, or both before and after the administering.

 In some embodiments, the poloxamer is P188. In further embodiments, the poloxamer is administered at a dosage level of approximately between 30 – 90 mg per kg
25 weight of the subject per hour for at least 5 weeks (e.g., at least 5, 6, 7, 8, 9, 10 ... 15 ... or 20 weeks). In certain embodiments, the poloxamer is administered for 5-10 weeks. In further embodiments, the dosage level is about 60 mg per kg weight of the subject per hour for at least 5 weeks. In some embodiments, the composition comprising a poloxamer is co-administered with one or more agents selected from the group consisting of streptomycin,
30 prednisone, deflazacort, azathioprine, cyclosporine, valproic acid, phenylbutyrate, sodium butyrate, M344, suberoylanilide hydroxamic acid, and PCT124.

 In some embodiments, the present invention provides systems comprising: a) a composition comprising a poloxamer; and b) an anesthetic agent. In certain embodiments, the anesthetic agent is an inhaled agent (e.g., desflurane, enflurane, halothane, isoflurane,

methoxyflurane, nitrous oxide, sevoflurane, and xenon). In other embodiments, the anesthetic agent is a non-opioid intravenous agent (e.g., barbiturates (e.g., methohexital and thiopental), benzodiazepines (e.g., diazepam, lorazepam, and midazolam), etomidate, ketamine, and propofol). In further embodiments, the anesthetic agent is a opioid intravenous agent (e.g., alfentanil, fentanyl, remifentanyl, and sufentanil).

The methods and systems are not limited to a particular type of poloxamers. In some embodiments, the poloxamer is a purified or fractionated poloxamer. In some embodiments, the poloxamer is P188, P138, P237, P288, P124, P338, and/or P407. In some embodiments, poloxamines and/or polyglycidols are used instead of, or with, poloxamers. The methods are not limited to a particular type of subject. In some embodiments, the subject is a human subject. In some embodiments the subject is a non-human subject (e.g., dog or other larger mammal). In some embodiments, the subject is a dystrophin deficient subject. In some embodiments, the subject has Duchene's muscular dystrophy.

The methods are not limited to a particular form of administration of the composition. In some embodiments, the composition is administered via intravenous administration. In some embodiments, the administration is local (e.g., intracardiac).

The methods are not limited to a particular dosage level for poloxamer administration to a subject. In some embodiments, it is expected that each dose of a composition comprising a poloxamer comprises between 0.1 mg – 200 mg (e.g., 0.1 to 5000; 0.2 to 4000; 0.3 to 3000; 0.4 to 2500; 0.5 to 2000; 0.6 to 1500; 0.7 to 1000; 1 to 800; 10 to 500; 100 to 450; 200 to 400; 300 to 350; etc.) of poloxamer per kg weight of the subject being treated. In some embodiments, each dose comprises between 0.46 mg to 500 mg of poloxamer per kg weight of the subject being treated. In some embodiments, each dose comprises 0.46 mg of poloxamer per kg weight of the subject being treated. In some embodiments, each dose comprises between 200-400 mg of poloxamer per kg weight of the subject being treated. In some embodiments, each dose comprises between 400 – 500 mg of poloxamer per kg weight of the subject being treated. In some embodiments, each dose comprises 500-2000 mg of poloxamer per kg weight of the subject being treated. In some embodiments, each dose comprises less than 100 mg of poloxamer per kg weight of the subject being treated. In some embodiments, each dose comprises more than 2000 mg of poloxamer per kg weight of the subject being treated. In some embodiments, each dose comprises between 400-520 mg of poloxamer per kg weight of the subject being treated. In some embodiments, each dose comprises more between 425-495 mg of poloxamer per kg weight of the subject being treated. In some embodiments, each dose comprises between 450-470 mg of poloxamer per

kg weight of the subject being treated. In some embodiments, each dose comprises 460 mg of poloxamer per kg weight of the subject being treated.

The methods are not limited to a particular manner of administration of poloxamer to a subject. Examples of administration include, but are not limited to, intravenous,
 5 intraarterial, subcutaneous, intraperitoneal, intramuscular injection or infusion, intrathecal and intraventricular administration.

In some embodiments, the composition comprising a poloxamer is co-administered with one or more agents useful in treating skeletal muscle deficiencies. Examples of such agents include, but not limited to, streptomycin, prednisone, deflazacort, azathioprine,
 10 cyclosporine, valproic acid, phenylbutyrate, sodium butyrate, M344, suberoylanilide hydroxamic acid, and PCT124, or a combination of these agents.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows (A) the backbone structure of a poloxamer and (B) examples of
 15 commercially available poloxamers useful in compositions, systems, and methods of the present invention.

Figure 2 shows chronic administration of poloxamer 188 (P188) is safe. a, Outline of study design for the chronic administration of P188 or saline in GRMD animals. b, Serum markers of muscle damage taken shortly after the initial induction of anesthesia. c-f, Serum
 20 chemistry data from samples take biweekly throughout the chronic infusion period, shown are (c) cardiac troponin I, (d) creatine kinase (CK), (e) aspartate transferase (AST) and alkaline phosphatase (ALP), and (f) blood urea nitrogen (BUN) and creatinine. Dashed lines indicate normal canine values for these parameters. Note that normal CK levels are too low to be visualized in d. Values are mean $\pm$ SEM, 4-8 animals per group. † indicates significant
 25 difference by t-test; * indicates a significant treatment effect by Two-way ANOVA analysis.

Figure 3 shows chronic P188 treatment limits myocardial fibrosis and blocks elevation in BNP. a, Sirius red staining of myocardial sections reveals extensive fibrosis in GRMD hearts. Collagen appears red in brightfield images (top) and yellow-green in polarized images (bottom). Bar represents 400 μ m. b, Quantification of collagen content
 30 from 16-24 sections from four dogs in each group. c, Serum brain natriuretic peptide (BNP) levels taken before and after the infusion protocol. Values are mean $\pm$ SEM, 4-8 animals per group. * indicates significant ($P < 0.05$) difference from control, † indicates significant ($P < 0.05$) difference from GRMD (saline) group. All data analyzed with a one-way ANOVA and a Bonferroni's multiple comparison post-test.

Figure 4 shows chronic P188 administration prevents left ventricular remodeling. Catheter based hemodynamics demonstrates significant ventricular remodeling in GRMD dogs receiving saline infusion. a,b, Representative tracings of pressure-volume loops from before the infusion (a) and after the 9 week infusion protocol (b). Monitoring of left ventricular end diastolic volume during the terminal hemodynamic protocol. Offset at the far left is baseline data from the initial hemodynamic protocol prior to chronic infusion. * indicates a statistical difference between treatments. d-f, Comparisons of hemodynamic data taken during the peak of the dobutamine response (≈ 10 min post infusion); left ventricular pressure tau (d), end diastolic volume (e), and end systolic volume (f). Groups consist of 3-4 dogs. † indicates a significant ($P < 0.05$) difference between the two groups by t-test. For d,e,f, values are mean $\pm$ SEM.

Figure 5 shows in vitro passive tension-extension relationships in dystrophic cardiac myocytes. Passive tension-extension relationships of acutely isolated membrane intact single cardiac myocytes reveal poor passive compliance in GRMD dogs (a). The passive tension-extension relationships of dystrophic cardiac myocytes in the absence (a) and presence (b) of acute application of 150 μ M P188. The black curve (in b) is derived from the control myocyte data in (a) and is included as a reference. c, Comparisons of maximum stable sarcomere length (Max SL) between the groups. * indicates significant ($P < 0.05$) difference from control, † indicates significant ($P < 0.05$) difference from chronic saline GRMD group, and ‡ indicates significant ($P < 0.05$) difference from chronic P188 GRMD group. Values are mean $\pm$ SEM. All points are derived from myocytes isolated from 4-5 dogs. All data analyzed with a one-way ANOVA and a Bonferroni's multiple comparison post-test.

DEFINITIONS

As used herein, the phrase "composition comprising a poloxamer" refers to compositions containing a poloxamer (e.g., P188), or combination of poloxamers, used for the treatment or prevention of cardiac type injury. A therapeutic composition comprising a poloxamer may also comprise one or more other compounds or agents including, but not limited to, streptomycin, prednisone, deflazacort, azathioprine, cyclosporine, valproic acid, phenylbutyrate, sodium butyrate, M344, suberoylanilide hydroxamic acid, and PCT124, or a combination of these agents, and/or other therapeutic agents, physiologically tolerable liquids, gels, carriers, diluents, excipients, salicylates, immunosuppressants, antibiotics, binders, fillers, preservatives, stabilizing agents, emulsifiers, and buffers.

As used herein, the terms "host," "subject" and "patient" refer to any animal, including but not limited to, human and non-human animals (e.g. rodents), non-human primates, ovines, bovines, ruminants, lagomorphs, porcines, caprines, equines, canines, felines, etc.), that is studied, analyzed, tested, diagnosed or treated (e.g. administered therapeutically or prophylactically a composition comprising a poloxamer of the present invention). The terms "host," "subject" and "patient" are used interchangeably, unless indicated otherwise herein.

As used herein, the terms "therapeutically effective amount" and "effective amount" when used in reference to a composition comprising a poloxamer of the present invention refer to an amount (e.g., a dosage level) sufficient to effect beneficial or desired results. An effective amount can be administered in one or more administrations, applications or dosages and is not intended to be limited to a particular formulation or administration route.

As used herein, the terms "administration" and "administering" refer to the act of giving a drug, prodrug, or other agent, or therapeutic treatment (e.g., compositions of the present invention) to a subject (e.g., a subject or *in vivo*, *in vitro*, or *ex vivo* cells, tissues, and organs).

As used herein, the terms "co-administration" and "co-administering" refer to the administration of at least two agent(s) (e.g., a composition comprising a poloxamer and one or more other agents - e.g., prednisone, streptomycin) or therapies to a subject. In some embodiments, the co-administration of two or more agents or therapies is concurrent. In other embodiments, a first agent/therapy is administered prior to a second agent/therapy. Those of skill in the art understand that the formulations and/or routes of administration of the various agents or therapies used may vary. The appropriate dosage for co-administration can be readily determined by one skilled in the art. In some embodiments, when agents or therapies are co-administered, the respective agents or therapies are administered at lower dosages than appropriate for their administration alone. Thus, co-administration is especially desirable in embodiments where the co-administration of the agents or therapies lowers the requisite dosage of a potentially harmful (e.g., toxic) agent(s), and/or when co-administration of two or more agents results in sensitization of a subject to beneficial effects of one of the agents via co-administration of the other agent.

As used herein, the term "treatment" or grammatical equivalents encompasses the improvement and/or reversal of the symptoms of a cardiac injury. The term "treatment" refers to both therapeutic treatment and prophylactic or preventative measures. For example, those who may benefit from treatment with compositions and methods of the present invention include those already with a disease and/or dysfunction (e.g., muscle injury, DMD

and/or a skeletal muscle deficiency) as well as those in which a disease and/or dysfunction is to be prevented (e.g., using a prophylactic treatment of the present invention).

DETAILED DESCRIPTION OF THE INVENTION

5 The present invention provides methods for treating and preventing cardiac deficiencies and injuries (e.g., cardiac injury and ventricular dilation) in dystrophic subjects using poloxamer containing compositions. In some embodiments, the poloxamers are administered to dystrophic subjects prior to cardiac stress, such as that caused by anaesthesia. In other embodiments, the present invention provides systems comprising poloxamer
10 compositions and at least one anesthetic agent.

Work conducted during the development of embodiments of the present invention showed chronic administration of the chemical-based membrane sealant Pluronic F 68 (Poloxamer 188; P188) to severely affected dystrophin-deficient golden retriever muscular dystrophy (GRMD) dogs in vivo is safe and effective in blocking the development of marked
15 cardiac disease. Administration of 60 mg/kg/hour P188 for 8 weeks to GRMD animals resulted in significant reductions in myocardial fibrosis, blocked increases in serum cTnI and BNP, and fully prevented left ventricular remodeling.

Duchenne muscular dystrophy (DMD) is a progressive disease of striated muscle deterioration that results from the loss of the protein dystrophin(1, 2). Mechanical disruption
20 of the sarcolemmal membrane has been implicated as a primary molecular defect in dystrophin deficient myocytes(3). Numerous therapeutic strategies have been proposed to impact disease progression in DMD. Previous studies have primarily focused on skeletal muscle manifestations of the disease, often leaving the deteriorating heart untreated. The present invention shows that chronic application of chemical-based membrane sealants will
25 block cardiac disease in the severely affected canine model of DMD. Acute application of membrane sealant Pluronic F 68 (Poloxamer 188; P188) is known to confer acute cardiac protection in the mildly affected mouse model of DMD(3). The successful transition from acute to long-term efficacy using a clinically relevant large animal model of disease is, however, an important and highly challenging achievement in the muscular dystrophy field.

30 The present invention is not limited to any particular poloxamer. The present invention is not limited to use of P188. Indeed, any poloxamer that possesses similar characteristics and traits (e.g., biological effects) with those of P188 find use in the present invention including, but not limited to, P138, P237, P288, P124, P338, and P407.

P188 is one of a family of poloxamer molecules originally developed by BASF in the

1950s. It is a nonionic triblock co-polymer made of poly (ethylene oxide)₈₀-poly (propylene oxide)₃₀-poly (ethylene oxide)₈₀ (molecular mass $\approx$ 8.4 Kda). The molecule has several names including PLURONIC F68, RheothRx, and FLOCOR.

Poloxamers (also termed PLURONIC block polymers, available from BASF Corp., Wyandotte, MI) generally comprise ethylene oxide (EO) and propylene oxide (PO) blocks arranged in a basic A–B–A structure: EO –PO –EO. This arrangement results in an amphiphilic copolymer, in which the number of hydrophilic EO_(x) and hydrophobic PO_(y) units can be altered (See, e.g., Reeve, pgs. 231-249, in Handbook of Biodegradable Polymers, Harwood Academic Pub., Eds. Domb et al., (1997)). The backbone structure of various poloxamers is shown in FIG. 1A. A list of selected PLURONIC copolymers available from BASF Corp. is shown in FIG 1B. Copolymers with various *x* and *y* values are characterized by distinct hydrophilic–lipophilic balance (HLB). Poloxamers can be synthesized by sequential addition of PO and EO monomers in the presence of an alkaline catalyst, such as sodium or potassium hydroxide (See, e.g., Schmolka, J. Am. Oil Chem. Soc. 54 (1977) 110–116). The reaction is initiated by polymerization of the PO block followed by the growth of EO chains at both ends of the PO block. Anionic polymerization usually produces polymers with a relatively low polydispersity index (M/M).

In some embodiments, a composition comprising a poloxamer of the present invention comprises a purified and/or fractionated poloxamer (e.g., purified and/or fractionated using gel filtration or chromatographic fractionation (See, e.g., Emanuele et al., Expert Opin Investig Drugs. 1998; 7:1193-20, U.S. Pat. Nos. 6,977,045 and 6,761,824). In some embodiments, poloxamers are used that have admixtures (e.g., PO homopolymer and/or block copolymer admixtures) removed. In some embodiments, a poloxamer (e.g., polyoxypropylene/polyoxyethylene copolymer) is used that is optimized for improved biological activity (See, e.g., U.S. Pat. No. 6,747,064). In some embodiments, chemically modified forms of one or more poloxamers are utilized in the compositions and methods of the present invention. Chemical modifications of poloxamers include, but are not limited to, radiolabelling, acetylating, biotinylation, addition of a fluorophore, and other chemical modifications.

A variety of poloxamers can be used in (e.g., in a composition comprising a poloxamer) the present invention that possess similar characteristics and traits (e.g., biological effects) with those of P188 (e.g., based on characteristics described in FIG. 1b). These poloxamers include, but are not limited to, P138, P237, P288, P124, P338, and P407.

In some embodiments, a poloxamer with a molecular weight of between 5000 and 9000 daltons is used (e.g., in a composition (e.g., pharmaceutical composition) of the present invention). In some embodiments, a poloxamer with a molecular weight of between 9000 and 12000 daltons is used (e.g., in a composition (e.g., pharmaceutical composition) of the present invention). In some embodiments, a poloxamer with a molecular weight of between 12000 and 15000 daltons is used. A poloxamer with a molecular weight below 5000 or greater than 15000 daltons may also find use in the present invention (e.g., in a composition (e.g., pharmaceutical composition) of the present invention).

In some embodiments, a poloxamer with a polyoxyethylene content greater than 50% is used (e.g., in a composition (e.g., pharmaceutical composition) of the present invention). In some embodiments, a poloxamer with a polyoxyethylene content between 50 and 60% is used. In some embodiments, a poloxamer with a polyoxyethylene content between 60 and 70% is used. Poloxamers with a polyoxyethylene content below 50% and above 70% may also find use in the present invention (e.g., in a composition (e.g., pharmaceutical composition) of the present invention).

Some common biological uses of P188 include use as a stool softener in several commercially available laxatives, as an ingredient in cosmetics and as an emulsifier for pharmaceutical agents. It is a powerful surfactant. P188 has been shown to insert into lipid monolayers (See, e.g., Maskarinec et al., 2002 Biophys. J. 82: 1453-1459). It has many biological effects *in vivo* including the repair of electrically damaged cell membranes (See, e.g., Lee et al., (1992) Proc. Natl. Acad. Sci. USA 89: 4524-4528), in controlled drug delivery, for sensitizing tumors to chemotherapy (See, e.g., Kabanov et al., Adv Drug Deliv Rev 2002, 54, 759-779), and for delivery of gene therapies, among others. Additionally, P188 was shown to have an effect on blood flow and viscosity as well as platelet adhesiveness. (See, e.g., Grover et al., (1969) Circ. 39 and 40: 1249, (Suppl. I)). It was developed as a therapeutic agent under the name of RheothRx by Glaxo Wellcome (See, e.g., Adams-Graves et al., (1997), Blood 90: 2041-2046) and by CytRx under the name of FLOCOR for vaso-occlusive crisis in sickle cell disease and has been in phase III clinical trials (See, e.g., Emanuele, (1998) Expert Opin. Investig. Drugs 7:1193-1200). It was also in Phase III trials to assess thrombolytic activity in patients with acute myocardial infarction (MI) (CORE), with mixed results (Schaer et al., (1996) Circ. 94: 298-307; Chareonthaitawe et al., (2000) Heart 84: 142-148). It has been in Phase II trials as an adjunct to primary percutaneous transluminal coronary angioplasty for acute MI (See, e.g., O'Keefe, et al., 1996 Am. J. Cardiol. 78: 747-750). Thus, the present invention contemplates use of poloxamers

(e.g., P138, P237 and P288) that enjoy similar characteristics and biological effects to those of P188.

P188 is safe when given acutely for up to 72 hr (See, e.g., Adams-Graves et al., (1997), Blood 90: 2041-2046) and is well tolerated in children and adults upon repeated exposure (See, e.g., Gibbs and Hagemann, 2004 Ann. Pharmacother. 38: 320-324). The most significant adverse effect in studies with RheothRx was renal dysfunction but this was not seen with the more highly purified form FLOCOR. The most frequently experienced adverse effects were pain, injection site abnormalities and nausea. It has a half-life in plasma of 7.5 hr in rodents and 18 hr in human subjects. Pharmacokinetic studies have shown that <5% of purified poloxamer is metabolized. A single metabolite of higher molecular weight and slower clearance has been detected (See, Gibbs and Hagemann, 2004 Ann. Pharmacother. 38: 320-324). Renal clearance is the primary route of elimination.

In certain embodiments of the invention, compositions may further comprise one or more alcohols, zinc-containing compounds, emollients, humectants, thickening and/or gelling agents, neutralizing agents, and surfactants. Water used in the formulations is preferably deionized water having a neutral pH.

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions. Thus, for example, the compositions may contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as dyes, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, preferably do not unduly interfere with the biological activities of the components of the compositions of the present invention. The formulations can be sterilized and, if desired, mixed with auxiliary agents (e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like) that do not deleteriously interact with the poloxamer of the formulation.

The present invention also includes methods involving co-administration of a composition comprising a poloxamer with one or more additional active agents. Indeed, it is a further aspect of this invention to provide methods for enhancing prior art treatment methods and/or pharmaceutical compositions by co-administering a composition of the present invention. In co-administration procedures, the agents may be administered concurrently or sequentially. In one embodiment, the compositions described herein are

administered prior to the other active agent(s). The pharmaceutical formulations and modes of administration may be any of those described herein. In addition, the two or more co-administered agents may each be administered using different modes (e.g., routes) or different formulations. The additional agents to be co-administered can be any of the well-known agents in the art, including, but not limited to, those that are currently in clinical use.

EXPERIMENTAL

The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

EXAMPLE 1

Use of Poloxomers to Reduce Cardiac Injury and Ventricular Dilation in Dystrophic Dogs

This example describes the use of poloxomer compositions to prevent cardiac injury and ventricular dilation in dystrophic dogs.

METHODS

Animals. Adult GRMD dogs were obtained from a colony maintained at the University of North Carolina-Chapel Hill. GRMD dogs were identified based on elevation of serum creatine kinase and subsequently developed characteristic clinical signs. Genotype was confirmed by PCR in most dogs. Age matched, heartworm negative normal dogs of mixed breeds were provided by R & R Research (Howard City, Michigan). At the initiation of this Example, the GRMD animals averaged 15 months of age. All dogs were used and cared for according to principles outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Vascular access port placement and hemodynamics. Anesthesia was induced with propofol and maintained with either isoflurane or sevoflurane. Following the placement of a femoral arterial catheter, the left jugular vein and carotid artery were isolated. A 6Fr introducer was inserted into the left carotid and secured with a purse string suture. Under fluoroscopic guidance, a 5Fr 10 electrode pressure-volume catheter (SPR-554-11, Millar Instruments, Houston TX) was advanced through the aortic valve into the lumen of the left ventricle and positioned to give strong signals in all seven segments. The catheter interfaced

with a MPVS Ultra signaling conditioning unit (Millar Instruments, Houston, TX) and data was collected by a DAQ-16 acquisition unit and Ponemah software package (DSI, St. Paul, MN). During the hemodynamic protocol measurements were taken at baseline, following an infusion of dobutamine (15ug/kg/min), following a return to baseline values, following an infusion of P188 (460 mg/kg), and following a second dobutamine infusion. Concurrently with the hemodynamic study, a stainless steel vascular access port (VAP; CP4AC-5IS-SS, Access Technologies, Skokie, IL) was implanted $\approx$ 5cm caudal to the spine of the scapula and $\approx$ 10cm from the dorsal midline. An 22g right angle Huber needle was introduced into the VAP and secured with 2-0 Prolene. A 5Fr silicone catheter was tunneled subcutaneously to the neck incision. The heparin flushed catheter was then introduced into the jugular vein such that the tip was near the right atrium. At the completion of the hemodynamic protocol, the carotid was repaired, all skin incisions were closed, and the animals were allowed to recover. Once fully recovered, animals were placed in tight fitting jackets (Lomir Biomedical, Malone, NY) that protected the VAP site.

Infusion protocol. An ambulatory infusion pump (Ambit, Sorenson Medical, West Jordan, UT) was inserted into a jacket pocket and a 500 ml IV bag was placed into a second pocket. In total, the jacket, pump, and a full bag of fluid weighed slightly more than 1 kg (6-9% of body weight). Saline infusions began the morning following the implantation of the VAP and were continued for seven days, at which time baseline blood samples were taken and dogs in the P188 treatment group started receiving P188 infusion.

Myocyte Isolation. Following the terminal hemodynamic study, the dogs were euthanized by a barbiturate overdose, the chest was rapidly opened through a lateral thoracotomy and the heart removed and placed into ice cold Krebs solution. Sections of myocardium were cut into 5 mm cubes and digested in a solution containing nominal calcium, type-2 collagenase (250-300 units/ml, Wothington Biochemical Corp, Lakewood, NJ), and hyaluronidase (250-500 units/ml, H3506, Sigma, St. Louis, MO). Following an initial period of digestion, the heart sections were gently triturated to further disperse the isolated cells. Calcium was reintroduced in a stepwise manner; calcium tolerant myocytes were then utilized.

Image acquisition and analysis. Images of sectioned tissues were obtained on an inverted Axio Observer Z-1 with a motorized stage (Zeiss, Thornwood, NY) Montage images were collected and stitched by AxioVision software (Zeiss, Thornwood, NY). Composite images were analyzed by a custom written edge-detection routine using MatLab (R2008a, MathWorks, Natick, MA).

Statistics. Statistical analysis was performed using Prism v 5.0 (GraphPad, La Jolla, CA) and a custom SAS-PROC MIXED model of covariance accounting for both fixed and random effects was used to perform analysis of repeated measures (SAS, Cary, NC).

5 RESULTS

Golden retriever muscular dystrophy (GRMD) animals closely recapitulate disease progression, both in timing and severity, of that in human DMD patients (4-7). GRMD arose from a spontaneous mutation in intron 6 that eliminates a splice acceptor site, resulting in the skipping of exon 7 and a subsequent frame shift and premature truncation of the dystrophin protein(8). In addition to significant skeletal muscle pathology, GRMD animals have cardiac lesions present as early as 6 months of age(6).

Eight adult GRMD animals were randomized into either a control saline infused group or a chronic P188 treatment group (Table 1; Fig. 2a).

Table 1 GRMD age, weight, randomized treatment and initial hemodynamics.

Name	Age (Months)	Weight (kg)	Treatment Group	Left Ventricular End-Diastolic Volume (ml)	Left Ventricular Systolic Pressure (mmHg)
Beretta	12.0	12.5	Saline	18.7	94.7
Billie	17.0	13.4	Saline	56.7	114.6
Cinderella	13.8	11.2	P188	23.7	88.5
Ella	17.3	13.2	P188	46.0	130.4
Karla	16.2	14.2	P188	29.9	123.8
Kimiko	17.9	17.1	Saline	28.7	121.1
Perla	13.2	13.1	P188	24.9	114.3
Una	13.8	12.7	Saline	N/A	95.7

Animals were anesthetized and baseline blood samples were drawn. Serum levels of cardiac troponin I (cTnI) were significantly elevated in GRMD dogs immediately following induction of anesthesia compared to unaffected anesthetized control dogs (Fig 2b), indicating

that general anesthesia alone provokes significant cardiac damage in GRMD but not in normal dogs. Animals underwent an initial hemodynamic assessment, vascular access port implantation, and jugular catheterization procedure. The hemodynamic protocol consisted of the fluoroscopy guided placement of a multi-segmented pressure-conductance catheter in to the left ventricle via the carotid artery. Following baseline measurements, dobutamine was infused (15ug/kg/min) until a peak effect was observed, at which time dobutamine infusion was stopped. Prior to treatment, there were no significant hemodynamic differences between dogs randomized to saline or P188 treatment groups (Table 1). Upon return to baseline hemodynamic function, an acute dose of P188 (460 mg/kg) was administered and followed by a second dobutamine stress test. In contrast to the mdx mouse data(3), there were no acute effects of P188 on GRMD ventricular geometry (data not shown). Following this surgical procedure the animals were recovered and both groups received 0.9% saline (0.4 ml/kg/hour) for one week. The serum half life of P188 is 18 hours in dogs(9), thus one week is sufficient to wash out any P188 remaining from the acute administration. Following this washout and surgery recovery period, the saline group continued receiving 0.9% NaCl at a rate of 0.4ml/kg/hour, and the P188 treatment group began receiving 60mg/kg/hour P188 in 0.9% NaCl at the same rate (0.4ml/kg/hour) for 8 weeks total treatment.

During the eight week chronic infusion period, blood samples were taken to monitor cardiac injury biomarkers. Saline treated GRMD animals had significantly elevated serum concentrations of cTnI, and is evidence of cardiac injury. In contrast, serum cTnI levels were completely normal in the P188 treated GRMD animals (Fig 2c $P < 0.05$ vs saline). Serum CK levels were unchanged by P188 (Fig. 2d). The safety of P188 for both the kidney and liver function was examined. Figures 2e and f show that during the course of the 8 week infusion, the dogs receiving P188 had no change in either liver enzymes or evidence of azotemia. These results indicate that administration of P188 at 60 mg/kg/hr for eight weeks does not induce renal or liver damage in GRMD animals.

Myocardial fibrosis is a consistent finding in both DMD patients and GRMD dogs. Fibrotic lesions result from myocyte necrosis and subsequent inflammatory response. Fibrotic lesions can increase the mechanical strain on neighboring healthy myocytes, causing further myocyte necrosis and subsequent fibrosis over time. To assess the ability of chronically administered P188 to limit fibrosis, cardiac tissue was stained with pico-sirius red to identify mature fibrotic lesions. Upon quantification of the fibrotic area, GRMD animals receiving P188 had significantly less ($P < 0.05$) fibrosis than the GRMD group receiving saline (Fig 3a,b). This reduction in fibrosis is consistent with P188 providing protection to

individual cardiac myocytes from mechanical damage. Cardiac lesions are progressive in DMD and adult GRMD dogs have noted cardiac lesions and evidence of significant cardiac fibrosis(6, 10, 11). While P188 blunted lesion formation in GRMD animals it did not restore myocardial lesion content back to wild-type levels. Chronic P188 administration to adult GRMD animals did however fully block increases in the heart failure biomarker BNP (Fig 3c; $P<0.05$).

Prior to GRMD randomization for the chronic study there was no significant difference in left ventricular geometry among GRMD saline vs P188 animals (Fig. 4a). During the course of the eight-week infusion period, GRMD dogs receiving saline developed a significant left ventricular (LV) dilated phenotype ($P<0.05$; Fig. 4). Most dramatically, GRMD animals receiving P188 had unchanged LV geometry and did not progress to dilated cardiomyopathy. LV geometry differences between saline and P188 -treated GRMD animals were evident throughout the hemodynamic testing protocol (Fig 4c) and were particularly pronounced following the administration of dobutamine (Fig 4d-f). The dilation of ventricular geometry in the saline GRMD group is consistent with on-going cardiac injury and necrosis/fibrosis. Hemodynamic end-point data also showed a significant enhancement in the isovolumic relaxation function in P188-treated GRMD animals compared to GRMD/saline (Fig 4d). Chronic P188-mediated improvements in diastolic function would be of benefit in DMD patients where diastolic dysfunction is an early marker of cardiac disease(12).

To determine the effects of chronic P188 administration on the primary cardiac cellular defect of dystrophin deficiency, the passive tension- extension properties of isolated, single adult cardiac myocytes were examined using micro-carbon fiber technology(3). Cardiac myocytes isolated from wild-type animals well tolerated sarcomere length passive extensions throughout the physiological range (1.8 – 2.2 μm) (Fig 5a). In marked contrast, single myocytes isolated from GRMD animals were highly sensitive to even mild passive extensions in length, and much worse than reported in mouse dystrophic myocytes(3). In addition to poor myocyte compliance, GRMD myocytes were highly susceptible to terminal contracture following mild sarcomeric extension (Fig. 5a). Myocytes isolated from GRMD dogs receiving chronic P188, but with P188 washed out in the in vitro assay, had similar passive extension properties to untreated dystrophic myocytes (Fig. 5a). However, acute administration of P188 to isolated GRMD myocytes, regardless of previous treatment, corrected the passive tension-extension relationship to normal canine myocytes (Fig 5b,c). These data indicate that the primary action of P188 on dystrophic cardiac myocytes is

reversible and that the beneficial actions of chronic P188 infusion require the presence of P188.

This example reports the demonstration of a therapeutic agent with long-term cardiac protection in a severely affected model of muscular dystrophy in vivo. Membrane sealants are proposed to function by inserting into the disrupted membranes of dystrophic cardiac myocytes(3, 13), suggesting that they may be particularly beneficial during times of cardiac stress. One important and predictable cardiac stress results from general anesthesia. DMD patients commonly undergo major surgical procedures to correct orthopedic dysfunction. These procedures can result in significant cardiac stress secondary to blood loss and hypotension. As noted here, general anesthesia alone was sufficient to induce significant increases in markers of myocardial necrosis and cardiac injury (Fig. 2). Membrane sealant administration may be particularly effective during these times of additional stress in DMD patients.

In summary, this example shows that chronic application of P188 is both safe and effective in preventing cardiac injury and ventricular remodeling in a severely affected large animal model of DMD. These data are evidence that P188 may slow or halt the progression of the cardiac disease associated with DMD, a finding with potential for both acute and chronic application in DMD patients. In addition, we contemplate that membrane sealants may extend to a myriad of acquired cardiac diseases in which dystrophin structure-function and membrane integrity is compromised, including viral myocarditis, myocardial ischemia and heart failure(14-17).

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15 All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described
20 modes for carrying out the invention that are obvious to those skilled in the relevant fields are intended to be within the scope of the present invention.

CLAIMS

We Claim:

- 5 1. A method of treating a dystrophic subject to prevent or reduce cardiac injury or ventricular dilation comprising: administering to said dystrophic subject a composition comprising a poloxamer under conditions such that said cardiac injury or said ventricular dilation is reduced or prevented.
- 10 2. The method of claim 1, wherein said administering is conducted prior to cardiac stress.
3. The method of claim 2, wherein said cardiac stress is caused by anaesthesia.
- 15 4. The method of claim 1, wherein said subject is a human subject.
5. The method of claim 1, further comprising measuring cardiac damage in said subject before, or after, or both before and after said administering.
- 20 6. The method of claim 1, wherein said subject is a dystrophin deficient subject.
7. The method of claim 6, wherein said subject has Duchene's muscular dystrophy.
8. The method of claim 1, wherein said administering is under conditions such that
25 said cardiac injury is reduced or prevented.
9. The method of claim 1, wherein said administering is under conditions such that said ventricular dilation is reduced or prevented.
- 30 10. The method of claim 1, wherein said poloxamer is P188.
11. The method of claim 1, wherein said poloxamer is administered at a dosage level of approximately between 30 – 90 mg per kg weight of said subject per hour for at least 5 weeks.

12. The method of claim 11, wherein said poloxamer is administered for 5-10 weeks.

13. The method of claim 11, wherein said dosage level is about 60 mg per kg weight of
5 said subject per hour for at least 5 weeks.

14. The method of claim 1, wherein said composition comprising a poloxamer is co-
administered with one or more agents selected from the group consisting of streptomycin,
prednisone, deflazacort, azathioprine, cyclosporine, valproic acid, phenylbutyrate, sodium
10 butyrate, M344, suberoylanilide hydroxamic acid, and PCT124.

15. A system comprising:

- a) a composition comprising a poloxamer; and
- b) an anesthetic agent.

15

16. The system of claim 15, wherein said anesthetic agent is an inhaled agent.

17. The system of claim 15, wherein said anesthetic agent is a non-opioid intravenous
agent.

20

18. The system of claim 15, wherein said anesthetic agent is a opioid intravenous agent.

19. The system of claim 1, wherein said poloxamer is P188.

25 20. The system of claim 15, further comprising one or more agents selected from the
group consisting of streptomycin, prednisone, deflazacort, azathioprine, cyclosporine,
valproic acid, phenylbutyrate, sodium butyrate, M344, suberoylanilide hydroxamic acid, and
PCT124.

FIG. 1A

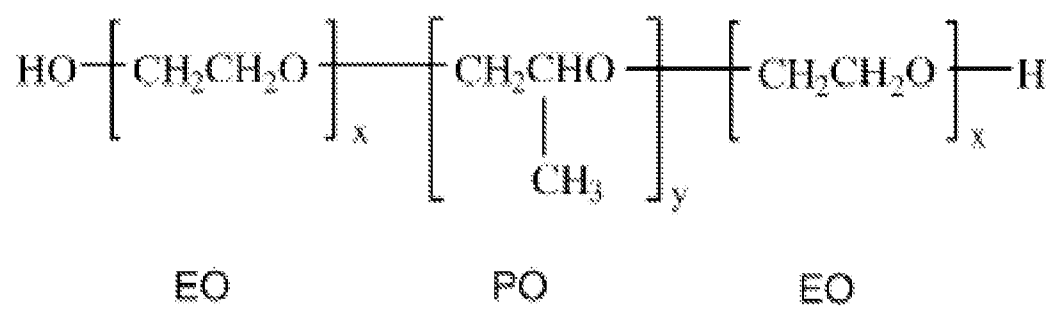


FIG. 1B

Copolymer	MW ^a	Average no. of EO units ($\bar{x}$) ^b	Average no. of PO units ($\bar{y}$) ^b	HLB ^c	Cloud point in 1% aqueous solution (°C) ^d	CMC (M) ^d
L35	1900	21.59	16.38	19	73	5.3×10^{-3}
L43	1850	12.61	22.33	12	42	2.2×10^{-3}
L44	2200	20.00	22.76	16	65	3.6×10^{-3}
L61	2000	4.55	31.03	3	24	1.1×10^{-4}
L62	2500	11.36	34.48	7	32	4.0×10^{-4}
L64	2900	26.36	30.00	15	58	4.8×10^{-4}
F68	8400	152.73	28.97	29	>100	4.8×10^{-4}
L81	2750	6.25	42.67	2	20	2.3×10^{-3}
P84	4200	38.18	43.45	14	74	7.1×10^{-3}
P85	4600	52.27	39.66	16	85	6.5×10^{-3}
F87	7700	122.50	39.83	24	>100	9.1×10^{-3}
F88	11 400	207.27	39.31	28	>100	2.5×10^{-4}
L92	3650	16.59	50.34	6	26	8.8×10^{-3}
F98	13 000	236.36	44.83	28	>100	7.7×10^{-5}
L101	3800	8.64	58.97	1	15	2.1×10^{-6}
P103	4950	33.75	59.74	9	86	6.1×10^{-6}
P104	5900	53.64	61.03	13	81	3.4×10^{-6}
P105	6500	73.86	56.03	15	91	6.2×10^{-6}
F108	14 600	265.45	50.34	27	>100	2.2×10^{-5}
L121	4400	10.00	68.28	1	14	1.0×10^{-6}
P123	5750	39.20	69.40	8	90	4.4×10^{-6}
F127	12 600	200.45	65.17	22	>100	2.8×10^{-6}

^a The average molecular weights provided by the manufacturer (BASF, Wyandotte, MI).

^b The average numbers of EO and PO units were calculated using the average molecular weights.

^c HLB values of the copolymers; the cloud points were determined by the manufacturer.

^d Critical micellization concentration values were determined previously using pyrene probe (See, e.g., Kozlov et al., Macromolecules 33 (2000) 3305–3313).

FIG. 2

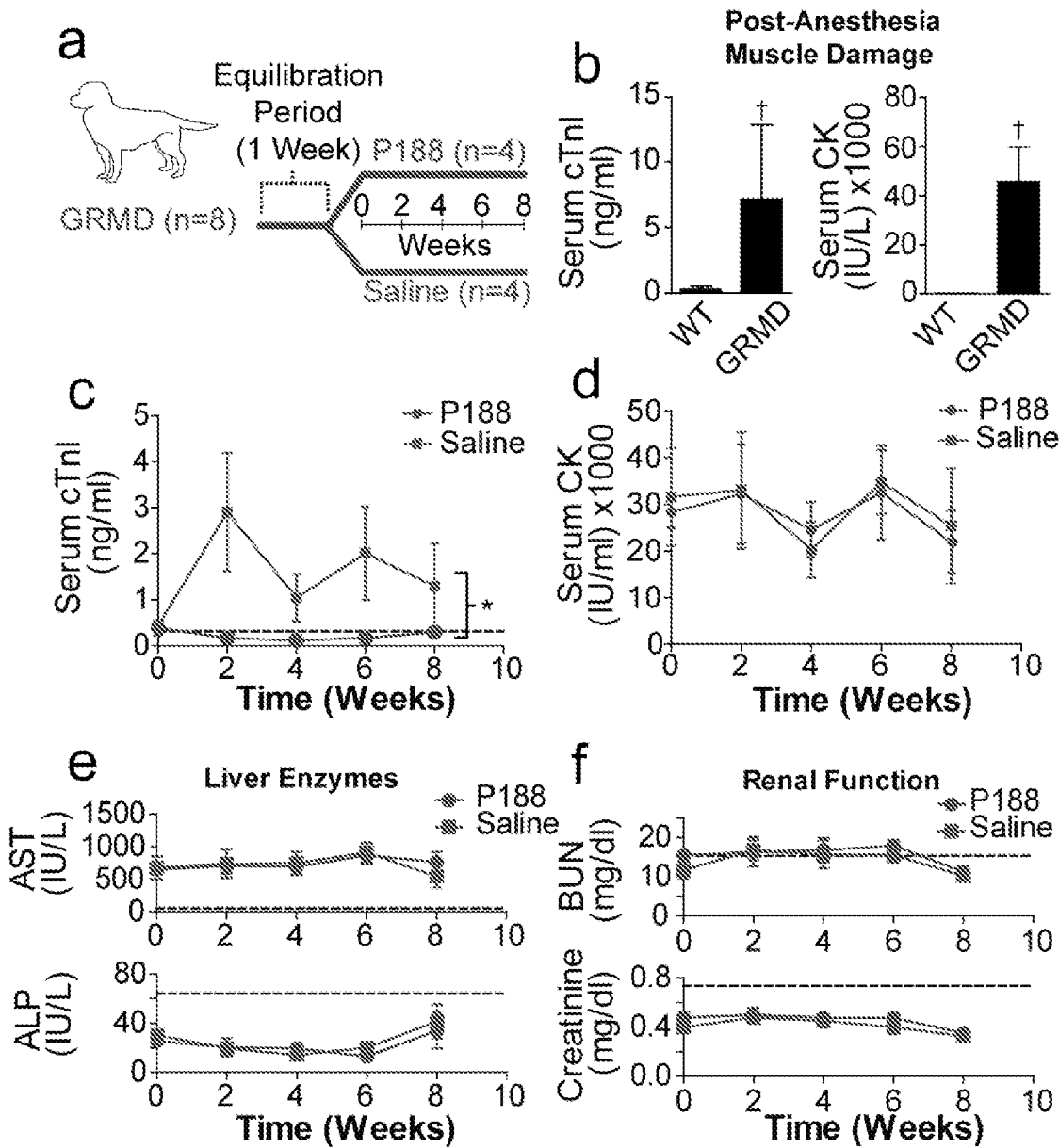


FIG. 3

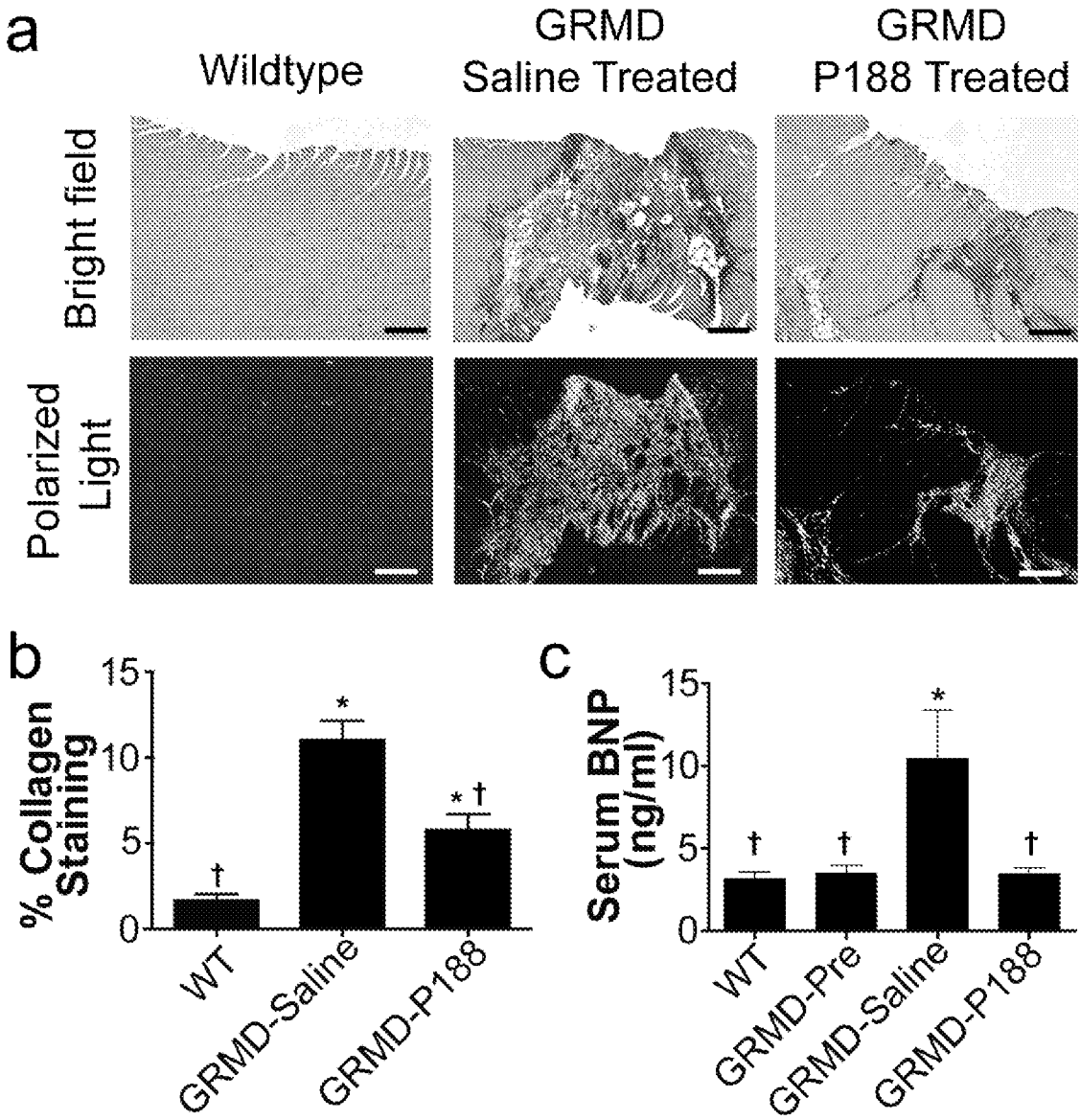


FIG. 4

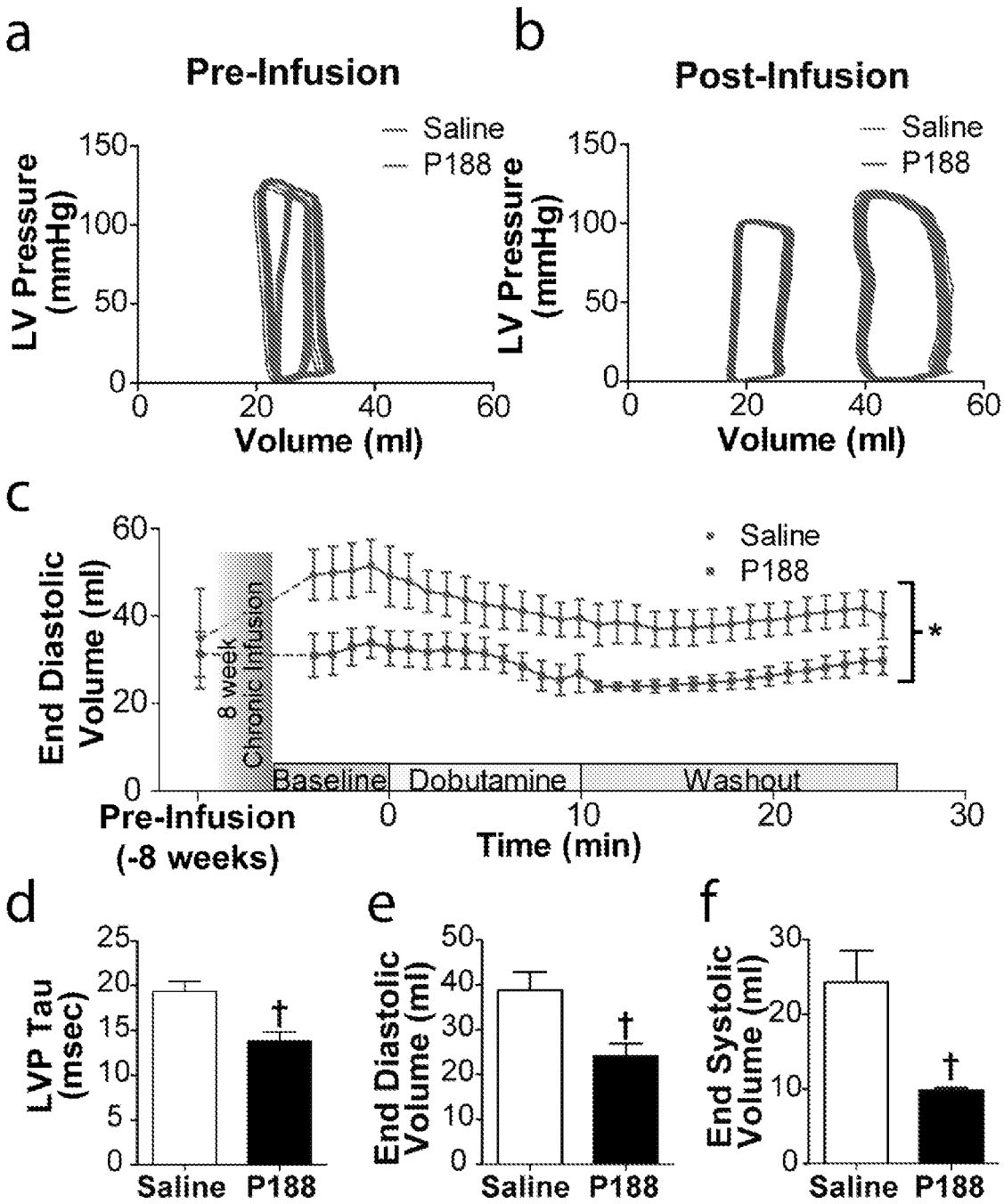


FIG. 5

