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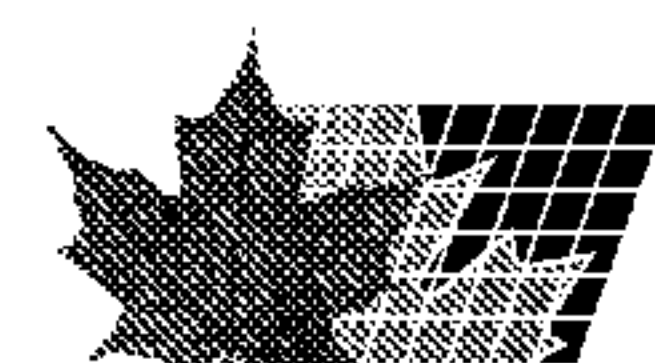
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(54) Titre : INHIBITION DU LYSOZYME DANS LE TRAITEMENT DU DYSFONCTIONNEMENT MYOCARDIQUE INDUIT
PAR UNE SEPTICEMIE

(54) Title: INHIBITION OF LYSOZYME IN TREATING SEPSIS-INDUCED MYOCARDIAL DYSFUNCTION

(57) **Abrégé/Abstract:**

Methods and compositions for treating myocardial dysfunction or inflammation are described. The methods of the invention involve administering an agent that can inhibit lysozyme to an animal in need thereof. Preferred lysozyme inhibitors include chitotriose and chitobiose.



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ABSTRACT OF THE DISCLOSURE

Methods and compositions for treating myocardial dysfunction or
5 inflammation are described. The methods of the invention involve
administering an agent that can inhibit lysozyme to an animal in need thereof.
Preferred lysozyme inhibitors include chitotriose and chitobiose.

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INHIBITION OF LYSOZYME IN TREATING SEPSIS-INDUCED MYOCARDIAL DYSFUNCTION

FIELD OF THE INVENTION

5 The invention relates to methods and compositions for treating myocardial
dysfunction or inflammation by inhibiting lysozyme, an enzyme released during inflammatory
response.

BACKGROUND OF THE INVENTION

Sepsis is a clinical syndrome that results from an activated systemic host
inflammatory response to infection. In its more severe form, sepsis affects almost 800,000
10 North Americans each year, and results in the deaths of approximately 30% of these. It is the
leading cause of morbidity for patients admitted to a contemporary intensive care unit (ICU),
through the development of a syndrome of disseminated organ injury, known as the multiple
organ dysfunction syndrome. Sepsis is characterized by an overwhelming systemic response
to infection and may lead to septic shock. Septic shock is a life threatening immunological
15 reaction to a severe infection. Septic shock is caused by the presence of large numbers of
bacteria in the blood stream; symptoms include a fall in blood pressure, impaired ability of the
blood to clot and damage to major organs including kidneys, liver and lungs.

Septic shock is becoming increasingly common in the North American population
because of an increasing population at risk: a larger proportion of elderly individuals including
20 many with chronic debilitating disease, and increasing numbers of people with impaired
immunity due to disease such as cancer and AIDS. About 400,000 cases of sepsis, 200,000
cases of septic shock and 100,000 deaths from both occur each year in the U.S.

Researchers continue to experiment with medication to fight the infection and control
the inflammatory response associated with sepsis and septic shock. However, the 50%
25 mortality rate associated with septic shock has improved little in the last 30 years despite
innovations in antibiotic therapy and life support modalities. To combat the condition,
hospitals currently administer drugs and fluids to patients maintain blood pressure and use
antibiotics to fight the infection.

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Epidemiologic surveys have demonstrated that tissue injury secondary to activation of the inflammatory system may also complicate noninfectious disorders (eg, acute pancreatitis and ischemia-reperfusion). The term systemic inflammatory response syndrome (SIRS) is used in this setting to refer to the consequences of a dysregulated host inflammatory response when infection is not present. SIRS is a widespread inflammatory response to a variety of severe clinical results.

It is important to distinguish between an underlying disease (infection or pancreatitis) and the host's response (sepsis or SIRS). This distinction is important clinically since it is the latter, not the primary disease, that is responsible for the multiple organ dysfunction syndrome (MODS). MODS is the usual explanation for the high mortality rates associated with these syndromes.

Several investigators have shown that a depression in cardiac contractility is an important component of hemodynamic collapse in sepsis (1,2). Some have attributed this cardiac dysfunction to a low molecular weight substance that is present in septic plasma (3-4). Lefer and coworkers suggested, on the basis of a large body of work, primarily in animal models of hemorrhagic shock, that myocardial depressant factor was a small molecular weight peptide originating from the pancreas (3,4). Others suggested that this substance was a cytokine released into the circulation as part of the inflammatory reaction induced by the infecting organism (5). However, there is no clear consensus about the origin or biochemical nature of myocardial depressant substance- (s) in sepsis. Indeed, the very existence of such a factor has remained controversial.

The inventors previously provided additional evidence for the existence of a myocardial depressant factor in sepsis (6). In an *Escherichia coli* model of induced sepsis in dogs, Gomez et al (6) showed that myocardial depression developed after 4 h of bacteremia and that this depression could be reversed by removal of a circulating substance of <30,000 molecular weight from the plasma by continuous arteriovenous hemofiltration. Myocardial depressant activity in plasma [filterable cardiodepressant

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substance (FCS)] was detected by a bioassay that included a right ventricular trabecular preparation. FCS activity was detected in the plasma as early as 1 h after sepsis and increased further at the 4 h interval. Hemofiltration returned FCS activity to pre-septic levels.

5 In a subsequent study, the inventors further characterized the nature of FCS (7). By pore filtration techniques, the inventors found that FCS was contained in the 10- 30 kilodalton (KD) fraction of plasma, was found in the acetone-insoluble portion of plasma, and that its activity could be diminished by the proteolytic enzyme proteinase- K. This suggested that FCS was likely
10 to be a protein.

SUMMARY OF THE INVENTION

The present inventors have purified the filterable cardiodepressant substance (FCS) and determined its identity using microcapillary reverse phase high pressure liquid chromatography (HPLC), tandem mass
15 spectrometry (MS/MS) and protein sequencing techniques. The results indicated that this protein is lysozyme C (LzmS). The inventors further determined that lysozyme C decreased myocardial contraction and the adrenergic response to neural stimulation and the β - agonist isoproterenol in respective right ventricular trabecular preparations. The inventors also
20 showed that these effects could be blocked by a competitive inhibitor of lysozyme, the results providing further evidence that lysozyme may be important in the pathophysiology of myocardial dysfunction in sepsis.

Accordingly, the present invention provides a method of preventing or inhibiting myocardial dysfunction comprising administering an effective
25 amount of an agent that can inhibit lysozyme to an animal in need thereof. The animal is preferably one with sepsis.

The present invention also provides a method of treating an inflammatory condition comprising administering an effective amount of an agent that can inhibit lysozyme to an animal in need thereof.

30 In a specific embodiment, the agent that can inhibit lysozyme is a competitive inhibitor such as chitotriose (N,N',N'' triacetylglucosamine) or chitobiose (N, N'-diacetylglucosamine).

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The present invention also includes pharmaceutical compositions for use in treating an inflammatory condition or in preventing or inhibiting myocardial dysfunction comprising an effective amount of an agent that can inhibit lysozyme in admixture with a suitable diluent or carrier.

5 According to an aspect of the invention, there is provided use of an agent that can inhibit lysozyme to reduce sepsis-induced myocardial dysfunction, wherein the agent that can inhibit lysozyme is selected from the group consisting of: N,N' diacetylglucosamine (chitobiose), N,N', N'' triacetylglucosamine (TAC), an anti-sense oligonucleotide to lysozyme mRNA and an antibody to lysozyme.

10 According to another aspect of the invention, there is provided use of an agent that can inhibit lysozyme to reverse myocardial depression in an animal with sepsis, wherein the agent that can inhibit lysozyme is selected from the group consisting of: N,N' diacetylglucosamine (chitobiose), N,N',N'' triacetylglucosamine (TAC), an anti-sense oligonucleotide to lysozyme mRNA and an antibody to lysozyme.

15 According to a further aspect of the invention, there is provided use of an agent that can inhibit lysozyme to treat septic shock, wherein the agent that can inhibit lysozyme is selected from the group consisting of: N,N' diacetylglucosamine (chitobiose), N,N',N'' triacetylglucosamine (TAC), an antisense oligonucleotide to lysozyme mRNA and an antibody to lysozyme.

20 Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

25 BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described in relation to the drawings in which:

15 Figures 1A, 1B and 1C are graphs showing plasma fractions were divided into three components by SEC- HPLC techniques. Aliquots of the presepsis and septic plasma fractions
30 were placed into the right ventricular trabecular preparation and the decrease in isometric tension (mean $\pm$ SE) as compared with baseline was measured. There was a significant increase in depressant activity in the F2 septic fraction as compared with the nonseptic plasma fraction, while the other fractions showed no increase in activity after sepsis.

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Note that even in the preseptic sample, there is some background depressant activity (BDA) that is found in all samples, but BDA appears to be of lipid moiety with different physical properties as compared with FCS (7). For this reason, it has been our approach in defining the nature of FCS to compare pre and septic plasma fractions taken from the same dog. An
5 increase in depressant activity found in a sepsis fraction relative to that found in the presepsis fraction is indicative of the formation of FCS. By two way ANOVA and SNK, *P<.05 vs baseline; +P<.05 septic vs presepsis fraction.

Figure 2 shows a one dimensional --gel electrophoresis. The control (i.e. presepsis) FI, F2, and F3 plasma fractions are shown in the left lanes, while the septic fractions are
10 shown in the right lanes. A broad band is

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observed in the F2 septic plasma fraction at approximately 15 KD that was not observed in the presepsis plasma fraction. ST is standard.

Figure 3A, 3B and 3C are graphs showing that FCS activity was measured in the spleen, the proposed source of FCS, as well as the heart, the proposed target organ of FCS (see Example 1). In addition, FCS activity was measured in lymphocytes, a cell type that would not be associated with production of this protein and would therefore serve as a negative control. Neutrophils, another source of FCS were not measured, since these cells would be found in the spleen. Mean (± 1 SE) myocardial depressant activity measured in the in-vitro preparation was increased in hearts and spleens taken from septic animals as compared with nonseptic animals, while there was no change of activity in lymphocytes. By two way ANOVA and SNK, *P<.05 vs baseline; +P<.05 sepsis vs presepsis.

Figure 4A is a graph showing that Lysozyme (Lzm-S) was prepared from the spleens of animals subjected to 4 hrs of sepsis (septic) and spleens of animals subjected to normal saline infusion (nonseptic). Lzm-S was placed into the in-vitro bath and the change in tension was recorded. There was no difference in the depressant effect (mean ± 1 SE) found between the two splenic preparations. In the time control experiments, an identical amount of KH was placed into the bath over the interval of the experiment. Accordingly, as assessed by per gram weight of spleen in Figure 3, the septic spleens showed a greater degree of depression than the nonseptic spleens, but when purified lysozyme was compared between septic and nonseptic spleens on a milligram basis, no difference was observed between the two preparations. Since there may be more polymorphonuclear cells found in septic spleens, the quantity of lysozyme would be greater than that found in nonseptic spleens, but the potency of depressant activity may not be different between preparations (see Discussion in Example 1). By two way ANOVA and SNK, *P<.05 vs baseline; +P<.05 vs time control group. Figure 4B is a graph showing that proteinase K treated Lzm-S caused less depression than the untreated sample at the lower lysozyme concentrations. Mean (± 1 SE) values

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are given. By two way ANOVA and SNK, * $P < .05$ vs baseline; + $P < .05$ treated vs nontreated groups.

Figure 5A, 5B and 5C are graphs showing the effect of chitotriose on lysozyme activity. The effect of chitotriose on attenuating lysozyme induced depressant activity was examined at 0.5 Hz (Fig 5A) and 1 Hz (Fig 5B). Either chitotriose or KH placebo was placed into the bath before the addition of lysozyme in respective groups. The chitotriose treated group showed less depression than the nontreated group. In Fig 5C, chitotriose was placed into the in-vitro preparation after the addition of lysozyme. Chitotriose treatment attenuated the decline in tension as compared with the nontreated (KH) group. By two way ANOVA and SNK, * $P < .05$ vs baseline; + $P < .05$ treated vs nontreated groups.

Figure 6 shows the sympathetic response to field stimulation in the ventricular trabecular preparation is shown at baseline (upper panel) and after treatment with lysozyme (lower panel) in which isometric tension is plotted against time. Adrenergic response is indicated by interval between arrows. Small dip in isometric tension at beginning of stimulation is believed to represent effect of synchronization, and may be due to abnormal conduction of action potential when sympathetic stimulation is initially applied and then stopped (18). Horizontal dashed line represents basal tension. At baseline, the adrenergic response increased approximately 25%, while there was no increase when lysozyme was added to the preparation.

Figures 7A, 7B and 7C are graphs showing the mean (± 1 SE) values obtained for the adrenergic response. In Fig 7A, lysozyme decreased the adrenergic response as compared with the time control group. In Fig 7B, chitotriose treatment prevented the decrease in adrenergic response observed with lysozyme. In Figure 7C, lysozyme also decreased the response to the β - agonist isoproterenol in the right ventricular trabecular preparation. * $P < .05$ vs baseline; + $P < .05$ between groups.

Figure 8 shows hemodynamics for the treated and nontreated groups at the different measurement intervals in the Pretreatment Study. At the 5 h interval, pretreatment with chitotriose resulted in significant increases in mean

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arterial pressure, stroke-volume, and stroke-work as compared with the nontreated group. * $P < .05$ vs baseline within a group by one way ANOVA and SNK; + $P < .05$ treated vs nontreated groups, in which the change from baseline was compared between the two groups by two way repeated measures

5 ANOVA and SNK.

Figure 9 shows hemodynamics for the treated and nontreated groups at the different measurement intervals in the Early Sepsis Study. At the 6 h interval, treatment with chitotriose resulted in significant increases in cardiac output and stroke-work as compared with the nontreated group. * $P < .05$ vs

10 baseline within a group by one way ANOVA; + $P < .05$ between groups by two way ANOVA in which the response to sepsis (A x B interaction) was significantly different between the two groups (factor A) over the 6 h period (factor B).

Figure 10 shows hemodynamics for the treated and nontreated groups

15 at the different measurement intervals in the Late Sepsis Study. There was no effect of chitotriose on hemodynamics in this study. * $P < .05$ vs baseline within a group by one way ANOVA and SNK.

Figure 11 shows isometric tension plotted as a function of lysozyme concentration under different conditions in the right ventricular trabecular preparation. In the left upper panel, the effect of lysozyme on isometric

20 tension in deglycosylated and native ventricular trabeculae is shown. In the native muscle, lysozyme produced a marked decline in isometric tension, while in the deglycosylated muscle the reduction in isometric tension was attenuated as compared with the native muscle. When chitotetraose (right

25 upper panel) or N-acetyl glucosamine (right lower panel) was added to the preparation, there was no inhibitory effect on lysozyme's depressant activity. However, chitobiose (left lower panel) completely blocked lysozyme's depressant activity. * $P < .05$ vs baseline by one way ANOVA and SNK; + $P < .05$ between groups by two way ANOVA and SNK.

30 Figure 12 are graphs. In the upper panel, percent isometric tension is plotted on the ordinate against Datura stramonium lectin concentration or equal volume of Krebs Henseleit solution (KH) on the abscissa. Datura

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stramonium lectin caused a marked decrease in isometric tension as compared with the time control group. In the lower panel, pre-treatment of the preparation with chitotriose blocked the isometric depression as compared with the nontreated preparation. *P<.05 vs baseline; +P<.05 between groups
5 by two way ANOVA and SNK.

Figure 13 are graphs. In the upper panel, percent isometric tension is plotted on the ordinate against Lycopersicon esculentum lectin or equal volume of Krebs Henseleit solution (KH) on the abscissa. Lycopersicon esculentum caused a marked decrease in isometric tension as compared with
10 the time control group. In the lower panel, Triticum vulgaris lectin produced a lesser degree of depression than that found with Datura Stramonium lectin and Lycopersicon esculentum lectin. *P<.05 vs baseline; +P<.05 between groups by two way ANOVA and SNK. #P<.05 vs Datura Stramonium lectin and Lycopersicon esculentum lectin in which the change in tension from
15 baseline was compared among the three groups by two way ANOVA and SNK.

Figure 14 is a graph. Percent isometric tension is plotted on the ordinate against Erythrina corallodendron lectin, Helix pomatia lectin, or Krebs Henseleit solution on the abscissa. Both Erythrina corallodendron lectin and
20 Helix pomatia lectin produced a lesser degree of depression than that found with Datura Stramonium lectin and Lycopersicon esculentum lectin. *P<.05 vs baseline; +P<.05 between groups by two way ANOVA and SNK. #P<.05 vs Datura Stramonium lectin and Lycopersicon esculentum lectin in which the change in tension from baseline was compared among the three groups by
25 two way ANOVA and SNK.

DETAILED DESCRIPTION OF THE INVENTION

I. Therapeutic Methods

The present inventors isolated and purified the filterable cardiodepressant substance (FCS) by size exclusion high pressure liquid
30 chromatography, until a single band was identified on one dimensional gel electrophoresis. This band was then subjected to tandem mass spectrometry and protein sequencing techniques. Both physical and biochemical

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sequencing techniques identified FCS as lysozyme c (Lzm-S), consistent with that originating from the canine spleen. Confirmatory tests showed that purified Lzm-S produced myocardial depression in the ventricular trabecular preparation at concentrations achieved during sepsis in the *in vivo* preparation. Other studies performed in the *in vitro* preparation demonstrated that myocardial depression caused by Lzm-S was inhibited by proteinase K, a nonspecific proteolytic enzyme, as well as chitotriose and chitobiose, competitive inhibitors of Lzm-S activity. In addition, it was shown that Lzm-S inhibited the adrenergic responses induced by field stimulation and isoproterenol in the *in vitro* preparation, these results suggesting that inhibit the sympathetic response in sepsis. The present findings indicate that Lzm-S originating from disintegrating leukocytes from organs such as the spleen contributes to myocardial dysfunction in this model. The mechanism may relate to its binding or hydrolysis of a cardiac membrane glycoprotein with an extracellularly oriented carbohydrate tree thereby interfering with myocardial excitation contraction coupling in sepsis.

Accordingly, the present invention provides a method of preventing or inhibiting myocardial dysfunction comprising administering an effective amount of an agent that can inhibit lysozyme to an animal in need thereof. The present application also includes a use of an effective amount of an agent that can inhibit lysozyme to treat myocardial dysfunction.

The term "lysozyme" as used herein includes lysozyme C or Lzm-S which is a bacteriolytic enzyme that can catalyze the hydrolysis of N-acetyl-D-glucosamine (NAG) to N-acetylmuramic acid (NAM) linkages of bacterial cell walls.

The term "agent that can inhibit lysozyme" means any molecule or compound that can inhibit the expression of the lysozyme gene or that can inhibit the activity of lysozyme protein. Examples of agents that can inhibit lysozyme are provided in Section II. Preferred agents that can inhibit lysozyme include chitobiose and chitotriose.

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The term "effective amount" as used herein means an amount effective, at dosages and for periods of time necessary to achieve the desired result.

5 The term "animal" as used herein includes all members of the animal kingdom, including humans. Preferably, the animal to be treated is a human.

The term "treatment or treating" as used herein means an approach for obtaining beneficial or desired results, including clinical results. Beneficial or desired clinical results can include, but are not limited to, alleviation or amelioration of one or more symptoms or conditions, diminishment of extent
10 of disease, stabilized (i.e. not worsening) state of disease, preventing spread of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. "Treating" can also mean prolonging survival as compared to expected survival if not receiving treatment.

15 The results in Example 2 demonstrate that when chitotriose is given either before sepsis or in early sepsis there is a significant prevention of myocardial depression as compared to the non treated animals. Consequently, lysozyme inhibitors may be useful in preventing the onset of myocardial dysfunction in an animal with sepsis.

20 Accordingly, the present invention provides a method of preventing or reducing the onset of myocardial dysfunction in an animal with sepsis comprising administering an effective amount of an agent that can inhibit lysozyme to the animal. The present invention also includes a use of an effective amount of an agent that can inhibit lysozyme to prevent or reduce
25 the onset of myocardial dysfunction

The inventors have demonstrated that lysozyme contributes to myocardial dysfunction in an animal model of septic shock. Sepsis is the result of an overwhelming inflammatory response to a severe infection. Therefore, agents that can inhibit lysozyme may also be useful in treating
30 inflammation.

Accordingly, the present invention provides a method of preventing or treating an inflammatory condition comprising administering an effective

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amount of an agent that can inhibit lysozyme to an animal in need thereof. The invention also includes a use of an effective amount of an agent that can inhibit lysozyme to treat an inflammatory condition.

The inflammatory condition that can be treated using the method of the invention includes, but is not limited to, bacterial infection (such as sepsis) or one of a group of diseases called systematic inflammatory response syndrome (SIRS). SIRS is a wide-spread inflammatory response to a variety of severe clinical insults that include pancreatitis.

II. Agents That Inhibit Lysozyme

The finding by the present inventors that lysozyme plays a role in cardiac dysfunction and inflammation allows the discovery and development of agents that inhibit for use in modulating these conditions.

The present invention includes the use of any and all agents that modulate lysozyme in the methods of the invention. The agent can be any type of substance, including, but not limited to, nucleic acids (including antisense oligonucleotides), proteins (including antibodies), peptides, peptide mimetics, carbohydrates, small molecules (including organic and inorganic compounds) and lectin inhibitors. Examples of some of the agents that modulate lysozyme are provided below.

(i) Carbohydrates

In one embodiment, the agent that can inhibit lysozyme is a carbohydrate. In a specific embodiment, the agent that inhibits lysozyme is a carbohydrate having at least two N-acetyl glucosamine (NAG) units, preferably 2 or 3 NAG units. In a specific embodiment, the lysozyme inhibitor is chitobiose (N,N' diacetyl-glucosamine) or chitotriose (N,N',N'' triacetyl-glucosamine). The natural substrate of lysozyme is the hexasaccharide NAG-NAM-NAG-NAM-NAG-NAM (corresponding to monosaccharides in an arrangement A-B-C-D-E-F). The enzyme-lysozyme- can hydrolyse only between positions D and E yielding as natural reaction products NAG-NAM-NAG-NAM and NAG-NAM. Both the tetrasaccharide NAG-NAM-NAG-NAM and the disaccharide NAG-NAM will act as product inhibitors of the enzyme. After the natural hexasaccharide is bound to lysozyme, the monosaccharide

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ring D is subjected to distortion by the enzyme: Ring D is forced from the chair conformation into the half-chair conformation before hydrolysis of the beta 1-4 glycosidic bond between rings D and E occurs. The hexasaccharide with a distorted ring D is referred to as the transition state or highly reactive
5 state of the substrate. A transition state inhibitor of lysozyme is NAG-NAG-NAG-NAG*, where NAG* is the lactone analog of NAG. NAG* has a conformation closely resembling the half-chair conformation of ring D of the natural substrate in the transition state. A transition state inhibitor of the composition NAG-NAM-NAG-NAM*, where NAM* is a lactone analog of NAM,
10 should be still more effective. Transition state inhibitors, where available, are most powerful inhibitors of lysozyme.

The carbohydrates used in the methods of the invention can be prepared as neoglycoconjugates in order to prolong their serum half-life. For example, chitotriose or chitobiose can be chemically coupled to a large
15 molecule such as human serum albumin (HSA) or bovine serum albumin (BSA). Neoglycoconjugates can be prepared using techniques known in the art. (See for example Glycoprotein synthesis: From glycobiological tools to tailor-made catalysts, by BG Davis and JB Jones, Synlett 1990; 9:1495-1507.)

(ii) Binding Inhibitors

20 In another embodiment, the agent that can inhibit lysozyme is an agent that inhibits the binding of lysozyme to its cellular target.

The inventors show in Example 2 that lysozyme interacts with a glycoprotein on the cardiac membrane. Removal of the carbohydrate portion of the cardiac membrane reduces the effect of lysozyme on myocardial
25 dysfunction. Further proof that binding of lysozyme to glycoproteins is required for its function is provided in Example 3 in which lectins were used to mimic lysozyme's depressant effect on the myocardium. Accordingly, the lysozyme inhibitor can be any agent that can interfere with the binding of lysozyme to a glycoprotein on the surface of the cell to be treated such as a
30 cardiac cell. Techniques known in the art can be used to determine if an agent interferes with the binding of lysozyme to a carbohydrate. The inventors previously used one of the principles of elucidating the binding of

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lysozyme to carbohydrate in Example 2 (Figure 4) in which enzymatic deglycosylation was used. The inventors used endoglycosidase action to remove larger blocks of carbohydrate and showed that the depressant effect of lysozyme was much attenuated as compared with the native preparation. It is also possible to use exoglycosidases, where individual sugars are removed from the outside positions of the carbohydrate tree and to test for the effect of lysozyme in the *in vitro* preparation.

One could also determine that lysozyme binds to the cardiac surface membrane by means of immunohistochemistry techniques. In the *in vitro* preparation, one would instill lysozyme into the *in vitro* preparation that would bind to the cardiac surface membrane. One would then add a fluorescent labeled antibody that would in turn bind to the lysozyme already located on the membrane. The ventricular trabecular muscle would be removed from the bath and quickly frozen and standard techniques used for pathological examination. Under a confocal microscope or other imaging techniques, one could detect the fluorescent labeled antibody located adjacent to the cardiac surface membrane.

Finally, one could use nuclear magnetic resonance (NMR) techniques. Both, the free lysozyme and the free carbohydrate have typical nuclear magnetic resonance spectra. On interaction of the two, there will be a characteristic change of the NMR spectrum.

(ii) Antibodies

In another embodiment, the agent that can inhibit lysozyme is a lysozyme specific antibody. Antibodies to lysozyme may be prepared using techniques known in the art such as those described by Kohler and Milstein, Nature 256, 495 (1975) and in U.S. Patent Nos. RE 32,011; 4,902,614; 4,543,439; and 4,411,993. (See also Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses, Plenum Press, Kennett, McKearn, and Bechtol (eds.), 1980, and 30 Antibodies: A Laboratory Manual, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988). Within the context of the present invention, antibodies are understood to

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include monoclonal antibodies, polyclonal antibodies, antibody fragments (e.g., Fab, and F(ab')₂) and recombinantly produced binding partners.

(iv) Antisense oligonucleotides

In another embodiment, the agent that can inhibit lysozyme is an
5 antisense oligonucleotide that inhibits the expression of the lysozyme gene. The term "antisense oligonucleotide" as used herein means a nucleotide sequence that is complementary to its target, e.g. the lysozyme gene. The sequence of the lysozyme gene is known in the art, for example, go to [www.tigr.org/docs/tigr-scripts/tgi/geneprod search.pl](http://www.tigr.org/docs/tigr-scripts/tgi/geneprod_search.pl). 5 different lysozyme
10 genes are deposited.

The term "oligonucleotide" as used herein refers to an oligomer or polymer of nucleotide or nucleoside monomers consisting of naturally occurring bases, sugars, and intersugar (backbone) linkages. The term also includes modified or substituted oligomers comprising non-naturally occurring
15 monomers or portions thereof, which function similarly. Such modified or substituted oligonucleotides may be preferred over naturally occurring forms because of properties such as enhanced cellular uptake, or increased stability in the presence of nucleases. The term also includes chimeric oligonucleotides that contain two or more chemically distinct regions. For
20 example, chimeric oligonucleotides may contain at least one region of modified nucleotides that confer beneficial properties (e.g. increased nuclease resistance, increased uptake into cells), or two or more oligonucleotides of the invention may be joined to form a chimeric oligonucleotide.

The antisense oligonucleotides of the present invention may be
25 ribonucleic or deoxyribonucleic acids and may contain naturally occurring bases including adenine, guanine, cytosine, thymidine and uracil. The oligonucleotides may also contain modified bases such as xanthine, hypoxanthine, 2-aminoadenine, 6-methyl, 2-propyl and other alkyl adenines, 5-halo uracil, 5-halo cytosine, 6-aza uracil, 6-aza cytosine and 6-aza thymine,
30 pseudo uracil, 4-thiouracil, 8-halo adenine, 8-aminoadenine, 8-thiol adenine, 8-thiolalkyl adenines, 8-hydroxyl adenine and other 8-substituted adenines, 8-halo guanines, 8-amino guanine, 8-thiol guanine, 8-thiolalkyl guanines, 8-

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hydroxyl guanine and other 8-substituted guanines, other aza and deaza uracils, thymidines, cytosines, adenines, or guanines, 5-trifluoromethyl uracil and 5-trifluoro cytosine.

Other antisense oligonucleotides of the invention may contain modified
5 phosphorous, oxygen heteroatoms in the phosphate backbone, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. For example, the antisense oligonucleotides may contain phosphorothioates, phosphotriesters, methyl phosphonates, and phosphorodithioates. In an embodiment of the invention there are
10 phosphorothioate bond links between the four to six 3'-terminus bases. In another embodiment phosphorothioate bonds link all the nucleotides.

The antisense oligonucleotides of the invention may also comprise nucleotide analogs that may be better suited as therapeutic or experimental reagents. An example of an oligonucleotide analogue is a peptide nucleic
15 acid (PNA) wherein the deoxyribose (or ribose) phosphate backbone in the DNA (or RNA), is replaced with a polyamide backbone which is similar to that found in peptides (P.E. Nielsen, et al Science 1991, 254, 1497). PNA analogues have been shown to be resistant to degradation by enzymes and to have extended lives *in vivo* and *in vitro*. PNAs also bind stronger to a
20 complementary DNA sequence due to the lack of charge repulsion between the PNA strand and the DNA strand. Other oligonucleotides may contain nucleotides containing polymer backbones, cyclic backbones, or acyclic backbones. For example, the nucleotides may have morpholino backbone structures (U.S. Patent No. 5,034,506). Oligonucleotides may also contain
25 groups such as reporter groups, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an antisense oligonucleotide. Antisense oligonucleotides may also have sugar mimetics.

The antisense nucleic acid molecules may be constructed using
30 chemical synthesis and enzymatic ligation reactions using procedures known in the art. The antisense nucleic acid molecules of the invention or a fragment thereof, may be chemically synthesized using naturally occurring nucleotides

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or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed with mRNA or the native gene (e.g. phosphorothioate derivatives and acridine substituted nucleotides). The antisense sequences may be produced
5 biologically using an expression vector introduced into cells in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense sequences are produced under the control of a high efficiency regulatory region, the activity of which may be determined by the cell type into which the vector is introduced.

10 (v) Peptide Mimetics

The present invention also includes peptide mimetics of the lysozyme protein. Such peptides may include competitive inhibitors, peptide mimetics, and the like. All of these peptides as well as molecules substantially homologous, complementary or otherwise functionally or structurally
15 equivalent to these peptides may be used for purposes of the present invention.

"Peptide mimetics" are structures which serve as substitutes for peptides in interactions between molecules (See Morgan et al (1989), Ann. Reports Med. Chem. 24:243-252 for a review). Peptide mimetics include
20 synthetic structures which may or may not contain amino acids and/or peptide bonds but retain the structural and functional features of a lysozyme peptide, or enhancer or inhibitor of the lysozyme peptide. Peptide mimetics also include molecules incorporating peptides into larger molecules with other functional elements (e.g., as described in WO 99/25044). Peptide mimetics
25 also include peptoids, oligopeptoids (Simon et al (1972) Proc. Natl. Acad. Sci USA 89:9367), and peptide libraries containing peptides of a designed length representing all possible sequences of amino acids corresponding to a peptide of the invention.

Peptide mimetics may be designed based on information obtained by
30 systematic replacement of L-amino acids by D-amino acids, replacement of side chains with groups having different electronic properties, and by systematic replacement of peptide bonds with amide bond replacements.

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Local conformational constraints can also be introduced to determine conformational requirements for activity of a candidate peptide mimetic. The mimetics may include isosteric amide bonds, or D-amino acids to stabilize or promote reverse turn conformations and to help stabilize the molecule. Cyclic amino acid analogues may be used to constrain amino acid residues to particular conformational states. The mimetics can also include mimics of inhibitor peptide secondary structures. These structures can model the 3-dimensional orientation of amino acid residues into the known secondary conformations of proteins. Peptoids may also be used which are oligomers of N-substituted amino acids and can be used as motifs for the generation of chemically diverse libraries of novel molecules.

(vi) Other substances

In addition to the above substances, other substances that can modulate lysozyme can also be identified and used in the methods of the invention. For example, substances which can bind lysozyme may be identified by reacting lysozyme with a substance which potentially binds to lysozyme, then detecting if complexes between the lysozyme and the substance have formed. Substances that bind lysozyme in this assay can be further assessed to determine if they are useful in modulating or inhibiting lysozyme and useful in the therapeutic methods of the invention.

Accordingly, the present invention also includes a method of identifying substances which can bind to lysozyme comprising the steps of:

(a) reacting lysozyme and a test substance, under conditions which allow for formation of a complex between the lysozyme and the test substance, and

(b) assaying for complexes of lysozyme and the test substance, for free substance or for non complexed lysozyme, wherein the presence of complexes indicates that the test substance is capable of binding lysozyme.

Conditions which permit the formation of substance and lysozyme complexes may be selected having regard to factors such as the nature and amounts of the substance and the protein.

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The substance-lysozyme complex, free substance or non-complexed proteins may be isolated by conventional isolation techniques, for example, salting out, chromatography, electrophoresis, gel filtration, fractionation, absorption, polyacrylamide gel electrophoresis, agglutination, or combinations thereof. To facilitate the assay of the components, antibody against lysozyme or the substance, or labelled lysozyme, or a labelled substance may be utilized. The antibodies, lysozyme, or substances may be labelled with a detectable substance.

The lysozyme or the test substance used in the method of the invention may be insolubilized. For example, the lysozyme or substance may be bound to a suitable carrier. Examples of suitable carriers are agarose, cellulose, dextran, Sephadex, Sepharose, carboxymethyl cellulose, polystyrene, filter paper, ion-exchange resin, plastic film, plastic tube, glass beads, silica, polyamine-methyl vinyl-ether-maleic acid copolymer, amino acid copolymer, ethylene-maleic acid copolymer, nylon, silk, etc. The carrier may be in the shape of, for example, a tube, test plate, beads, disc, sphere etc.

The insolubilized lysozyme or substance may be prepared by reacting the material with a suitable insoluble carrier using known chemical or physical methods, for example, cyanogen bromide coupling.

The lysozyme or test substance may also be expressed on the surface of a cell in the above assay.

The lysozyme gene or protein may be used as a target for identifying lead compounds for drug development. The invention therefore includes an assay system for determining the effect of a test compound or candidate drug on the activity of the lysozyme gene or protein.

Accordingly, the present invention provides a method for identifying a compound that modulates lysozyme gene or protein activity comprising:

- (a) incubating a test compound with a lysozyme protein or a nucleic acid encoding a lysozyme protein; and
- (b) determining the effect of the test compound on lysozyme protein activity or lysozyme gene expression and comparing with a control (i.e. in the absence of a test compound) wherein a change in the lysozyme protein

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activity or lysozyme gene expression as compared to the control indicates that the test compound is a potential modulator of the lysozyme gene or protein.

III. Compositions

The present invention also includes pharmaceutical compositions
5 containing the agents that can inhibit lysozyme for use in the methods of the invention. Accordingly, the present invention provides a pharmaceutical composition for inhibiting inflammation comprising an effective amount of an agent that can inhibit lysozyme in admixture with a suitable diluent or carrier. The present invention further provides a pharmaceutical composition for
10 preventing or inhibiting cardiac dysfunction comprising an effective amount of an agent that can inhibit lysozyme in admixture with a suitable diluent or carrier. The present invention also provides a pharmaceutical composition for treating sepsis comprising an effective amount of an agent that can inhibit lysozyme in admixture with a suitable diluent or carrier.

15 Such pharmaceutical compositions can be for intralesional, intravenous, topical, rectal, parenteral, local, inhalant or subcutaneous, intradermal, intramuscular, intrathecal, transperitoneal, oral, and intracerebral use. The composition can be in liquid, solid or semisolid form, for example pills, tablets, creams, gelatin capsules, capsules, suppositories, soft gelatin
20 capsules, gels, membranes, tubelets, solutions or suspensions.

The pharmaceutical compositions of the invention can be intended for administration to humans or animals. Dosages to be administered depend on individual needs, on the desired effect and on the chosen route of administration.

25 The pharmaceutical compositions can be prepared by per se known methods for the preparation of pharmaceutically acceptable compositions which can be administered to patients, and such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in
30 Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985).

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On this basis, the pharmaceutical compositions include, albeit not exclusively, the active compound or substance in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids. The pharmaceutical compositions may additionally contain other agents such as other agents that can prevent the inhibition of apoptosis or that are used in treating inflammatory conditions or sepsis.

The following non-limiting examples are illustrative of the present invention:

10 EXAMPLES

Example 1

Lysozyme: a mediator of myocardial depression and adrenergic dysfunction in septic shock

The objective of the present study was to identify the nature of a filterable cardiodepressant factor (FCS) that contributes to myocardial dysfunction in a canine model of *Escherichia coli* septic shock. In a previous study, it was found that FCS increased in plasma after 4 h of bacteremia (Am J Physiol 1993; 264: H1402) in which FCS was identified by a bioassay that included a right ventricular trabecular preparation (RVT). In that study, FCS was only partially identified by pore filtration techniques and was found to be a protein of molecular weight between 10-30K. In the present study, FCS was further purified by size exclusion high pressure liquid chromatography, until a single band was identified on one dimensional gel electrophoresis. This band was then subjected to tandem mass spectrometry and protein sequencing techniques and both techniques identified FCS as lysozyme c (Lzm-S), consistent with that originating from the canine spleen. Confirmatory tests showed that purified Lzm-S produced myocardial depression in the RVT preparation at concentrations achieved during sepsis in the in-vivo preparation. In addition, Lzm-S inhibited the adrenergic response induced by field stimulation in the in-vitro preparation, these results suggesting that Lzm-S may inhibit the neural sympathetic response in sepsis. The present findings indicate that Lzm-S originating from disintegrating leukocytes from organs

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such as the spleen contributes to myocardial dysfunction in this model. The mechanism may relate to its binding or hydrolysis of a cardiac membrane glycoprotein thereby interfering with myocardial excitation contraction coupling in sepsis.

5 In the present study, the inventors further purified FCS to the extent that a single broad band could be found on one-dimensional-gel electrophoresis. The inventors then determined the nature of this substance by microcapillary reverse phase high pressure liquid chromatography (HPLC) tandem mass spectrometry (MS/MS) and protein sequencing techniques
10 (8,9). Once this substance was isolated, the inventors showed that it decreased myocardial contraction and adrenergic responses to neural stimulation and the β - agonist isoproterenol in respective right ventricular trabecular preparations. The inventors also showed that these effects could be blocked by a competitive inhibitor of FCS, the results providing further
15 evidence that this substance may be important in the pathophysiology of myocardial dysfunction in sepsis.

METHODS

These experiments were approved by the University Animal Care Committee and conform with the Guide for the Care and Use of Laboratory
20 Animals published by the US National Institutes of Health (NIH Publication No 85-23, 1996) (10).

The model of *E. coli* sepsis used in this study has previously been described (6). In brief, the dogs (20 to 30 kg) were anesthetized with pentobarbital sodium (30 mg/kg); the trachea was intubated with an
25 endotracheal tube; and the lungs were mechanically ventilated. The animal was given 3 ml of heparin (1000 units/ml) intravenously. After 1 hr of stability, sepsis was induced by intravenous infusion of 10^{10} colony-forming units (CFU) of live *E. coli* (0111:B4) that were suspended in normal saline and were administered over 1 h. Approximately 5×10^9 CFU/h of *E. coli* were then
30 infused for 3 additional hours, at which time left ventricular contractility and mean arterial pressure have been shown to be depressed to approximately 60-75 % of baseline in this model (6).

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Isolation of FCS

In a previous study, FCS activity was identified in the 10-30 KD plasma fraction by pore filtration techniques (7) and this fraction served as a starting point for further purification. In the dogs described above, sixty milliliters of blood were taken from the femoral artery before and after 4 h of sepsis, and the samples centrifuged. Because of the large size of the animals, this phlebotomy did not affect their hemodynamic status. The plasma samples were sequentially passed through filters with cut-offs of 30,000 and 10,000 molecular weight (Millipore, Bedford, MA), respectively, to isolate the 10- 30 KD plasma fraction. Approximately three ml of this fraction could be obtained from each of the presepsis and sepsis samples.

Aliquots of the 10- 30 KD plasma fraction (0.05 to 1 ml) were then added to a right ventricular (RV) trabecular preparation to test for the presence of FCS activity. The RV preparation has previously been described (6,7). Briefly, mongrel dogs (3 to 10 kg) were anesthetized with pentobarbital, and their hearts were removed. Three to four thin trabeculae (< 1 mm diameter) were obtained from the right ventricle and were suspended in a vertical constant temperature bath (5 ml) that contained Krebs Henseleit (KH) solution. The muscle was stimulated electrically at 0.5 Hz. Aliquots of plasma fractions (from 0.05 to 1 ml) were added to respective organ baths at 5 minute intervals. The changes observed in the pre and septic plasma fractions were expressed relative to tensions found at baseline (i.e. prior to the addition of the sample). An increase in the depressant activity in the septic plasma fraction relative to that found in the presepsis sample was indicative of an increase in FCS.

Since pore filtration techniques are an imperfect method of purification, size -exclusion high pressure liquid chromatography (SEC-HPLC) was additionally used in the present study to further purify the 10-30 KD plasma fraction. In this procedure, 50 µl protein markers (Boehringer Mannheim Biochemica) were first run through a column (Progel- TSK G2000SWXL) in 0.1 M phosphate buffer, pH 6.7, flow rate at 1.0 ml/min to derive the standard curve. The standard markers used were 465 KD (5.58 min), 150 KD (8.75

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min), 50 KD (10.98 min), 17 KD (11.98 min), 38D (14.45 min). Then the 10-30 KD plasma fraction (100 µl) was injected into the column. The eluent obtained from the column was divided into three fractions termed F1, F2, and F3 in which the molecular weight of the substances contained in the F1
5 fraction was > 30 KD, in the F2 fraction was between 10- 30 KD, and in the F3 fraction was < 10 KD. F1, F2, and F3 fractions were then tested by the right ventricular muscle bioassay to confirm that FCS activity was contained in the 10- 30 KD plasma fraction.

In addition, the respective F1, F2, and F3 pre and sepsis fractions from
10 all of the dogs were pooled, and aliquots of the samples were then run on a single dimension polyacrylamide gel electrophoresis (SDS- PAGE, 12.5%) and stained with silver nitrate. The volume and protein concentration of the samples were 20 µl and 56 µg, respectively. There were no differences in the bands found in the F1 and F3 fractions on SDS-PAGE between presepsis
15 and sepsis samples (see Result Section). On the other hand, in the F2 fraction, a broad band was found in the sepsis sample located at approximately 15 KD that could not be observed in the presepsis sample. This band, as well as a comparable area in the presepsis sample was cut from the gel and sent to the Harvard Microchemistry Facility (Cambridge,
20 Mass) for sequence analysis.

This analysis included microcapillary reverse phase HPLC tandem mass spectrometry (µLC/MS/MS) on a Finnigan LCQ quadrupole ion trap instrument (8,9). In this procedure, a reverse-phase HPLC run is directly coupled to the nano-electrospray ionization source of an ion trap mass
25 spectrometer. This configuration is capable of acquiring individual sequence (MS/MS) spectra on-line at high sensitivity (<<<10 femtomole) for multiple peptides in the chromatographic run. These fragmentation spectra are then correlated with known sequences in which the algorithm Sequest developed at the University of Washington and the programs developed at the Harvard
30 Microchemistry Facility were used. In addition, a HPLC chromatogram of the enzymatically digested sample was obtained from the pre and sepsis samples. Two chromatogram peaks in the sepsis sample that were not found

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in the presepsis sample were subsequently subjected to chemical sequencing analysis (11, 12).

Since results of the sequencing suggested that the FCS was produced in the spleen, FCS activity was measured in the spleen, *the proposed source*
5 *of FCS*, as well as the heart, the proposed target organ of FCS. In addition, FCS activity was measured in lymphocytes, a cell type that would not be associated with production of this protein and would therefore serve as a negative control. Neutrophils, another source of FCS were not measured, since these cells would be found in the spleen. In these experiments, spleens
10 and hearts were removed from dogs subjected to 4 hrs of E coli sepsis (septic group), and the results were compared to organs of animals subjected to 4 hrs of infusion of normal saline (nonseptic group). 10 gram aliquots of the spleens and hearts were obtained and were placed in 50 ml KH buffer. The mixture was homogenized and centrifuged at 1500 g for 10 minutes. The
15 supernatant was passed through respective 30 and 10 KD filters to isolate the 10-30 KD fraction. Aliquots of the 10- 30 KD sample were then placed into the organ bath, and the reduction in isometric tension relative to baseline was compared between the septic and nonseptic preparations. In addition, lymphocytes were isolated from plasma samples of dogs by cell separation
20 media placed in buffer solution (Organon Teknika Corporation, Durham NC), and the cells were analyzed for FCS activity in the pre and septic samples. A modification of the method of Boyum was used (13) in which centrifugation through a Ficoll sodium diatrizoate solution causes differential migration that results in the formation of several cell layers. In the lymphocyte layer, the
25 procedure produces 5×10^6 cells/ml that was composed of lymphocytes after staining and by light microscopy analysis.

Purification of canine spleen lysozyme and assay of activity

Since tandem mass-spectrometry showed that cardiac depressant activity was due to spleen lysozyme (Lzm-S) (see Results), Lzm-S was
30 purified in a manner described by Grobler et al (14). In the present study, note that the term Lzm-S is used rather than lysozyme c, since from the standpoint of the present results, the lysozyme identified by sequencing could

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arise from the source organ, ie the spleen or leukocytes arising from that organ, or possibly from other organs (see Discussion). In the preparatory process used, the spleen was homogenized in a blender in 2 vol of water and the homogenate was centrifuged at 7000 rpm for 15 minutes at 4°C. The
5 pellet was reextracted with water and centrifuged again. The supernatants were pooled and the pH was adjusted to 4.5 with 10% acetic acid. The solution was heated to boiling for a short period (3 minutes) and then centrifuged as before. The pH of the supernatant was adjusted to 7.0 with 2 M Tris, and the fraction that precipitated between 30 to 80% saturation with
10 ammonium sulfate was collected by centrifugation. The pellet was dissolved in 0.1 M Tris-HCl, pH 7.4 and dialyzed against the same buffer. Lzm- S was subsequently purified by cation-exchange chromatography (CEC) with CM-cellulose (Whatman CM-52, Kent United Kingdom). The columns were equilibrated with a linear gradient (200 to 600 ml) from 0 to 0.4 M NaCl in 0.1
15 M Tris pH 7.4. The protein was further purified by SEC-HPLC. Purification was confirmed by means of SDS gel and two-dimensional electrophoresis.

In addition, the amount of Lzm-S activity found in the preseptic and septic plasma fractions was estimated by the turbidometric method of Shugar (15). Aliquots of suspension of 25 mg heat killed *Micrococcus lysodeikticus* in
20 100 ml of phosphate buffer, pH 6.4 with 0.1% NaCl and 0.3ml of standard or presepsis and sepsis plasma fraction samples were used. Lzm-S activity was measured in a spectrophotometer as change in percent transmission at 450 nm for an interval of 1 minute following the addition of the sample to the bacterial suspension.

25 **Effect of Lzm- S on myocardial isometric tension**

The effect of purified Lzm-S on myocardial depression was then studied in the ventricular trabecular preparation. In initial experiments, the objective was to compare Lzm-S prepared from septic and nonseptic spleens to see if there was any difference between the two preparations. Although no
30 differences were apparent by SDS- PAGE gel, different isoforms of the enzyme could have developed in sepsis thereby increasing its potential to cause myocardial depression. Aliquots of the septic and nonseptic

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preparations were added (at 5 minute intervals) to respective organ baths, and the changes in isometric tension were then compared between Lzm-S obtained from spleens of septic and nonseptic animals.

In subsequent experiments, the objective was to determine whether pretreatment of Lzm-S with the proteolytic enzyme proteinase K reduced the extent to which myocardial depression developed in the in-vitro preparation (7). Proteinase K (attached to beaded agarose; Sigma Chemical, St Louis, MO) was added to the pure Lzm-S preparation. The effects of proteinase-K treated and nontreated Lzm-S on isometric tension were compared between the samples. Since proteinase -K is a non-specific proteolytic enzyme, a specific competitive inhibitor of lysozyme, chitotriose was subsequently examined to determine whether it prevented the depression in isometric tension observed in the in-vitro preparation (16,17). Chitotriose is the trisaccharide of N-acetylglucosamine (NAG) that has three NAG residues linked by beta (1-4) glycosidic linkages that competitively inhibits the enzymatic activity of Lzm-S.

In one set of experiments, it was determined whether chitotriose prevented the reduction in tension observed with Lzm-S. A dose of 10^{-3} M chitotriose (based on preliminary experiments) was added to the organ bath before the addition of Lzm-S. Lzm-S was then added to bring the final concentrations to 10^{-10} M, 10^{-9} M, 10^{-8} M, and 10^{-7} M in sequential steps. In a related set of experiments, the effect of chitotriose was studied under a similar protocol in which the ventricular trabecular preparation was paced at 1 Hz instead of 0.5 Hz. The objective was to determine whether the higher frequency altered the inhibitory response of chitotriose. In a third set of experiments, the sequence of adding chitotriose and Lzm-S was reversed. Lzm-S (10^{-7} M) was initially added to the preparation, after which chitotriose at bath concentrations of 10^{-6} M, 10^{-5} M, 10^{-4} M, 10^{-3} M were sequentially added. The objective was to determine whether chitotriose could attenuate the effect of Lzm-S on reducing the isometric response after an initial depression in isometric tension had already been attained.

Effect of Lzm-S on adrenergic function

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Since myocardial depression in sepsis may also be related to a diminished sympathetic response, the possible role of Lzm-S in contributing to this effect was additionally investigated in the right ventricular preparation as previously described (18). Neural adrenergic stimulation was produced in which the pulse width of the electrical stimulus-trains was increased from 2 ms to 20 ms, keeping other stimulus parameters unchanged. The increase in tension seen with sympathetic nerve stimulation was calculated as a percent increase from basal twitch amplitude. In the protocol used, the adrenergic response was measured before and after 10^{-10} M, 10^{-9} M, and 10^{-8} M Lzm-S were added to the organ bath. The results obtained with Lzm-S were compared with those obtained in time control experiments in which equal volumes of KH were added to the preparation at identical intervals. In a second set of experiments, it was observed whether chitotriose prevented the decrease in the adrenergic response produced by Lzm-S. Either chitotriose (10^{-4} M) or KH was added to an organ bath, and the results obtained with chitotriose treatment were compared with those obtained without the addition of the inhibitor.

In a third set of experiments, the effect of lysozyme (10^{-8} M) on inhibiting the adrenergic response to the β - agonist isoproterenol (10^{-8} M) was examined in the right ventricular preparation in which the muscle was paced at 0.5 Hz. The change in basal twitch tension after isoproterenol was added to the lysozyme treated preparation was compared to that found in the nontreated preparation. The objective was to determine if the β adrenergic effect of exogenous catecholamines could be inhibited by lysozyme in the *in vitro* preparation.

Statistical Analysis

Statistics comparisons between groups were made by means of a two way repeated measures analysis of variance (ANOVA) (factor A, different treatment groups; factor B, different time periods). A Student-Newman-Keuls' (SNK) multiple range test was used to determine where differences occurred. In the analysis of plasma fractions F1, F2, F3 (see Figure 1), 11 animals were used. Based on ref 7, this number gave us 80% power to detect a difference

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of 10% between pre and sepsis samples. In the gel experiments, 24 animals were necessary because many types of experiments were performed on the plasma samples obtained, and a lot of the protein was lost in the preparation of the samples. Results are reported as mean $\pm$ 1SE.

5 RESULTS

In the separation procedures outlined, the 10-30 KD pre and septic plasma fractions, initially obtained by pore filtration techniques, were passed through a SEC -HPLC column for further purification, and the eluents from the respective samples were divided into three fractions termed F1, F2, and F3.

10 The results shown in Figure 1 indicate that there was a significant increase in depressant activity found in the F2 fraction, but not in the other fractions. In the F3 fraction, depression in the septic fraction was slightly less than that found in the preseptic fraction. It is interesting to note that even in the preseptic sample, there is some background depressant activity (BDA) that is

15 found in all samples. As described in a previous study (7), the nature of BDA is not yet defined, but it appears to be of lipid moiety with different physical properties as compared with FCS. The increase in depressant activity in sepsis therefore does not represent an increase in BDA, but the formation of a new substance, ie. FCS. For this reason, it has been our approach in

20 defining the nature of FCS to compare pre and septic plasma fractions taken from the same dog. An increase in depressant activity found in a sepsis fraction relative to that found in the presepsis fraction is indicative of the formation of FCS.

From the respective F1, F2, and F3 fractions, a SDS- PAGE gel was

25 prepared that represented the pooled sample of 24 dogs obtained from the pre and sepsis samples (see Figure 2: note that some of the dogs used in the gel preparation were not studied in the organ bath shown in Figure 1). In Figure 2, the F1 and F3 fractions showed no differences in bands between the two fractions. However, in the F2 fraction, a broad band was identified at ~15

30 KD in the septic sample, but not in the nonseptic (control) sample and as noted above that contained FCS activity. The substance depicted in the gel

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was identified as canine spleen lysozyme with a high degree of confidence by both physical and chemical sequencing techniques (8,9,11).

In addition to the plasma, evidence of myocardial depressant activity was also examined in the spleen, heart, and lymphocytes. In terms of the
5 inventors' hypothesis (see Discussion), the heart was thought to be the target organ of FSC; the spleen was considered to be the source organ of FCS, while lymphocytes were not thought to be involved either in the formation or the action of FCS. When expressed per ml of organ homogenate, the results showed that as compared with the presepsis sample, in the septic sample,
10 increased depressant activity was found in the spleen and heart, while no increase was found in the lymphocyte fraction (see Fig 3). Purified Lzm-S was additionally prepared from spleens taken from septic and nonseptic dogs, and the results showed a marked decrease in isometric tension that occurred to a similar extent in both preparations (Fig 4A); time control experiments in
15 which KH was placed into the organ bath over an identical interval showed only small changes in tension. Thus, when standardized on a milligram basis of pure Lzm-S, no differences in activity could be observed between the septic and nonseptic preparations, these results suggesting that more leukocytes contained in the organ sample contributed to the increased depressant activity
20 found in septic spleens (see Discussion).

The activity of purified Lzm- S, as determined by the turbidometric method of Shugar (15), was found to be 4.4×10^4 international units per mg of Lzm-S. Lzm-S activity was then determined in the pre and septic plasma fractions, and the results were 20 units/ml and 70 units/ml, respectively. The
25 latter values would correspond to a negligible amount detected in the presepsis sample (i.e beyond our ability to define a specific amount below 10^{-10} M) while in the septic sample, this activity would correspond to a Lzm-S concentration of 10^{-8} M (see below).

Non-specific and specific inhibitors of Lzm-S were used to determine
30 whether the depression by Lzm-S could be attenuated in the in-vitro preparation. In one group of experiments (see Figure 4B), Lzm-S was treated with proteinase K which is a nonspecific proteolytic treatment. Proteinase K

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treatment caused a reduction in depressant activity as compared to the untreated sample that was most apparent at the lower concentrations of the treated sample.

In Figure 5A, chitotriose (10^{-3}M), a competitive inhibitor of Lzm-S, was
5 added to the bath prior to the addition of Lzm-S. As compared with the baseline condition, chitotriose itself had no effect on isometric tension. However, with subsequent addition of Lzm-S, the depression in isometric tension observed in the chitotriose treated group was significantly less than that found in the nontreated group. In Figure 5B, the pacing rate of the
10 trabecular preparation was increased from 0.5Hz to 1 Hz. The objective was to determine whether the effect of chitotriose (10^{-3}M) on attenuating the depressant activity of Lzm-S was still observed at this higher heart rate and this indeed was the case. In Figure 5C, Lzm-S was added to the organ bath before rather than after the addition of chitotriose, and the results showed that
15 even after myocardial depression had been initiated by Lzm-S, treatment attenuated the reduction in isometric tension as compared with the nontreated group.

In another set of experiments, the effect of Lzm-S on the adrenergic response was determined. In the example in the upper panel in Figure 6, no
20 Lzm-S treatment was administered, and the increase in adrenergically mediated contractile response was approximately 25%. In the lower panel, Lzm-S was administered and the response was near zero. In Figure 7A, mean values are shown. In the time control group, the adrenergic response was unchanged as compared to the baseline value, while in the Lzm-S
25 treated group, the adrenergic response progressively decreased when higher concentrations were added to the preparation. In Figure 7B, pretreatment with chitotriose prevented the reduction in adrenergic response otherwise found when Lzm-S was added without the inhibitor. In Figure 7C, the adrenergic response to the β - agonist isoproterenol was associated with
30 significantly less increase in isometric tension in the lysozyme treated preparation as compared with the nontreated preparation.

DISCUSSION

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The significant finding of this study is that Lzm-S contributes to myocardial dysfunction in experimental sepsis. It is important to note that our approach to identifying this depressant factor was different from that used by other investigators (19,20). Rather than to postulate a priori that a specific substance, such as endotoxin, cytokines, etc, was accountable for causing myocardial depression in sepsis, the inventors initiated this study without preconceived notion about what the nature of this substance could be. The inventors obtained plasma from the septic animal and used the right ventricular trabecular assay to determine whether a given plasma fraction developed cardiac depressant activity over 4 h of bacteremia. The inventors always compared pre and septic plasma fractions that were obtained from the same dog because the presence of background myocardial depressant activity would otherwise confound the interpretation of the data. Eventually, the inventors were able to purify myocardial depressant factor, and this factor was identified as Lzm-S. Grobler et al (14) sequenced canine spleen lysozyme and the molecular weight calculated from the amino acid sequence was 14,578 and thus fits well with the molecular weight identified as FCS in the present study.

Once the nature of FCS was identified, the inventors were able to show that purified Lzm- S caused myocardial depression in a manner identical to that produced when the 10- 30 KD septic plasma fraction was added to the in-vitro preparation. The inventors then showed that its effect could be blocked by the nonspecific proteolytic enzyme proteinase K, and more importantly by chitotriose, a competitive inhibitor of Lzm-S. The inventors also demonstrated that chitotriose was able to attenuate the depressant effect of Lzm-S when given either before or after Lzm-S was added to the in- vitro preparation. The inventors also confirmed that the concentration of Lzm-S required to cause myocardial depression in the in-vitro preparation was comparable to that found in-vivo. In the presepsis plasma fraction, the concentration of Lzm-S was low and insufficient to cause myocardial depression while it increased to 10^{-8} M in the septic plasma fraction, a concentration that was shown to produce a decline in isometric tension of approximately 50% in the *in vitro*

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preparation. Therefore, based on the in-vitro findings, the concentration found in-vivo would be sufficient to cause left ventricular dysfunction in our model.

In an earlier study, Burgess et al (21) used elevations in serum lysozyme as a marker to diagnose the development of an occult
5 intraabdominal abscess in critically ill patients following trauma. Lysozyme activity was found to increase over the course of hospitalization in septic patients with intraabdominal abscess, while activity did not increase in nonseptic control groups. Burgess et al (21) measured serum lysozyme activity in a manner similar to the present study and found that a mean value
10 of 39 units/ml was reached in their septic patients. Although their value was lower than the mean value of 70 units/ml found in the septic plasma fraction in the present study, the patients described by Burgess et al (21) were not in septic shock and hemodynamic measurements were not obtained.

In the present study, the decline in myocardial tension observed with
15 Lzm-S occurred within 5 minutes, and thus its mechanism of action would favor a direct effect of this agent on the myocardium. Many mechanisms of cardiac depression induced by sepsis have previously been postulated. Among others, these involve the effects of inflammatory mediators on releasing cytokines that would in turn cause myocardial depression through
20 changes in signal transduction, or through mechanisms that would promote cell death by means of apoptotic pathways in the heart (19,20,22). However, such mechanisms would occur in hours rather than minutes and would not fit with the rapid effect observed in the in-vitro preparation. The inventors know, moreover, that an intact membrane is required for action of Lzm-S (23). The
25 inventors showed in a skinned ventricular preparation, that the addition of the septic plasma fraction did not affect myofilament tension, while it did so in the intact muscle. The inventors think, therefore, that Lzm-S probably decreases myocardial contraction by interfering with membrane function and that this interference must occur at an extracellularly orientated site in accordance with
30 the biochemical properties of the enzyme described below.

Lysozyme is a bacteriolytic agent originally identified by Fleming in 1922 in mucus (24). Lysozyme has been shown to catalyse the hydrolysis of

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N-acetyl-D- glucosamine (NAG) to N-acetylmuramic acid (NAM) linkages of bacterial cell walls thereby disrupting the bacterial cell (16,17). It also exists among the cells of the blood exclusively in neutrophilic granulocytes and monocytes, whereas in tissues, the greatest concentrations are found in the bone marrow, lungs, intestines, spleen, and kidneys (25). Plasma lysozyme is believed to stem mainly from disintegrating neutrophilic granulocytes, and the high concentrations that are found in bone marrow, lung, spleen, and intestine have been suggested to relate to the breakdown of granulocytes located in these organs (25-27). In the present study, the inventors have used the term Lzm-S rather than lysozyme c, since from the standpoint of the present results, lysozyme identified by the sequencing analyses could have arisen from the source organ, ie the spleen or leukocytes arising from that organ, or possibly from other organs.

In Figure 3, the inventors compared the cardiodepressant activity in spleens taken from septic vs nonseptic animals and demonstrated that depressant activity increased in the septic spleens. As assessed by per gram weight of spleen, the septic spleens showed a greater degree of depression than the nonseptic spleens. However, when purified lysozyme was compared between septic and nonseptic spleens on a milligram basis, no difference was observed between the two preparations. Since there may be more polymorphonuclear cells found in septic spleens, the quantity of lysozyme would be greater than that found in nonseptic spleens, but the potency of depressant activity would not be different between preparations (see Fig 4).

The mechanism of action of lysozyme in causing cardiac depression is a very interesting question. In the heart, most membrane proteins are posttranslationally modified, are heavily N-glycosylated, and therefore are glycoproteins (28-30). In turn, changes in the N-glycosylated regions may affect membrane function and may alter myocardial contraction. The inventors consider two possibilities about how Lzm-S may affect membrane function by acting on a glycoprotein. In one mechanism, lysozyme binds to an extracellularly saccharide- bearing structure and hydrolyses the bond between two monosaccharide units, such that this hydrolysis causes the biological

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effect of a decrease in myocardial contraction. Alternatively, lysozyme binds to an extracellular orientated saccharide bearing structure, is unable to hydrolyse it, but because of its binding induces a conformational change of the carbohydrate tree and the glycoprotein bearing it. This conformational
5 change causes the biological effect of a depression in myocardial function. For lysozyme to cause hydrolysis, the presence of a vicinal NAG-NAM structure is required, while for binding alone, a structure containing NAG-NAG should be sufficient (16,17). The inventors believe that binding to a NAG-NAG membrane structure is the most likely possibility.

10 In terms of the relation of our results to previous studies, Hansen et al (26,27) measured plasma lysozyme concentrations in nonseptic humans and found a mean plasma concentration of 2.4 µg/ml in 24 individuals, although more recent studies suggest that the amount is much less and approximates 1 µg/ml (31). The higher concentrations found in some individuals in the
15 studies of Hansen et al (26,27) may be close enough to cause myocardial depression as ascertained by our in-vitro preparation. However, when previous studies are considered, some additional aspects need to be entertained. Firstly, myocardial function was never systematically investigated in these patients, so that changes may not have been detected. Secondly,
20 the sensitivity of the myocardium of human subjects to lysozyme may be less than that found for canine myocardium and this aspect needs to be elucidated. Thirdly, it is not clear the extent to which plasma lysozyme represents the actual concentration found in the myocardium. Since lysozyme is a relatively large molecule, some permeability of the vascular
25 endothelium as that which occurs in sepsis may be required for an increase in myocardial concentrations to occur.

In summary, the present findings indicate that Lzm-S originating from disintegrating leukocytes (ie predominantly neutrophils) from organs such as the spleen contributes to myocardial dysfunction in this model. In other
30 studies (32), leukocytes have also been shown to be an important component in causing myocardial dysfunction in sepsis. Although histology was not performed in this study, Granton et al (32) found neutrophils in hearts of

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animals subjected to bacteremia, and close proximity of activated neutrophils would expose myocardium to high concentrations of Lzm-S. The inventors hypothesize that Lzm-S causes myocardial dysfunction by binding to a carbohydrate portion of a membrane glycoprotein. This binding leads to a conformational change of the carbohydrate portion and the anchoring protein portion of the glycoprotein. Alternatively, Lzm-S can cleave part of the carbohydrate structure which would also lead to a conformational alteration of the glycoprotein. Such actions could interfere with myocardial excitation contraction coupling in sepsis.

10 **Example 2**

N,N',N'' triacetylglucosamine, an inhibitor of lysozyme, prevents myocardial depression in E coli sepsis in dogs

OBJECTIVE

The inventors previously found that lysozyme (Lzm-S) consistent with that originating from the spleen was a mediator of myocardial depression in an Escherichia coli model of septic shock in dogs (Example 1). The inventors further showed in a right ventricular trabecular preparation (RVT) that Lym-S's depressant activity could be blocked by N,N',N'' triacetylglucosamine (tri NAG: chitotriose), a competitive inhibitor of Lzm-S. The inventors hypothesized that Lzm-S binds to or cleaves a cardiac membrane glycoprotein thereby interfering with myocardial contraction in sepsis. In the present study, the inventors examined whether chitotriose could prevent myocardial depression in an *in vivo* preparation and whether other related NAG structures could also inhibit Lzm-S's effect in RVT.

25 In one part of the present study, the effect of chitotriose on myocardial depression when this treatment was intravenously administered at various time intervals in the E coli model was examined. Treatment was administered prior to the induction of sepsis (Pretreatment study), 1.5 hrs after sepsis was induced (Early sepsis study), and after 3.5 hrs of sepsis was induced (Late Sepsis Study) in respective experiments. The rationale of the different studies was that in the Pretreatment and Early Sepsis studies, myocardial depression would not have as yet occurred in the present model, while myocardial

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depression would have been present in the Late Sepsis Study (6). It could therefore be determined whether chitotriose treatment could prevent the development of myocardial depression in the Pretreatment and Early Sepsis Studies and whether it could also reverse myocardial depression that had
5 already occurred in the Late Sepsis Study.

In a second part of this study, a series of NAG oligosaccharides and variants to the NAG structure as inhibitors of Lzm-S's depressant activity in the right ventricular trabecular preparation (6,18) was tested. It was also determined whether deglycosylation of N linked oligosaccharides from cardiac
10 membrane glycoproteins to which Lzm-S would bind would result in a lessening of Lzm-S's effect on myocardial depression in this preparation. Whereas chitotriose is the trisaccharide of NAG (i.e NAG-NAG-NAG), other related compounds might also be useful as inhibitors of Lzm-S. Among others, the inventors tested N-acetylglucosamine (NAG) which consists of a
15 single NAG unit; N,N' diacetylglucosamine (NAG-NAG: chitobiose) which is composed of two NAG units, and N,N',N'',N''' tetraglucosamine (NAG-NAG-NAG-NAG: tetrachittraose) which is composed of four NAG units (17). The inventors therefore tried to determine the structural specificity of the sugar required to inhibit lysozyme's activity in sepsis.

20 The primary objective of the present study was to examine whether chitotriose or related sugars would be potentially useful in the treatment of cardiovascular collapse in sepsis.

METHODS

These experiments were approved by the University Animal Care
25 Committee and conform with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No 85-23, 1996).

In-vivo Experiments

In all of the studies outlined, the animals were randomized into
30 treatment and nontreatment placebo groups (see below). The E coli model used in these studies was identical to that previously described (6). Sepsis was induced by the intravenous infusion of 10^{10} colony forming units of live E

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coli (designation 011:B4). The bacteria were suspended in normal saline solution and were given over 0.5 hour. A constant infusion of approximately 5×10^9 colony forming units/hour of E coli was then maintained for the remainder of the experiment.

5 During the experiment, the animals (16-24 kg) were initially anesthetized with thiopental sodium (20 mg/kg, intravenously) and constantly infused with sufentanil citrate (1 ug/min) and midazolam (5 ug/kg/min) (33). Drug rates were adjusted as required to abolish the palpebral reflex. The animals were placed in the supine position; the trachea was intubated with an
10 endotracheal tube; and the lungs were mechanically ventilated at a tidal volume of 20 ml/kg. The ventilator rate was initially set to maintain blood pH at approximately 7.35 and was increased as necessary to reverse the metabolic acidosis that developed over the course of the study. Approximately 100% oxygen was inspired to maintain arterial oxygen tension
15 greater than 500 mmHg, so that hypoxemia did not affect the results of the study.

 Under sterile conditions, the right jugular vein was percutaneously cannulated with an introducer (Arrow International, Reading, PA). A thermister tipped catheter was advanced from the jugular vein into the
20 pulmonary artery to measure mean pulmonary arterial pressure (Ppa), mean pulmonary capillary wedge pressure (Pwp), mean right atrial pressure (Pra), thermodilution cardiac output (CO, Columbus Instruments, OH), and to obtain mixed venous oxygen blood samples. Another introducer was placed into the left jugular vein for administration of intravenous saline solution and the
25 treatment or placebo drug (see below). A polyethylene catheter was also percutaneously placed into the femoral artery to measure mean blood pressure (MAP), and to withdraw samples of blood for blood gas analysis, blood hematocrit (Hct), lactate concentrations, and blood cultures in some cases. Lactate determination was made by an automated lactate
30 dehydrogenase based assay (34) and was performed only in the Early and Late Sepsis Studies because of equipment difficulties in the Pretreatment Study.

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All transducers were connected to transducers (Cobe Laboratories), were referenced relative to the left atrium and were connected to a chart recorder (Astro-Med, W Warwick, RI). Heart rate (HR) was measured from the recorder tracing. Stroke-volume (SV) was calculated as CO/HR.

5 Systemic vascular resistance (SVR) was calculated from $[(MAP - P_{ra})/CO] \times 80$. At each measurement interval (see below), a full set of measurements included MAP, CO, P_{pa} , P_{ra} , P_{wp} , Hct, HR, SV, arterial and mixed venous sample for blood gases, Hct, and lactate.

In the analysis, stroke work (SW) was our primary index of cardiac function and the primary hemodynamic endpoint chosen in the in-vivo studies. SW is a useful physiological index to assess myocardial contractility and has been used in this model in previous studies (18,35). At constant preload, SW reflects both the decreases in afterload (as assessed by MAP) and the changes in contractile function (as assessed by SV) that accompany sepsis.

15 SW was determined at approximately the same P_{wp} at each measurement interval. The baseline P_{wp} usually averaged ≈ 9 - 10 mmHg, and normal saline solution was given as necessary to maintain P_{wp} constant over the experimental period. SW was calculated from SV (MAP- P_{wp}).

Experimental Protocols

20 **Pretreatment Study.** In the Pretreatment Study, chitotriose was given before the bacteria were infused. Based on previous studies, it takes approximately 3 to 4 hours of constant bacteremia in order for myocardial depression to develop in this model (6,18). A three to five hour course of bacteremia was therefore considered necessary to determine if chitotriose

25 could block the decline in SW observed at this interval. In the pretreatment protocol, measurements were obtained at baseline, after administration of treatment or placebo, and then after 3 h and 5 h of continuous E coli infusion.

The dose of chitotriose used in the pretreatment study was based on calculations previously obtained from the ventricular trabecular experiments in which it was estimated that a plasma concentration of at least 10^{-5} M would be

30 required to inhibit the lysozyme's depressant activity in-vivo. Also included in the assumptions for the in-vivo study was that chitotriose would be distributed

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in the extracellular space and would be excreted somewhat in the urine and possibly the stool over the course of the study. Based on the molecular weight of chitotriose (628 MW), the average size of the dog (20 kg), and the estimated distribution space of approximately 50% of total body weight, the dose chosen was 100 mg (Sigma Corp, Canada, Oakville, Ontario) mixed in 200 ml 5% dextrose in water (D₅W) that was given intravenously over 0.5 hr. After this initial dose, a dose of 50 mg mixed in 50 cc D₅W was slowly infused over each hour for the remainder of the study. An identical infusion of D₅W as placebo was given in the nontreated septic group. In the pretreatment group, blood cultures were obtained to make sure that chitotriose did not affect the viability of the organism infused.

Late Sepsis Study. In the Late Sepsis Study, the objective was to see if chitotriose treatment would reverse myocardial depression once it had already been produced. Chitotriose was administered after 3.5 hours after the initiation of bacteria and was given over a 0.5 hour in 200 ml D₅W. Because it was also noted in the in-vitro trabecular preparation that once lysozyme was already placed into the bath, a higher concentration of chitotriose was required to inhibit the effect of lysozyme, the initial dose was increased to 250 mg in the Late Sepsis Study. After this initial dose, as in the pretreatment study, a dose of 50 mg mixed in 50 cc D₅W was given slowly per hour over the remainder of the study. An identical infusion of D₅W was given as placebo in the nontreated group.

In the Late Sepsis Study, measurements were obtained at baseline, after 3.5 hours of bacteremia, immediately after treatment or placebo, and 2 hours after chitotriose or placebo treatment was administered.

Early Sepsis Study. The purpose of this study was to assess the effect of chitotriose when treatment was administered during sepsis, but before myocardial depression developed. Treatment was administered at 1.5 hours after bacteremia was begun (6). Based on the lack of response of some animals in the Late Sepsis Study, the initial dose of chitotriose was increased to 400 mg. After this initial dose, as in the other studies, a dose of 50 mg mixed in 50 cc D₅W was given per hour over the remainder of the

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study. An identical infusion of D₅W was given as placebo in the nontreated group. In the Early Sepsis Study, measurements were obtained at baseline and after 6 hours of bacteremia.

In-vitro experiments

5 The right ventricular (RV) preparation used in this study has also been described (6). Briefly, mongrel dogs (3 to 10 kg) were anesthetized with pentobarbital. The hearts were removed, flushed with 50 ml of Krebs-Henseleit solution (KH), and placed in ice-cold KH bubbled with a mixture of 95%O₂ and 5% CO₂. Three to four thin trabeculae (< 1 mm diameter) were
10 obtained from the right ventricle and were tied at each end with 6-O silk thread. Each thin muscle was suspended in a vertical constant temperature bath (5 ml) that contained KH (in mM: NaCL, 118; KCl, 4.7; CaCl₂ 2.5; MgSO₄, 1.2; KH₂PO₄, 1.4; NaHCO₃, 25; and dextrose, 11). The muscle was stimulated electrically via punctate platinum bipolar electrodes with
15 rectangular pulses (1 ms duration) at an intensity of 50% above threshold delivered at intervals of 2,000 ms. The trabeculae were stretched to optimal length.

 In the first part, the objective was to provide further evidence that interaction of Lzm-S with the cardiac membrane glycoprotein was an essential
20 mechanism through which the enzyme mediated myocardial depression. Since the sugar moiety to which Lzm-S would attach would be removed in the deglycosylated muscle, less reduction in depressant activity would be observed when Lzm-S was added to the preparation. Deglycosylation of the N-linked oligosaccharides from cardiac membrane glycoproteins was
25 performed under native conditions with a Native Protein Deglycosylation Kit (Product Code N-Degly, Sigma Corporation Canada, Oakville, Ontario). The endoglycosidase used was Endo-B-Nacetylglucosaminidase F1 (Endo F1 from *Chryseobacterium meninosepticum*). Endo F1 cleaves between the two N-acetylglucosamine residues in the diacetylchitobiose core of the
30 oligosaccharide, generating a truncated sugar molecule with one N-acetylglucosamine residue remaining on the asparagines (36). The ventricular trabeculae were incubated in the enzyme for 2 h. The effect of

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Lzm-S at a range of concentrations between 10^{-9} M to 10^{-5} M was compared between deglycosylated and nonglycosylated trabeculae. The concentration of Lzm-S previously found in septic plasma was 10^{-8} M which was approximately in middle of the range used in the glycosylation experiments
 5 (5). Lzm-S was purified from the spleens of nonseptic dogs as previously described (14).

The second objective was to determine whether saccharides that were structurally related to chitotriose could also inhibit Lzm-S's myocardial depressant activity in the in-vitro preparation. Inhibitory activities of N-
 10 acetylglucosamine (NAG), N,N' diacetylglucosamine (NAG-NAG: chitobiose), and N,N',N'',N''' tetraacetylglucosamine (NAG-NAG-NAG-NAG: tetrachitrose) were examined (Sigma Corp Canada, Oakville Ontario). These are structurally similar to chitotriose, but have a different number of NAG units (17). Two somewhat related sugars were also examined to see if there was potential for
 15 blocking the activity of lysozyme. These included D-celotriose and N-acetylglucosamine. D-celotriose includes three glucose residues linked by beta (1-4) glycosidic linkages, and while N acetylglucosamine includes N-acetylglucose and galactose residues linked by beta (1-4) glycosidic linkages (12).

20 After baseline tension was determined, either the sugar or placebo was placed into respective organ baths. The concentration of the sugar used was 10^{-3} M based on our previous experiments with chitotriose (Example 1), while the concentrations of lysozyme were 10^{-9} , 10^{-8} , and 10^{-7} M, respectively. Measurements were obtained at 5 minute intervals. The changes in isometric
 25 tension measured from baseline were compared between the treated and placebo baths.

Statistics

Statistical analyses included one and two way - analysis of variance for repeated measures and Student Neuman Keuls' multiple comparison test.
 30 Results are reported as mean $\pm$ 1SE.

RESULTS

In-vivo Studies

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There were no differences in the baseline variables between the treatment and nontreatment groups in any of the three studies. The hemodynamic results obtained in the Pretreatment Study are shown in Figure 8. In the placebo treated group, MAP (left upper hand panel) decreased over 5 hours of sepsis as compared with baseline. In the treatment group, chiotriose had no effect on baseline MAP, while it prevented the decrease in MAP observed at the 5 hour period in the nontreated group. The change in MAP observed between baseline and the 5 h interval was significantly different between the two groups.

10 The changes in CO and SV observed in the treatment and nontreatment groups are also shown in Figure 8 (see left lower panel and right upper panel, respectively). In the treatment group, pre-treatment with chiotriose had no significant effect on CO, but on the mean CO increased slightly as compared with baseline, and this increase was associated with a
15 slight accompanying increase in Pwp in some dogs (see Table 1; note that it was decided not to remove fluid in this condition to achieve Pwp identical to that at baseline because this change in Pwp was relatively small and transient). A similar increase in Pwp with a lesser increase in CO was observed with placebo administration in the nontreatment group, so that the
20 results were not different between the two groups (see Table 1 and Figure 8). In the treatment group, as compared with baseline, CO increased further at the 3 h period, while it remained unchanged in the nontreatment group. The change in CO observed between baseline and 3 h was significantly different between the two groups. The changes in SV followed those in CO, and the
25 change in SV observed between baseline and 5 h was significantly different between the two groups.

The changes in SW observed in the treatment and nontreatment groups are shown in Figure 8 (right hand lower panel). In the nontreatment group, SW decreased at the 5 h period as compared with baseline, while
30 there was no change over the course of the experiment in the treatment group. The change in SW observed between baseline and 5 h was significantly different between the two groups.

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The results obtained in Pwp, HR, Hct, arterial pH and SVR over the course of the study were not different between the two groups and are shown in Table 1. PH declined over time in all groups in all experiments. This was due to the development of a hyperchloremic metabolic acidosis due to the infusion of normal saline solution. The amount of normal saline administered was not different between the two groups (2.0 ± 1 L in the treated vs $1.3 \pm .34$ L in the nontreated group). Blood cultures were positive for E coli in all experiments in both the treatment and nontreatment groups.

In the Early Sepsis Study, chitotriose was infused after 1.5 h of sepsis, and the major hemodynamic results are shown in Figure 9. In the treatment group, MAP (left hand upper panel) increased in 4 of the 5 dogs over the 6 hrs of sepsis, while it decreased in 4 of the 5 dogs in the nontreatment group; however, the results were not different between the two groups. In the treatment group, CO (right upper panel) increased over the course of sepsis as compared with the nontreated group, while SV (right upper hand panel) showed a similar difference between groups. In the nontreatment group, SW (right lower hand panel) decreased approximately by 50% as compared with baseline, while it remained unchanged in the treatment group. The changes in SW were significantly different between the two groups. The results obtained in Pwp, HR, Hct, lactate, arterial pH, and SVR over the course of the study were not different between the two groups and are shown in Table 2. The amount of normal saline infused was also not different in the treated ($2 \pm .7$ L) and nontreated groups ($1.3 \pm .5$ L).

In the Late Sepsis Study, chitotriose was administered after 3.5 h of sepsis. The major hemodynamic findings are shown in Figure 10. Once myocardial depression was observed, there was little effect of treatment on SW in this model. Although SW increased on the mean after chitotriose was administered, this increase was relatively small as compared to the decline in SW observed between the baseline and 3.5 h interval. This increase was also not different from that found in the nontreatment group. The results obtained in Pwp, HR, Hct, lactate, arterial pH, and SVR over the course of the study were also not different between the two groups (see also Table 3), as

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was the amount of normal saline solution administered (3.6 ± 1 L in the treated vs 4.3 ± 1 L in the nontreated groups).

In-vitro Studies

The effect of Lzm-S on deglycosylated trabeculae is shown in Figure 11 (left upper panel). There was significantly less depression observed in the deglycosylated muscle as compared with the native preparation when Lzm-S was added to the preparation.

Other experiments were performed to test whether sugars that were related to chitotriose or had slight variations in structure would prevent the depressant effect of Lzm-S on the myocardium. The results obtained for N-acetyl glucosamine, chitobiose, and chitotetraose are also shown in Figure 11. As previously reported, in the nontreated group, there was a dose response decrease in isometric tension as compared with baseline when Lzm-S was added to the trabecular preparation. The decline in isometric tension observed with Lzm-S at a concentration of 10^{-7} M was approximately 50% of baseline in all experiments. There was no affect of N-acetyl glucosamine (right lower panel) or chitotetraose (right upper panel) on the decline in isometric tension caused by Lzm-S in the in-vitro preparation. However, in a manner similar to that described for chitotriose, chitobiose completely inhibited the depressant effect of Lzm-S in the in-vitro preparation (left lower panel).

In other experiments, there were also no inhibitory effects of N-acetyllactosamine or celluotiose observed in the in-vitro preparation. Following pretreatment with N-acetyllactosamine, isometric tensions were $60 \pm 0\%^*$, $54 \pm 1\%^*$, and $46 \pm 1.5\%^*$ of baseline ($n=2$) at respective Lzm-S concentrations of 10^{-9} , 10^{-8} , and 10^{-7} M, while following pretreatment with D-cellotriose ($n=3$), respective isometric tensions were $83 \pm 9\%^*$, $76 \pm 13\%^*$, $57 \pm 17\%^*$ of baseline ($*P < .05$ vs baseline). Although only a small number of experiments were performed with these sugars, the values were so close to what was found in the nontreated preparation, that further experiments were not pursued. Accordingly, any inhibitory effect of these sugars would be relatively small as compared to that found with chitobiose or chitotriose.

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DISCUSSION

In Example 1, the inventors identified Lzm-S as a cause of myocardial depression in E coli sepsis and showed that chitotriose, a competitive inhibitor of this enzyme, blocked the myocardial depression caused by Lzm-S in the right ventricular trabecular preparation. In the present Example, the inventors extended these findings and showed that in an in-vivo model, chitotriose prevented the development of myocardial depression as well.

In the Pretreatment Study, the inventors gave the drug before E coli was infused and found that the reduction in SW usually observed at the 5 h interval was prevented as compared with the nontreated septic group. In the Early Sepsis Study, the inventors administered chitotriose in the middle of sepsis, before myocardial depression is found in this model, and the results again showed that chitotriose prevented the reduction in stroke-work as compared with the nontreated sepsis group. In the Late Sepsis Study, however, there was no response to treatment as compared with the nontreated septic group. Although there appeared to be a limited response to treatment in some experiments in the Late Sepsis Study, this increase was relatively small and SW remained quite reduced as compared with the presepsis measurement.

The findings observed in these in-vivo experiments are very similar to those previously reported in-vitro (Example 1) where a greater degree of inhibition by chitotriose was observed when the treatment was administered before myocardial depression had developed. Thus, whereas chitotriose is able to prevent the development of myocardial depression in sepsis even when bacteremia is ongoing as in the Early Sepsis Study, it is not clear whether once myocardial depression has already developed whether it is possible to reverse this depression. In a model of *Pseudomonas aeruginosa* bacteremia and pneumonia in dogs (35), the inventors previously used continuous arterial venous hemofiltration (CAVH) to remove myocardial depressant activity from the circulation and compared the effects of early vs late hemofiltration on the subsequent changes in myocardial function that occurred in the two groups. SW was again used as the primary index of

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myocardial function. The inventors found that although CAVH removed myocardial depressant activity from plasma in both the early and late hemofiltration groups, early hemofiltration was associated with a greater degree of preserved myocardial function as compared with comparable
5 nonhemofiltered septic groups. Thus, prevention of myocardial dysfunction by CAVH or chitotriose appears easier to accomplish than a reversal of myocardial depression in these experimental models.

Nevertheless, there is other evidence to suggest that complete reversal of myocardial depression by chitotriose may be accomplished in sepsis. In
10 ventricular trabeculae that had been subjected to 4 h of bacteremia in a previous study (7), the inventors observed that there was a gradual improvement of isometric tension over time when the trabeculae were continuously washed over a period of 1 h. This observation would indicate that it possible to remove Lzm-S from the cardiac membrane under
15 appropriate conditions, and with its removal, myocardial function may improve. In the Late Sepsis Study, the dose of chitotriose may have been too small to reverse myocardial depression. Since chitotriose is a competitor inhibitor of Lzm-S, the inventors believe that a higher dose of treatment may have been able to competitively remove Lzm-S from the cardiac membrane
20 and with its removal, reversal of the myocardial depression may have been found in the Late Sepsis Study. Alternatively, hydrolysis of the membrane glycoprotein by Lzm-S in the Late Sepsis Study may have irreversibly injured the muscle (see further below).

The mechanism of action of Lzm-S on causing myocardial depression
25 also needs to be further evaluated. In the in-vitro preparation, the decline in myocardial tension observed with Lzm-S occurs within 5 minutes, and thus its mechanism of action would favor a direct effect on the myocardium. Many mechanisms of cardiac depression induced by sepsis have previously been postulated. Among others, these involve the effects of inflammatory
30 mediators on releasing cytokines that would in turn cause myocardial depression through changes in signal transduction, or through mechanisms that would promote cell death by means of apoptotic pathways in the heart

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(20,22). However, such mechanisms would occur in hours rather than minutes and would not fit with the rapid effect observed in the in-vitro preparation. The inventors know, moreover, that an intact membrane is required for action of Lzm-S, since in a skinned ventricular preparation, the
5 addition of septic plasma did not affect myofilament tension, while it did so in the intact muscle (23).

To provide further evidence that the carbohydrate portion of the glycoprotein was the mechanism through which Lzm-S may cause myocardial depression in sepsis, the inventors performed deglycosylation experiments in
10 which the N-linked oligosaccharides from glycoproteins on the cardiac membrane were cleaved. Deglycosylation would prevent the binding of Lzm-S to the membrane and would reduce the effect of Lzm-S on causing myocardial depression. As shown in Figure 11, the reduction in isometric tension caused by Lzm-S in the deglycosylated muscle was much less than
15 that found in the native muscle. This supports the notion that Lzm-S decreases myocardial contraction by interfering with membrane function and that this interference must occur at an extracellularly orientated site in accordance with the biochemical properties of the enzyme as described below.

20 The mechanism of action of Lzm-S in causing cardiac depression is a very intriguing question. In the heart, most membrane proteins are posttranslationally modified, are heavily N-glycosylated, and therefore are glycoproteins (28-30). In turn, changes in the N-glycosylated regions may affect membrane function and may alter myocardial contraction. The
25 inventors consider two possibilities about how Lzm-S may affect membrane function by acting on a glycoprotein. In one mechanism, Lzm-S binds to an extracellularly saccharide-bearing structure and hydrolyses the bond between two monosaccharide units, such that this hydrolysis causes the biological effect of a decrease in myocardial contraction. Alternatively, Lzm-S binds to
30 an extracellularly orientated saccharide bearing structure, is unable to hydrolyse it, but because of its binding induces a conformation change of the carbohydrate tree and the glycoprotein bearing it. This conformational

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change causes the biological effect of a depression in myocardial function. For Lzm-S to cause hydrolysis, the presence of a vicinal NAG-NAM structure is required, while for binding alone, a structure containing NAG-NAG should be sufficient. The inventors believe that binding to a NAG-NAG membrane
5 structure is the most likely possibility leading to depression in sepsis, although hydrolysis of the carbohydrate structure is still a possibility. Binding of Lzm-S to the membrane could be overcome by an inhibitor, such as chitotriose, while a cleaved bond would not be restored by it. This is one explanation about why myocardial function was not reversible once depression had already
10 occurred in the Late Sepsis Study, and this possibility needs to be addressed in future studies.

In the present study, the inventors tested numerous possible inhibitors in order to learn something about the specificity of Lzm-S in causing myocardial depression (17). The inventors tested on the one hand a series of
15 NAG oligosaccharides as well as variants to the NAG structure. N-acetylglucosamine is composed of a single NAG unit and demonstrated no inhibition of Lzm-S's myocardial depression in the in-vitro preparation; the tetrasaccharide chitotetraose is composed of four NAG units and also showed no effect. On the other hand, chitobiose is composed of two NAG
20 units and demonstrated a very substantial inhibitory effect in a manner identical to that previously described for chitotriose. The inventors also tried N-acetyllactosamine and D-cellotriose to see if these compounds could inhibit the effect of Lzm-S in the in-vitro preparation. D cellotriose includes three glucose residues linked by beta (1-4) glycosidic linkages, and while N
25 acetyllactosamine includes N-acetylglucose and galactose residues linked by beta (1-4) glycosidic linkages (12). Neither of the latter treatments showed any inhibitory effect. Accordingly, it appears that Lzm-S is strongly inhibited by the NAG-NAG and NAG-NAG-NAG structure, but does not tolerate any variation to the NAG structure. Of the inhibitors tried, only chitotriose and
30 chitobiose showed a beneficial effect, these results pointing to the specificity of the inhibitor in preventing Lzm-S's depressant activity on the myocardium.

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In the design of the study, the inventors did not use nonseptic treated and nontreated control groups in the experimental protocols. This design was chosen because we previously showed that hemodynamic parameters and cardiac mechanic mechanics are stable in nonseptic control groups over the 6 h period of observation (6,18), and furthermore, the inventors did not find in a previous study (Example 1) that chitotriose caused changes (increases or decreases) in isometric tension at the many concentrations tested in the right ventricular trabecular preparation. When chitotriose was administered in the nonseptic condition in the Pretreatment Study, other than to observe a slight increase in Pwp related to the added volume, there was little change in hemodynamic results noted. These findings would suggest that chitotriose improves myocardial function only by blocking the effect of Lzm-S on the myocardium. Since the inventors previously showed that only trace concentrations of Lzm-S were found in nonseptic plasma, the inventors thought that there was little rationale for the inclusion of treated and nontreated nonseptic control groups in the present study.

It is also noteworthy that the inventors previously showed that Lzm-S inhibited the myocardial adrenergic response to neural stimulation and exogenous β catecholamines in the right ventricular preparation, and furthermore, that neural inhibition could be blocked by chitotriose. Since the β adrenergic receptor is a heavily glycosylated protein of which its carbohydrate tree would be susceptible to Lzm-S's binding or cleavage, the inventors hypothesize that Lzm-S also interferes with adrenergic function in sepsis. In terms of the findings observed in the Pretreatment Study and ESS, it is possible that an enhanced adrenergic response with chitotriose could have also contributed to the improved myocardial function observed in the treated groups, although one would have expected evidence of an enhanced sympathetic response, such as a higher HR, in the treated groups.

The inventors previously showed that in E coli sepsis, Lzm-S increases in plasma over the course of bacteremia and the concentrations reached are significantly high enough to produce myocardial depression in the right ventricular trabecular preparation. The inventors further found that this

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depression could be blocked by chitotriose in this preparation, but it was unclear whether this finding was relevant to the setting of sepsis. The present study extends these findings and shows that chitotriose also prevents myocardial depression in the in-vivo preparation. Once myocardial depression had already developed, however, there was little reversal of this depression. The inventors also showed that Lzm- S is strongly inhibited by chitibiose as well as chitotriose, but not to variations in these structures. This is consistent with what enzymologists have found in studies of structure and function where the inhibitors for this enzyme were found to be quite specific. It is also important to note that this study does not exclude other mechanisms that have previously been proposed to cause myocardial depression in sepsis. Whereas it is recognized that the relevance of animal models to the human condition must be interpreted cautiously, the present results suggest that inhibition of Lzm-S by chitotriose or chitobiose may be useful in the treatment of cardiovascular collapse in sepsis.

Example 3

Specific lectins mimic the myocardial dysfunction caused by lysozyme in canine ventricle

In the present Example, the inventors hypothesized that lectins with a carbohydrate specificity similar to that described for lysozyme with primary affinity for oligomers of N-acetylglucosamine (NAG-NAG-NAG structure) (eg Datura stramonium lectin and Lycopersicon esculentum lectin) would cause myocardial depression in a right ventricular trabecular preparation (RVT). The inventors compared the latter lectins with those with affinities to variants to the NAG-NAG-NAG structure, that included affinities for the chitobiose structure (NAG-NAG: Triticum vulgaris lectin), for the N-acetyllactosamine structure (Erythrina corallodendron lectin), and for terminal N-acetyl galactosaminyl residues (Helix pomatia lectin). The results showed that lectins with an affinity for the NAG-NAG-NAG structure demonstrated a depression in isometric tensions similar to that described for lysozyme which was significantly greater than that found for the other lectins. The inventors conclude that binding to rather than hydrolysis of the carbohydrate tree on the cardiac membrane is all

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that is required for lysozyme to cause myocardial depression. Lectins that bind with a specificity of lysozyme also show comparable depression. These data provide further evidence that the release of mediators, such as lysozyme, that bind to cardiac membrane glycoproteins may lead to myocardial dysfunction in sepsis.

METHODS

These experiments were approved by the University Animal Care Committee and conform with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No 85-23, 1996).

The right ventricular (RV) preparation used in this study has also been described. Briefly, mongrel dogs (3 to 10 kg) were anesthetized with pentobarbital. The hearts were removed, flushed with 50 ml of Krebs-Henseleit solution (KH), and placed in ice-cold KH bubbled with a mixture of 95%O₂ and 5% CO₂. Three to four thin trabeculae (< 1 mm diameter) were obtained from the right ventricle and were tied at each end with 6-O silk thread. Each thin muscle was suspended in a vertical constant temperature bath (5 ml) that contained KH (in mM: NaCL, 118; KCl, 4.7; CaCl₂ 2.5; MgSO₄, 1.2; KH₂PO₄, 1.4; NaHCO₃, 25; and dextrose, 11). The muscle was stimulated electrically via punctate platinum bipolar electrodes with rectangular pulses (1 ms duration) at an intensity of 50% above threshold delivered at intervals of 2,000 ms. The trabeculae were stretched to optimal length.

The lectins chosen were based on their specificity for binding to carbohydrate structures similar to those to which lysozyme would bind as well to slight variants in the NAG structure. The latter would indicate whether binding to specific carbohydrate structures affected the degree of myocardial depression that occurs in the in-vitro preparation. Lycopersicon esculentum lectin (MW 71 K) has an affinity for NAG oligomers and is a glycoprotein that contains approximately equal amounts of protein and carbohydrate (37). Datura stramonium lectin (MW: 86K) has affinity for oligomers of N-acetylglucosamine and N-acetyllactosamine (38). This lectin is a glycoprotein

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that contains approximately 35% carbohydrate. *Triticum vulgaris* lectin (MW: 36K) has an affinity for N-acetyl-glucosamine residues and N-acetyl-glucosamine oligomers (39). This lectin contains no protein bound carbohydrate. *Erythrina corallodendron* lectin (MW: 60 K) has an affinity for
5 N-acetyllactosamine, N acetyl-galactosamine, lactose, and D-galactose (40). *Helix pomatia* lectin (MW: 79K) has affinity for terminal N-acetyl galasoaminyll residues (41).

After baseline tension was determined, either the lectin or placebo was placed into respective organ baths. The concentrations of the lectins used
10 were 10^{-8} M, 10^{-7} M, and 10^{-6} M based on the concentration of lysozyme found in sepsis that averaged approximately 10^{-8} M. In the case of *Datura stramonium* lectin, moreover, the effect of chitotriose on inhibiting the myocardial depression caused by this lectin was also examined. Chitotriose was found to be an inhibitor of lysozyme in the in-vitro preparation in previous
15 experiments, and since *Datura stramonium* lectin has similar affinity for the NAG-NAG-NAG structure as does lysozyme, it was determined whether chitotriose (10^{-4} M) blocked the depressant activity of this lectin in the in-vitro preparation. Measurements were obtained at 5 minute intervals. The changes in isometric tension measured from baseline were compared
20 between the lectin treated and placebo preparations.

Statistics

Statistical analyses included two way analysis of variance and Student Neuman Keuls' multiple comparison test.

RESULTS

25 The results obtained with *Datura stramonium* are shown in Figure 12 (upper panel). *Datura stramonium* lectin caused a dose response reduction in myocardial tension. At a lectin concentration of 10^{-6} M, isometric tension decreased to approximately 35% of baseline. There were no changes in isometric tension observed in the time control group over this interval. In
30 Figure 12 (lower panel) the preparation was treated with either chitotriose or KH in respective organ baths. The degree of myocardial depression caused

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by *Datura stramonium* in the chitotriose treated preparation was markedly attenuated as compared with the placebo preparation.

In Figure 13 (upper panel), the results obtained with *Lycopersicon esculentum* lectin are shown. At a lectin concentration of 10^{-6} M, *Lycopersicon*
5 *esculentum* again reduced isometric tension to approximately 35% of baseline, while there were no changes in isometric tension found in the time control group over this interval. In Figure 13 (lower panel), *Triticum vulgaris* lectin caused much less reduction in isometric tension as compared with either *Datura stramonium* lectin or *Lycopersicon esculentum* lectin. At a lectin
10 concentration of 10^{-6} M, *Triticum vulgaris* lectin reduced isometric tension to 70% of baseline, while there were no changes observed in the time control group.

In Figure 14, the results obtained with *Erythrina corralloides* lectin and *Helix pomatia* lectin are shown. Although some decline in isometric
15 tension was observed with these lectins, these reductions were small as compared with *Datura stramonium* lectin and *Lycopersicon esculentum* lectin. At lectin concentrations of 10^{-6} M, the reductions observed with *Erythrina coralloides* and *Helix pomatia* lectin were to approximately 75% of baseline, which were just slightly greater than the results obtained in the time
20 control group.

DISCUSSION

There were two reasons for performing this study. On the one hand, the inventors wanted to determine if the mechanism by which lysozyme causes myocardial depression in sepsis is related to the binding of the
25 enzyme to the carbohydrate portion of cardiac membrane or whether hydrolysis of the bond between two monosaccharide units is an important requirement. For lysozyme to cause hydrolysis, the presence of a vincinal NAG-NAM structure on the cardiac membrane would be necessary, while for binding alone, a structure that contained NAG-NAG or NAG-NAG-NAG units
30 would be sufficient. Lectins are carbohydrate binding proteins that display no enzymatic activity (42). *Datura stramonium* lectin and *Lycopersicon esculentum* lectin (see Figures 12 and 13, respectively) have predominant

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affinity for the NAG-NAG-NAG structure and resulted in marked decreases in isometric tension in the in-vitro preparation. According, these findings would support the notion that binding alone is all that is required for lysozyme to cause myocardial depression in sepsis.

5 The second reason for performing this study was to determine the degree to which lectins with different carbohydrate binding specificities would cause myocardial dysfunction in the right ventricular trabecular preparation. The inventors looked at lectins with specificities to oligomers of NAG (NAG-NAG-NAG), residues of NAG (NAG-NAG), and to variants of the NAG
10 structure (ie N-acetyllactosamine and terminal N-acetyl galactosaminy residues) and determined the extent to which each compound would reduce isometric tension in the in-vitro preparation. The results showed that lectins with a primary affinity for the NAG-NAG-NAG structure (e.g. Lycopersicon
15 esculentum lectin and Datura stramonium lectin) (37,38) caused a much greater reduction in isometric tension than that caused by the lectin whose primary affinity was to the NAG-NAG residue (eg Triticum vulgaris lectin) (39). Furthermore, there was only a minimal reduction in isometric reduction caused by the lectins with specificities to the variants to the NAG structure, such as Erythrina corallodendron lectin and Helix pomatia lectin.

20 In the previous Examples, the inventors examined the effect of lysozyme on isometric tension in the right ventricular trabecular preparation and found that at a concentration of 10^{-6} M, the reduction in isometric observed was to approximately 30% of baseline. This degree of depression is very similar to that found with either Datura stramonium lectin and
25 Lycopersicon esculentum lectin in the present study. Since, on a molar bases, all three compounds- Datura stramonium lectin, Lycopersicon esculentum lectin, and lysozyme- produced similar degrees of depression, one could argue that they all preferentially targeted the NAG-NAG-NAG structure on the cardiac membrane to the same extent. Although feel that this
30 is the case, the inventors recognize the need to be cautious about this interpretation. Individual lectins have peculiar tendencies that affect their binding to various carbohydrate structures and these cannot readily be

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discerned by their structure; for instance *Datura stramonium* lectin may target the NAG-NAG structure and N-acetyllactosamines, while *Triticum vulgaris* lectin may also have specificity to N-acetylneuraminic acid. Such differences may complicate comparisons among the various lectins. Nevertheless, from
5 the standpoint of the present study, it appears that myocardial depression in the in-vitro preparation can best be explained by compounds, such as lysozyme, *Lycopersicon esculentum* lectin and *Datura stramonium* lectin that favor the NAG-NAG-NAG structure on the cardiac membrane.

Although many of the cardiac membrane proteins are glycosylated, we
10 are still unclear about how changes in glycosylation may alter function in the myocardium. Others have found that changes in N-glycosylation may alter the function of L-type calcium channels, regulation of potassium channels, and activation of sodium channels (28-30). Changes in the function of these channels may lead to myocardial depression in sepsis. The inventors
15 hypothesize that binding by lysozyme causes a functional change in the properties of a membrane glycoprotein with an extracellularly orientated carbohydrate tree thereby interfering with myocardial excitation contraction coupling in sepsis.

It therefore seems reasonable to assume that the binding by lectins
20 would result in a similar functional change in the cardiac membrane glycoprotein. The structures of *Datura stramonium* lectin and *Lycopersicon esculentum* lectin are quite distinct (37,38), and the only common denominator between them is that they target the NAG-NAG-NAG structure on the cardiac membrane. Although the physiological functions of many
25 lectins are not clear, these range from affecting cell-cell interactions, intracellular routing of glycoproteins and vesicles, cellular mitosis, among others (42). However, it is unlikely that any such mechanisms would be applicable to the results reported in the present study, since the aforementioned mechanisms would require a relatively long time frame to
30 occur. In the present study, the decline in myocardial tension observed in the *in vitro* preparation when the lectins were instilled occurred within 5 minutes, and thus their mechanism of action (like lysozyme) would favor a direct effect

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on the cardiac membrane glycoprotein. Future experiments are required to discern the specific physiological changes that occur related to this binding.

5 The inventors previously showed that in E coli sepsis, lysozyme increased in plasma over the course of bacteremia and the concentrations reached are significantly high enough to produce myocardial depression in the right ventricular trabecular preparation. The inventors further found that this depression could be blocked by chitotriose in in-vitro and in-vivo preparations (Examples 1 and 2). The present study extends these findings. It shows that lectins that bind to the carbohydrate portion of cardiac membrane with a specificity of
10 lysozyme may also cause comparable myocardial depression. Taken together, these data provide further evidence that during the inflammatory response, the release of mediators, such as lysozyme, by binding to an extracellular orientated saccharide bearing structure, induces a conformation change of the carbohydrate tree and the glycoprotein bearing it, that in turn leads to myocardial dysfunction in sepsis.

15 While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the invention is not limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

20

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TABLE 1**Hemodynamics and blood parameters in the Pretreatment Study**

5

Treatment Group (n=6)

	Baseline	Treatment	3h	5h
HR	74±11	78±5	105±5*	90±5
PwP	9.3±.8	10.4±1	9±.9	9.6±.9
SVR	2472±320	1872±320	1272±221*	1504±249*
Arterial pH	7.35±.01	7.28±.01*	7.25±.001*	7.22±.01*
Hct	33±1	34±2	28±2	26±3

10

Placebo Group (n=6)

	Baseline	Treatment	3h	5h
HR	82±16	82±16	97±5	90±5
PwP	8.9±1	10.3±1	8.9±.6	8.3±.8
SVR	2072±310	1420±164	1274±141	1711±348
Arterial pH	7.33±.01	7.31±.01	7.25±.01*	7.23±.01*
Hct	33±2	31±2	25±2*	24±2*

15

Mean (± SE). HR is heart rate (beats/min); Pwp is pulmonary capillary wedge pressure (mmHg), SVR is systemic vascular resistance (dynes s cm⁻⁵), Hct is hematocrit (%). *P<.05 vs baseline by one way ANOVA and SNK.

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TABLE 2**Hemodynamics and blood parameters in the Early Sepsis Study****Treatment Group (n=5)**

5

	Baseline	6h posttreatment
HR	68±9	94±8
Pwp	10±5	10.9±1
SVR	2160±581	1792±233
Arterial pH	7.32±03	7.24±03
Hct	31±2	30±4
Lactate	1±.1	1±.1

Placebo Group (n=5)

	Baseline	6h posttreatment
HR	71±15	96±8
Pwp	9±1	9.8±1
SVR	1888±164	1600±389
Arterial pH	7.35±02	7.27±03
Hct	33±3	32±3
Lactate	.7±.1	.9±.1

10

Mean (± SE). HR is heart rate (beats/min); Pwp is pulmonary capillary wedge pressure (mmHg), SVR is systemic vascular resistance (dynes s cm⁻⁵), Hct is hematocrit (%), lactate is in mM. *P<.05 vs baseline by one way ANOVA and SNK.

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TABLE 3**Hemodynamics and blood parameters in the Late Sepsis Study****Treatment Group (n=5)**

	Baseline	3.5h Post sepsis	Posttreatment	2h posttreatment
HR	60±4	113±9	96±8*	90±9*
PwP	9.4±1	9.8±1	10.5±1.4	10±1.6
SVR	2437±343	1200±262*	1268±196*	1280±182*
Arterial pH	7.34±.015	7.24±.05*	7.20±.04*	7.20±.04*
Hct	34±2	26±5	27±5	29±6
Lactate	.9±.2	1.2±2.5	1.3±.2	1.3±.4

5

Placebo Group (n=4)

	Baseline	3.5h Post sepsis	Posttreatment	2h posttreatment
HR	61±8	99±10*	89±6*	89±9*
PwP	9.6±1	9.6±1	9.7±1	9 6±1
SVR	1992±124	800±87*	832±128*	1380±153*
Arterial pH	7.36±.001	7.23±.025*	7.22±.03*	7.20±.04*
Hct	35±1	30±5	28±5	28±5
Lactate	1.15±.16	1.5±25	1.7±.25*	1.46±.2

10 Mean (± SE). HR is heart rate (beats/min); Pwp is pulmonary capillary wedge pressure (mmHg), SVR is systemic vascular resistance (dynes s cm⁻⁵), Hct is hematocrit (%), lactate is in mM. *P<.05 vs baseline by one way ANOVA and SNK.

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CLAIMS

1. Use of an agent that can inhibit lysozyme to reduce sepsis-induced myocardial dysfunction, wherein the agent that can inhibit lysozyme is selected from the group consisting of: N,N' diacetylglucosamine (chitobiose), N,N', N'' triacetylglucosamine (TAC), an anti-sense oligonucleotide to lysozyme mRNA and an antibody to lysozyme.
5
2. The use according to claim 1 wherein the agent is N,N' diacetylglucosamine (chitobiose).
3. The use according to claim 1 wherein the agent is N,N',N'' triacetylglucosamine (TAC).
10
4. The use according to claim 1 wherein the agent is an antisense oligonucleotide to lysozyme mRNA.
5. The use according to claim 1 wherein the agent is an antibody to lysozyme.
6. Use of an agent that can inhibit lysozyme to reverse myocardial depression in an animal with sepsis, wherein the agent that can inhibit lysozyme is selected from the group consisting of: N,N' diacetylglucosamine (chitobiose), N,N',N'' triacetylglucosamine (TAC), an anti-sense oligonucleotide to lysozyme mRNA and an antibody to lysozyme.
15
7. The use according to claim 6 wherein the agent is N,N' diacetylglucosamine (chitobiose).
20
8. The use according to claim 6 wherein the agent is N,N',N'' triacetylglucosamine (TAC).
9. The use according to claim 6 wherein the agent is an antisense oligonucleotide to lysozyme mRNA.
25
10. The use according to claim 6 wherein the agent is an antibody to lysozyme.

FIGURE 1A

Fraction 1: >30,000 Daltons

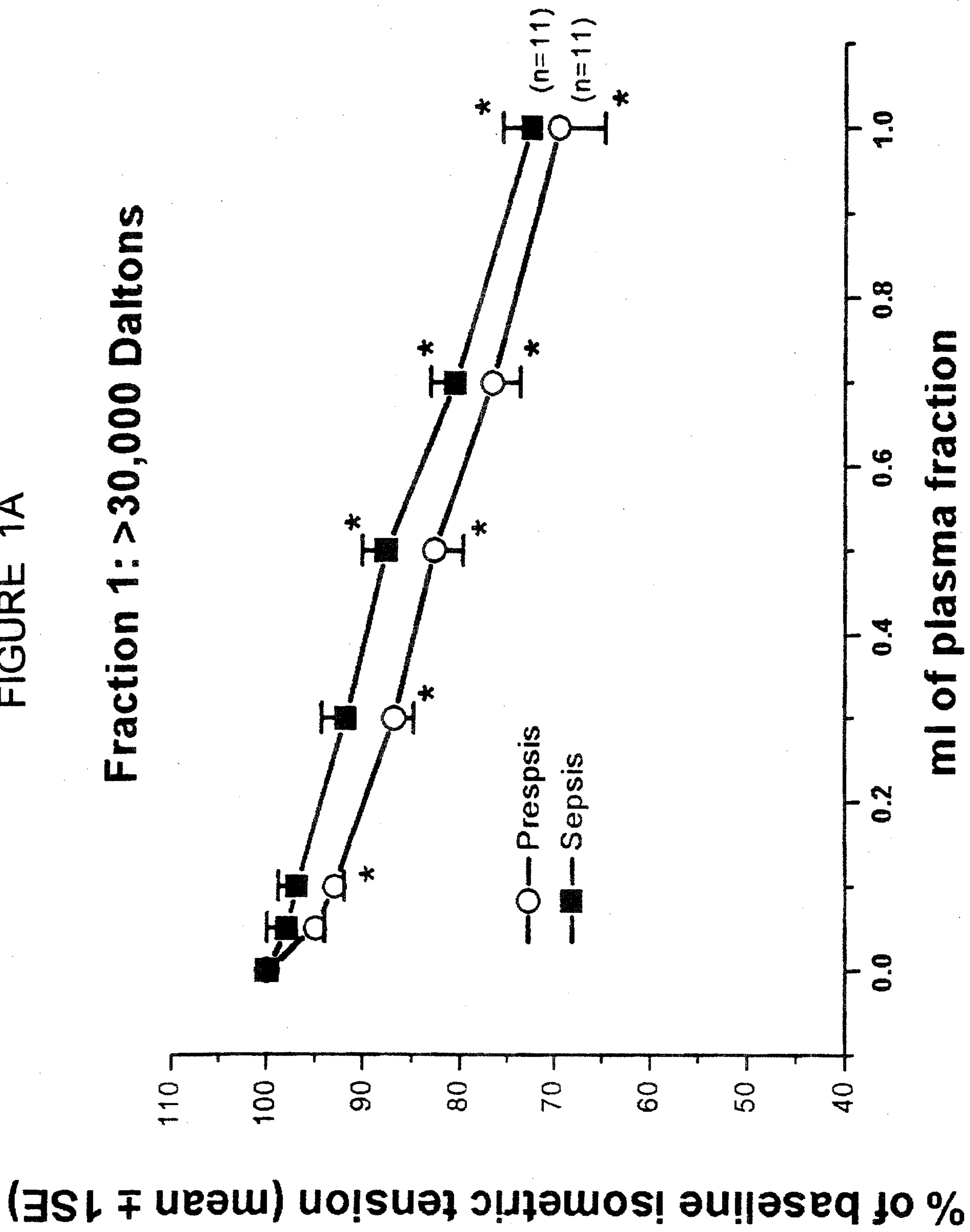
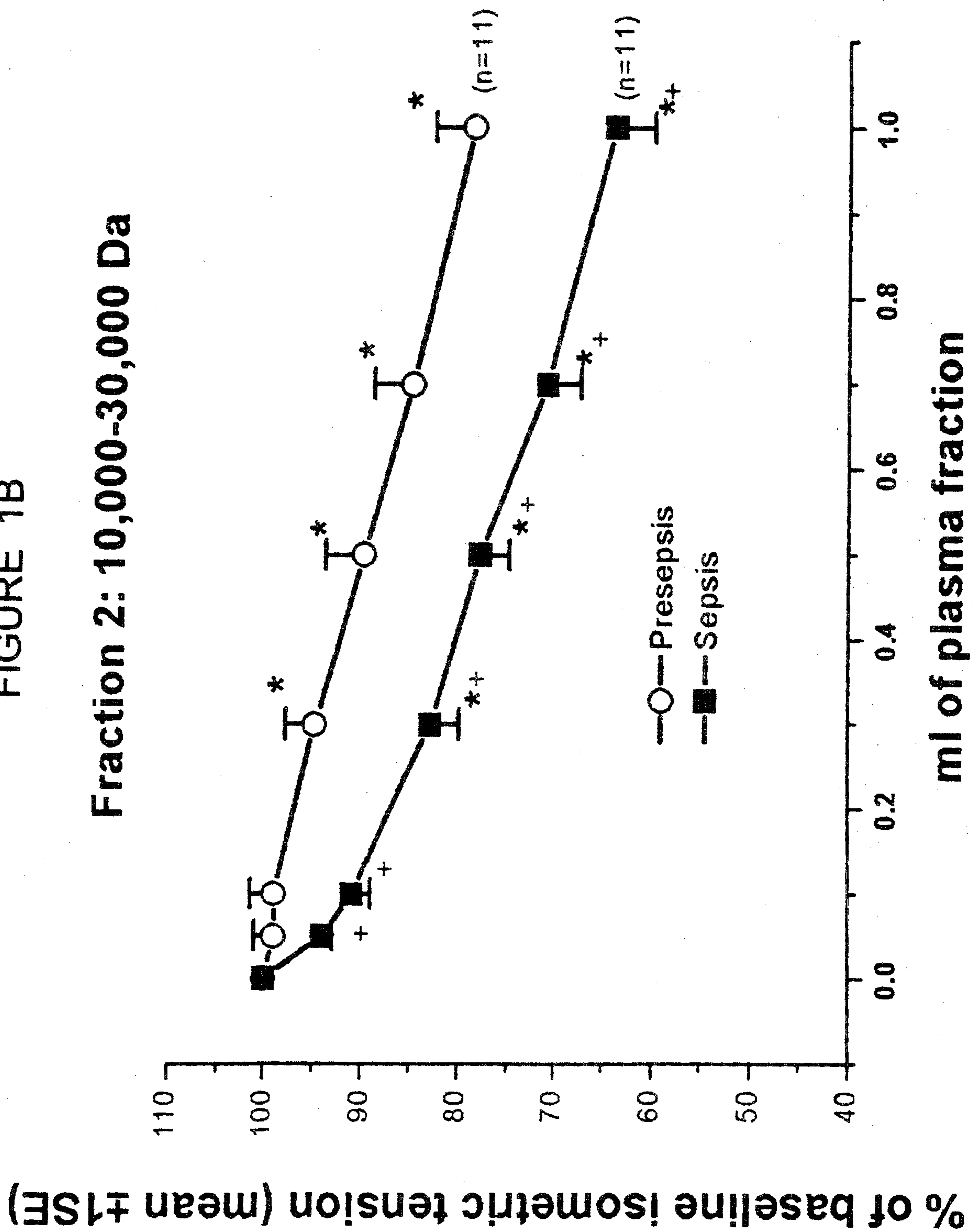


FIGURE 1B

Fraction 2: 10,000-30,000 Da



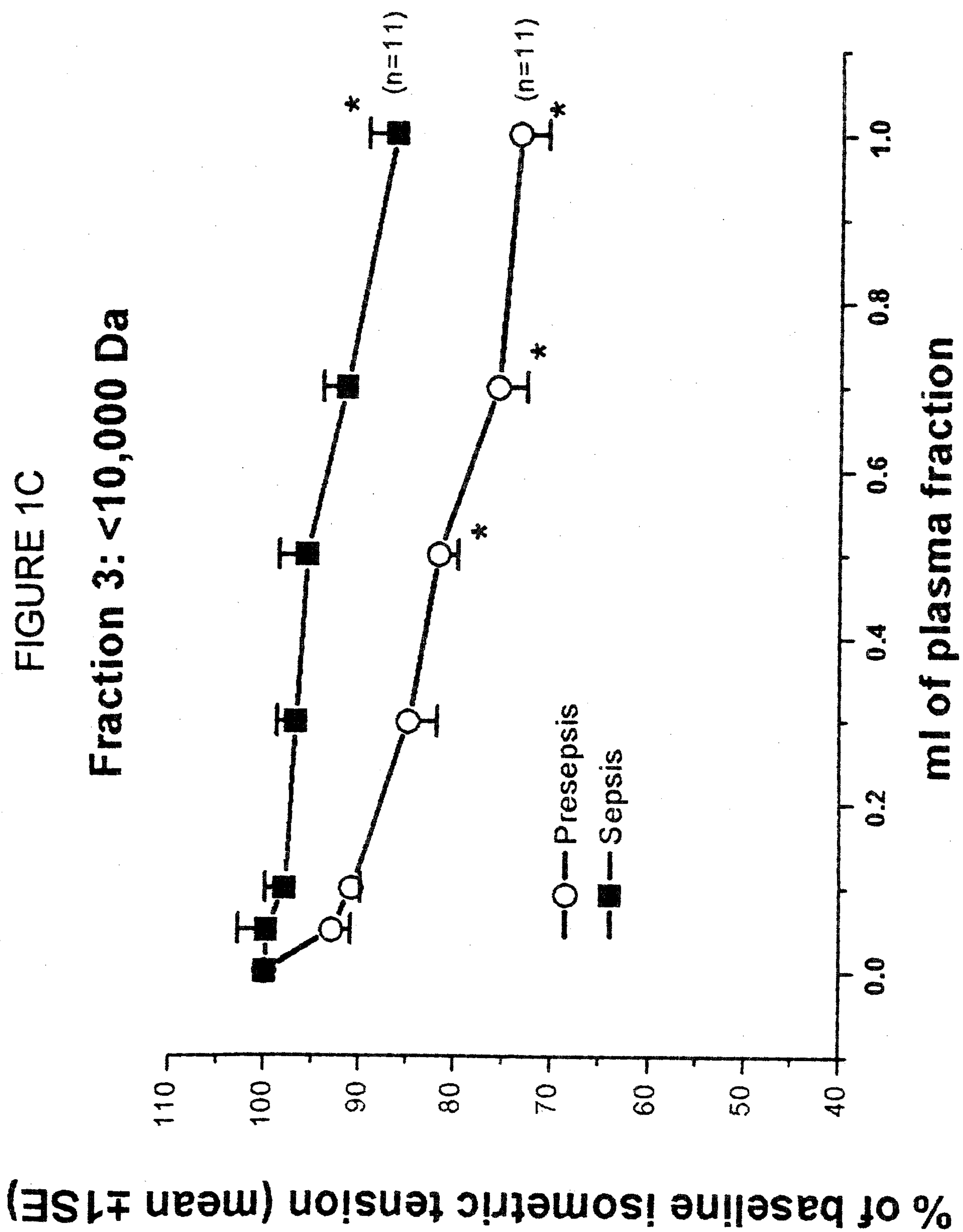
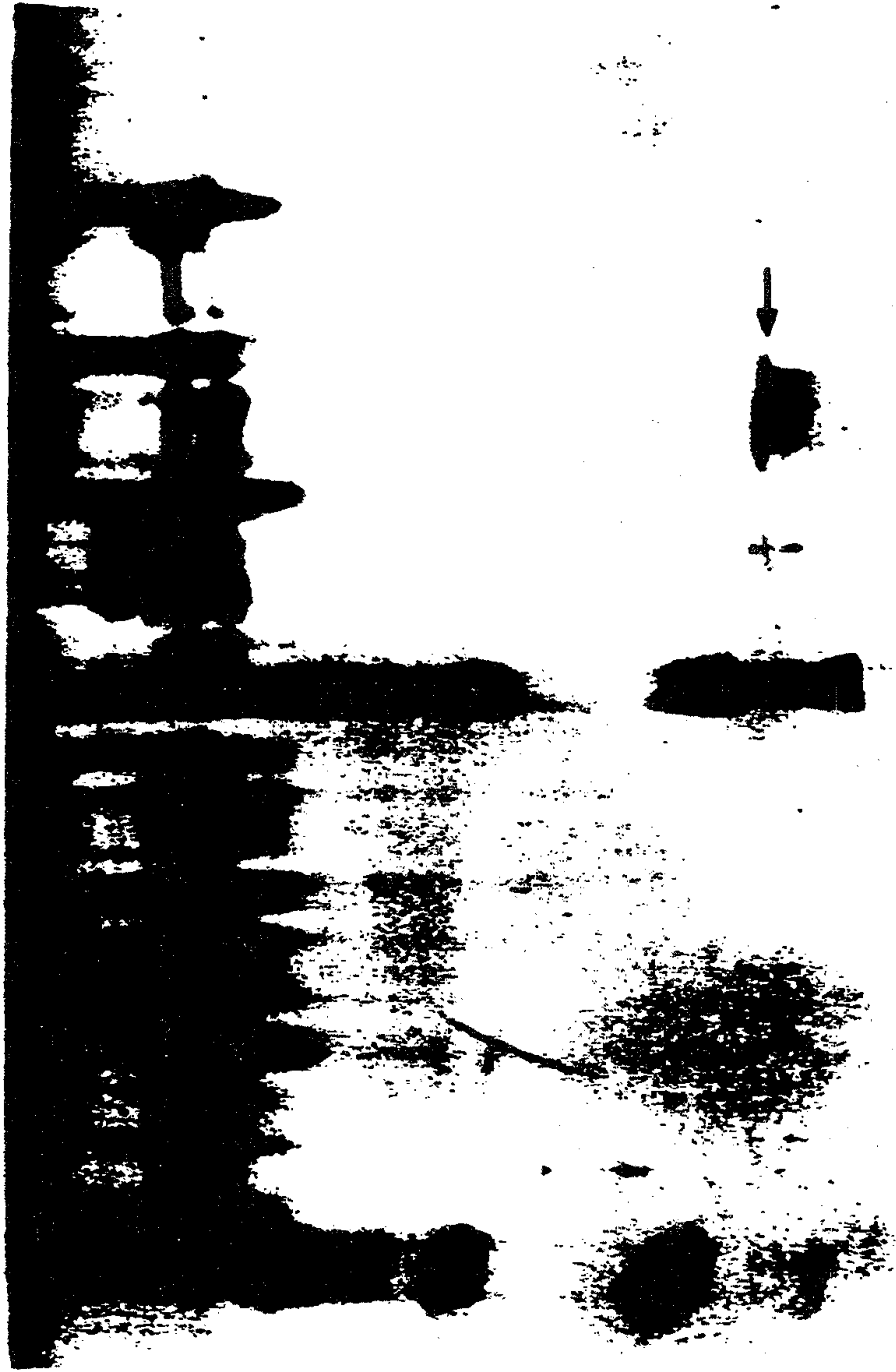


FIGURE 2

Control Sepsis

ST F₁ F₂ F₃ ST F₁ F₂ F₃

Molecular Weight (KD)
42.6
31.0
21.6



ST = Standard

FIGURE 3A

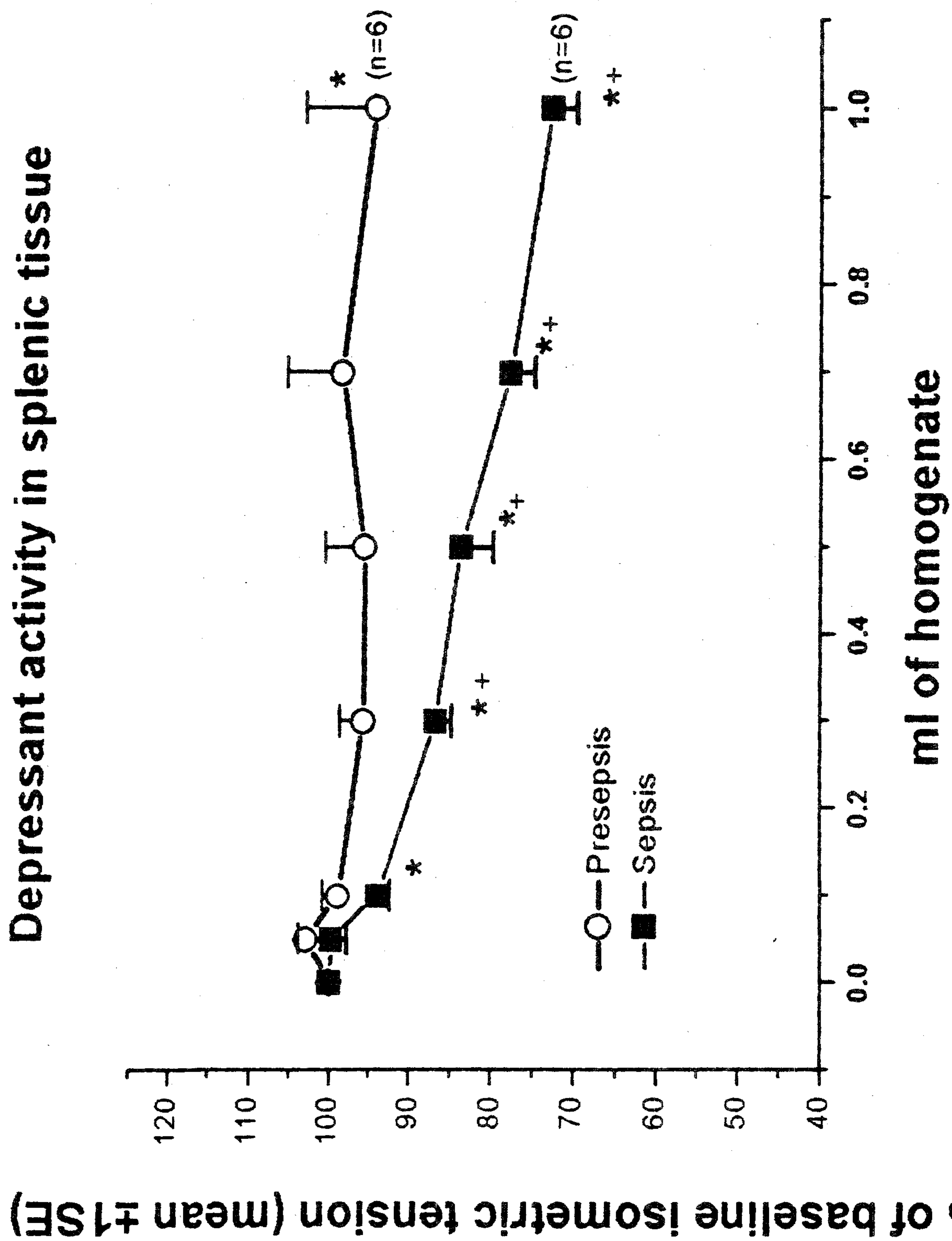


FIGURE 3B

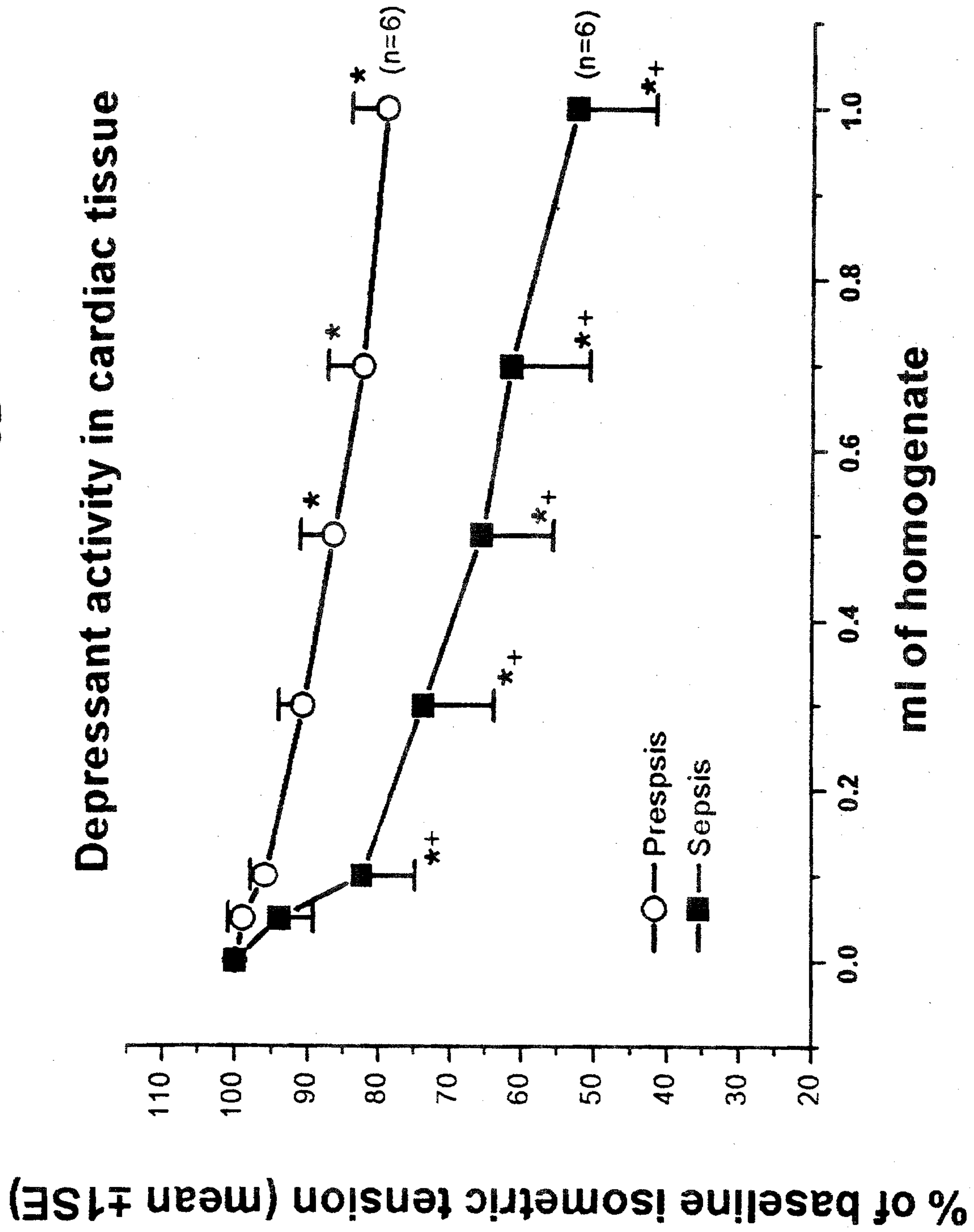


FIGURE 3C
Depressant activity in lymphocytes

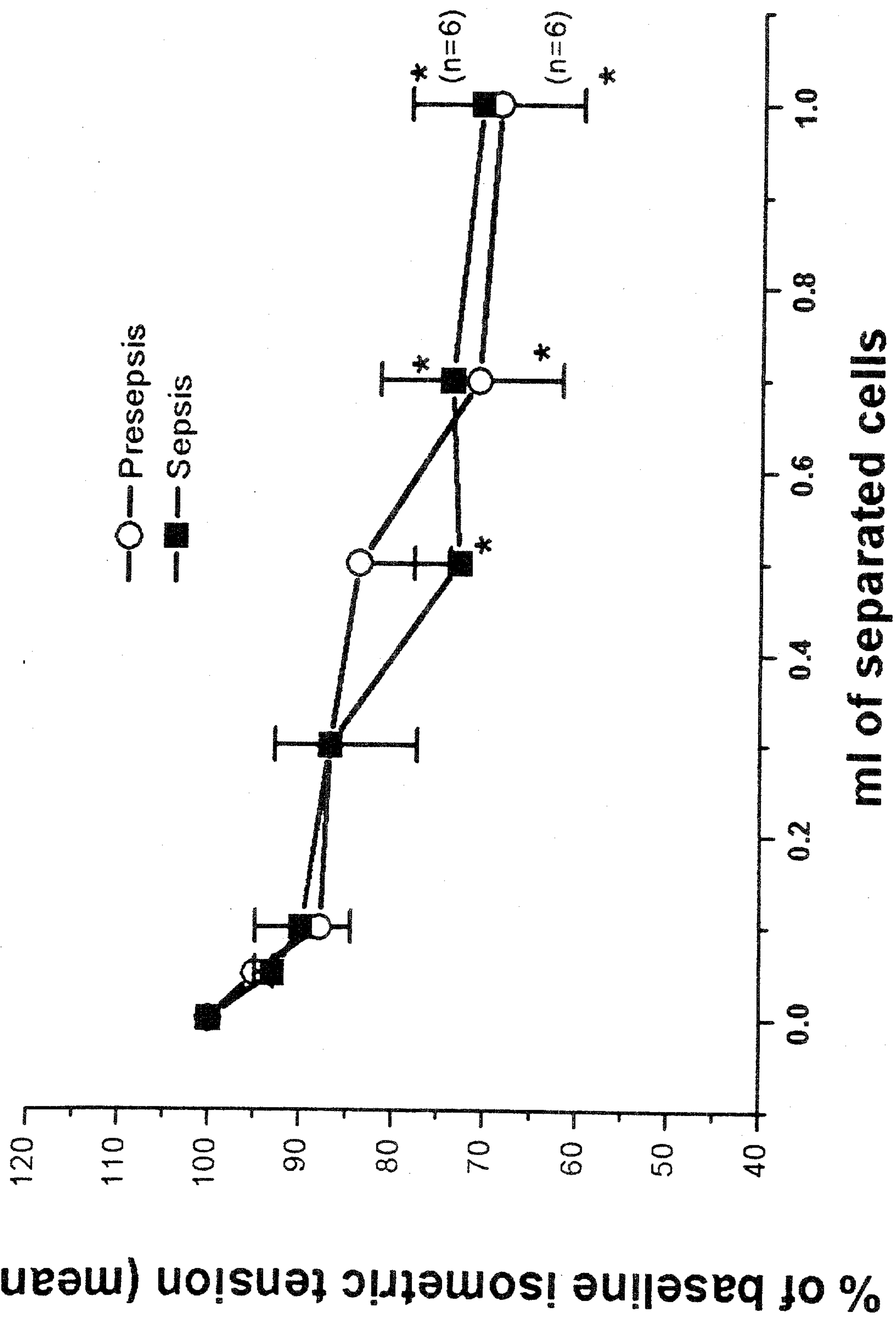


FIGURE 4A

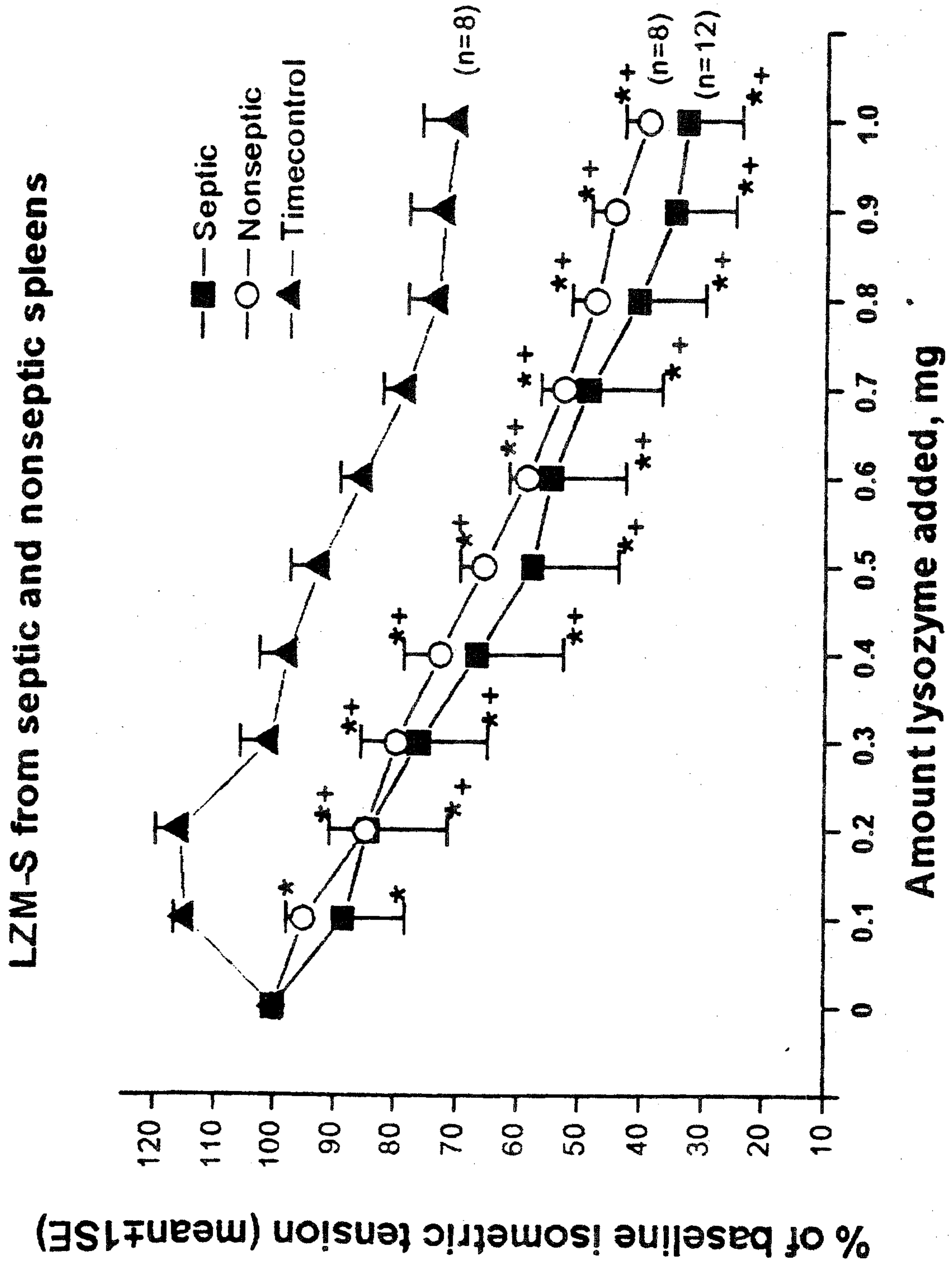


FIGURE 4B

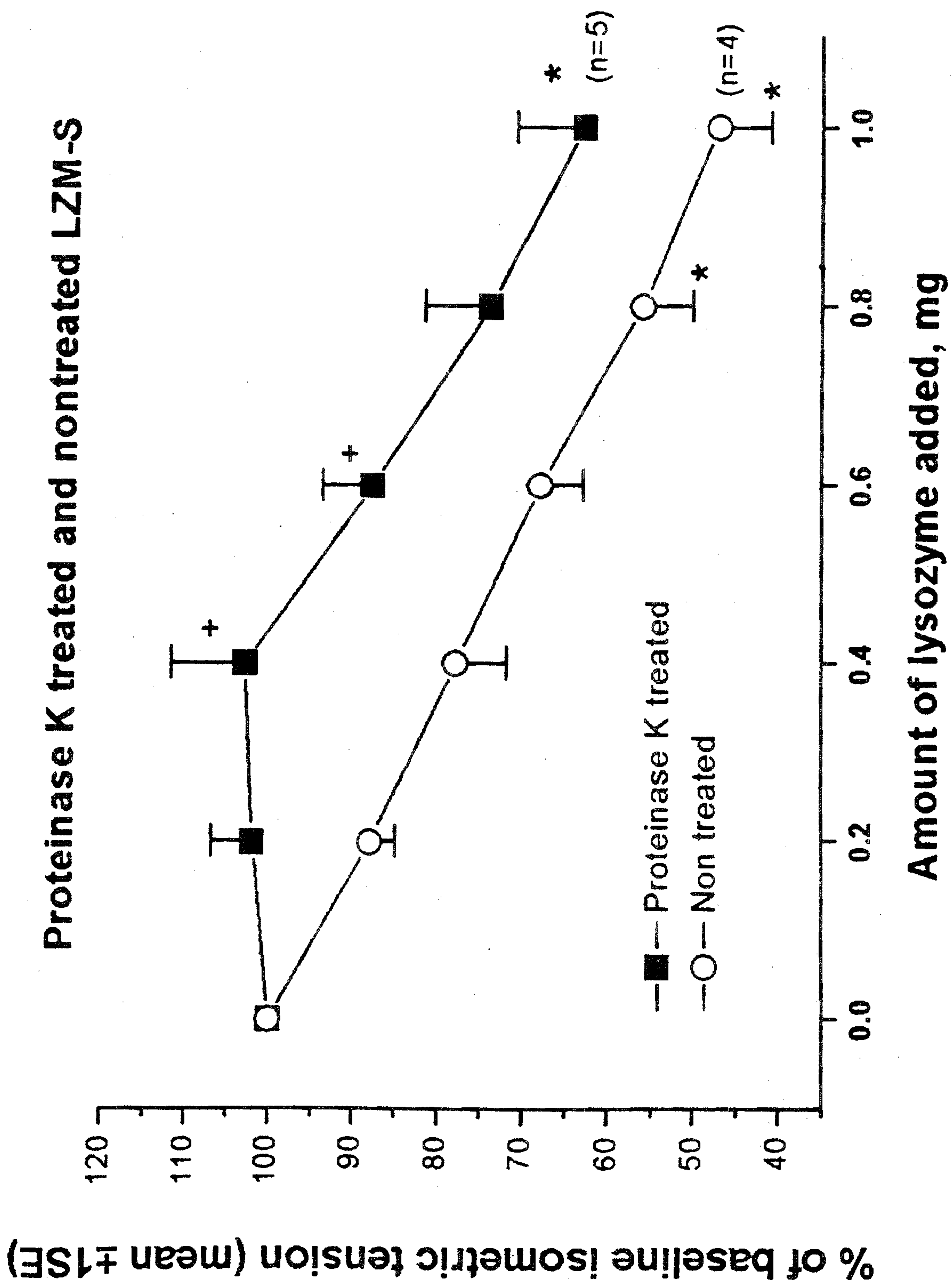


FIGURE 5A

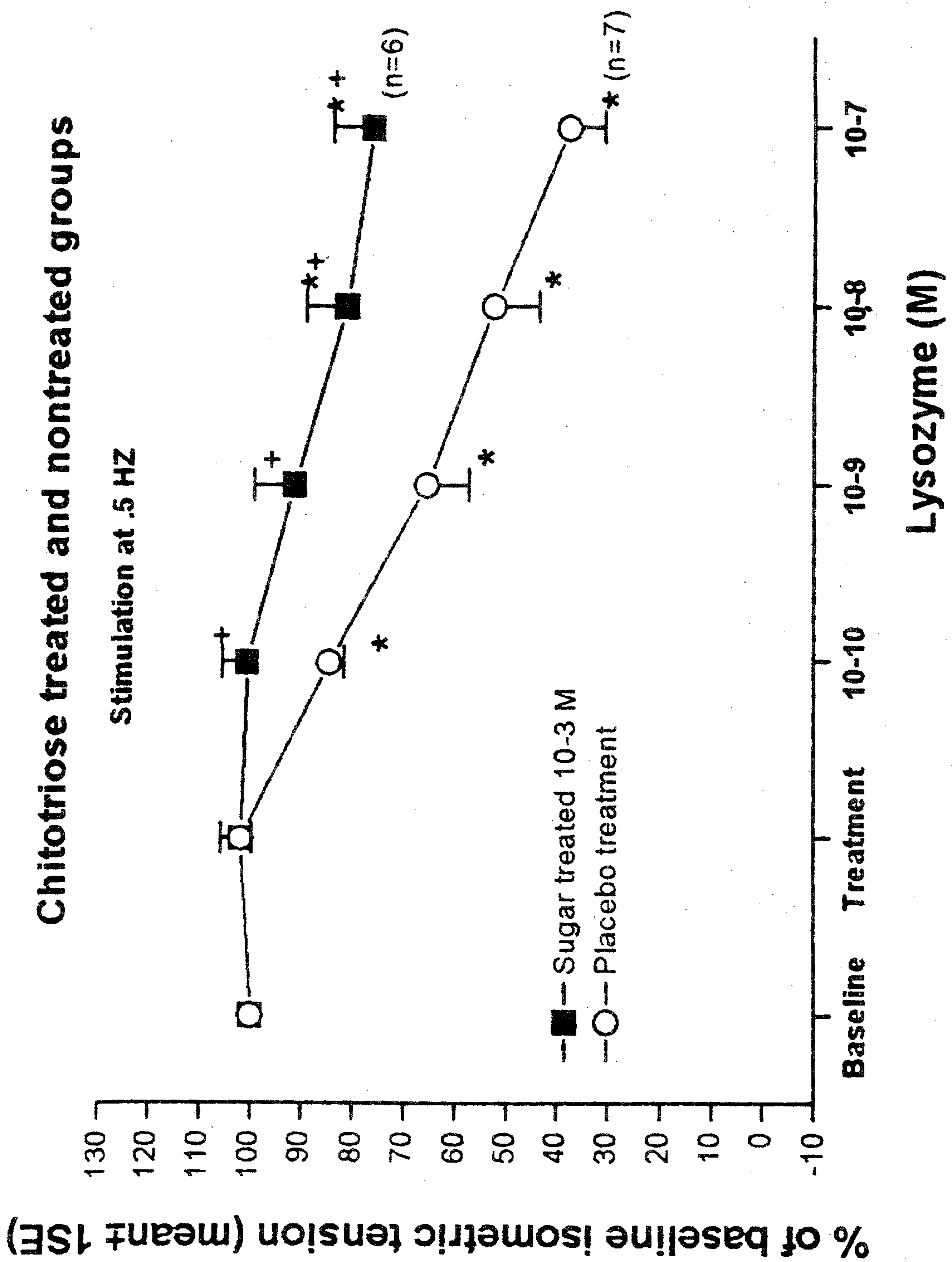


FIGURE 5B

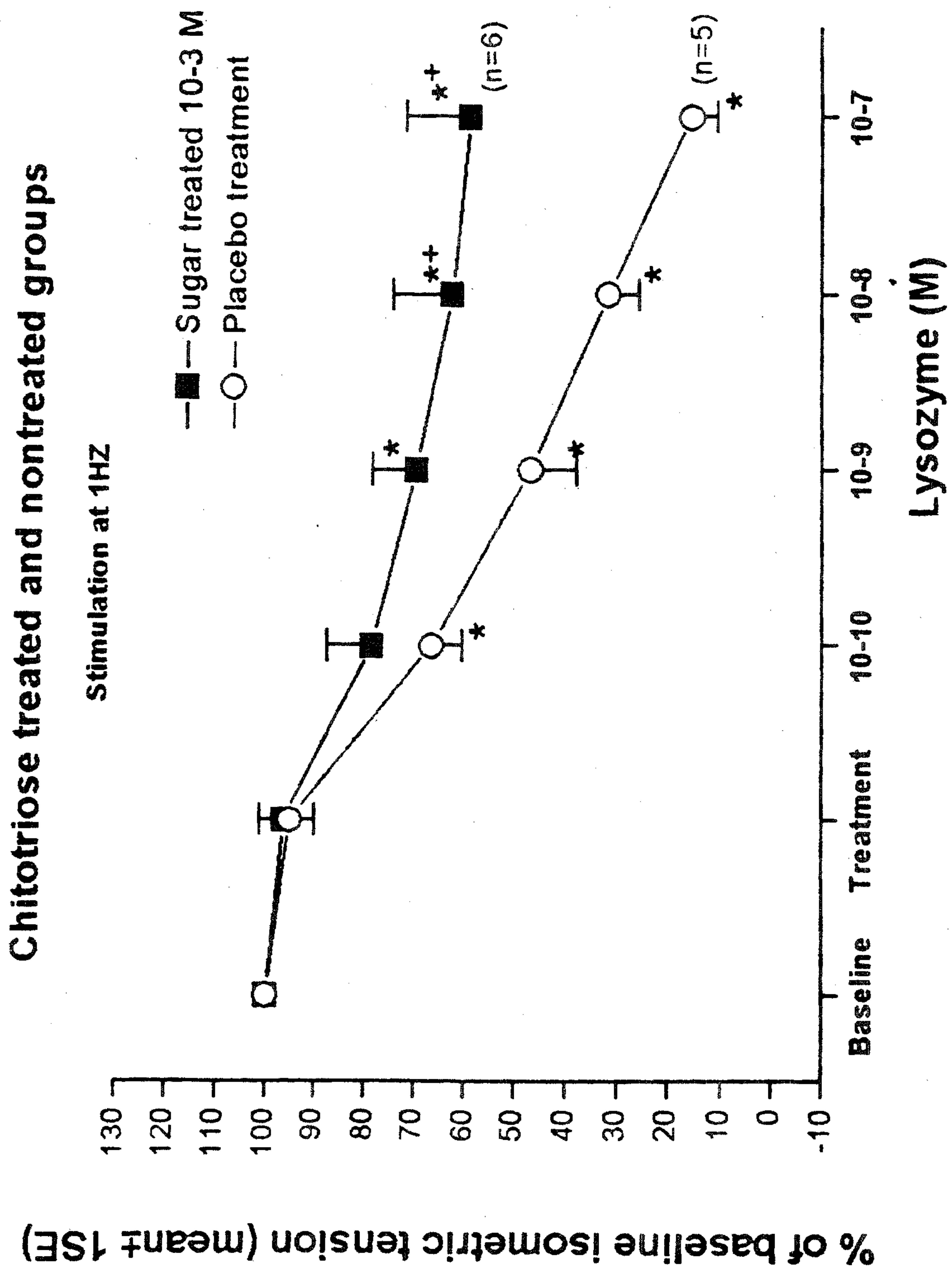
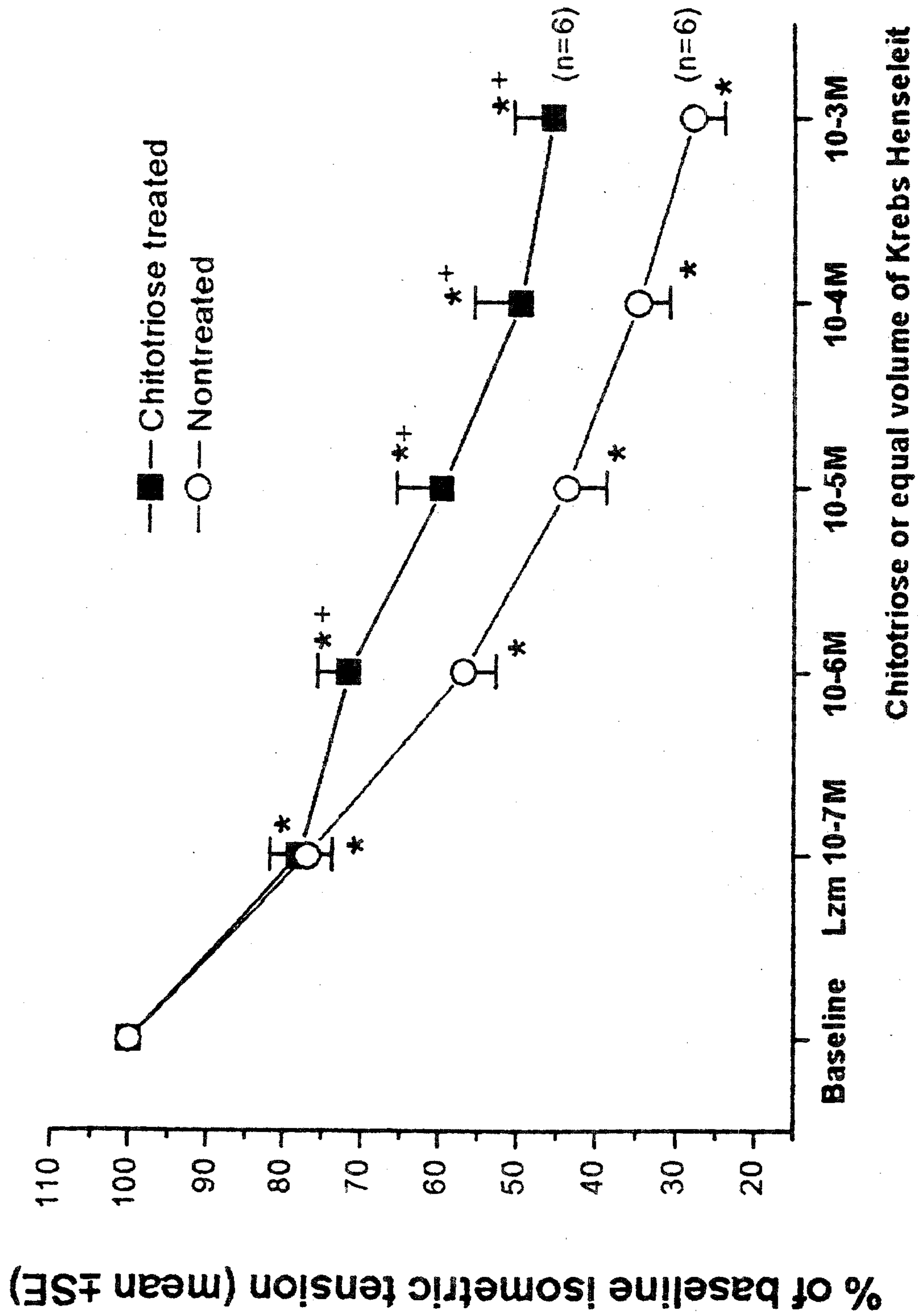


FIGURE 5C

Chitotriose effect on isometric tension post Lzm-S administration



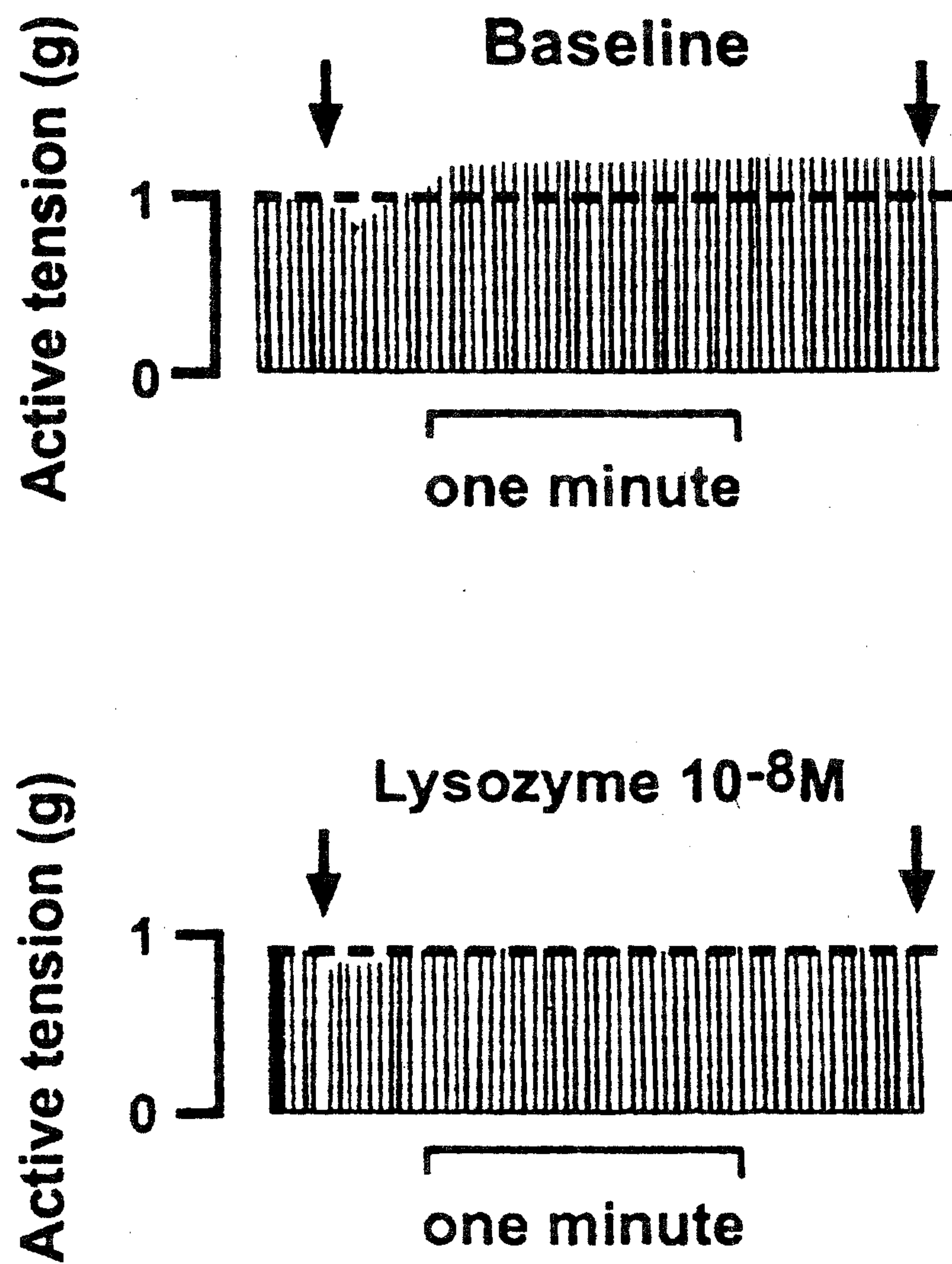


FIGURE 6

FIGURE 7A

Sympathetic response

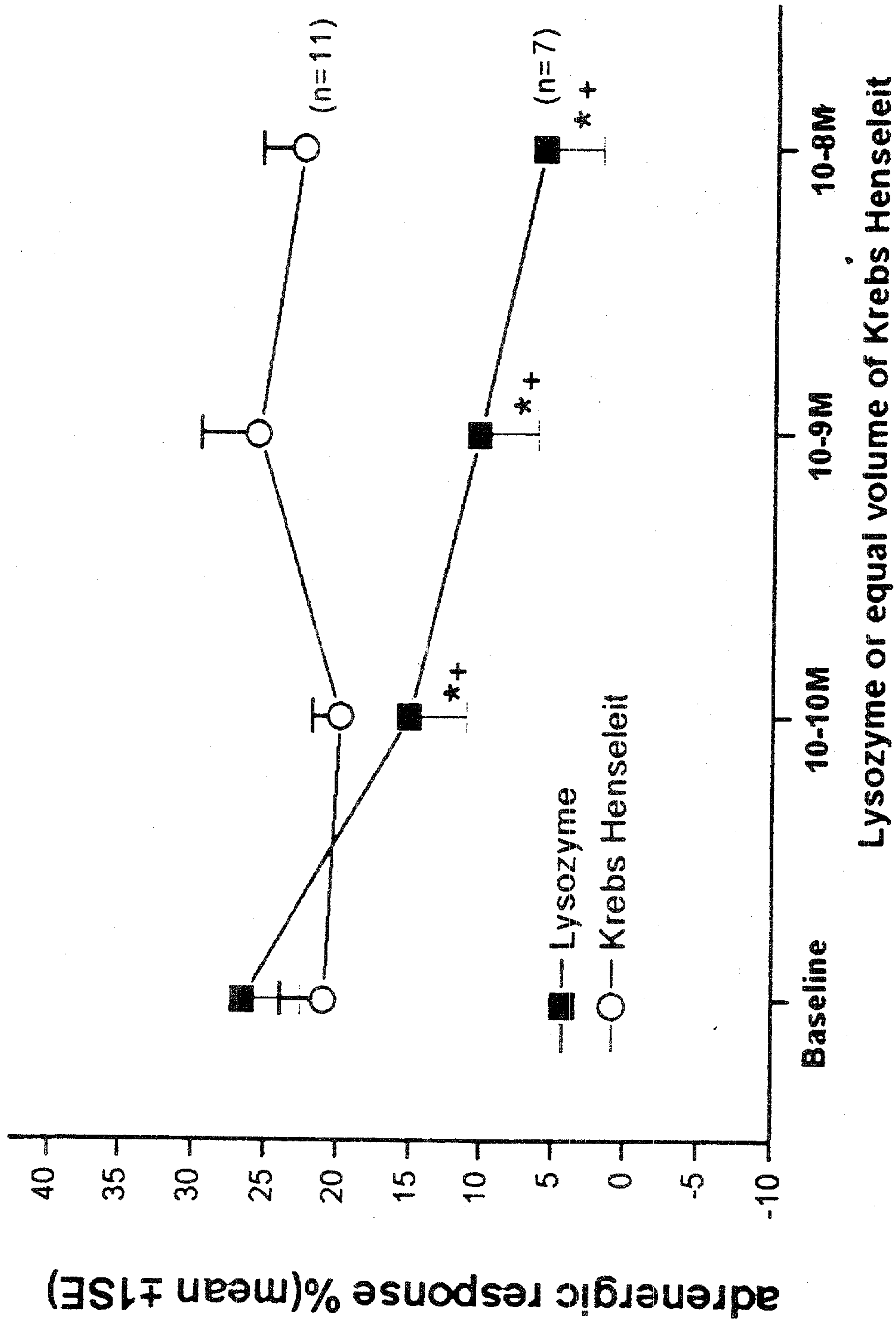


FIGURE 7B

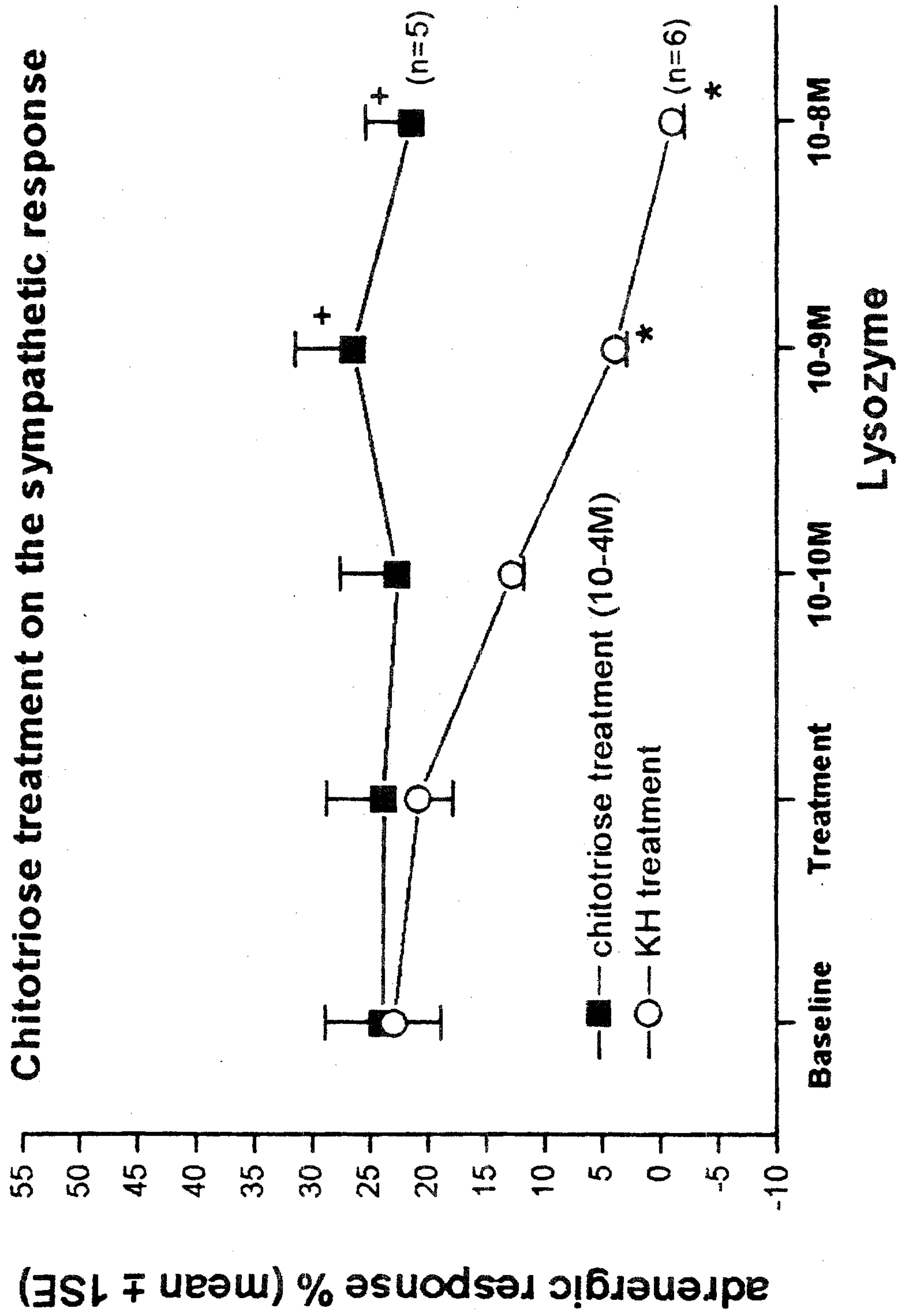


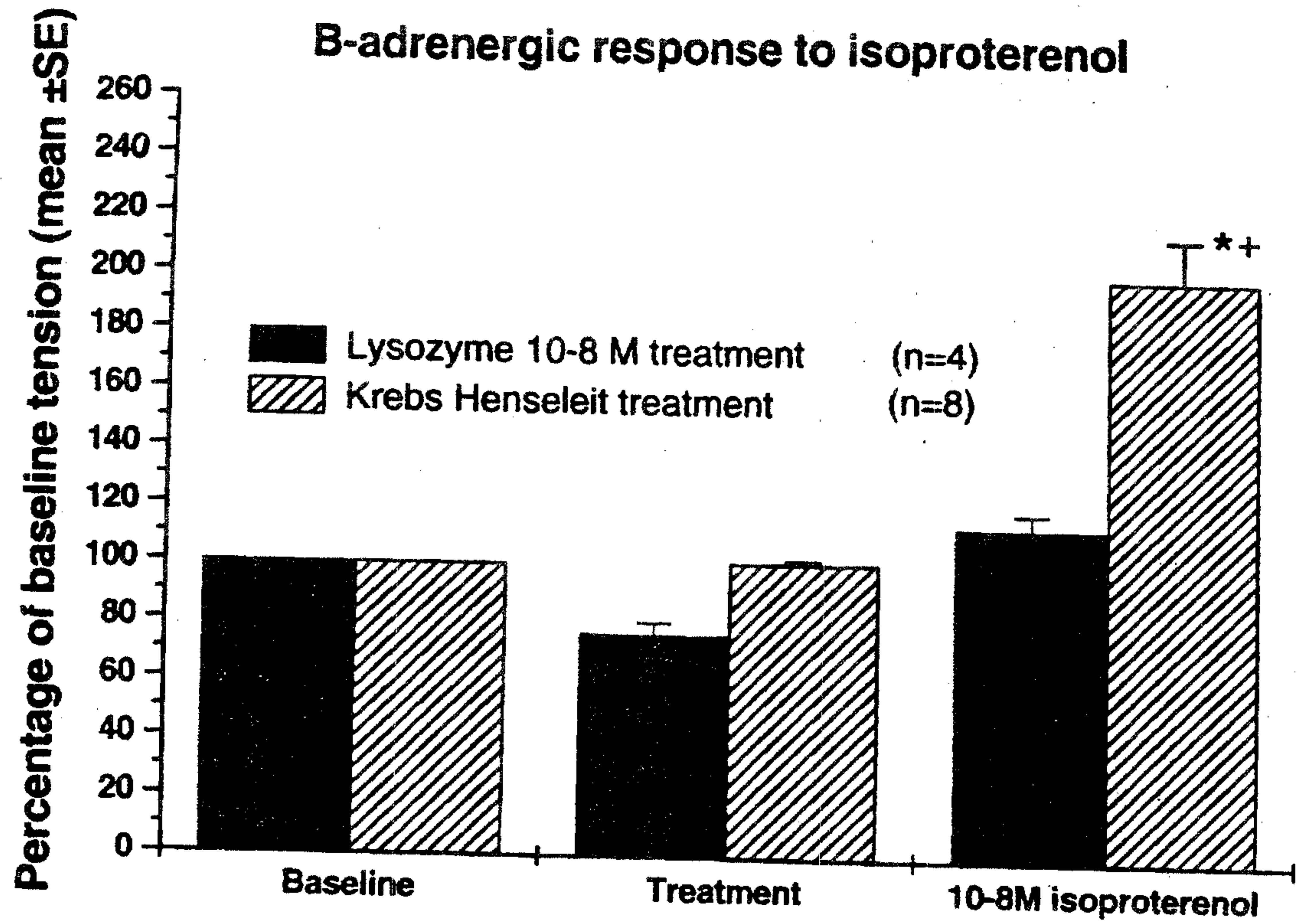
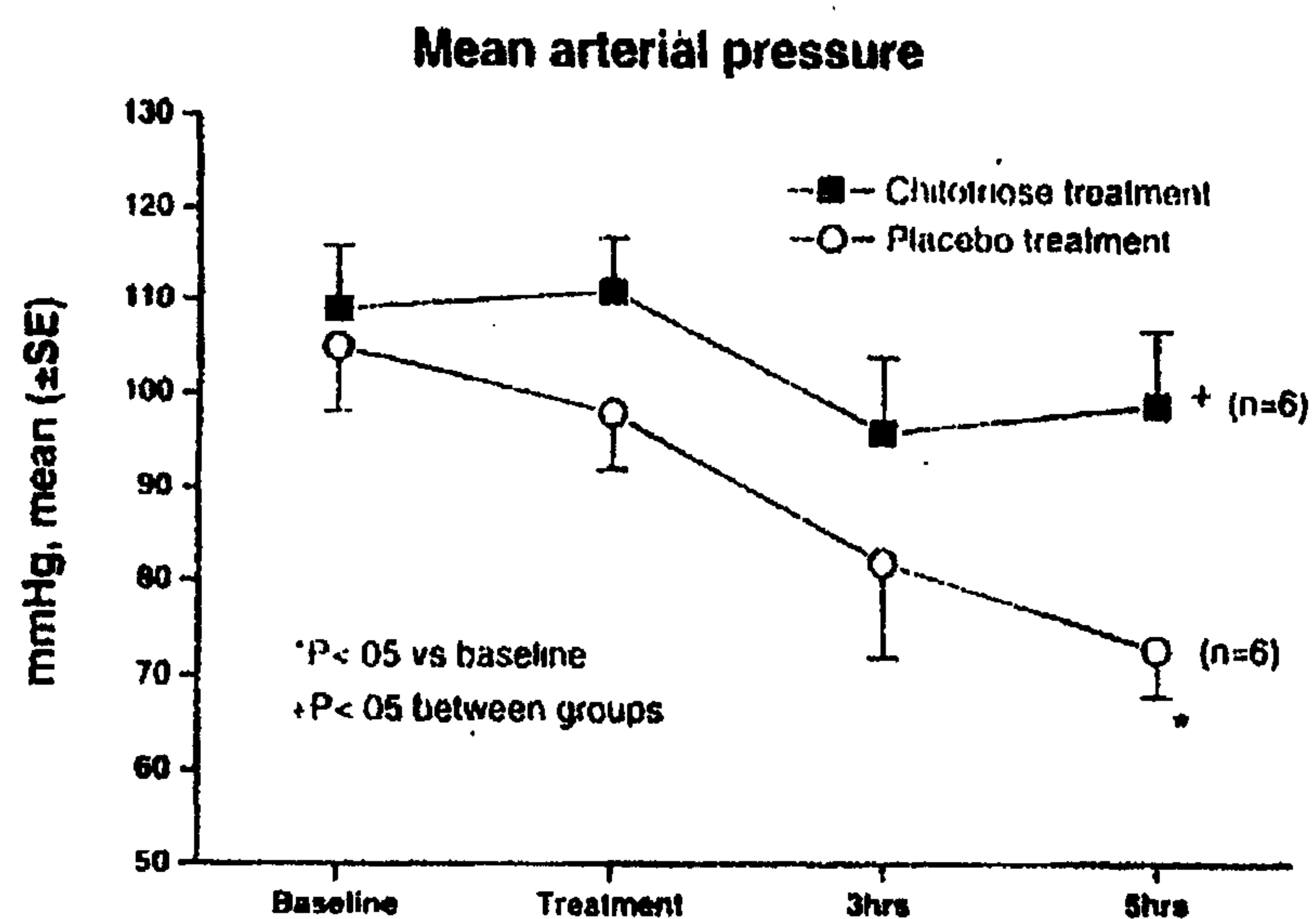
FIGURE 7C

FIGURE 8A

PRETREATMENT STUDY



PRETREATMENT STUDY

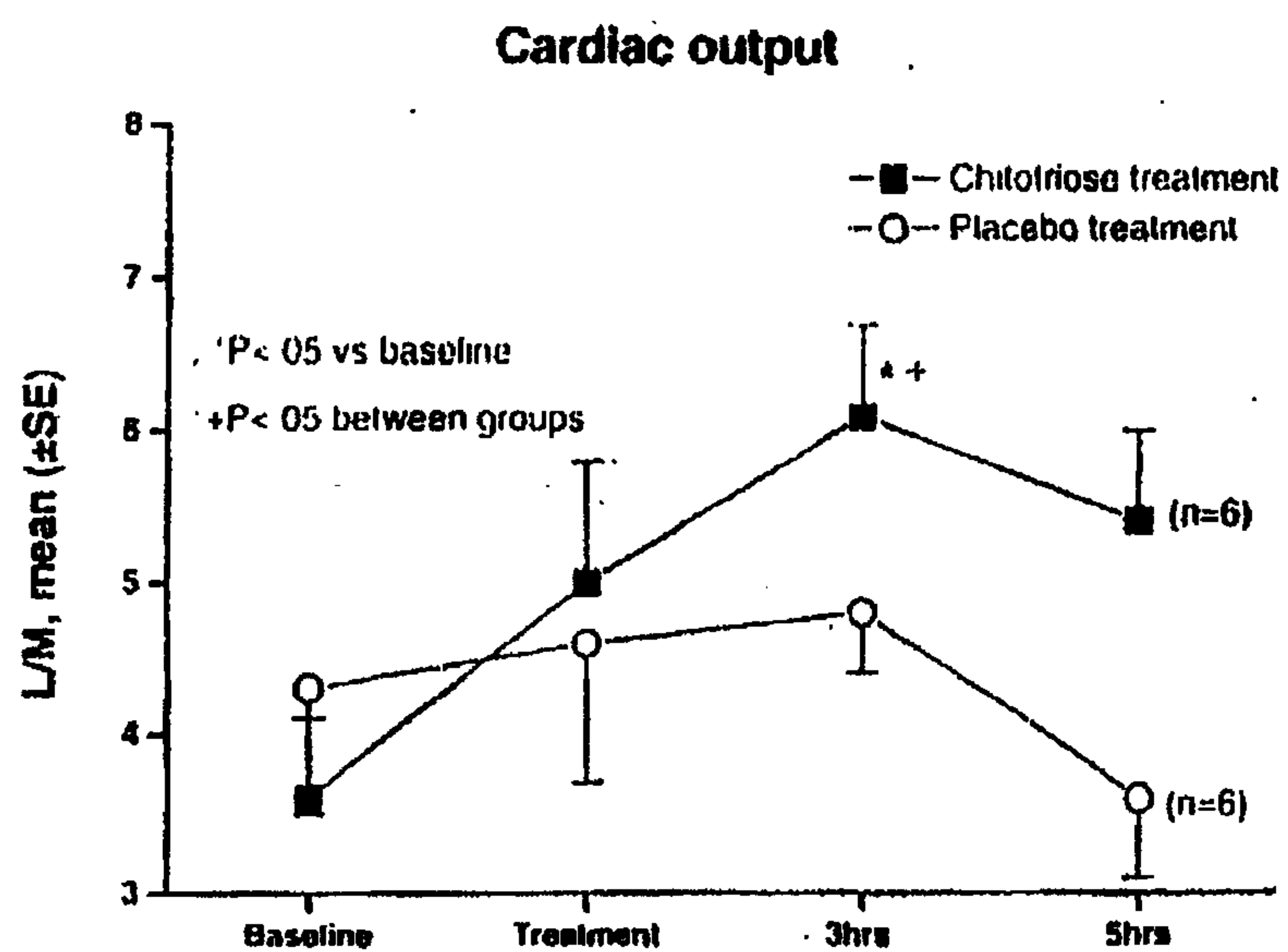


FIGURE 8B

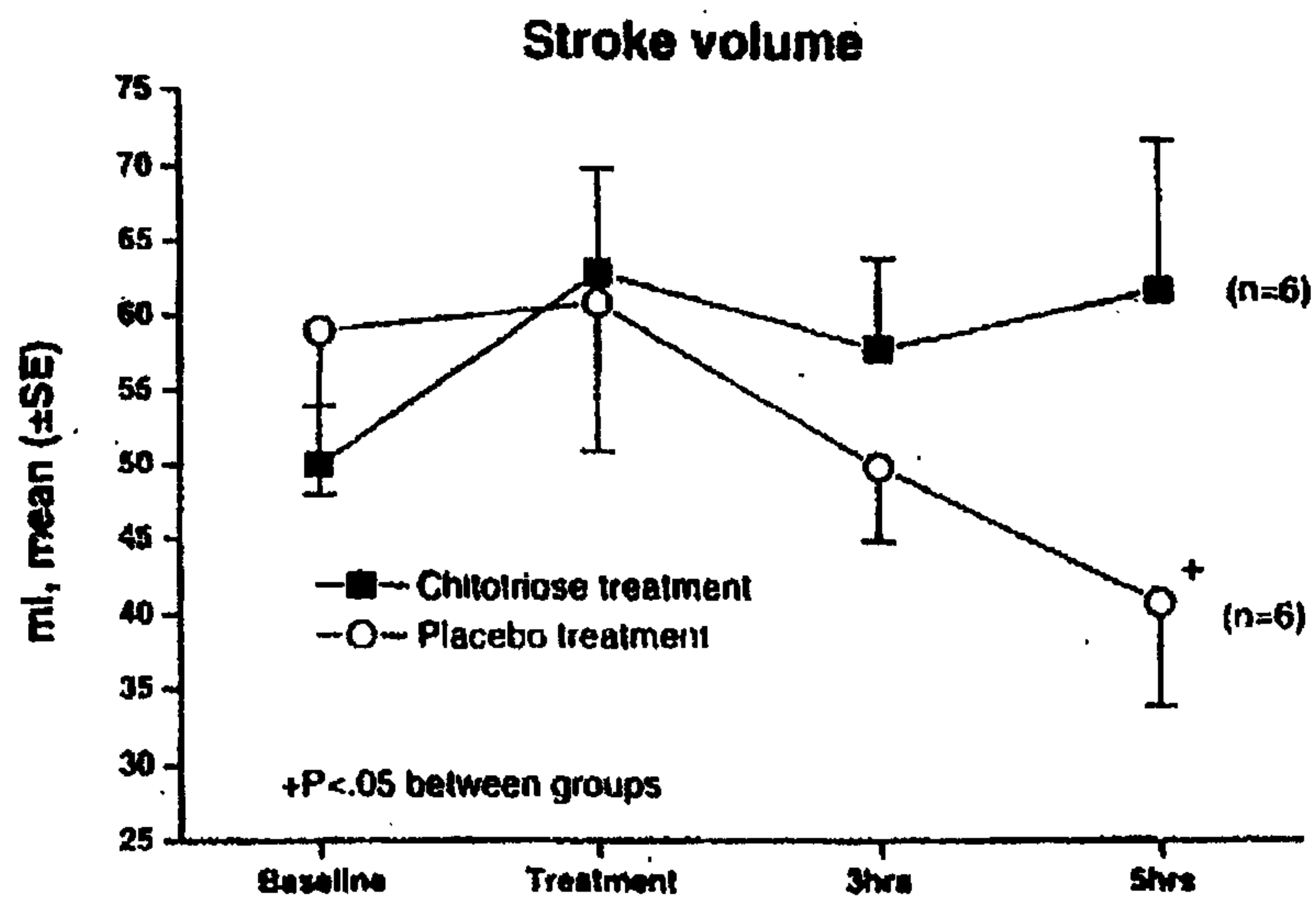
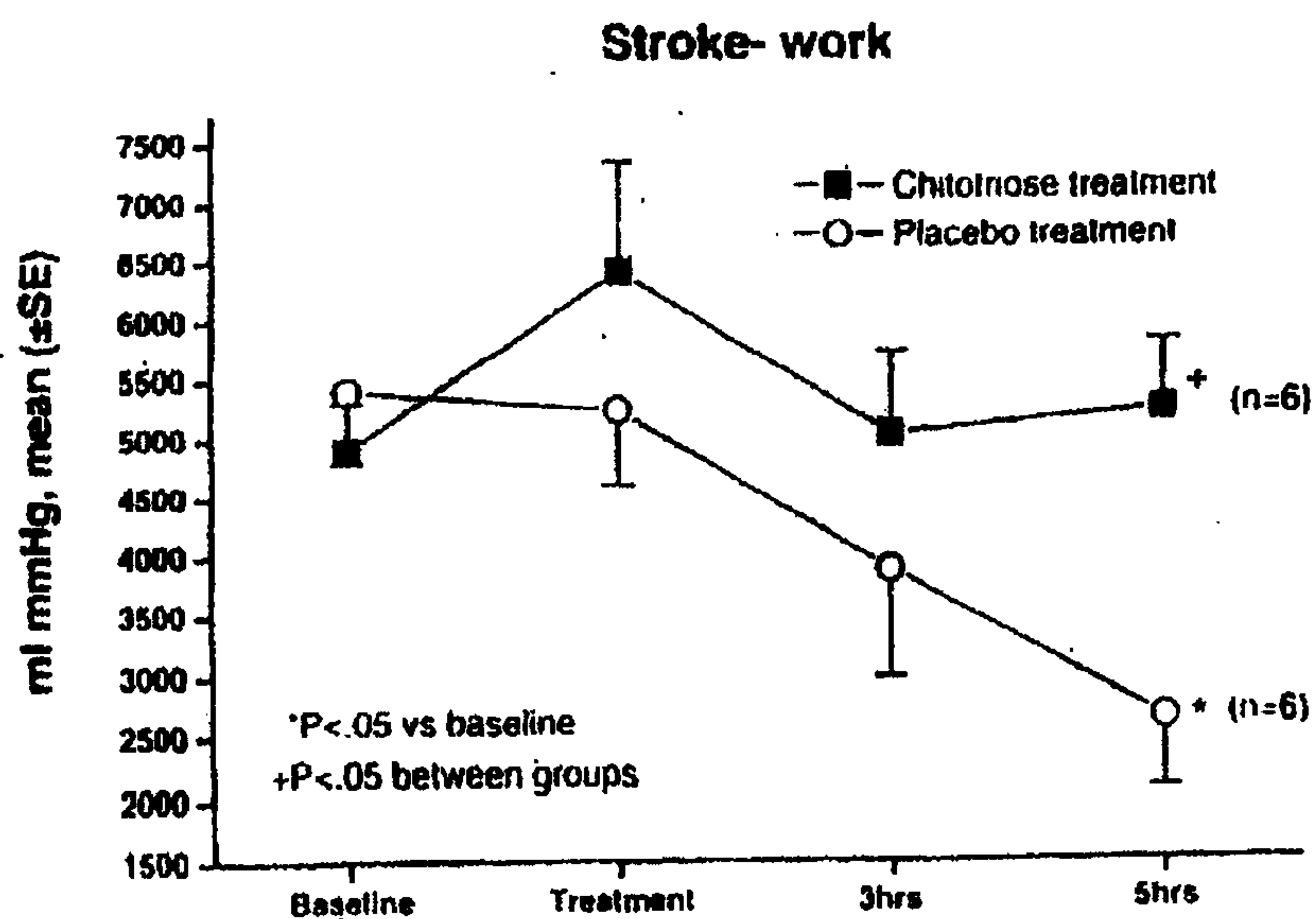
PRETREATMENT STUDY**PRETREATMENT STUDY**

FIGURE 9

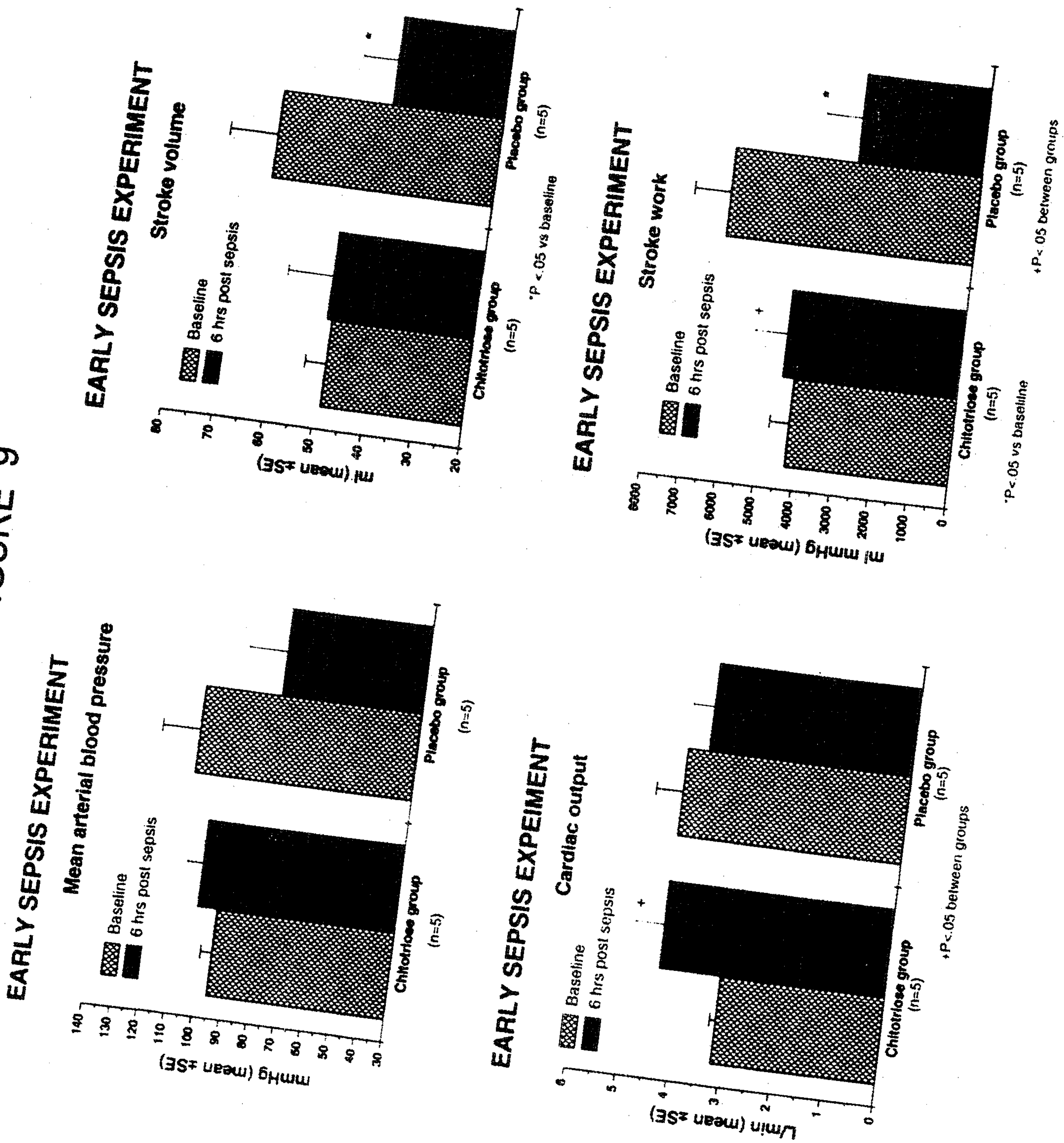
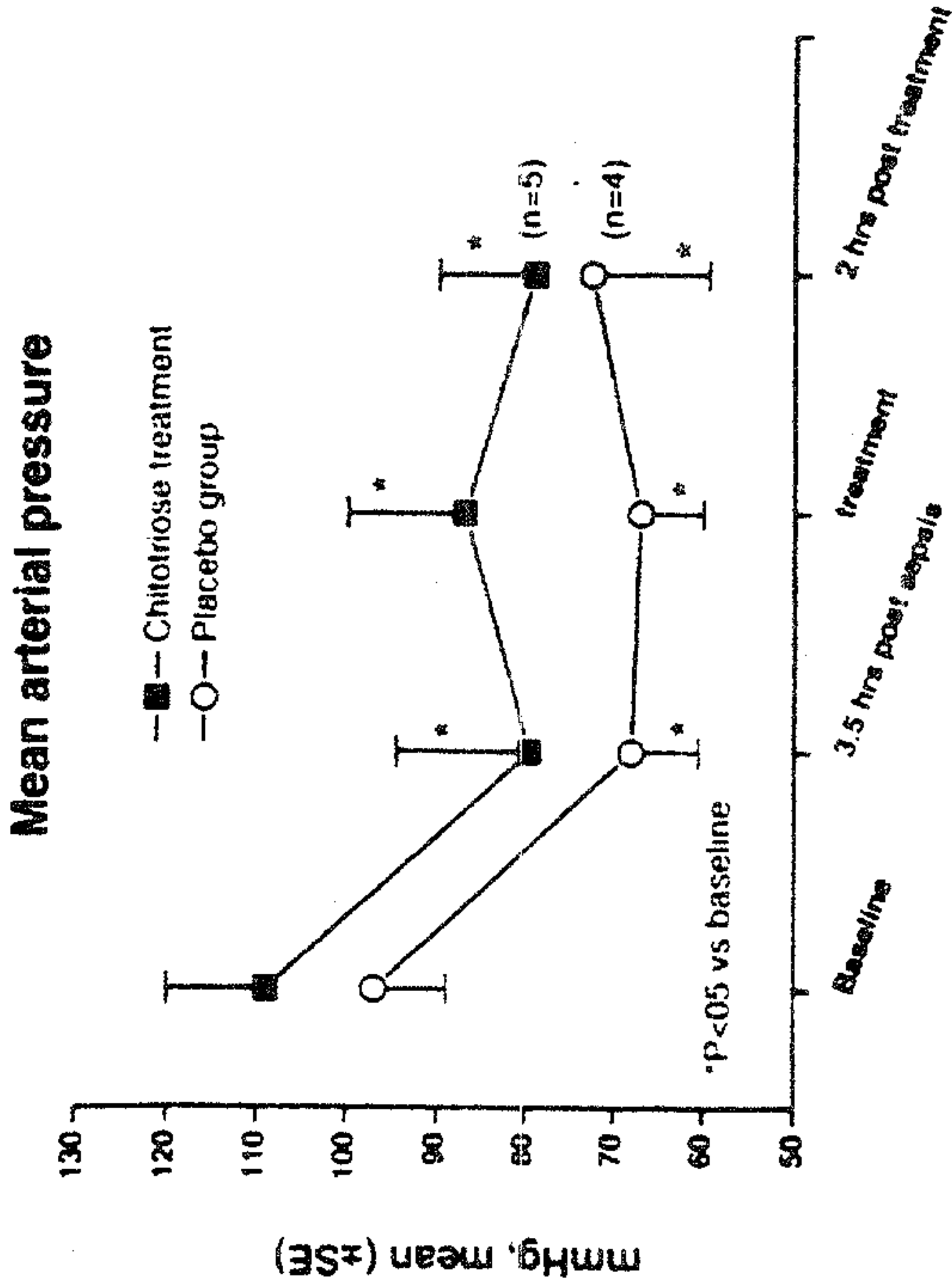
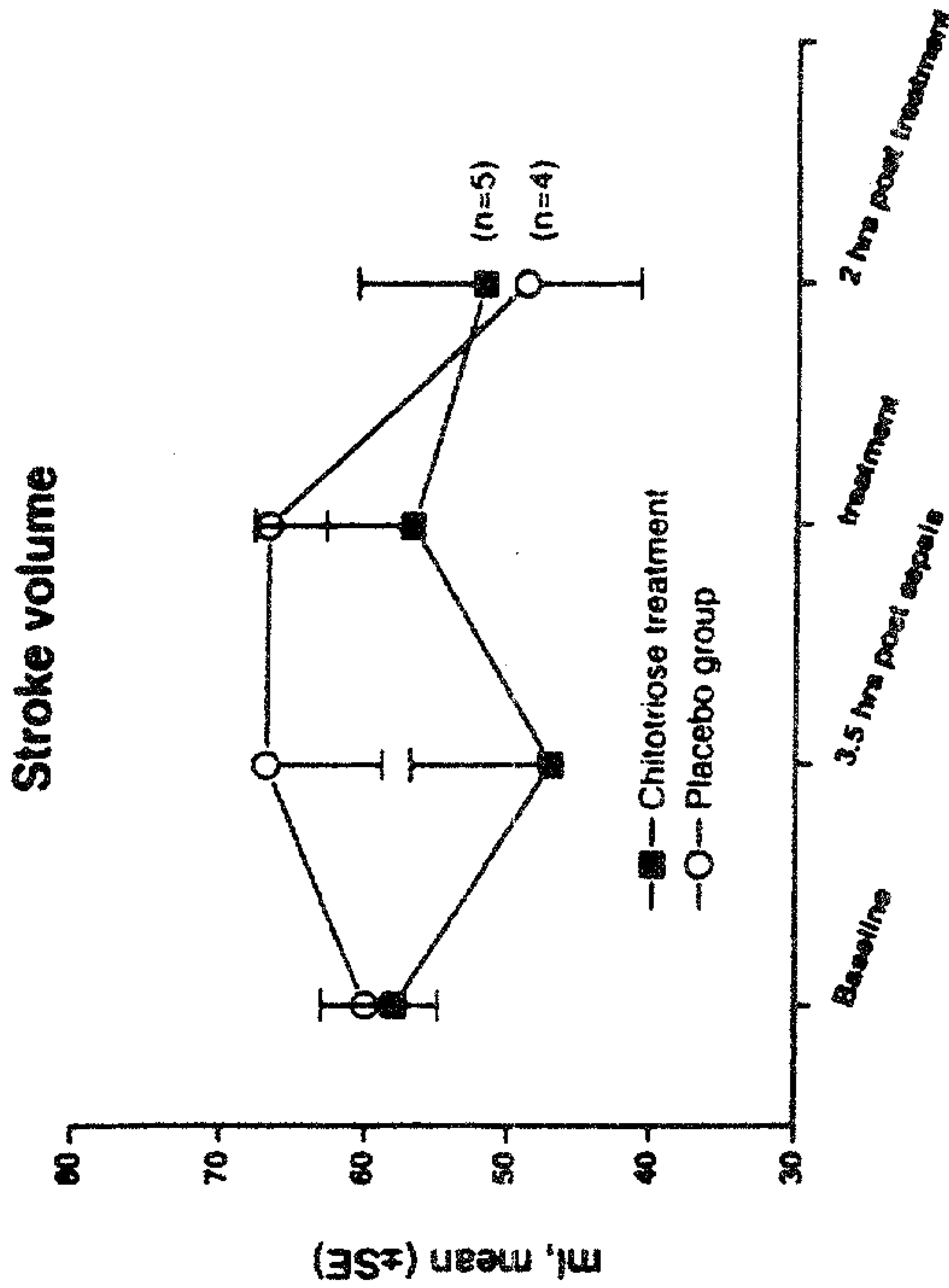


FIGURE 10

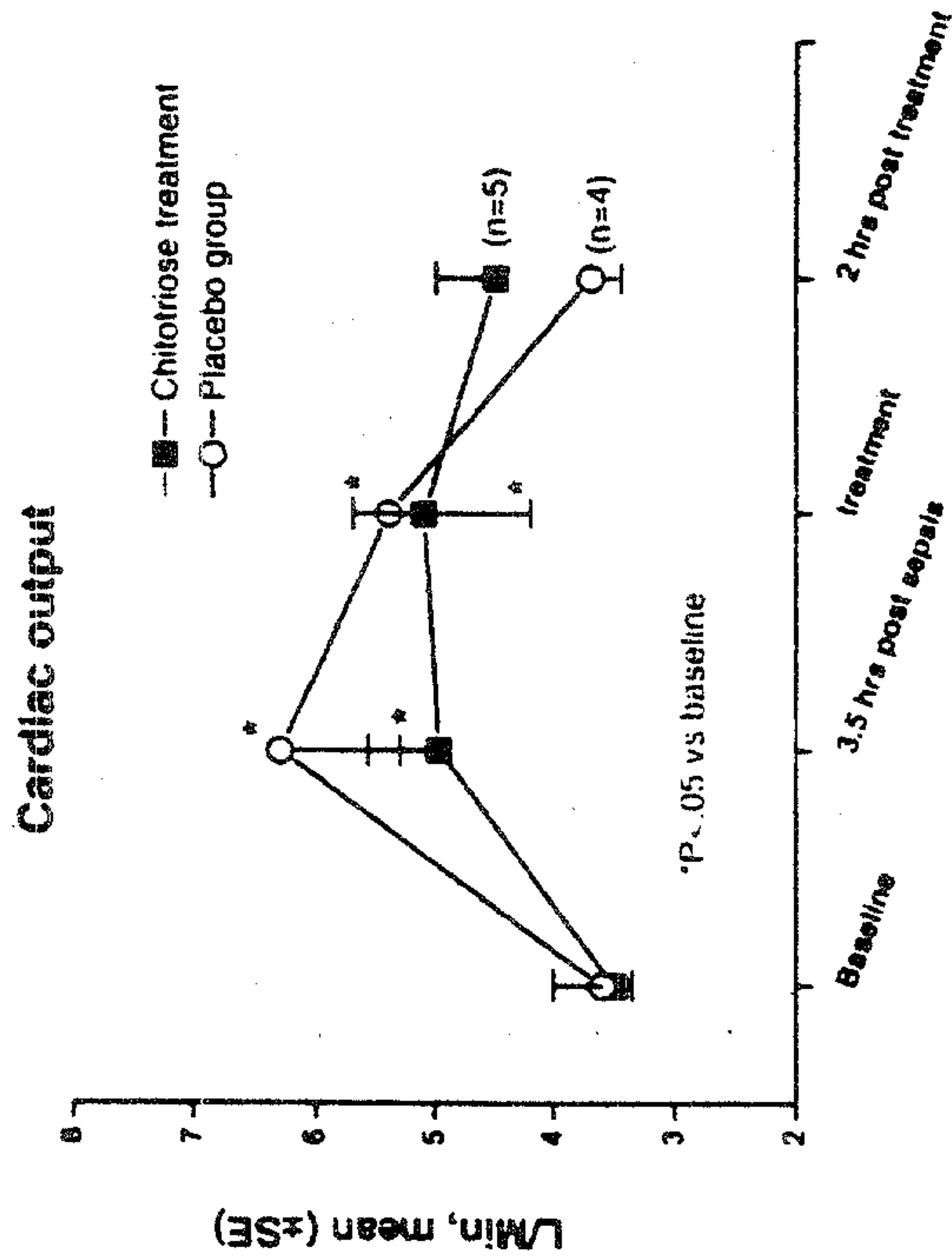
LATE SEPSIS EXPERIMENT



LATE SEPSIS EXPERIMENT



LATE SEPSIS EXPERIMENT



LATE SEPSIS EXPERIMENT

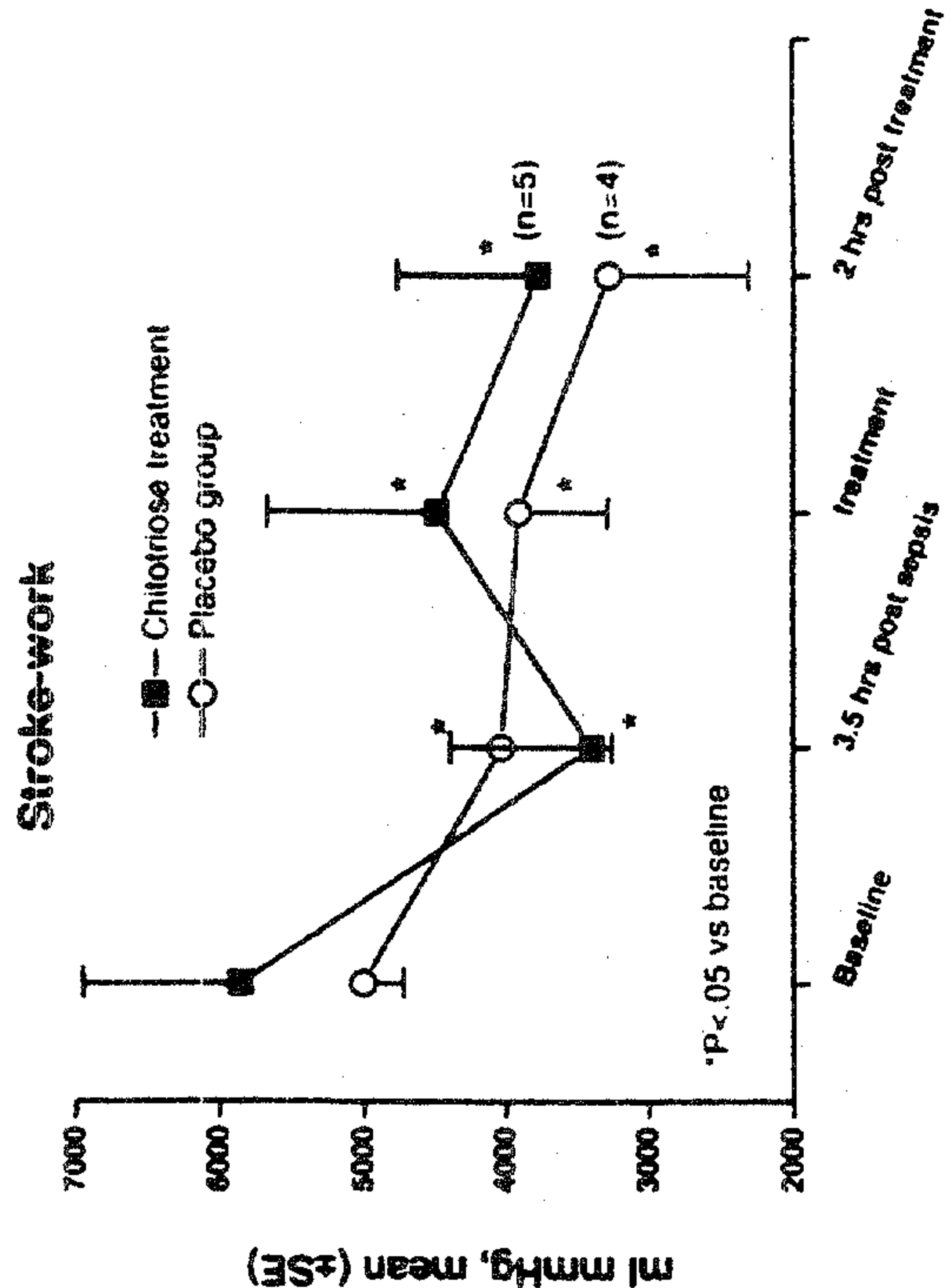
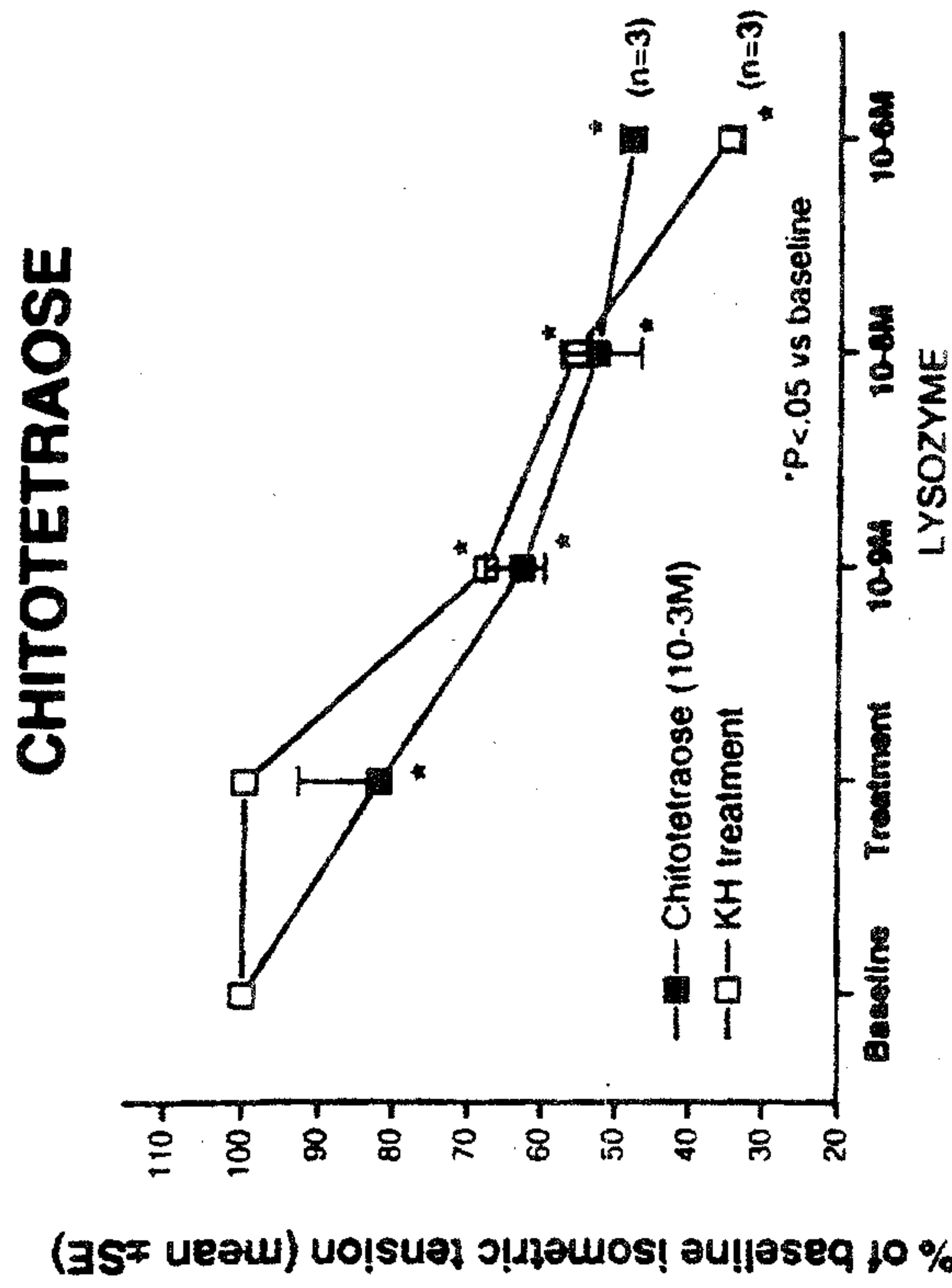
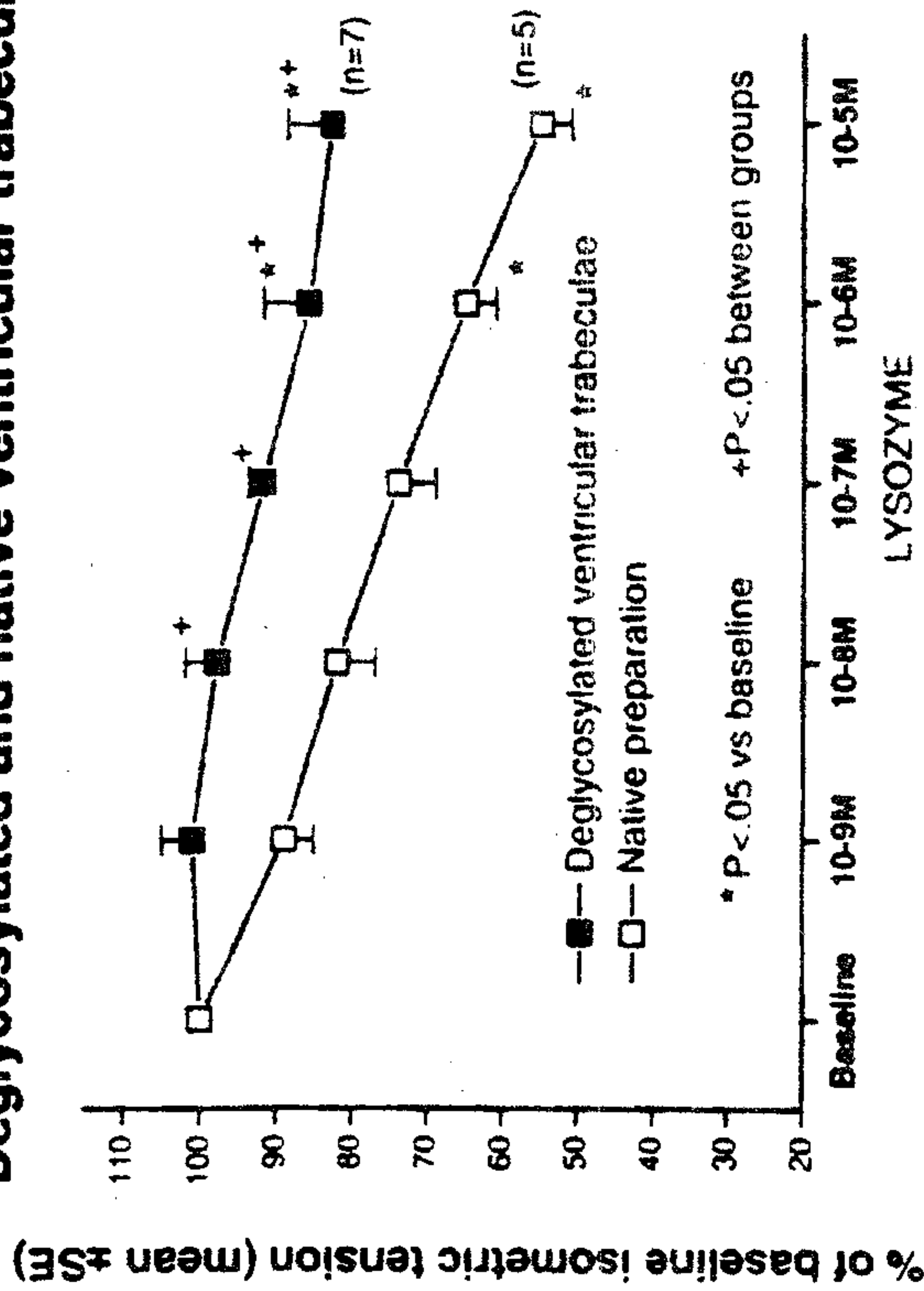
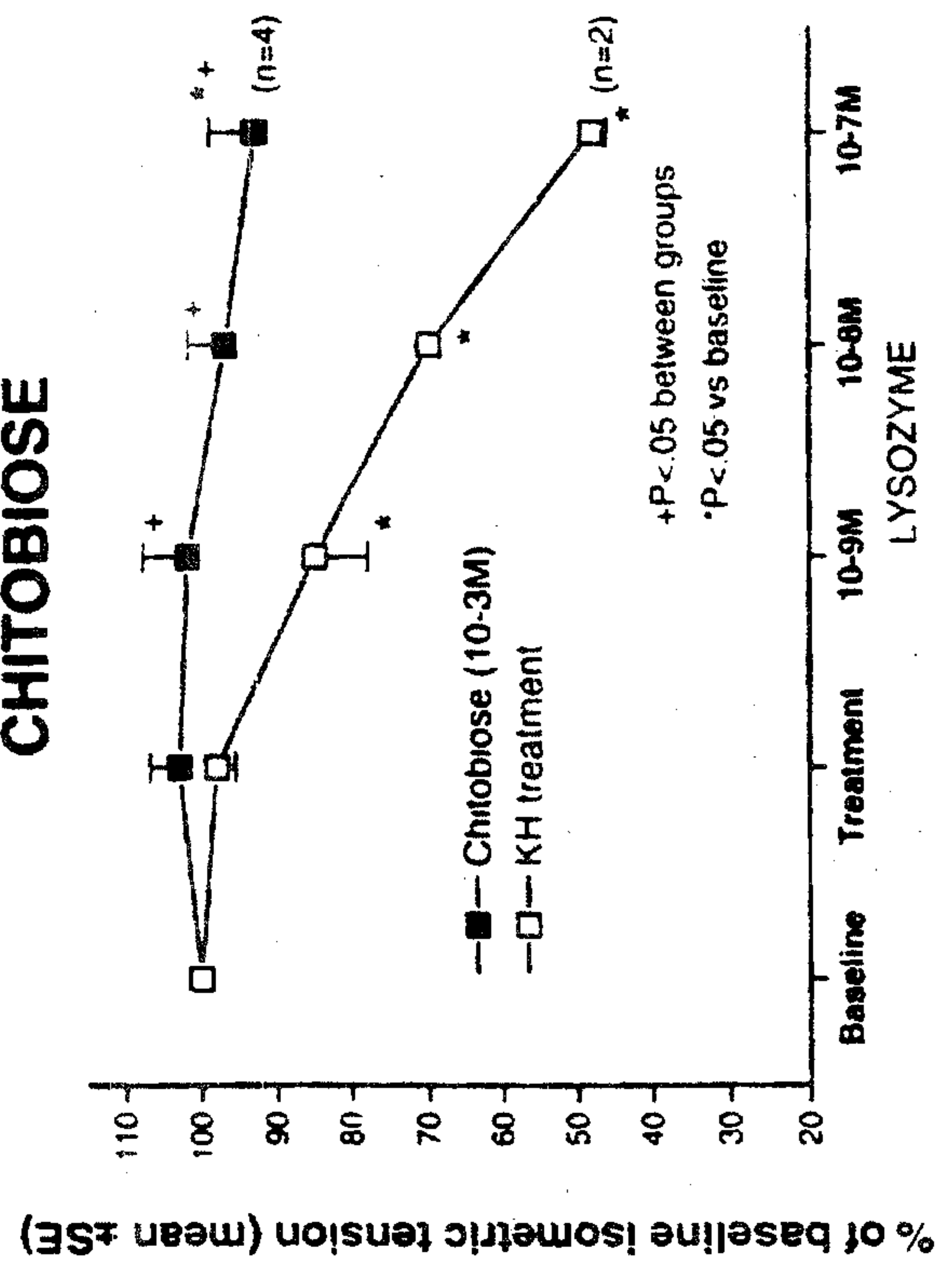


FIGURE 11

Deglycosylated and native ventricular trabeculae



CHITOBIOSE



N-ACETYL GLUCOSAMINE (NAG)

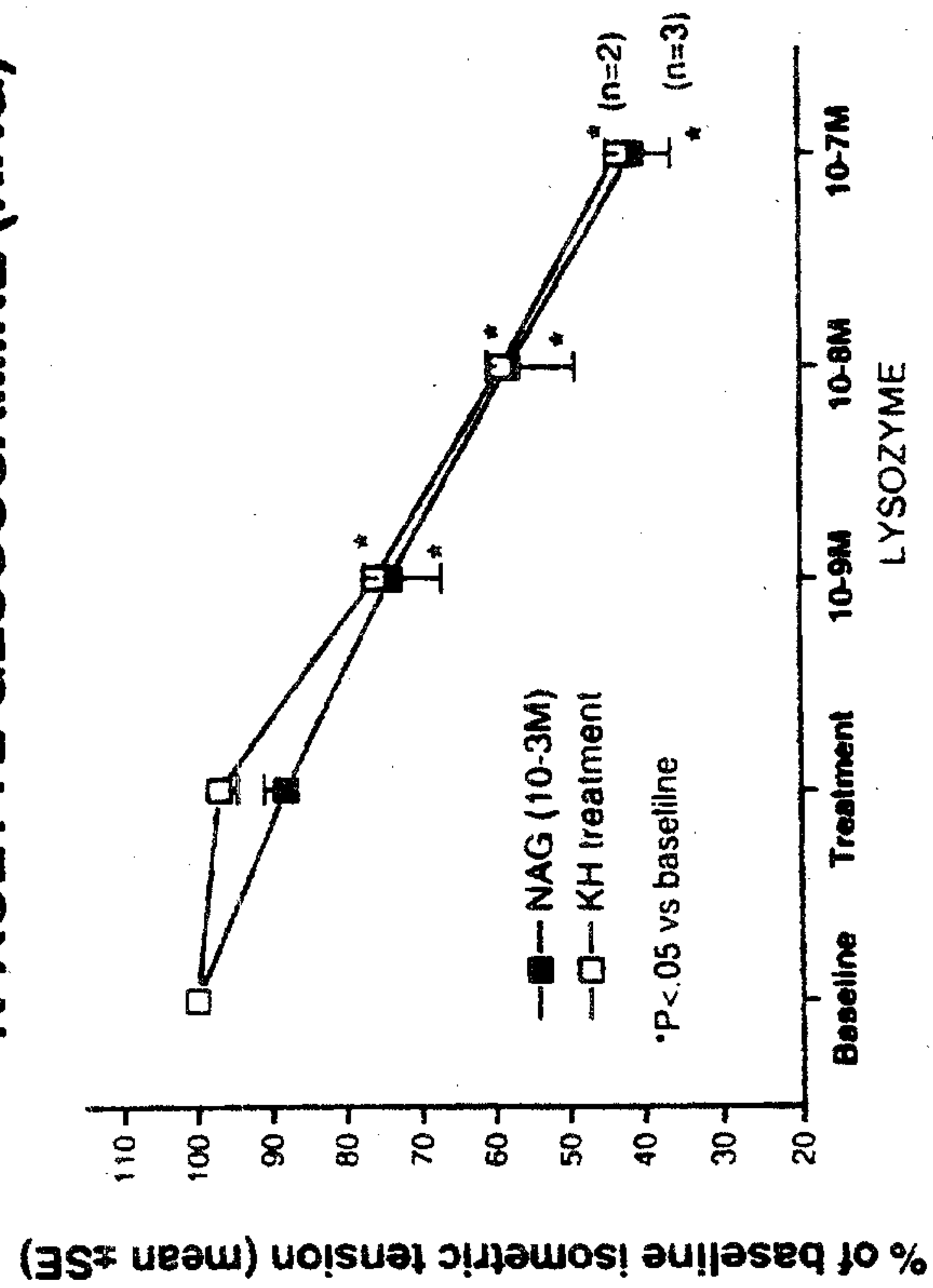
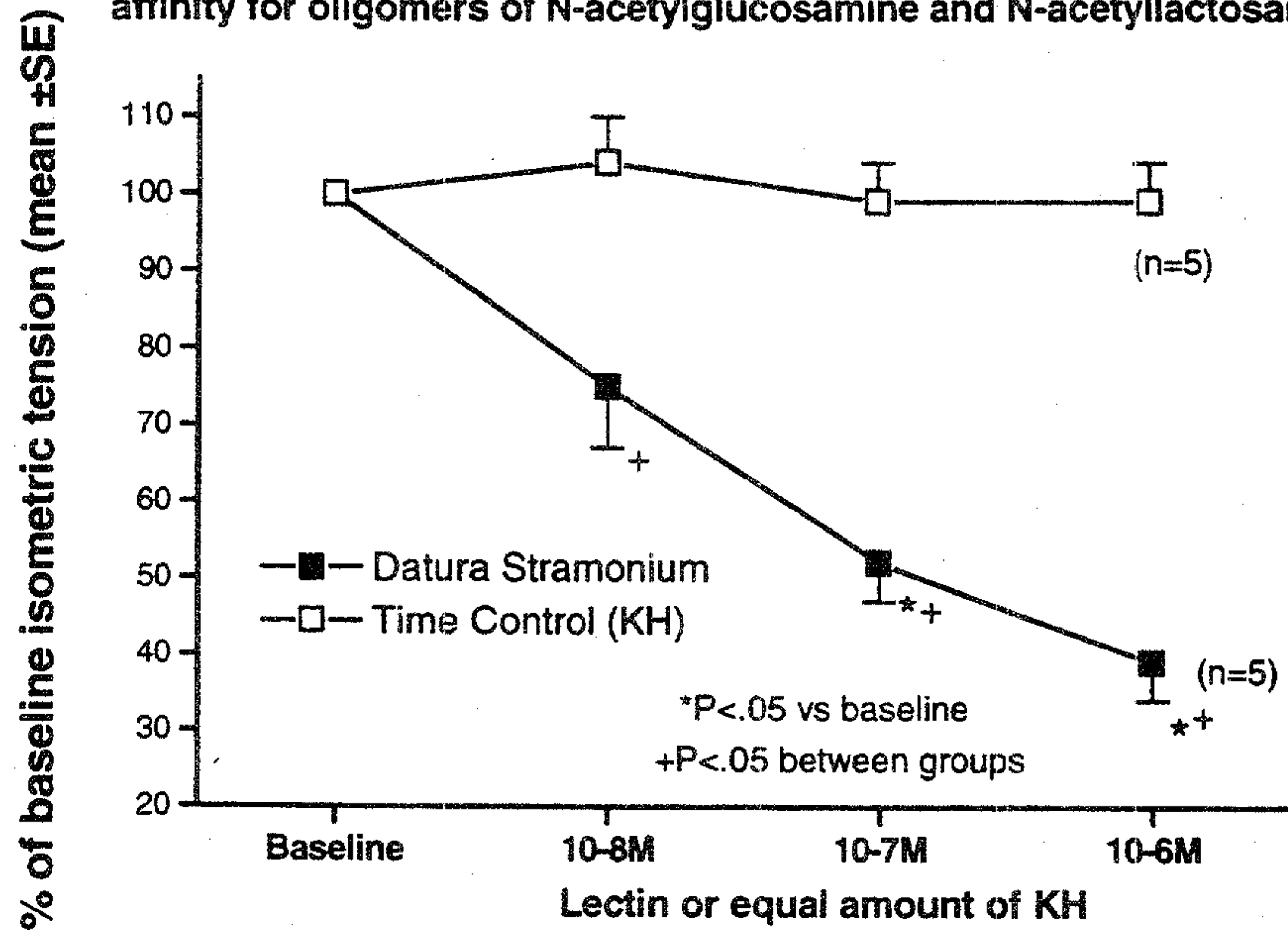
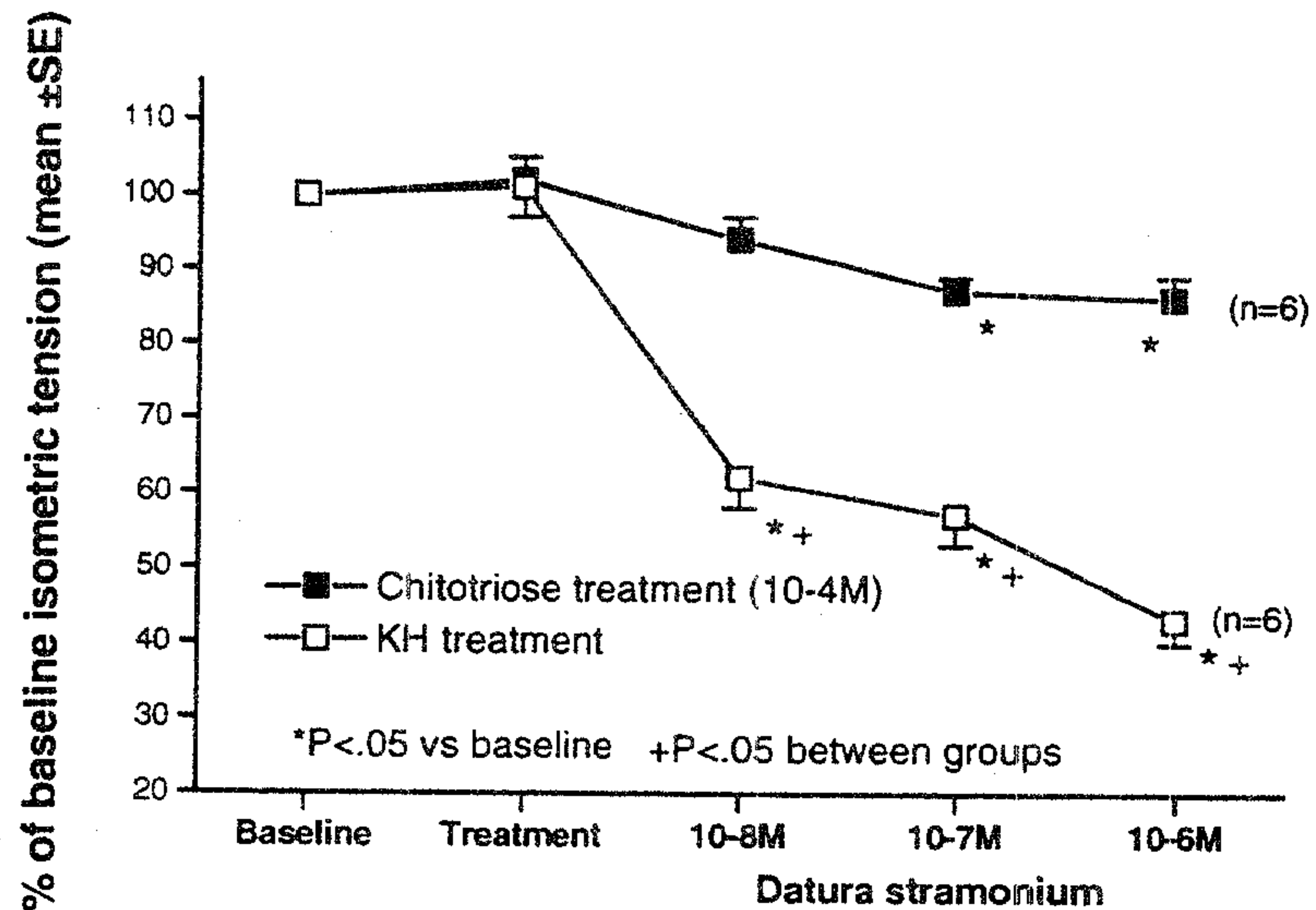


FIGURE 12

DATURA STRAMONIUM

affinity for oligomers of N-acetylglucosamine and N-acetyllactosamine

**DATURA STRAMONIUM and CHITOTRIOSE**

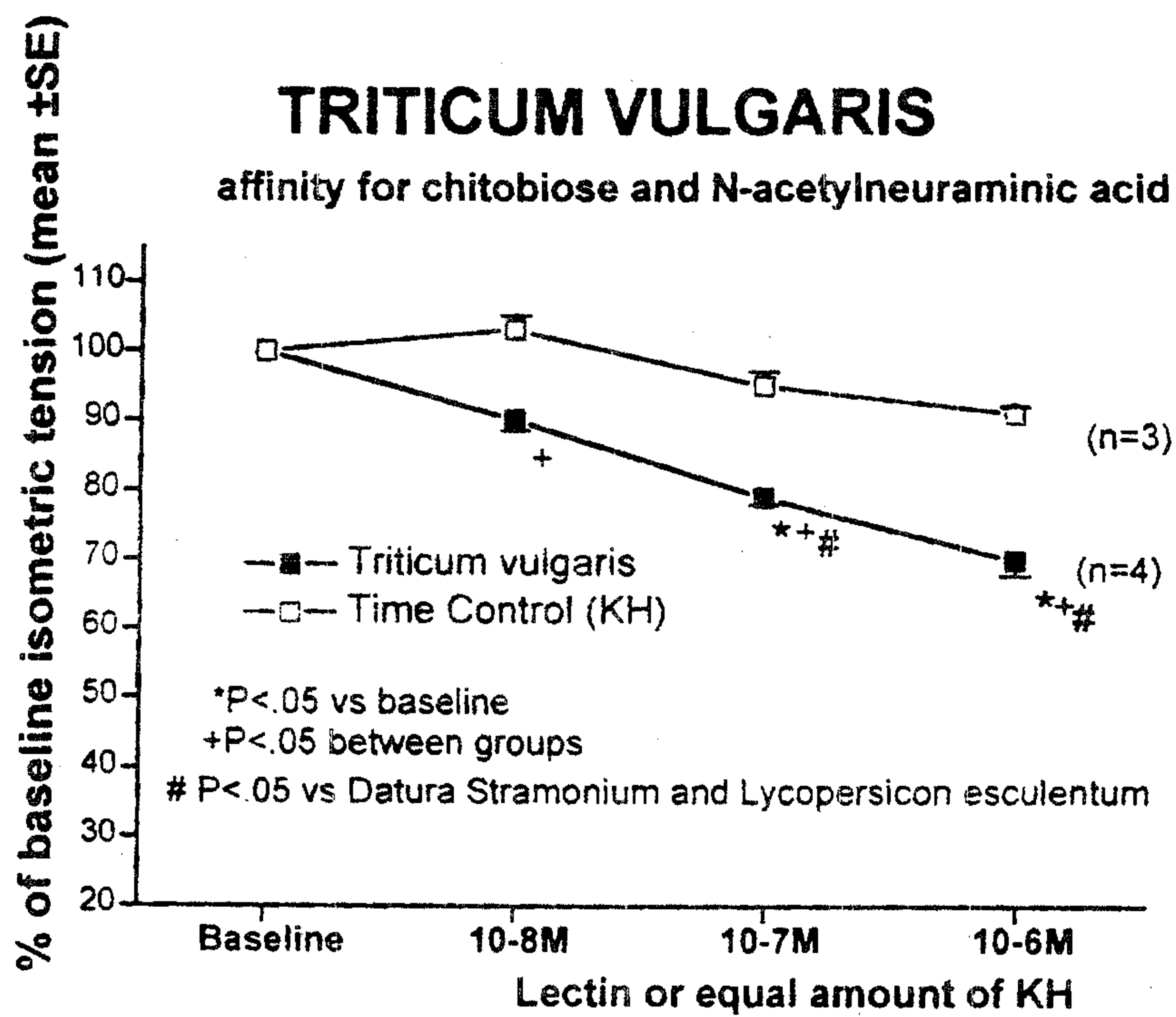
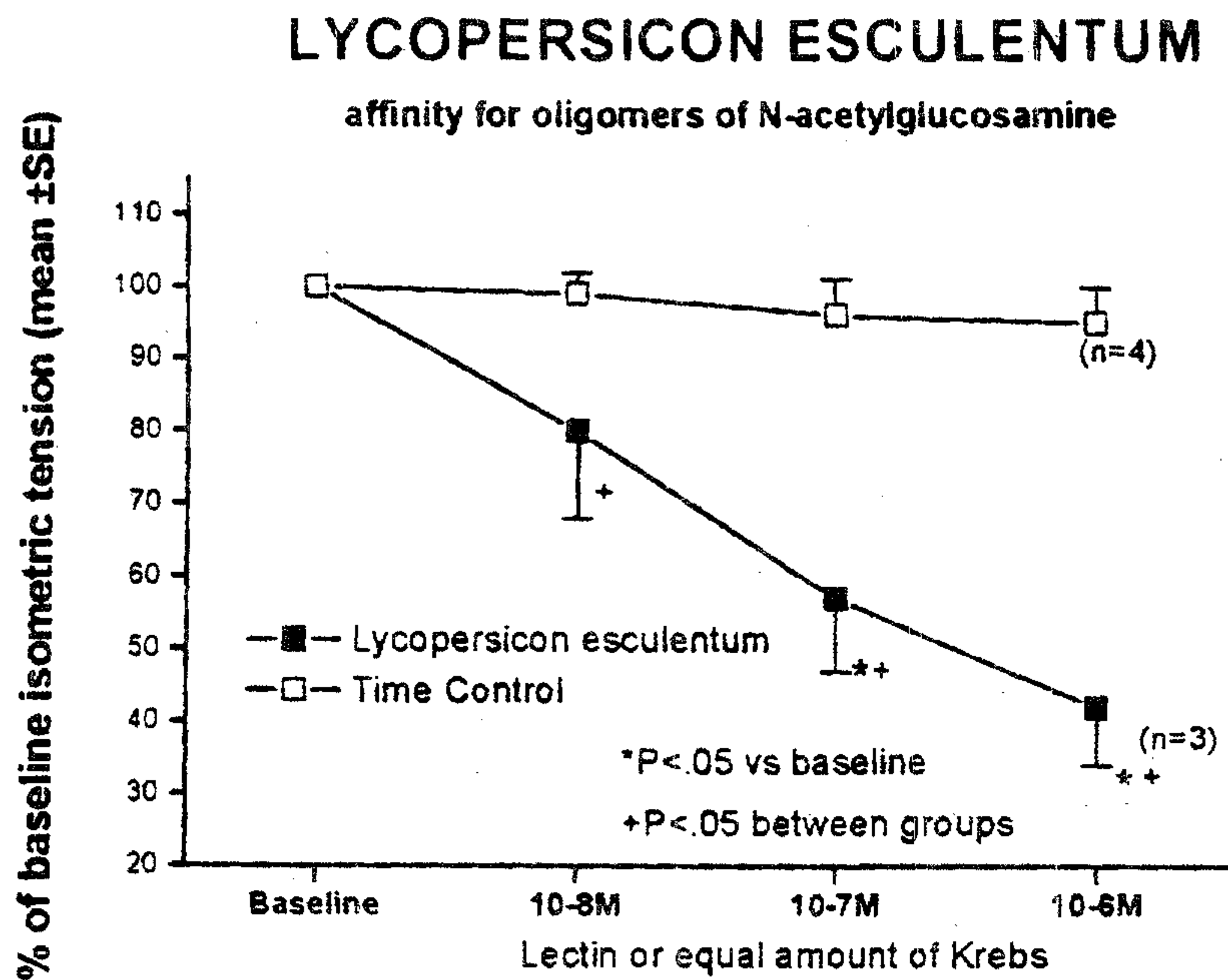


FIGURE 13

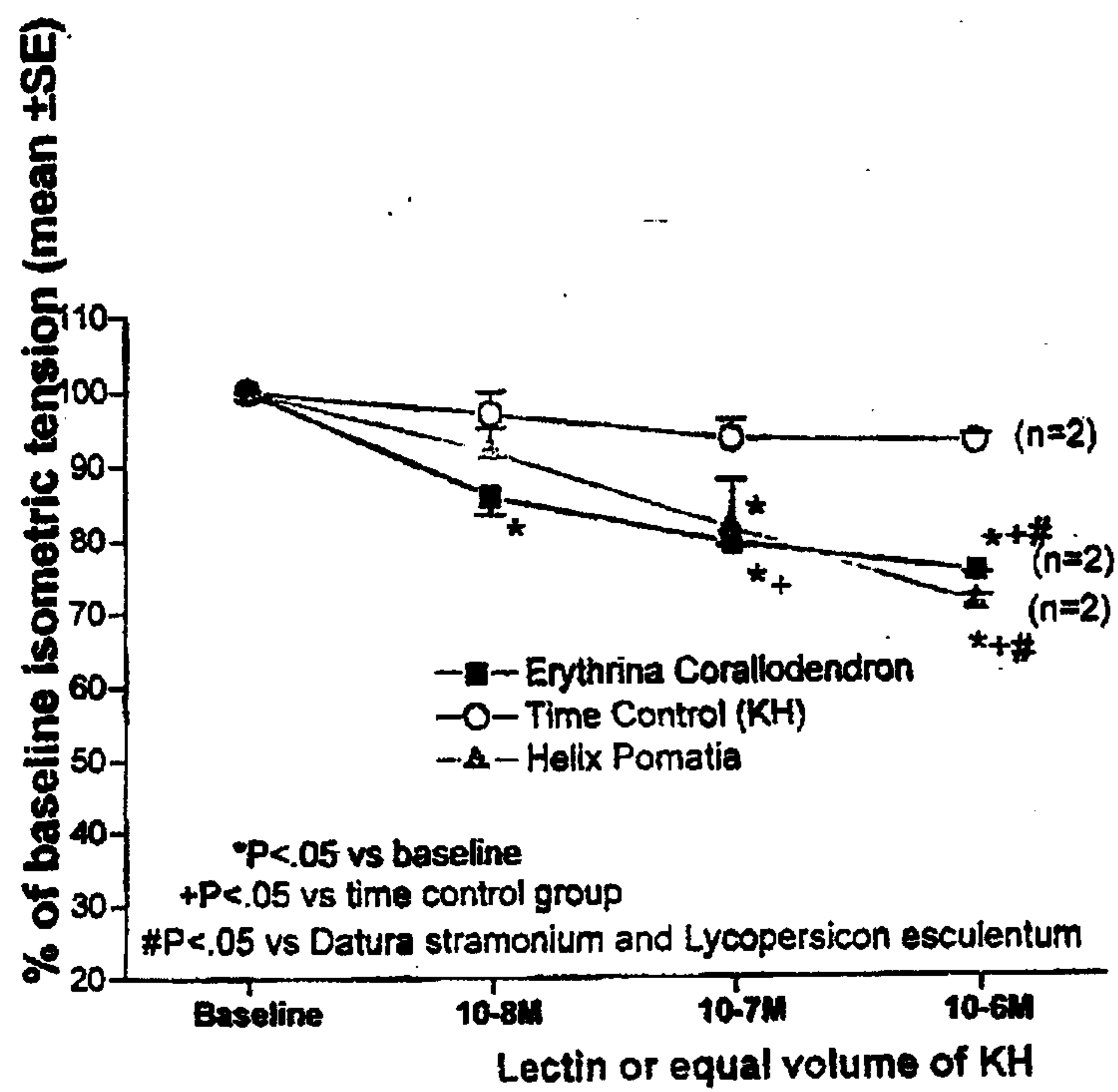


FIGURE 14