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THE UNIVERSITY OF ALBERTA

Endothelium Dependent Relaxation In A Spontaneous Model of Atherosclerosis

BY

CIARAN JOSEPH MALACHY MCNAMEE

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled:

Endothelium Dependent Relaxation in a Spontaneous Model of Atherosclerosis

submitted by Ciaran Joseph Malachy McNamee

in partial fulfilment of the requirements for the degree of

Master of Science in Experimental Surgery

Supervisor

.....

Supervisor

.....

Date: June 20/1987.

DEDICATION

THIS THESIS IS SINCERELY DEDICATED

TO MY WIFE ALLISON JOANNE MCNAMEE

FOR HER SELFLESS ENCOURAGEMENT AND

HER SUPPORT IN THIS WORK

AND

TO MY FATHER DR. JOHN MCNAMEE

WHO EXEMPLIFIES A LIFE DEVOTED

TO SCIENTIFIC RESEARCH

ABSTRACT

The effect of a genetically determined form of atherosclerosis on endothelium dependent relaxation (EDR) was assessed in LA/N-CP rats. The homozygous corpulent rat (cp/cp) on a normal diet exhibits a hypertriglyceridemia with mildly elevated cholesterol and glucose levels compared to homozygous normal (lean +/+) rats. Corpulent male rats have been demonstrated to develop atherosclerotic and myocardial lesions.

Male and female corpulent rats were compared to lean rats at 6 and 9 months of age using rings of their thoracic aorta removed following pentobarbitone anaesthesia (20 mg/kg) and suspended in tissue baths under isometric tension. All drug manipulations were preceeded by a 2 hour incubation period and EDR by acetylcholine (ACH -10 to -4 log mol/l) were expressed in concentration curves following a contraction by norepinephrine (NE -7 log mol/l).

At 6 months of age the maximum relaxation by ACH in the male LA/N-CP (70%±4% (SEM) at ACh of -4 log mol/l) was attenuated as compared to the female LA/N-CP rats and the lean control rats (100%±5% at ACH of -4 log mol/l). Concentration curves for relaxation showed a significant difference between the male corpulent rats and the female corpulent rats which were similar to the lean male and female rats. Male corpulent 9 month old rats did not demonstrate inhibiton of EDR as compared to lean controls or female corpulent or lean rats. Comparisons of contractile agents showed that the sensitivity to alpha adrenergic agents did not change between 6 and 9 months. However male corpulent rats retained a strong contracile force at 9 months while lean males and corpulent females exhibited a reduced maximum force of contraction to alpha agonists. Male

and female corpulent rats demonstrated a significant inhibition of relaxation ($p<0.05$) with the endothelium independent agents isoproterenol and sodium nitrite as compared to lean controls.

Female corpulent rats were able to overcome the smooth muscle defect from the hyperlipidemia when relaxation was induced by acetylcholine (ACH) which is an endothelium dependent agent. Male corpulent rats at 6 months of age demonstrated an attenuation of EDR which was induced by ACH. However at 9 months of age male corpulent rats had fully regained their ability for EDR with no attenuation of relaxation as compared to lean males. Results of this study indicate a smooth muscle relaxation problem in hyperlipidemic states which is corrected by the presence of the endothelium in female rats.

Table of Contents

List of Figures

I Endothelium Dependent Relaxation (History)

EARLY HISTORY OF ENDOTHELIUM DEPENDENT RELAXATION

In 1905 Sollman and Brown discovered that intravenous injections of ergot into in situ veins caused a sudden fall in blood pressure. They reviewed the literature and showed that several investigators had shown a similar fall in blood pressure with intravenous ergot. This was in the face of the general conviction at that time that ergot was a vasoconstrictor. They also outlined the problems with other ergot preparations which could account for the vasoconstriction. Sollman and Brown also noted one of the effects of intravenous ergot was a reversal of the vasoconstriction induced by adrenalin[1].

In July of 1906 H.H. Dale in a review of the physiological effects of ergot concluded that it had an ambiguous effect on the peripheral circulation. He agreed with the review of Sollman and Brown concerning the divergent effects of ergot, due to the impure preparations up to this time. Dale also confirmed the phenomenom noted by Sollman and Brown of the reversal of vasoconstriction following adrenalin administration by perfusion with ergot[2].

In December of 1906 Hunt and Tavēau from Harvard discovered the compound acetyl-choline when working with choline derivatives. They observed that small amounts of this compound caused a fall in blood pressure[3].

By 1914 H.H. Dale discovered small amounts of acetyl-choline in preparations of ergot. He also observed vasodilation of rabbit ear arteries with perfusion studies using acetyl-choline. The vasodilation was

accomplished with minute amounts of acetyl-choline and Dale attributed this phenomenom to a direct vascular event[4].

In the same year Hunt debated the vasodilation seen by Dale on the basis of his experiments using high doses of acetyl-choline in perfusates of rabbit ear arteries. To further confuse the issue Hunt attributed the fall in blood pressure with low concentrations of acetyl-choline to a cardio-inhibitory rather than a vaso-dilatory effect[5].

However by 1917, Hunt after several further experiments with acetyl-choline perfusions of isolated limbs was inclined to accept the vasodilation view of Dale. He also tested histamine and yohimbine, both of which when given after acetyl-choline and atropine caused vasodilation. He also reported the reversal of the vasodilation induced by acetyl-choline following the addition of atropine to the perfusate indicating a muscarinic effect[6].

In 1950 J. Burn and J. Robinson repeated Dale's experiments with isolated perfused rabbit ear arteries. These two investigators again noted vasodilation at low concentrations with changes to vasoconstriction at higher concentrations of acetyl-choline. This effect was attributed to two different receptors on arterial smooth muscle wall with different affinities for acetyl-choline. One receptor with a high affinity for acetyl-choline could bind acetyl-choline at low concentrations; while a vasoconstrictor receptor with low affinities would bind acetyl-choline at high concentrations[7].

It remained for S. Kottegoda in 1953 to show that the vasoconstrictor response to high doses of acetyl-choline was due to a nicotine like effect releasing an adrenalin-like substance from nerve endings[8].

By 1955 R. Furchgott, in a classic review of the literature, contrasted the vasodilation effect of acetyl-choline on perfused vascular beds[4,6,7], with vasoconstriction by acetyl-choline on isolated arterial segments or strips[9,10,11]. Furchgott also observed that in isolated rabbit aortic strips the predominant effect of acetyl-choline in his preparations was vasoconstriction[12].

However the physiological effects of acetyl-choline on vascular tissue had not been settled, as in 1962 R. Jelliffe repeated Furchgott's experiments on isolated rabbit aortic strips. Despite Furchgott's findings, Jelliffe consistently reported vasodilation of aortic strips by acetyl-choline following an established contraction by epinephrine, norepinephrine, or serotonin[13].

In 1974 Vanhoutte, in a further review of the effects of acetyl-choline on vascular arteries, observed an ambiguous response in his experiments. However, he felt that the most common reaction to acetyl-choline was a slight relaxation at low concentrations with constriction at higher concentrations. The relaxing effect was exaggerated by pre-constricting the arteries with noradrenalin. This was in accordance with the results from other current investigators[14].

In 1979 Furchgott resolved the confusion concerning the effects of acetyl-choline on vascular-tone. He showed that the endothelial surface mediated the relaxation phenomenom and that endothelial damage resulted in vasoconstriction when acetyl-choline was added to the perfusate[15].

This paper was soon followed by a further report from Furchgott where he showed graded loss of the relaxation property to acetyl-choline with increasing endothelial cell damage as assessed by electron microscopy studies of aortic strips. Unrubbed preparations giving 80-100% relaxation

of moderate tone retained 60-75% of endothelial cells by the end of the experiment. Complete loss of relaxation was associated with virtually 100% loss of endothelial cells[16].

It remained for De Mey and Vanhoutte in 1982 to show that in comparison to the case in veins, endothelium dependent inhibitory responses to acetyl-choline, adenosine triphosphate, thrombin, and arachadonic acid were much more pronounced in arteries. In veins, only transient endothelial dependent relaxations to these substances were observed. Endothelial independent inhibitory effects of isoproterenol and adenosine and the excitatory effects of acetyl-choline and A.T.P. were more pronounced in veins than in arteries. In both arteries and veins, anoxia increased the contractility induced by noradrenalin and this potentiation was decreased when the endothelium was removed. Furthermore, endothelial relaxation caused by acetyl-choline was abolished under anoxic conditions[17].

It should be noted that hypoxic conditions will induce endothelial dependent dilatations which are completely reversible upon reoxygenation of the perfusate. However, the mechanism of this dilation is prostacyclin related and is inhibited by indomethacin which acetyl-choline induced dilations are not[18].

Furchgott has also conducted experiments on several arteries of the rabbit, the thoracic aorta of the rat and guinea pig, and a variety of arteries of the dog and cat. In all the above arteries which were tested with acetyl-choline, relaxation was governed by the presence of intact endothelial cells[16].

II Recent Discoveries Concerning Endothelium Dependent Relaxation

ACETYLCHOLINE AS AN AGENT OF ENDOTHELIUM DEPENDENT RELAXATION

In 1979 R. Furchgott discovered that relaxation of strips of rabbit aorta by acetylcholine was determined by the presence of endothelial cells. Previous in vitro experiments showing either contraction or relaxation of arteries to exogenous acetylcholine probably resulted from rubbing of the intimal surface of the vessel[19]. Using scanning electron microscopy and an en face silver staining method for light microscopy Furchgott demonstrated the complete loss of the endothelium by either mechanical rubbing or by a collagenase treatment. Rings of rabbit aorta which demonstrated good relaxation to acetylcholine were seen to have at least 70-80% of the endothelial surface undamaged on histological examination[16].

Acetylcholine was subsequently shown to have a relaxing effect on pre-contracted arteries of canine renal vessels[20], canine pulmonary vessels[21], canine coronary arteries[22], and rat aortic strips[16,23]. Other species studied are: the canine pulmonary, saphenous, femoral, and spleenic arteries as well as veins[17], rabbit superior mesenteric, pulmonary, and ear arteries as well as thoracic arteries, guinea pig thoracic aorta, cat thoracic and abdominal aorta, cat cerebral[24], superior mesenteric, pulmonary and external iliac arteries[16]. The relaxation occurs by the activation of muscarinic receptors on the endothelial cell by acetylcholine[16,17,24,26]. This was shown in the rabbit aorta by the high potency of atropine as a blocking agent as well as by the relative potencies of the muscarinic agonists (acetylcholine > methacholine > carbachol)[16]. Atropine has been shown subsequently to block relaxation by

acetylcholine in a variety of tissues[17,24,25,27]. Higher concentrations of acetylcholine can elicit contractions acting directly via muscarinic receptors on smooth muscle cells[16,24,27].

In some arteries at very high concentrations of acetylcholine (>0.1mmole) noradrenaline is released from adrenergic nerve terminals stimulated by nicotinic receptors for acetylcholine[30]. Different species may be subserved by different muscarinic receptors as in the isolated canine femoral artery[25,26], and isolated rabbit thoracic aorta[25,29], relaxations are mediated by a low affinity muscarinic receptor. However in intact canine femoral arteries with intra-luminal perfusion, acetylcholine binds to both high and low affinity muscarinic receptors[26]. Experiments in resistance vessels (rabbit ear artery or rat mesenteric bed) indicate that acetylcholine will provoke proportionatly greater relaxations the smaller the vessel caliber[31,32].

The presence of the endothelium can modulate the basal tone of arterial preparations. Studies with the effluent from rabbit aortas[33] or canine femoral arteries[34] will relax bioassay deendothelialized coronary arteries. Vasodilation can be induced by flow increments through arteries in situ[35,36] or in vitro[37]. It is known that hemodynamic stress will increase production of prostacyclin (a prostaglandin vasodilator) from endothelial cells[38] but the vasodilation accompanying perfusion studies of arteries is not prevented by inhibition of prostacyclin synthesis[37]. Furthermore endothelial removal will abolish the vasodilation in arteries due to fluid stress[37].

The active arterial tone is also modulated by the presence of the endothelial cell; selective alpha-2 agonists have 5 to 7 fold enhancement of their vasoconstrictive properties by endothelial removal while alpha-1

agonists are increased 2 fold after endothelial denudation[39]. Martin has also shown that smooth muscle cells of rat aorta are under the spontaneous tonic influence of an endothelial dependent relaxation factor preventing contraction of the vessel wall[40].

Intraluminal hypoxia also will stimulate endothelial cells to secrete both PGI_2 and endothelial dependent relaxation factor (E.D.R.F.)[18]. This might provide endothelial cells a local mechanism of increasing oxygen delivery in hypoxic situations.

Removal of the Endothelium

It has been shown that gentle rubbing of the intimal surface of blood vessels cut into rings will remove all of the endothelial cells. This method will not affect the sensitivity of endothelial independent relaxants such as glyceryl trinitrate, sodium nitrite, sodium azide, adenylic acid, isoproterenol, and near U.V. irradiation[29]. The contractility of the preparation is augmented after endothelial removal[19,39,41]. A second method for removal of the endothelial cells from preparations is by preincubating the intimal surface with a collagenase. This method also will not interfere with contraction sensitivity or the relaxation effect of endothelial independent agents[16]. Other reported methods for endothelial removal with in vivo preparations is by fogarty endarterectomy or by a 5 minute perfusion with a high K^+ buffer (40 mMole.)[33]. All methods cause the complete loss of the endothelial cell layer as visualized by an en-face silver staining technique[29,33]. Visualization by scanning electron microscopy in systemic blood vessels will also accurately demonstrate the presence or absence of the endothelial cell layer[42]. Mechanical removal of the endothelium will trigger an immediate and sustained mobilization of arachadonic acid which is converted transiently by the subendothelium to

prostacyclin. This effect lasts only 2 hours after which self-inactivation of cyclooxygenase occurs with a resultant fall in PGI_2 levels[43].

Demonstration of the release of E.D.R.F.

In cultured endothelial cells of the rabbit aorta, acetylcholine has been shown to cause hyperpolarization and to augment the levels of cyclic nucleotides. This indicates that a transmitter is released or there is cell to cell conduction to inhibit the contractility of the intimal structures in the vessel wall[44]. Furthermore, the effect of acetylcholine or it's mediators is not simply hyperpolarization of cell membranes because ouabain or potassium free solutions partially inhibit relaxation but completely prevent hyperpolarization. Thus, despite the inactivation of the Na^+/K^+ pump in vascular smooth muscle, the endothelial dependent relaxation factor (E.D.R.F.) still can induce relaxation[45].

It has been shown that endothelial cells when exposed to acetylcholine will release a diffuseable substance that will relax deendothelialized blood vessels. This was done with "sandwich" or layered preparations where a transverse strip freed of endothelial cells was tested with it's intimal side facing a similar strip of blood vessel with endothelium present on it's intimal surface. The deendothelialized preparation will then regain it's ability to relax when acetylcholine is added to the preparation[16,46,47].

Further evidence for the release of a relaxing factor comes from super-perfusion studies where the perfusate first passes through a strip of blood vessel with endothelium and then runs downstream to a deendothelialized preparation. The deendothelialized preparation relaxes when the perfusate has first contacted the blood vessel containing

endothelium. It does not relax when the direction of the perfusate is reversed[33,48,49].

When cultured porcine endothelial cells are incubated with cultured porcine smooth muscle cells a rise in cyclic GMP is found in response to exposure by both A.T.P. or methacholine. These two agents are both endothelial dependent vasodilators. This rise in cyclic GMP has been shown to be the final common pathway for smooth muscle relaxation by endothelial dependent and independent relaxing factors. Furthermore this rise in cyclic GMP is blocked by E.T.Y.A. (5,8,11,14-eicosatetreynoic acid) which is a cyclooxygenase and lipoxygenase inhibitor; these are the main pathways for arachadonic acid metabolism[50].

Cocks has also demonstrated that cultured bovine endothelial cells seeded onto endothelium denuded rings of canine coronary artery will produce relaxation to bradykinin and A23187 (a calcium ionophore). The production of prostacyclin by these cells was blocked by a cyclooxygenase inhibitor while this relaxation occured. Although bradykinin and A23187 are both endothelial dependent relaxants, acetylcholine and substance P could not induce these relaxations[51].

Effect of Contracting Agent

Most preparations have used noradrenalin as a contracting agent against which acetylcholine will induce relaxation. In the rabbit aorta at approximatly 25-50% of maximal contraction by noradrenalin, acetylcholine at 1 μM. will produce 90-100% relaxation in unrubbed preparations. If the strip of aorta is fully contracted by noradrenalin (30 μM. with rabbit aorta) acetylcholine will usually produce about 30% relaxation[47].

The sensitivity of many other arteries (rabbit celiac, superior mesenteric, ear, and renal arteries, cat thoracic and abdominal aorta,

superior mesenteric, renal, pulmonary, and external iliac arteries, and dog femoral superior mesenteric, inferior mesenteric, celiac, renal, pulmonary, circumflex and left anterior descending coronary artery) to the inhibitory effect of acetylcholine is equal or greater than the rabbit aorta[47]. Studies have demonstrated that cultured endothelial cells can evoke relaxations to contracted strips of rabbit aorta. The relaxation was initiated by A23187 with a cyclooxygenase inhibitor in the bath[52].

Further studies have shown that acetylcholine will produce endothelium dependent relaxation in human splanchnic, pulmonary, cerebral, and periperal arteries[53]. Garry has demonstrated that acetylcholine will relax normal coronary vessels in vivo but will constrict the coronary vessels of patients with a stenosis. All vessels relaxed normally to nitroglycerin which demonstrated that the relaxing ability of the vessel was not destroyed by the process causing the stenosis[54]. This study indicates that disease processes can affect the endothelial cells ability to allow relaxation to occur.

Agents other than noradrenalin which have been used for contraction of aortic strips are: the use of a high K^+ buffer (18-24 mM.)[47], any alpha-1 or alpha-2 agonist[39], or $P.G.F._{2\alpha}$[55]. It has also been shown that removal of the endothelium will enhance contractility to alpha agonists (alpha-2 more so than alpha-1)[41,39]. Furthermore, using in vivo preparations of rabbits, following endothelial denudation, intimal hyperplasia occurs and by day 14 there is a 2.6 fold increase in the affinity of alpha-1 receptors with no change in receptor density[56].

III Nature of the Endothelium Dependent Relaxation Factor (E.D.R.F.)

To date the exact nature of the E.D.R.F. is still not known, however several of it's characteristics have been determined.

1) Half-life: The half-life of the relaxing factor is fairly short; it ranges between 6-80 seconds depending on the species studied[33,48,49]. E.D.R.F. has been found to bind extensively to albumin[33].

2) Path of formation for E.D.R.F.

In 1980 Furchgott demonstrated that E.D.R.F. was not formed by the enzyme cyclooxygenase because inhibitors of that enzyme (indomethacin 40 μM. or aspirin 100 μM.) did not prevent acetylcholine induced relaxations[16]. He postulated that E.D.R.F. was liberated by the activation of muscarinic receptors by acetylcholine which initiated a reaction in the endothelial cell using arachadonic acid or some other unsaturated fatty acid. Furchgott arrived at this conclusion because E.T.Y.A. (5,8, 11, 14-eicosatetraynoic acid) a lipoxygenase and cyclooxygenase inhibitor would block the relaxations of acetylcholine[16].

Involvement of Arachadonic Acid

Exogenous arachadonic acid will evoke endothelial dependent relaxations when cyclooxygenase is blocked[57,58,59]. Furthermore, if the lipoxygenase pathway is formative in the synthesis of E.D.R.F., studies have shown that most of the lipoxygenase activity in vascular smooth muscle is limited to the endothelial layer[58].

Prostacyclin (PGI_2), a known vasodilator, is the main product of prostaglandin synthesis from arachadonic acid in endothelial cells[58,60]. However despite the potential for vasodilation by PGI_2, the predominate agent causing vasodilation from endothelial cells in canine ring arterial

preparations is not prostacyclin[58]. Furthermore this vasodilation can be blocked by E.T.Y.A. (a lipoxygenase and cyclooxygenase inhibitor) and by N.D.G.A. (nordihydroguairaretic acid) which is a lipoxygenase inhibitor[58]. Thus this study strongly suggests a role for lipoxygenase in the formation of E.D.R.F.[16]. Inhibitors of phospholipase A_2 (the calcium sensitive enzyme that liberates arachadonic acid from membrane phospholipids) will prevent endothelial dependent relaxations from occuring after acetylcholine is added to the preparation. Quinacrine (Mepacrine) a reported phospholipase A_2 inhibitor[61] will prevent acetylcholine induced relaxations in rabbit aorta. There is no difference if it is added prior to or after the addition of acetylcholine[16,62].

In rat aortas relaxation by acetylcholine or histamine was only partially reversed by quinacrine. However, the final endpoint by all endothelial dependent and independent relaxants of an elevated cyclic GMP level in the tissue was prevented by quinacrine[63]. In bioassay experiments on rabbit aortas, quinacrine irreversibly inhibited acetylcholine induced relaxations[33]. However, the other possible sites of quinacrine inactivationare the endothelial cell muscarinic receptor or the calcium channels governing the activity of phospholipase A_2[62].

B.P.B. (α-p-Dibromoacetophenone) is another phospholipase A_2 inhibitor[64] and a potent inhibitor of acetylcholine induced relaxations in arteries of rats, dogs, cats, rabbits, and humans. Unfortunatly, it also causes some endothelial cell desquamation which interferes with the usefulness of this drug as an inhibitor[47]. Melliten is a bee venom polypeptide which activates phospholipase A_2 on cell surfaces to form arachadonic acid and other unsaturated fatty acids. Melliten when added to unrubbed preparations will induce endothelial dependent relaxations similar

to acetylcholine. Furthermore the relaxations are inhibited by B.P.B. and mepacrine and by lipoxygenase inhibitors but not by cyclooxygenase inhibitors. However, exogenous phospholipase A_2 is ineffective in inducing relaxations[65]. Glucocorticoids which are reported to induce an inhibitor to phospholipase A_2[66] do not prevent acetylcholine induced relaxations in rat aorta after pretreatment for seven days with dexamethasone[50].

Other known inducers of E.D.R.F. which are not inhibited by mepacrine are A.T.P., bradykinin, or A23187 in rabbit aorta[62]; or A.T.P. or thrombin in canine femoral arteries[67]. This may indicate that there are several E.D.R.F. molecules or that multiple pathways for it's formation exist.

Lipoxygenase Pathway

Endothelial cells can metabolize arachadonic acid through either the cyclooxygenase pathway leading to formation of PGE_2, PGI_2, $PGF_{2\alpha}$, PGD_2, and thromboxane A_2; or it can be metabolized through the lipoxygenase pathway[60] which is localized to the intimal layer of the artery[67]. Unfortunately experiments with known endproducts of the lipoxygenase pathway have demonstrated inconclusive results as regards their relaxing abilities. The labile 15-hydroperoxy intermediates (15-HPETE) have some relaxing effects on blood vessels[58] but the stabile 12- and 15- HETE do not[68]. Furthermore, inhibitors of the sulfidopeptide leukotrienes (LTC_4, LTD_4, LTE_4,) do not alter acetylcholine induced relaxations in rabbit aorta[69].

Fatty Acids and E.D.R.F. Release

The unsaturated fatty acids, oleic, elaidic, cis-vaccenic and cis-4,7,10,13,26,19-docosohexaenoic acid will produce endothelial dependent relaxations in both dog and rabbit aortas. The relaxations are not inhibited by cyclooxygenase inhibitors. However it is suggested that these agents may actually facilitate enzymatic reactions in endothelial cell

membranes by increasing membrane fluidity rather than promuting a pathway like that of arachadonic acid. It has been shown that the membrane stabilizing agent A_2C can produce endothelial dependent relaxations in rabbit aorta and canine superior mesenteric arteries. These relaxations are not inhibited by cyclooxygenase inhibitors[59].

Oxidation State of EDRF

It had earlier been noted that complete anoxia could inhibit the relaxation induced by acetylcholine[47]. Furthermore several anti-oxidants and non-specific radical scavengers could inhibit the acetylcholine induced relaxation[33,68]. Catecholamines also possess anti-oxidant properties and can inactivate the relaxing factor in transit[49]. It has also been shown in vascular rings of rabbit aorta[70] and canine femoral arteries[71], that oxygen derived free radicals are not the E.D.R.F., since scavengers of these radicals do not prevent relaxation. However oxidized free radicals can facilitate the release of an E.D.R.F. and affect it's actions on smooth muscle[72]. Furthermore, superoxide anions can accelerate the destruction of E.D.R.F.[71] and depress acetylcholine induced relaxations.[72]. From these studies, it is concluded that the E.D.R.F. is probably an oxidized substance depending on it's oxidized state for it's activity[73].

Epoxygenase Pathway for Formation of EDRF

It was known since 1981 that arachadonic acid could also be processed through an epoxygenase pathway which is dependent on cytochrome P_{450} monooxygenase[60,74]. Inhibitors of the above enzyme like SKF 525A and metyrapone will antagonize the endothelial dependent relaxations of acetylcholine in the rabbit aorta[70] but not in the rat aorta[75]. E.T.Y.A. has also been shown to inhibit arachadonic acid metabolism by the cytochrome P_{450} pathway in rat renal cortex[76], and N.D.G.A. will block

microsomal P_{450} pathways[77]. Thus it is possible that E.D.R.F. is formed by the epoxygenase pathway and this is the site of inhibiton for E.D.R.F. by E.T.Y.A. and N.D.G.A.[73]

However SKF-525A and metyrapone have been shown to prevent activation of relaxation by calcium ionophores. It is possible they inhibit relaxation by coupling to Ca^{++} channels[78] or by affecting the cholinergic receptor linked to these channels[79].

Involvement of Calcium in the formaton of EDRF

It has been known since 1980 that the Ca^{++} ionophore A23187 would elicit an endothelium dependent relaxation of arterial preparations which was even more potent than that of acetylcholine. Furthermore this relaxation like that of acetylcholine, was inhibited by anoxia, E.T.Y.A., N.D.G.A., and B.P.B.. Bioassay experiments with A23187 in sandwhich preparations would elicit relaxation from deendothelialized preparations.[79]. However, arterial preparations with A23187 will not be prevented from relaxing if quinacrine is added to the medium[47]. This action of quinacrine might be explained as interference with ionic fluxes on Ca^{++} coupling which would explain it's ineffectiveness with A23187[47]. It has been known that membrane Ca^{++} channels are somehow linked to muscarinic receptors in most cells[80]. Furthermore, it is known that phospholipase A_2 (the enzyme that liberates arachadonic acid from membrane phospholipids) is calcium dependent[61].

On the basis of the above results, it was postulated that Ca^{++} acts as a regulator for the production or the release of E.D.R.F.. It was thought that A32187 might act as an ionophore to transport Ca^{++} across the cell membrane and acetylcholine might open a Ca^{++} channel through activation of a muscarinic receptor[73].

Preparations with Ca^{++} eliminated from the medium demonstrated inhibition of choline induced relaxation by 67% and A23187 induced relaxation of 92%. Furthermore verapamil and nifedepine (calcium channel blockers for smooth muscle[81]) reduced methacholine and A23187 relaxation by about 40%. This finding is unexpected, since Ca^{++} blockers should not inhibit Ca^{++} membrane fluxes in the presence of ionophores. It is as yet not fully explained[79].

Agents Other Than Acetylcholine or A23187 Inducing the Release of E.D.R.F.

1) A.T.P. and A.D.P. -

A.T.P. and A.D.P. will exert most of their relaxing effect on arteries from rabbits, dogs, and pigs, by the release of an E.D.R.F. from endothelial cells[28,67,82]. In the dog and the rabbit, A.M.P. and adenosine will induce endothelial independent relaxations while in the pig the relaxations by A.M.P. and adenosine were endothelial dependent, but 120 times less so than A.T.P. or A.D.P.[28]. The effect of A.T.P. and A.D.P. on the smooth muscle cell may be a direct action through it's metabolites A.M.P. and adenosine, whereas it still has a more powerful effect via the endothelial cell[47].

Inhibitors of A.T.P. Induced Relaxation

a) Cyclooxygenase inhibitors do not prevent A.T.P. induced relaxation in the rabbit[47] or the dog femoral artery[67].

b) E.T.Y.A. (cyclo and lipoxygenase inhibitor) will partially prevent A.T.P. induced relaxation in the rabbit aorta[47], but do not prevent such relaxation in the dog femoral artery[67].

c) Lanthanum (an agent that blocks trans-membrane calcium movement[83]) blocks ^{86}Rb efflux in cultured porcine aortic endothelial cells when bradykinin and A.T.P. were added to the medium. It has previously been

shown that ^{86}Rb efflux (an indicator of K^+ transport) from pre-loaded smooth muscle cells was increased by relaxing agents[84].

It has been shown that A.T.P., bradykinin, A23187, (all known endothelial dependent relaxants) increased the rate of ^{86}Rb efflux from cultured endothelial cells while acetylcholine or carbachol did not[28]. A23187 was the most powerful stimulus for ^{86}Rb efflux from cultured endothelial cells[28]. It is suggested indirectly from these observations that A.T.P., A.D.P., A23187, and bradykinin may activate E.D.R.F. by a calcium sensitive channel while acetylcholine acts by a different pathway or by releasing another E.D.R.F.[28].

2) Bradykinin

IN 1981 the relaxatant effect of bradykinin on the canine superior mesenteric artery was reported[85]. This was soon followed by reports that bradykinin induced endothelial dependent relaxations in pig arteries[28], and a wide variety of canine arteries. These relaxations were not prevented by cyclooxygenase inhibitors but were blocked by inhibitors similar to those blocking acetylcholine induced relaxations[86]. In man (mesenteric and ovarian vessels) bradykinin will ellicit endothelial dependent vasodilation with cyclooxygenase inhibitors present[47]. However, in the supeior mesenteric or celiac arteries of the rabbit and cat, bradykinin relaxes the artery by formation of PGI_2, probably through the smooth muscle[47]. This difference in relaxation via the prostaglandin pathway may be species related[47].

3) Thrombin

Thrombin has been known since 1978 to release PGI_2 from cultured endothelial cells[87]. Thus in 1982, it was no surprise that thrombin generated relaxations in canine arterial rings. However, the relaxations were predominatly generated by E.D.R.F. because they were fully present with cyclooxygenase inhibitors added to the preparation[72].

The relaxation by thrombin is counteracted by a direct contractile action on smooth muscle[67]. The binding of thrombin to endothelial cells is prevented by heparin[88] and heparin will prevent the relaxation response by thrombin[73].

4) Products of Platelet Aggregation and Serotonin

Experiments with precontracted canine coronary rings and platelets revealed that in endothelial intact strips, platelet aggregations induce a relaxation that is not cyclooxygenase dependent. In endothelial denuded strips the response of the tissue is a further contraction[89]. One of the mediators released by platelets which can cause this relaxation is serotonin. However antagonists of serotonin fail to completely inhibit the relaxation[89]. Serotonin induces relaxation by a S_1 receptor which is blocked by the S_2 and S_1 antagonist methiopthepin but not selective S_2 antagonists[90]. Furthermore it is known that A.T.P. and A.D.P. are released from platelets and may also induce endothelial dependent relaxations[28,82]. Apyrase an enzyme that hydrolyzes A.T.P. and A.D.P. to A.M.P. greatly attenuates the relaxation induced by platelets. Therefore, the consensus of opinion is that the predominant platelet agent causing relaxation is A.T.P., A.D.P., or thrombin[90].

5) Histamine

In 1983 it was shown that rat thoracic aorta would demonstrate endothelial dependent relaxation to histamine. Release of E.D.R.F. by histamine was confirmed by sandwich preparations. Histamine in the rat model demonstrated inhibition by the same drugs that would affect acetylcholine induced relaxation. Histamine exerts it's vasodilator effect on endothelial cells via H_1 receptors[91].

6) Substance P and related peptides

Isolated arteries from rats, rabbits, dogs, and cats will show endothelium dependent relaxation to substance P, vasoactive intestinal polypeptide, and calcitonin gene related peptide[23;92,93]. Hysteresis or desensitisization will occur with these compounds, but following a wash-out of the preparation, full sensitization would be restored. Furthermore after desensitization, there was no loss of sensitivity to relaxation by acetylcholine, A23187, or bradykinin. This would indicate receptor desensitization for substance P and related peptides. Inhibitors for substance P all have the same characteristics as for acetylcholine[47].

7) Vasopressin

The effect on peripheral vessels to vasopressin is contraction; however in canine cerebral vessels, and to a lesser extent in coronary vessels, vasopressin causes endothelial dependent relaxation via V_1 receptors. Teleologically this may favor the brain and the heart following shock or hemorrahge by redistributing blood to central compartments from the periphery[73].

IV Inhibitors of E.D.R.F.

Inhibitors of Formation

a) Lipoxygenase Pathway: N.D.G.A. and phenidone are lipoxygenase inhibitors and will block endothelial dependent relaxation to acetylcholine, A23187, bradykinin, and A.T.P.[20,33,47,57]. BW775C, another lipoxygenase inhibitor, did not prevent the relaxation induced by acetylcholine in rabbit aorta[59] but did so in rat aorta[94].

However, in transfer experiments it has been shown that N.D.G.A. and phenidone possess both anti-oxidant properties as well as being non-specific radical scavengers and these agents can affect the E.D.R.F. in transit[33]. This might explain the failure of acetylcholine to induce relaxation with anoxia[47].

Furthermore, anoxia will augment the contractility of the vessel wall and removal of the endothelium will reduce this anoxic potentiation. These experiments suggest that a relaxation factor generated by the endothelium is interrupted under anoxic conditions[59,98]. Hypoxia (40 to 10 mm. Hg.), on the other hand, will induce dilatation by the formation of PGI_2[18]. As the Km of cyclooxygenase is 14 mm. of Hg. it is evident that under anoxic conditions the formation of PGI_2 is inhibited[18].

Stalcup et al. in 1982 have shown that angiotensin converting enzyme on endothelial cells is inhibited in a linear relationship below pO_2 of 100. At a pO_2 of 30mm. Hg. there was no detectable enzyme activity. The importance of this is that as well as converting Angiotensin I to Angiotensin II it degrades systemic bradykinin. Therefore under hypoxic situations bradykinin, a vasodilator and inducer of endothelial permeability, is increased. Furthermore when intraluminal pO_2 was

normoxemic with extraluminal hypoxia, no dilation of the vessel occured underscoring the role of the endothelium. Thus endothelium dysfunction occurs with pathologic conditions, and this may create unusual circulatory disturbances[95].

b) Lipoxygenase and Cyclooxygenase Pathway

E.T.Y.A. will block relaxation by almost all inducers of E.D.R.F. in a multitude of species, save the dog pulmonary artery with acetylcholine[21], the dog femoral artery with A.T.P.[17], and thrombin[58].

c) Phospholipase A_2 Inhibitors

Quinacrine will prevent endothelial dependent relaxtion in all animals tested with acetylcholine[16,17,47,63]. However, it does not prevent A.T.P. induced relaxation in the dog femoral artery[17], or relaxation by A23187 in the rabbit or rat aorta[47,63]. Quinacrine will prevent bradykinin relaxation in many canine vessels[86] and relaxation induced by Substance P in rabbit and cat vessels[92].

B.P.B. is a second inhibitor of phospholipase A_2 and it will prevent endothelial dependent relaxation in arteries from dogs, cats, rabbits, and man but it concomitantly caused endothelial desquamation which possibly obviates it's effectiveness on phospholipase A_2[47].

Epoxygenase Path from Arachadonic Acid

The agents N.D.G.A. and E.T.Y.A. have been demonstrated in some tissues to block the epoxygenase pathway from arachadonic acid[76,77]. SKF 525A and metyrapone are two other epoxygenase inhibitors which will prevent formation of E.D.R.F. in the rabbit by acetylcholine.[79]. However it has been sugested that SKF 525A and metyrapone can also block calcium channels which would block E.D.R.F. release[72]. The Ca^{++} ionophore A23187 is blocked from releasing E.D.R.F. by anoxia, E.T.Y.A., N.D.G.A., and BPB[47].

Furthermore, the calcium channel blockers (nifedepine and verapamil) will possibly mitigate the release of E.D.R.F.[79].

Inactivation in Transit

As explained above, anoxia[47] and agents with anti-oxidant properties or non-specific radical scavengers will inactivate E.D.R.F. in transit[33]. Catecholamines also possess anti-oxidant properties and can inactivate E.D.R.F. after it's release[49]. Free hemoglobin can inactivate E.D.R.F. in transit; since hemoglobin is a large molecule and can not pass cell membranes, it must interact with E.D.R.F. in it's passage through the extracellular space[59]. From the above observations, it is suggested that E.D.R.F. is probably derived from arachadonic acid and that there is involvement of the lipoxygenase pathway. Ca^{++} is important, perhaps in activating or accentuating a cell receptor or an enzymatic process in the formation of E.D.R.F.. The formation of E.D.R.F. may be through several pathways or alternatively there may be several E.D.R.F. molecules. (Prostacyclin is, in effect, an auxillary E.D.R.F.). However, the E.D.R.F. found is probably an oxidized molecule which depends on it's oxidized state for it's biological activity[73].

Inactivation at the Level of the Smooth Muscle

The relaxation induced by acetylcholine, bradykinin, and A23187 is associated with elevated levels of cyclic GMP in the smooth muscle cells of the arteries of rabbits, rats, bovine coronary arteries, and canine femoral arteries[59,80.96]. Furthermore, when co-cultured endothelial cells and smooth muscle cells are exposed to methacholine, elevated levels of cyclic GMP are found in the smooth muscle cells[52].

The endothelial independent vasodilators $NaNO_2$, glycerol trinitrate, and nitroprusside, will also cause elevations of cyclic GMP in muscle

concomitant with relaxation of the artery[97]. When endothelial denuded preparations are used, the relaxation and accumulation of cyclic GMP was prevented when acetylcholine was added to the bath. However sodium nitroprusside was able to relax deendothelialized preparations and in these preparations examination of the muscle cells revealed high levels of cyclic GMP[75]. Furthermore methylene blue is a known cyclic GMP inhibitor for $NaNO_2$, glycerol trinitrate, and sodium nitroprusside[97]. It has also been shown to prevent endothelial dependent relaxation and cyclic GMP accumulation in bovine coronary arteries[96]. Inhibitors of cyclic GMP phosphodiesterase (M-B 22,948) will augment the relaxation seen with acetylcholine and also increases the levels of smooth muscle cyclic GMP[96].

Thus the E.D.R.F. and endothelial independent vasodilators most likely have a similar endpoint: to create a rise in the smooth muscle cyclic GMP level. The elevated cyclic GMP probably then activates a protein kinase to lead to altered protein phosphorylation and dephosphorylation of the myosin light chain and thereby relax the artery[97].

V Contraction and Relaxation of Smooth Muscle

Contraction of Smooth Muscle

The contraction of vascular smooth muscle cells depends on an increase in cytoplasmic concentrations of Ca^{++}. At rest the intracellular Ca^{++} concentration is less than 10^{-7} M., while the extracellular concentration of Ca^{++} is about 1.5×10^{-3}M.. To initiate contraction of smooth muscle cells the intracellular concentration of Ca^{++} rises by a factor of 100. This is caused by an opening of Ca^{++} channels in the plasma membrane of the cell[99]. Smooth muscle depends largely on external sources of calcium for this internal increase in Ca^{++}. In skeletal muscle, the Ca^{++} is largely recycled between the sarcoplasmic reticulum and the cytoplasm[100]. However, there are several sites of calcium storage in smooth muscle cells. Mitochondria are known to store Ca^{++}, but studies by Somlyo et al. have revealed that the Ca^{++} content of mitochondria in smooth muscle cells is low. Somlyo has shown that in active smooth muscle cells mitochondria play little role in the rise of intracellular calcium associated with contraction[101]. Furthermore Somlyo et al. have described in smooth muscle cells close connections between the sarcoplasmic reticulum and the plasma membrane[102]. It is thought that calcium in the sarcoplasmic reticulum is released by agonists acting on the plasma membrane to initiate degranulation of the sarcoplasmic reticulum and thus induce a contraction through the increased intracytoplasmic Ca^{++}[102].

Another source of calcium present in the cell is that which is bound to contractile proteins and other calcium binding proteins. This calcium will exchange rapidly with external Ca^{++} during potassium induced smooth muscle contractions[100].

In 1979 Bolton proposed that there were two mechanisms of increasing myoplasmic calcium to cause a contraction. The influx of extracellular Ca_o^{++} could arise from two sorts of plasma membrane receptors. The first is called a potential operated channel and is opened by membrane depolarization. The second is linked to specific receptors and is called a receptor operated channel[103]. The opening of receptor operated channels would be called pharmaco-mechanical coupling as opposed to electro-mechanical coupling for membrane depolarization. Furthermore, the receptor operated channels would release intracellular calcium from the sarcoplasmic reticulum which would be refilled by absorbing Ca^{++} from extracellular stores. It is suggested that the initial phase of a noradrenalin contraction is caused by the receptor operated channel with the ensuing maintained contraction due to the release of Ca^{++} from the sarcoplasmic reticulum[100]. The rise in myoplasmic Ca^{++} then allows four calcium molecules to bind to a protein called calmodulin which then binds to a myosin light chain kinase. This Ca^{++}_4-calmodulin myosin light chain kinase complex then acts on the light heads of myosin molecules to phosphorylate them. It is only in this form that the myosin heads can interact with actin. Thus the thick filament binds to the thin filament in the contraction process. Termination of contraction occurs with dephosphorylation of the myosin light chain by a myosin light chain phophorylase after Ca^{++} has been removed from the contractile filaments[104].

The primary difference between smooth and striated muscle in the process of contraction is the lack of troponin on the actin filament of smooth muscle. The troponin of skeletal muscle will bind the Ca^{++} calmodulin complex and then switch on a region of the thin filament to allow actin to bind to phosphorylated myosin heads. In smooth muscle there

is no toponin to regulate the activation of actin by calcium in smooth muscle contraction[104].

There are a number of hypothesis for the control of smooth muscle contraction:

1) Experiments have shown that phosphorylation of myosin light chain kinase by a cyclic AMP dependent kinase will decrease it's affinity for binding to the Ca^{++}_4-calmodulin complex. It has been suggested that Beta receptor activation might lead to increased levels of cyclic AMP which would then mitigate the contraction process[105].

2) There have also been reports of an actin linked protein called leiotonin which function like troponin to act like an on-off switch[106]. A second troponin like molecule has also been isolated[107]. A further possible Ca^{++} binding site for sensitive control of contraction is the myosin light chain itself[104].

3) Another possible mechanism of Ca^{++} control is postulated by Sobue. He discovered a large protein called caldesmon which interacts with tropomyosin bound actin to prevent myosin binding. Ca^{++}_4-calmodulin complexes would bind to caldesmon and then liberate actin sites for myosin binding[108].

Relaxation of Smooth Muscle

IN 1979 Murad proposed elevated levels of myoplasmic cyclic GMP as the signal for muscle relaxation. He noted that exogenous cyclic GMP or 8-bromo-cyclic GMP would relax smooth muscle preparations. Furthermore, he went on to speculate that smooth muscle relaxation by nitrous compounds was mediated by cyclic GMP accumulation[109]. In closely related experiments it was noticed that phosphodiesterase inhibitors added to the medium would

shift the dose response curve for nitroprusside to the left when either relaxation or cyclic GMP levels were examined[110].

By 1983 evidence for the role of cyclic GMP in relaxation of smooth muscle was overwhelming:

a) Nitroprusside and other nitrate vasodilators produced time and dose dependent increases in cyclic GMP in smooth muscle cells.

b) 8-br-cyclic GMP given exogenously would produce relaxation in smooth muscle.

c) Activation of cyclic GMP protein kinase is observed with high concentrations of nitroprusside.

d) Substrates (proteins) for cyclic GMP dependent protein kinase have been observed in vascular smooth muscle.

e) Phosphodiesterase selective for the inhibition of cyclic GMP hydrolysis will relax coronary smooth muscle[110].

Furthermore distinction should be made about the effects of receptor stimulated cyclic GMP production which required the influx of Ca^{++} [110]. This process was probably caused by acetylcholine acting on smooth cells to initiate contractions which were attenuated by cyclic GMP elevations by E.D.R.F. release from endothelial cells[111].

Murad et al. have also discovered that nitroprusside and endothelial dependent vasodilators (acetylcholine, histamine, A23187, A.T.P., thrombin, and trypsin) will all increase cyclic GMP levels in smooth muscle cells of rat aorta. They will all also increase cyclic GMP dependent protein kinase activity and alter the incorporation of ^{32}P into numerous smooth muscle proteins. Furthermore, various inhibitors of phospholipase and lipoxygenase will prevent the endothelial dependent effects from occuring. Finally a smooth muscle protein identified as myosin light chain was found

to have decreased ^{32}P incorporation after either type of vasodilator was given[112]. What is still not known is how the myoplasmic Ca^{++} is decreased and whether the elevated cyclic GMP levels act to increase myosin light chain phosphatase or to decrease myosin light chain kinase.

VI Architecture of Arteries and Function of Endothelium

Architecture and Ultrastructre of Arteries

Arteries are generally subdivided into either elastic or muscular arteries. Elastic arteries comprise large diameter vessels with both smooth muscle cells and elastic lamina in the media. As vessels extend peripherally their caliber diminishes and the predominant arteries in the periphery are muscular arteries. These arteries have more smooth muscle and less elastic lamina in their media. As one extends peripherally along an elastic artery it may change into a muscular artery or remain elastic as in the vertebral arteries. The media of arteries decreases as the diameter decreases; where the media contains only 1-2 layers of smooth muscle cells the vessels are now called arterioles.

Blood vessel walls are composed of 3 layers: tunica intima, tunica media, tunica adventia. The intima consists of a single layer of endothelial cells, a basal lamina 80 nm. thick, and a subendothelial layer.

Tunica Intima.

The endothelium consists of a single layer of squamous cells with a thickness of 0.2-0.5 um. They form a continuous layer and are connected to each other by junctional complexes. The junctional complexes include occluding junctions and gap junctions which are possible sites of cell to cell comunication. There are pinocytic vesicles within the endothelial cell which are implicated in transendothelial transport.

The subendothelial layer is composed of collagenous bundlles, elastic fibrils, smooth muscle cells and occasional fibroblasts. With age, this layer undergoes a progressive thickening by hyalization and fibrosis. As vessels

become smaller to form arteries and arterioles the intima is composed of only endothelial cells and a basal lamina.

Tunica Media.

This layer consists of smooth muscle cells; elastic lamina, collagenous fibrils, and a network of elastic fibrils. The only cell present in the media is the smooth muscle cell.

In large arteries there are 40-60 fenestrated elastic lamina and this number decreases as the vessels become more peripheral. Smooth muscle cells are arraigned between the laminae in the shape of a spiral. The whole network of structures is held together by collagenous fibrils. In muscular arteries there is a distinct internal elastic lamina between the intima and the media and an external elastic lamina between the media and the adventitia. Between the two lamina are several layers of elastic lamina and as the vessel size decreases there are fewer layers of elastic laminae in the media. Smooth muscle cells of muscular arteries form a spiral and those close to the internal or external elastic laminae are attached to these laminae. There is less connective tissue present in the media of muscular as compared to elastic arteries. Small arteries and arterioles either lack elastic lamina or have indistinct internal elastic lamina.

Tunica Adventitia.

The outermost arterial layer is dependent on the location and type of the artery. In the cerebral circulation an adventitia is non-existant. In all other arteries the adventitia is composed of dense fibroelastic tissue without smooth muscle cells. In elastic arteries this layer comprises about 10% of the vessel wall. In muscular arteries the proportion of the wall consisting of the adventitia can be up to 50%. In arterioles and venules the adventitia

forms a continuous narrow sleeve of collagen fibers which merge imperceptibly with the surrounding connective tissue[113].

Physiological Function of Normal Endothelial Cells

Endothelial cells individually have a surface area of 20x50 micron[114], however, collectively their surface area is 1000 m^2.[114]. Ultrastructurally, there are two types of endothelial cells: continuous endothelium seen in elastic and muscular arteries and veins, and fenestrated endothelium which is characteristic of visceral capillaries[115].

Endothelial cells come in constant contact with the entire circulating blood volume. In the protection of their basal surface endothelial cells serve two functions:

1) a selective permeability for molecules, and a nonthrombotic covering of the basal surface from platelets and clotting factors[115].

2) synthesis of collagen, fibronectin, and other proteins for maintenance of the basal membrane as well as collagenase for remodelling of the basal lamina.

Markers of Endothelial Cells.

The Weibel - Palade body is a specific organelle for endothelial cells, however there is a variation in it's frequency and ease of demonstration from species to species. A more universal marker is Von Willebrand factor (VIII-VW.) antigen[114].

Permeability Properties of Endothelial Cells.

There are several studies which support the existence of 2 types of lipophylic pores from the luminal to adluminal surface of endothelial cells[115]. The smaller but more ubiquitous pore measures 60-90 A^o in diameter with larger but fewer pores of the order of 500 A^o[115]. There are two types of

junctions between cells: the gap junction and the tight junction (macula occludens). Studies have shown that these junctional areas may be functionally dynamic[115].

Furthermore, there are two known transport systems through cells: endocytosis and trancytosis[116,117]. Endocytosis may be receptor mediated or non-receptor mediated. Low density lippoproteins (LDL) may interact with the transport system via all three mechanisms[116,117]. The glycocalyx is a carbohydrate matrix surrounding the the endothelial cell. In the junction between cells it is known as intercellular cement[115]. It is known that certain alterations of the glycocalyx will result in increased permeability through the endothelial cell layer[118]. Endothelial cells can also be actively stimulated to increase their permeabilty by kinins, histamine, PGE_2, PGI^2, and the leukotrienes C_4, D_4, and E_4[119].

Prevention of Coagulation.

The major pathway for the endothelial cell to prevent platelet aggregation on it's surface is the synthesis of heparin sulfate on it's glycocalyx. These heparin molecules activate anti-thrombin III to deactivate thrombin. During platelet aggregation an endoglycosidase is released which degrades endothelial cell surface heparin. Thus a balance is struck whereby a communal relationship between platelets and endothelial cells exists. However imbalances favoring platelets could seriously harm endothelial cells. The endothelial cells also secrete Protein C which is activated by thrombin formation and which acts to limit coagulation by preventing the activation of factors V and VIII which accelerate the formation of thrombin. Patients who are deficient in Protein C are subject to spontaneous episodes of thrombophlebitis[179].

Furthermore PGI_2, a potent anti-platelet aggregator is synthesized and released from endothelial cells[114]. The endothelial cell also will secrete tissue plasminogen activator to form anti-thrombin III; this synthesis is downgraded by thrombin[114].

On the converse side, endothelial cells also secrete Von Willebrand factor (VIII VW.) which is necessary for the binding of platelets to collagen. Animals deficient in this factor have a marked reduction in spontaneous and diet induced atherosclerosis[115].

Metabolic Agents of Endothelial Cells.

Besides Von Willebrand factor, endothelial cells also synthesize angiotensin converting enzyme for the production of the vasoconstrictor angiotensin II and the inactivation of the vasodilator and permeability agent bradykinin[114]. They also secrete a growth factor similar to platelet derived growth factor (P.D.G.F.) and a growth factor for endothelial cells[114]. They will also synthesize colony stimulating activity for granulocyte - macrophage colonies; during endotoxemia this colony stimulating activity is increased[114].

Control of Vascular Tone

There are three well known mechanisms by which endothelial cells exert an influence over vascular tone. The first is extracellular and depends on an enzyme localized to the surface of the endothelial cell. This is called angiotensin converting enzyme and it combines the two functions of activating angiotensin I to the active form angiotensin II as well as degrading active bradykinin to an inactive form. The Km for oxygen for this enyme is 30 mm. Hg. and thus it is possible that under hypoxic conditions this enyme could be inhibited rendering the vessel susceptible to vasodilation from bradykinin which would be unopposed by angiotensin II. The other two known agents for control of vasodilation are PGI_2 and EDRF[18,180]. Rubanyi has reported that

hypoxia induces the endothelial cells to secrete a vasoconstrictor substance[181], although Busse has reported opposite findings[180].

Endothelial Cell Receptors.

Endothelial cells have receptors for mumerous agents such as: growth factors, beta-LDL, HDL, acetyl LDL,chylomicrons, insulin, angiotensin converting enzyme, and lipoprotein lipase. There are also binding sites for polymorphonuclear leukocytes and lymphocytes, and as well endothelial cells can express blood group antigens[114,120].

The hormonal receptors discovered so far on endothelial cells are: catecholamines (α_1, α_2, β_1, β_2,), serotonin ($5HT_1$, $5HT_2$), angiotensin II, oxytocin, vasopressin, kinins (bradykinin, metheonylysylbradykinin, lysylbradykinin,), histamine (H_1, H_2), prostanoids, adenosine, ATP, ADP, AMP, and two cholinergic receptors (M_1, M_2)[119].

VII Atherosclerosis

Since the mid - nineteenth century pathologists have argued about the pathogenesis of atherosclerosis. In 1844 Rokitansky proposed a thrombogenic theory whereby blood elements encrusted on the vessel wall could undergo atheromatous transformation. This was rebutted by the infiltrative theory of Virchow in 1853 in which early lesions of atherosclerosis were based on a loosening of the connective tissue ground substance of the intima due to an infiltration of plasma constituents. This begat a neoplastic proliferation of connective tissue and ground substance following which degenerative changes occured[121].

To understasnd the theories for the pathogenesis of atherosclerosis it is necessary to understand the morphology of lesions and the natural history of the disease. This is well reviewed by Haust in "The Natural History of Human Atherosclerotic Lesions[122].

Morphology of Lesions

There are three broad classifications of atheromatous lesions: early, advanced, and complicated lesions.

Early Lesions.

Early lesions can begin in the intima of either elastic or muscular arteries in one of three forms:

1) Gelatinous Elevations - These lesions are grey gelatinous circumscribed elevated areas consisting of focal edema of tissue due to an insudative process. In small intimal lesions, the edema consists of albumin containing fluid and stretches only beneath the superficial layers. In larger lesions the insudation may extend to the internal elastic lamina and may have fibrin mixed with it's serous component.

2) Fatty Dots or Streaks - These are tiny yellow dots or streaks and if large are elevated above the intimal surface. In the subendothelial layer are found smooth muscle cells with fat droplets within their cytoplasm. Fatty streaks have 9 times the lipid content of lesion-free intima; and 65-80% of this lipid is cholesterol[123]. Larger lesions may have numbers of intracellular fat droplets to the point where the cell is practically composed of fat droplets. These are called myogenic foam cells and may disintigrate spilling their fat droplets into the ground substance. Mononuclear cells and histiocytes may then phagocytose the fat to become macrophagic foam cells.

3) Microthrombi - These are either platelets, fibrin or mixed attachments to the intima by either a pedicle or extend over the surface in a mushroom shape.

Advanced Lesions: These are of two types:

1) Pearly - White Fibrous Plaques - These plaques consist of layers of avascular fiberous tissue consisting of collagen and elastic tissue fibers embedded in a glycoaminoglycan rich substance with smooth muscle cells interspersed throughout. The plaques may be covered by mural thrombii which may encourage granulation tissue to form at the base of the lesion. The origin of the capillaries in the granulation tissue is uncertain as no capillaries are present in the inner arterial media.

2) Atheromatous Plaques - These are composed of an atheromatous pool of accellular, amorphous, proteinaceous and fatty substances intermingled with lipoid bodies and cholesterol crystals. Smooth muscle cells in the vicinity of the atheroma contain fat droplets and have the appearance of foam cells. This pool is covered by a fiberous cap with an architecture akin to the pearly - white fiberous plaques.

Complicated Lesions: These can occur in three Forms:

1) Calcification - Dystrophic calcification occurs in either the atheroma or the fiberous plaque or both leading to stiffness and loss of elasticity of the vessel wall.

2) Ulceration - This occurs when there is an increase in the size of the atheroma relative to the fibrous plaque causing a break in the fiberous cap. It may occur by relatively greater atheroma enlargement than cap enlargement leading to breakdown by hemodynamic forces or by hemorrhage into an atheroma.

3) Thrombosis - Thrombosis of the artery can occur due to deposition of thrombii on an ulcerated or lacerated fiberous plaque.

4) Hemorrhage - Bleeding from capillaries in the core of the atheroma may occur since these capillaries lack structural support other than the atheroma. This may cause ulceration of the plaque by enlarging the atheroma or it may lead to vessel thrombosis.

Pathogenesis of Atherosclerosis

These lesions are believed to form a continuous spectrum of a disease process. This precursor product relationship is supported by studies by Stary who examined the coronary vessels in a series of children and young adults. He found fatty streaks at the same anatomical sites as fibrous plaques in older aged vessels[124]. Furthermore McGill has demostrated that severe coronary atherosclerosis is preceeded by fibrous plaques in adulthood which are also preceeded by fatty streaks in teenagers[125].

There are several theories concerning the pathogenesis of atherosclerosis and each one tends to focus on one aspect of cell injury. In all likelihood components of all these represent the ultimate cause of atherosclerosis.

1) Monoclonal Theory.

The smooth muscle cell by it's proliferation represents a large part of the intimal hyperplasia accompanying atherosclerosis. However examination of isoenzymes of glucose-6-phosphate dehydrogenase in women heterozygous for glucose-6-phosphate deficency have shown that of two possible isoenzymes only one was found in the proliferating smooth muscle cells in the atheroma[126]. This indicates a benign smooth muscle cell tumor selected by growth factors or a mutation for the invideous process that caused the atherosclerosis.

2) Lysosomal Theory.

Lysosomal dysfunction results in an accumulation of lipid in cells in the atheroma. Further increase in lipid would further impair lysosomal activity thus creating a viscous cycle until the cell bursts[126].

3) Clonal Senescence Theory.

This theory is based on a failure of cell to cell regulation of growth. Organ size is determined by growth factors and inhibitory factors called chalones. Advocates of this theory believe that there is a selective loss of chalone producing smooth muscle cells leading to an imbalance of growth promoting smooth muscle cells[126].

4) The Response to Injury Theory.

This theory championed by Russel Ross has received most of the attention from investigators of atherosclerosis. It's credibility stems from the fact that it can draw together most of the known theories for atherosclerosis and array them in a neat argument for the etiology of atherosclerosis.

In it's most basic form this theory proposes that endothelial injury is the initiating factor in the genesis of atherosclerosis. Following the endothelial injury a variety of factors can ensue to promote atherosclerosis. They have been recently reviewed by Ross[127] but will be mentioned here in a

condensed form.

Monocytes and Macrophages.

Studies by Faggiotto et al have demonstrated in animals with diet induced atherosclerosis the attachment of monocytes to junctional areas between endothelial cells throughout the aortic tree. These monocytes then seemed to move to the subendothelial spaces and then accumulate lipids to become foam cells. These lesions closely resemble the fatty streaks seen in humans. Progression of these lesions leads to further subendothelial migration by monocytes which later become macrophages. Enlargement of the lesion would also occur by the migration of smooth muscle cells into the lesion. Faggiotto also showed that these lesions first occured at branches and bifurcations of the aorta, however they later occured in other anatomic sites. Furthermore the extent and stage of the disease was directly correlated with the increase in hypercholesterolemia, the level of blood cholesterol, and the duration of the hypercholesterolemia[128,129]. The same sequence of events has been described in rats on a high cholesterol diet and the precipitating event is an accumulation of monocytes at junctional areas of the endothelium up to 50 times normal[130]. It has also been shown that the monocyte will adhere preferentially to injured or regenerating endothelial cells in culture[131].

Once macrophages have begun to migrate inside the endothelium they begin to scavenge low density lipoproteins (LDL). The cholesterol from these lipids undergoes cycles of esterification and hydrolysis and accumulates in the cell to form foam cells. High density lipoproteins (protectors against atherosclerosis) will act to expell the free cholesterol from macrophages[132].

From their subendothelial position, macrophages can injure neighboring cells by secreting toxic substances or promote cell populations by secreting potent growth factors[127].

Smooth Muscle Cells

These cells are prominent because of their proliferation in most of the lesions associated with atherosclerosis. Furthermore smooth muscle cells have receptors for LDL and growth factors and can accumulate lipids to form foam cells as well as secrete enormous amounts of connective tissue matrix[127]. It has been demonstrated that after endothelial injury smooth muscle cell replication occurs in the media with subsequent migration into the intima. Smooth muscle cell proliferation in the intima appears to be generated only from certain differentiated smooth muscle cells[133].

Smooth muscle cells from proliferating lesions in rats, which had previously had the endothelium removed from their aorta, responded to an endogenous mitogen like platelet derived growth factor (PDGF). These intimal smooth muscle cells, although expressing fewer PDGF receptors, grew at the same rate as medial smooth muscle cells[134]. This would possibly be an autocrine control on the tumorogenic growth of a population of proliferating smooth muscle cells. Finally endothelial cells secrete a heparin like glycosaminoglycan into the basal ground substance. Endothelial damage or platelet adherence and release of heparinase and PDGF damages this glycosaminoglycan layeer and possibly selects a phenotypic cell line corresponding to the monoclonal theory[135].

Platelets

In hypercholesterolemic states platelet interactions with the vessel wall are important in the genesis of atherosclerosis[128,129]. Undamaged endothelium normally prevents platelet adherence through the formation of heparin and

prostacyclin. Any morphologic or functional change in endothelial cells may allow platelet adherence[127]. Furthermore the endothelial cells protect the the underlying subendothelial connective tissue and foam cells (if present) from platelet adherence through factor VIII-VW. (Von Willebrand factor)[114]. Studies with platelet interactions and macrophages at areas of exposed endothelium suggest later proliferation to smooth muscle lesions due to the mitogen platelet derived growth factor (PDGF)[116].

PDGF may be important in atherogenesis because it is chemotactic and mitogenic for smooth muscle cells[127]. Several other platelet substances are chemotactic and can further the injury to vessel walls by attracting monocytes and smooth muscle cells[127]. PDGF like activity has also been observed in substances released from macrophages[127], smooth muscle cells[134], and endothelial cells[114].

In 19[illegible] studies by Moore with repeated endothelial injury in rabbits on a normal diet demonstrated the formation of atherosclerotic lesions. When anti-platelet anti-serum was administered before the endothelial trauma the platelet count was significantly diminished and no atherogenesis could be found[135]. A second study by Freidman in 1977 demonstrated that thrombocytopenia decreased proliferation of smooth muscle cells and prevented formation of atherosclerosis[136]. Thus a suspected mitogen from platelets is implicated in the genesis of atherosclerosis[127].

Lipids and Atherosclerosis

It has been repeatedly shown in human and experimental animal mopdels that elevated plasma lipoproteins particularily low density lipoproteins (LDL) and very low density lipoproteins (VLDL) are associated with an increased incidence of atherosclerosis[137]. Furthermore studies have demonstrated that

although the endothelial cell and smooth muscle cell can synthesize all the major lipids necessary for structure and function; most of the lipid and cholesterol in the necrotic core of plaques comes from plasma lipoproteins (mostly LDL and VLDL)[138,139].

Control of Plasma and Fatty Acid Metabolism

Exogenous cholesterol from intestinal absorption is transported in lipoproteins containing apolipoprotein B-48. The absorbed chylomicrons from the intestines are acted upon by lipoprotein lipase enzymes on the luminal side of capillary endothelium. This enzyme allows formation of chylomicron remnants and the exit of triglycerides to cells. The chylomicron remnants are processed by hepatocytes to VLDL which now contain apolipoprotein B-100 and these are now released into the plasma[140]. The VLDL molecules are then processed by lipoprotein lipase enzymes in capillaries to Intermediate Density Lipoproteins (IDL) and LDL and can exit plasma by binding to LDL receptors on liver and extrahepatic tissue[140].

Following contact with receptors, LDL particles are absorbed by a process of absorbtive endocytosis. These endocytic vesicles then travel through the cytoplasm to cell lysosomes where the LDL is broken down to cholesterol esters and protein moities by hydrolytic enzymes. The cholesterol esters then are hydrolyzed by an acid cholesterol esterase and the free cholesterol then can enter the cytoplasm. Cytoplasmic cholesterol is used for membrane synthesis but has three other functions:

1) to inhibit the enzyme hydroxymethylglutaryl-CoA (HMG-CoA) reductase thus suppressing endogenous production of cholesterol.

2) to activate acyl-CoA cholesterol acyltransferase (ACAT) which reesterefies cholesterol for storage.

3) to shut off synthesis of the LDL receptor which thereby prevents further uptake of LDL molecules and increases plasma LDL levels[139,140].

With suppression of the LDL receptor further accumulation of plasma LDL will occur as intestinal absorbtion occurs but little of the LDL is metabolized. Eventually removal of LDL molecules occurs through poorly understood and inefficient pathways[140].

Brown and Goldstein have demonstrated that in familial hyperlipidemic states there are few or nil LDL receptors on cells[141]. Furthermore they have shown that when LDL cell receptors are deficient, Intermediate Density Lipoproteins (IDL) shunt to LDL to further increase LDL levels[140]. HDL prevent the atherogenesis of LDL by promoting the efflux of cholesterol from cells and therby increase LDL surface receptors[139].

LDL and Atherogenesis.

LDL can produce both morphologic and functional changes in endothelial cells[143,144], and this might allow penetration of atherogenic molecules to the subendothelium. Furthermore, there are two methods by which LDL could be transported across endothelial cells to the subendothelial connective tissue. Endocytosis could occur by a receptor mediated or a non-receptor mediated process whereby LDL passes first to lysosomes and then to the abluminal side of the cell[116]. Trancytosis, an alternative method for trans-cellular transport of cholesterol, may occur. This transport system is mediated via plasmalemmal vesicles[117].

Hypercholesterolemia may also subtly affect the plasma membrane of the endothelial cell by altering it's cholesterol to phospholipid ratio. This might explain the increased malleability of endothelial cells allowing cellular retraction at branches and bifurcations to ultimatly expose the intimal connective tissue to platelets[127].

Studies have shown that following endothelial injury endothelial cells that were released from contact inhibition showed a great increase in LDL uptake at the periphery of the wound but not in cells that remained contact inhibited[134]. Thus any chemical, rheologic, or mechanical injury to the endothelium may promote LDL uptake in some endothelial cells therby compounding the atherogenesis.

It has been noted that cholesterol feeding (even as briefly as three days) will promote increased endothelial cell turnover well before cholesterol infiltrates the arterial endothelium[142]. Elevated cholesterol levels may thus damage endothelial cells and allow platelets and LDL to penetrate through to subintimal areas. Furthermore damage to the endothelial cell may alter it's functional capacity for transport or permeability without morphologically destroying the cell.

It is known that high levels of LDL[143] and cholesterol[144] have been found to functionally impair endothelial cells without morphologically damaging them[143]. This effect is greatly mitigated by HDL[145]. Furthermore endotoxemia which is known to damage endothelial cells[146] may also contribute to endothelial damage by affecting LDL oxidation and rendering it more toxic to endothelial cells[147]. This may be one way that increased permeability of the cell membrane occurs with sepsis where there are concomitant elevations of triglycerides[149]. Studies have also shown that if the reticulo - endothelial system is blocked, endothelial damage occurs and endothelial dependent vasodilator responses are attenuated[148].

It is known that hypertension alone has the capacity to alter endothelial morphology, permeability, and cell turnover[150,176]. The effects of hypertension may be multiple but even fluid sheer stress will result in endothelial cell dammage[151]. Even reducing the lumen of an artery by 25% of

it's normal caliber to increase fluid stress caused loss of most of the endothelium at the stenosis within 3 minutes of clipping the artery[161]. Unfortunatly this endothelial dammage could have been due to the clip itself on the adventitial side of the artery. However spasm alone mediated by noradrenalin can induce endothelial damage allowing gaps to form between endothelial cells[162].

Lipids and the Glycocalyx.

The glycocalyx is a carbohydrate - rich matrix which envelops all sides of the endothelial cell and at junctions between cells acts as intracellular cement. On the basal membrane it extends down into the extracellular matrix. This layer may represent a barrier to the permeation of plasma macromolecules[152]. Studies with hypercholesterolemic rabbits have demonstrated an increased permeability of this glycocalyx[118].

Lipids and Subendothelial Damage

Glycosaminoglycans in the connective tissue matrix of the subendothelial areas can selectively bind LDL. The increased intimal glycosaminoglycan content found in fatty streaks and fibromuscular lesions may be associated with intimal lipid accumulation[152]. In contrast, HDL has been shown to break up complexes between glycosaminoglycans and LDL and reduce lipid deposition in such areas[153]. Furthermore, proteoglycans in the extracellular matrix bind apo-B lipoproteins and are then taken up by macrophages and possibly smooth muscle cells leading to massive accumulation by these cells of cholesterol esters[154]. Walker has also shown by in vivo studies of hypercholesterolemic animals subjected to endothelial denudation that monocyte - macrophage like cells accumulate in the intima and lipid accumulated in both the intima and the media. This is in contrast to control animals with normal cholesterol levels also subjected to endothelial denudation in vivo. In these animals

there was no intimal accumulation of macrophages nor was there lipid accumulation. In both groups healing of the endothelium was of the same duration[163].

Studies by Duff have shown that the first change in the arterial wall of cholesterol fed rabbits is an increase in the ground substance between the endothelium and the internal elastic membrane. Following this there is deposition of extracellular [illegible] in the matrix[155].

It is also known that [illegible] (particularily that from hyperlipemic serum) may act as a mitogen and lead to smooth muscle cell proliferation[156]. Furthermore Imai has shown that oxidized products of cholesterol are particularily toxic for endothelial and smooth muscle cells[157]. With endothelial death, areas of exposed collagen would attract platelets which could degranulate and attract macrophages to the inflammatory area. Platelets adhere to the exposed collagen by Von Willebrand factor (factor VIII-VWF.) which is a necessary co-factor and is secreted by endothelial cells[152]. Functional impairment of platelet reactivity with collagen as in Von Willebrand's disease will prevent intimal proliferation[158]. However in the presence of diet induced hypercholesterolemia, animals made thrombocytopenic prior to endothelial dammage will develop intimal thickening[159].

Furthermore, experiments have shown that regenerating neo-endothelium more so than deendothelialized areas, will accumulate lipid with resulting intimal thickening. This neo-endothelium has been studied up to a year follwing regeneration and after one year still shows some functional derangements[160]. If the regenerated endothelial cell can transport the lipid to the intimal areas more effectively than normal cells a causal relationship between endothelial cell damage and atherogenesis is indicated.

In conclusion LDL and cholesterol have the ability to dammage endothelial cells. This can be through cell death or functional derangement[161,162]. Additional risk factors for atherosclerosis such as smoking, hypertension, homocystinuria, and endotoxemia may aggravate this damage[160,176]. This would expose the subendothelial areas to LDL, platelets, and macrophages with their toxic substances and mitogenic factors for smooth muscle cell proliferation[127].

VIII Endothelium Dependent Relaxation and the LA/N-CP Rat

Justification for Animal Models in Research

Almost all animal models of human atherosclerosis have as a principal etiological agent chronic hyperlipidemia. Reasons for this are:

1) Many hyperlipidemic models have lesion components similar to human atherosclerosis.

2) Non-hyperlipidemic models do not result in chronic lesions resembling atherosclerosis.

3) To date there are no reports of human populations with low serum lipids and a high incidence of advanced atherosclerosis.

4) Accelerated plaques in human familial hyperlipidemia closely resemble slowly developing lesions in humans who succumb to the severe effects of atherosclerosis.

5) Accelerated plaques resulting from chronic hyperlipidemia in animals resemble slow developing human lesions[164].

However there are species differences with respect to anatomically different areas which are involved by atherosclerosis. In rabbits the lower abdominal aorta is most heavily involved with atherosclerosis while in rats the lesions occur proximally on the thoracic aorta. In humans there is heavy involvement of the thoracic aorta with less involvement of the abdominal aorta[164].

Natural Versus Diet Induced Arterial Lesions.

In many animal models atherosclerosis is induced by a high cholesterol (3%) plus fat (4-8%) diet. In rabbits such a diet will induce atherosclerosis but this is associated with excessive fat and cholesterol in most animals and a dramatic hypercholesterolemia. Lesions in such animals are composed of

extracellular lipids, and fibromuscular caps are not common[165]. Furthermore in diet induced hypercholesterolemia because of the rapid increase in cholesterol there are biochemical changes in smooth muscle cells and lipophage movement into arterial walls from the blood[166].

The rat model used for this experiment is the corpulent rat which when first developed by Koletsky showed rampant atherosclerosis from the genetic equivalent of a Type IV hyperlipidemia and hypertension[167,177]. Because the combination of these two genes created breeding problems and experimental design problems, two cogenic strains of rats were developed to separate the hyperlipidemia from the hypertension. These two strains were the LA/N-CP and SHR/N-CP rats[168].

The LA/N-CP rats have been studied by Russell and coworkers and shown to be hyperphagous, hyperlipidemic, and hyperinsulinemic with only mild elevations of cholesterol on a normal diet. Studies of these rats at 9 months of age demonstrated early onset of atherosclerosis in males damaging the endothelium of the thoracic aorta and exposing sub-endothelial structures[168]. Furthermore at nine months of age the males suffer myocardial lesions while the females are protected in a manner analagous to that in humans[178].

Vascular Relaxation in Arteries with Endothelial Dammage

Studies with hypertensive rats as compared to normotensive rats have shown that there is an inhibition of the relaxation induced by endothelium dependent relaxants (acetylcholine and A23187). The authors suspected that this might be caused by endothelial dysfunction resulting from the hypertension[169].

Further studies by Criscione have demonstrated a reduction in the relaxation of mesenteric arteries of hypertensive rats as compared to normotensive rats with acetylcholine as a relaxing agent. Furthermore they

demonstrated that the endothelium of the hypertensive rats was much more labile and thus more easily damaged than control rats[170].

Animal studies on diet induced hypercholesterolemia in dogs, rabbits, and monkeys have demonstrated that there is decreased relaxation of arterial ring preparations with endothelium dependent vasodilators[171,172,173,174,175]. There was no failure of relaxation with endothelium independent relaxants (except in severely involved vessels) with no increase in contractility from alpha-agonists[171]. With progression of fatty streak formation endothelium dependent relaxants progressively failed to relax the vessels. The endothelium in these vessels was able to release E.D.R.F. in bioassay experiments and the authors proposed that the defect was possibly due to increased intimal thickness[171]. However Kappagoda has reported that there is reduced EDRF release in bioassay studies of cholesterol fed animals[182]. Freiman et al. have shown that hypercholesterolemia itself does not affect the E.D.R.F. formation, release or action. However they found that the failure of relaxation was due to an alteration of receptor sites for endothelial dependent agonists or the effector mechanism for formation of E.D.R.F.[172].

All the previous studies mentioned here have used diet induced atherosclerosis to determine if there is a reduction in endothelial dependent relaxation. The purpose of this study was to evaluate the effect of genetically induced atherosclerosis in LA/N-CP rats on a normal diet to determine if there is a failure of endochelium dependent relaxation in this animal model of spontaneous atherosclerosis.

IX METHODS

General Overview

In this study 8 male and 8 female LA/N-CP rats with equal numbers of lean control rats were compared at 6 months of age. In the second part of the experiment 4 male and 4 female LA/N-CP rats with comparable lean controls were examined at 9 months of age. All animals were maintained on a standard rat diet (Wayne Lab Blox, Allied Mills, Chicago, Il.). The rats were weighed before they were anaesthetized with sodium pentobarbital (20 mg/kg, I.P.). After a mediam sternotomy blood samples were taken from the right side of the heart and then the thoracic aorta was gently removed and immediately placed in iced Kreb's buffer solution (composition mmol/l: NaCl 116.0, KCl 5.4, $CaCl_2$ 1.2, $NaHCO_3$ 22.0, NaH_2PO_4 1.2, glucose 10.1, $MgCl_2 6H_2O$ 1.2, $CaNa_2EDTA$ 0.023, ascorbic acid 1.1). The blood samples were centrifuged at 2000 rpm for 10 min. and the serum was then collected and stored at -70° for analysis of glucose, cholesterol, and triglyceride.

All extraneous tissue was then removed from the thoracic aorta by careful dissection, and the aorta was then cut into small rings (average size 2-3 mm.). In the first series of experiments with 6 month rats (n=4 for all groups) the endothelium was removed from one of the rings by inserting a pin through the vessel lumen and gently rolling the aortic ring on a piece of filter paper which was moistened with Kreb's buffer (DeMey and Vanhoutte 1982.[17]). Following this, all rings were placed on small triangular clips for organ bath experiments. In the second series of experiments on the 6 month animals (n=4 for all groups) the endothelium was removed from all arterial rings by the above method. In both series a similar sized sample of the aorta was examined by scanning electron microscopy (to determine the presence or absence of endothelium) both

before and after the experiment was completed. A second sample was subjected to sudan red staining for lipid or cholesterol deposition.

In the nine month animals (n=4 for all groups) the second series of experiments were conducted on the same ring segments 2 hours after the first series was concluded. This was due to a shortage of animals in the 9 month group. Before commencing the experiment, samples of the aorta were submitted for scanning electron microscopy (SEM) and sudan red staining. Following the conclusion of the first series of experiments on the 9 month rings, a small sample was reserved for examination by scanning electron microscopy and then the three ring segments with endothelium present underwent the de-endothelialization proceedure mentioned above. They were then re-suspended on the metal clips for the second series of experiments and on completion of this series of experiments samples of the artery were examined by SEM to demonstrate the absence of endothelium.

Histological Examination

Sudan Red Staining:

The specimens were placed in 70% alcohol for 2 minutes and then placed in a Sudan red solution which was intermitantly agitated and maintained at room temperature. After 15 minutes, the tissues were then placed in a 80% ethanol solution for 20 minutes and then washed in cold running water for 1 hour. The specimens were then visually inspected under low power magnification for sudaphilic lipid deposits (Duff and Macmillan 1949[183]).

Scanning Electron Microscopy:

Scanning electron microscopy (SEM) was performed on all aortas to determine the presence or absence of endothelium. The luminal surface of all samples was exposed by a logitudinal cut in the arterial ring and then the tissues were fixed with 2.5% gluteraldehyde with Milloning's buffer ($NaH_2PO_4 \cdot H_2O$, NaOH, glucose, $CaCl_2$) for 48 hours. Post fixation with 1% osmium tetroxide was then performed after which the tissues were then dehydrated in a graded series of ethyl alcohol solutions (50-100%). The tissues spent 15 minutes in each alcohol bath followed by three 10 minute periods in the 100& alcohol bath. The samples were then placed in a critical point dryer at 41° C and 1200 psi of carbon dioxide for five minutes after which they were mounted on aluminum stubs with silver glue, sputter coated with gold and then examined in a Phillips SEM 505 scanning electron microscope.

Organ Chamber Experiments

The arterial ring preparations were suspended in Kreb's buffer solution under an isometric force of 1.5 grams which was applied to the rings by 2 triangular clips on opposite sides of the vessel through which tension could be applied. A tension of 1.5 grams had previously been determined as the optimum by length tension analysis. Kulite (Model-797159-3) force tranducers were used to maintain and sense force changes which were then recorded on a Gould (Model-8188-4400-00) recorder. Tissue oxygenation was achieved with continuous carbogen ($95\%O_2$, $5\%CO_2$) perfusion of the Kreb's buffer which was maintained at a temperature of 37° C. Drug manipulations commenced after a 2 hour incubation period during which the Kreb's solution was changed every 30 minutes.

Study Protocol

In series 1A of the study using the 6 month rats, one of the arterial rings with endothelium was incubated with indomethacin (10^{-5} mol/l) for 30 minutes before acetylcholine (ACH) curves were started (bath no. 4). This was done to prevent prostacyclin participation in the relaxation (Forstermann et al., 1984). Following this the following drugs were added to each of the tissue baths; norepinephrine (NE; -7 log mol/l) was used to precontract the arterial ring after which ACH was given from -10 to -4 log mol/l with increments of 0.5 log mol/l to acheive a concentration curve of arterial relaxation. After the last dose of ACH (10^{-4} mol/l) nordihydrograiaretic acid (NDGA, -4.4 log mol/l) was added to bath number 3 containing a ring with endothelium. The only difference between the arterial rings was that bath number 2 contained a de-endothelialized arterial ring. Thus bath number 1 and 2 contrasted the effect of endothelial presence on relaxation by acetylcholine; while baths number 3 and 4 assessed the effect of enzyme blockers on acetylcholine induced relaxations.

After the last drug was given, the tissues were washed several times with Kreb's solution and then allowed to equilibrate (30 min.) after which they were then washed again and series 1B commenced. In this series atropine 10^{-5} mol/l was added to bath number 4 and NDGA (-4.4 log mol/l) was added to bath number 3 to assess the effect of these inhibitors on ACH induced relaxation. Then NE -7 log mol/l was used to precontract all the tissues after which Na nitrite (-8 to -3 log mol/l) was added in increments to bath numbers 1 and 2 while ACH (-10 to -4 log mol/l) was added to baths 3 and 4.

The aortic rings from the nine month rats underwent an identical sequence of events save that following the completion of series 1B the de-endothelialization proceedure was performd on the 3 rings with endothelium present. The tissues were then re-suspended at 1.5 grams of tension and washed with Kreb's solution and finally re-incubated with frequent washes for 2 hours. After this was accomplished series 2 of drug manipulations was performed on these tissues.

Drugs Used

The following pharmacological agents used in these experiments were kindly supplied by Sigma Chemical Co. (St. Louis, Mo,U.S.A.): norepinephrine bitartate, acetylcholine chloride, indomethacin, nordihydrograiaretic acid (NDGA), Na nitrite, atropine sulfate, clonidine, isoproterenol, methoxamine, calcium disodium ethylenediaminetetraacetic acid ($CaNa_2EDTA$).

Distilled water was used to prepare drug solutions which are expressed as the final drug concentrations in the bath (13.1 ml). Indomethacin was dissolved in equimolar sodium carbonate and NDGA was dissolved in dimethyl sulfoxide (DMSO). The concentration of DMSO in the bath did not exceed 0.025 moles per cent; this concentration has no effect on the tissues nor does it interfere with the interactions of drugs and tissues.

Serum Glucose, Cholesterol, and Triglyceride Determinations

Serum glucose, cholesterol, and triglyceride levels were analyzed by an automated system (Multistat III, Instrumentation Laboratories, Lexington, Kentucky, U.S.A.) according to the methods of Pinter et al[184] and Allain et al[185].

Statistical Analysis

All data are expressed as mean±SEM. Student's t-test for unpaired observations was used for analysis of weight, serum levels of glucose, cholesterol, and triglycerides, and grams of either relaxation or contraction. The ability of the tissues to react to the various drugs was evaluated by the ED_{50} value and the slope of the concentration curve following linear regression. A probit transformation of the dose response curve was also used after linear regression to give a probit ED_{50} and a probit slope to the linear regression. All comparisons between tissues were evaluated by analysis of varience and the probability of a difference between concentration curves was expressed as significant ($p<0.05$, $p<0.01$) or not ($p>0.05$) (Cook et al[186]).

RESULTS

Results of Scanning Electron Microscopy and Sudan Red Staining

Scanning eletron microscopy confirmed that at least 60% of endothelial cells remained at the conclusion of the experiments designed to require the presence of endothelial cells. All de-endothelialized specimens were shown by SEM to be denuded of their endothelial cell layer with no apparent damage to underlying structures. None of the specimens had demonstrable atherosclerotic lesions by either SEM or by Sudan Red staining.

Blood Chemistry and Body Weights for 6 and 9 Month Rats (Figure,76-77)

Nonfasting blood values revealed no significant difference in glucose levels for corpulent or lean rats of either sex. There was only a mild elevation in cholesterol levels for corpulent male and female rats. Corpulent male rats showed triglyceride levels that were elevated to twice the level of lean coutetparts. However corpulent female rats had very high concentrations of triglycerides which rose to ten times the level of lean counterparts and were five times greater than those of corpulent male rats. Male corpulent rats weighed almost twice as much as lean male rats while corpulent female rats weighed twice as much as lean females. The body weights of corpulent females were up to 75% of those of corpulent male rats.

Results of Contraction Curves

Response to Noradrenalin (Figure,78-83)

Noradrenalin (Nor) sensitivity curves showed no difference between corpulent and lean males or females at either 6 or 9 months of age. There

was a non-significant increase in Nor sensitivity in corpulent male rats between 6 and 9 months while lean male rats showed a significant increase in Nor sensitivity over the same age span. Both corpulent and lean female rats showed a significant lower % contraction at higher doses of Nor compared to male rats at 6 months of age which was lost by 9 months of age. There was no increase in sensitivity to Nor between 6 and 9 months of age in female rats.

At 6 months absolute values for Nor contraction (-4 log mol/l) were highest for corpulent and lean males (2.43±0.35gm. and 2.26±0.25gm. respectively) which differed significantly ($p<0.05$) from the force of contraction of females (1.13±0.035gm. for corpulent females and and 1.11±0.25gm. for lean females). By 9 months of age lean males and females showed the same maximum force of contraction (1.42±0.062gm. for males and 1.39±0.253gm. for females). There was no significant difference at 9 months of age for corpulent males (2.29±0.345gm.) and corpulent females (1.94±0.259gm.); however there was a significant difference ($p<0.05$) between 9 month corpulent and lean males with respect to absolute force of contraction. The difference in force of contraction between 9 month corpulent and lean females was considerable but not statistically significant.

Comparisons between the active tension of precontraction (Nor -7 log mol/l) in corpulent and lean rats at 6 months of age showed that there was no significant difference between force of contraction in endothelialized corpulent males and de-endothelialized corpulent females and lean males and females. There was a significant difference ($p<0.05$) between the active tension of de-endothelialized rings from corpulent and lean male rats as

well as the active tension in rings with endothelium for corpulent males (1.81±0.152gm.) and for lean males (.912±0.150gm.).

At 9 months of age there was a difference in the active tension between the de-endothelialized ring of the corpulent male (2.08±0.165gm.) and the lean male (1.42±0.152gm.) which was not significant. However there was no differnce in the active tension of rings with endothelium between corpulent males (1.16±0.144gm.) and lean males (1.21±0.324gm.). There was also no difference in the active tension of endothelialized rings between corpulent females (.412±0.150gm.) and lean females (.662±0.123gm.); although these values were significantly less ($p<0.05$) than male values.

Direct comparisons of the active tension for de-endothelialized rings of 6 and 9 month old rats with Nor -7 log mol/l showed that there was a decrease for lean males and corpulent females while corpulent males and lean females contracted to the same amount. However in endothelialized rings there was a significant decrease in the active tension between 6 and 9 months with Nor -7 log mol/l for corpulent males and for corpulent females while lean males slightly increased and lean females contracted to the same extent.

Response to Methoxamine (Figure 84-88)

There was very little difference in sensitivity to methoxamine between corpulent or lean males at 6 or 9 months of age. Both corpulent and lean females also showed the same sensitivities at 6 and 9 months of age. Male lean rats had a significant ($p<0.05$) reduction in the maximum force of contraction between 6 months (1.74±0.150gm.) and 9 months (1.10±0.145gm.). Fat females also showed a reduction in the force of maximum contraction between 6 months (1.65±0.304gm.) and 9 months (1.25±0.150gm.) of age which

was not significantly different. There was no such decrease between 6 and 9 months for corpulent males or for lean females although the maximum forces of contraction at 6 months for females were consistently lower than the males. Nine month old lean males contracted to the same tension as females in response to maximal doses of methoxamine.

Response to Clonidine (Figure,89-93)

The male corpulent rats as compared to lean males were more sensitive to clonidine at 9 months of age but not at 6 months of age. There was little difference in the response to clonidine of corpulent males between 6 or 9 months of age nor was there a difference between 6 or 9 month lean rats. There was not a corresponding differnce in clonidine sensitivities between corpulent or lean females at either 6 or 9 months of age. Again there was a significant reduction in the maximum force of contraction for lean males between 6 months (1.05±0.102gm.) and 9 months (.418±0.106gm.), while lean females again showed a non-significant reduction from 6 months (0.48±0.224gm.) to 9 months (0.25±0.05gm.). There was not a concomitant reduction in the maximum force of contraction between 6 and 9 months for corpulent males or lean females. Females had lower maximum forces of contraction at 6 months than males but at 9 months the lean male group reduced the maximum contraction by clonidine to similar levels as the females.

Effect of Inhibitors on Acetylcholine Induced Relaxations

Effect of Nordihydroguateic acid(NDGA) (Figure,94-97)

The predominant effect of NDGA on male rats was a progressive and significant depression of relaxation starting at -7.5 log mol/l of Ach.

the only group where this is not true is in the 6 month old male corpulent rats which relaxed as well with NDGA as without it. Female rats suffered an inhibition of relaxation by NDGA at a lower concentration of Ach (-9 log mol/l).

Effect of Indomethacin (Figure,98-101)

There was no significant inhibition of relaxation by indomethacin in any of the groups of animals that were studied.

Effect of Atropine (Figure,102-107)

Atropine very significantly ($p<0.01$) inhibited acetylcholine induced relaxation for all groups studied. The aortic rings from lean 9 month male rats relaxed better on treatment with atropine than 6 month lean males or 6 or 9 month corpulent males. Six month corpulent and lean females overcame the atropine block at higher doses of Ach. However by 9 months of age there was poor relaxation in the presence of atropine for all females.

Effect of De-endothelialization (Figure,108-111)

The effect of the de-endothelialization proceedure was to prevent any relaxation greater than 30% for the arterial rings. This was strongly statistically different ($p<0.01$) to the case of arteries with intact endothelium.

Results of Arterial Relaxation Agents

Effect of Na Nitrite (Figure,112-115)

The effect of Na Nitrite was to produce a significant difference in arterial relaxation between corpulent and lean males at 6 months ($p<0.01$)

and at 9 months ($p<0.05$) of age. There was no difference in nitrite induced relaxation between 6 month corpulent and lean females. However by 9 months there was a right shift of the relaxtion curve for corpulent females which was not significant.

Effect of Isoproterenol(Figure,116-119)

There was no difference in relaxation by isoproterenol between 6 month corpulent and lean males. However, by 9 months there was a significant depression of relaxation with isoproterenol in the corpulent males. Comparisons between 6 and 9 month lean males showed an increase in relaxation by isoproterenol over time; the converse was true for corpulent males. There was a significant impairment of relaxation by isoproterenol, manifested by a right shift of the relaxation curve, in both 6 and 9 month corpulent female rats as compared to lean female rats.

Effect of Acetylcholine Induced Relaxation(Figure,120-124)

There was no difference in Ach induced relaxations between corpulent and lean female rats at either 6 or 9 months of age. The aortas of the 9 month lean females relaxed less well at the highest doses of Ach than the 6 month lean females, however this was not significantly different. There was no difference in Ach induced relaxation between 9 month corpulent and lean males, however the 6 month corpulent males showed a significant depression in relaxing ability to Ach as compared to lean males. There was little change in sensitivity of the arteries of lean males to Ach from 6 to 9 months. However as expected from the above data the 9 month corpulent males increased in sensitivity to Ach as compared to the 6 month corpulent males.

In absolute value there was little difference in the force of relaxation between 6 month corpulent males (1.59±0.171gm.) and 6 month lean males (1.06±0.161gm.). Six month females both corpulent (1.137±0.124gm.) and lean (0.956±0.227gm.) relaxed to the same extent and to the same absolute force of relaxation as male 6 month rats. At 9 months of age there was little difference between corpulent males (1.267±0.217gm.) and lean males (1.30±0.293gm.); nor was there a difference between corpulent females (.631±0.130gm.) or lean females (.681±0.137gm.). However the force of relaxation by Ach for the females was half that of the males.

Discussion

One of the major findings in this study is the decreased responsiveness of the smooth muscle in hyperlipidemic male and female animals to endothelium-independent relaxants. This inhibition of relaxation is seen to be present for both isoproterenol (a beta agonist) and by Na nitrite. In the male corpulent animals this could perhaps be due to a higher maximum contractility by alpha agonists, preventing effective relaxation by endothelium independent relaxants. Lean male rats would be able to relax better in response to these agents due to the decreased maximum contraction by alpha agents at both 6 and 9 months of age. Previous investigators have noted an increased arterial contractility in hypercholesterolemic rabbits to the constrictor agonists 5-HT and ergometrine[187]. However, contrary to our findings, Verbeuren et al found that in hypercholesterolemic rabbits there is a decreased responsiveness to alpha agonists[171]. In comparison to the males, corpulent females who are free of atherosclerotic lesions at 9 months of age do not change in sensitivity to alpha agonists but have a decreased maximum force of contraction by alpha agents at 9 months as compared to 6 months. Despite this decrease in the maximum force of contraction there was still a persistent impairment of relaxation at 9 months of age. This can only point to a failure of relaxation at the level of the smooth muscle in respose to endothelium independent relaxants with hypertriglyceridemic conditions. Corpulent males may have an associated enhanced contractivity which further impairs their smooth muscle cells from relaxing.

The other major finding is that there is no failure of endothelium dependent relaxation by Ach in female hyperlipidemic rats. Despite the

extremely elevated triglyceride levels with moderate elevations of cholesterol the female rats at 6 or 9 months are not known to significantly develop atherosclerotic myocardial or vascular lesions[177]. Thus despite the known deleterious effect of hyperlipidemia on endothelial cells[143], female hyperlipidemic rats have no loss of endothelium dependent relaxation by Ach.

Evaluation of endothelium dependent relaxtion in corpulent LA/N-CP male rats is very difficult. Results from this study show that there is attenuation of endothelium dependent relaxation at 6 months which has spontaneously corrected itself by 9 months of age. This is an anomalous finding because histological studies show a progressive disease from 6 to 9 months although most of the disease is confined to the aortic arch with less than 10% of the endothelium damaged distal to the arch in the 9 month male animals[188]. The segments of thoracic aorta used in this study were taken between the aortic arch and the incursion of the aorta through the diaphragm. There are several possible reasons why this endothelium-dependent relaxation might be corrected between 6 and 9 months of age.

One possible explanation for this observed difference in relaxation might be due to the power of the statistical tests used to assess the results. The power of a given statistical test depends on three factors:

1) the risk of error that is tolerated to reject the hypothesis that there was no difference in endothelium dependent relaxation. (In this study the null hypothesis (Ho) is rejected if $p<0.05$).

2) the size of the difference to be detected relative to the amount of variability in the population. (This is a function of the arterial

response of the LA/N-CP rat and the research technique that was used; it is largely beyond control in most studies).

3) the sample size (n=4) for all groups might have caused the difference in relaxation to acetylcholine between the corpulent and lean male rats. A small sample size would invite two types of error which could affect the power of our hypothesis testing[189]:

a) Type I error where the null hypothesis is true but rejected. In this situation we might conclude that the null hypothesis is rejected when in reality there is not a true difference in endothelium dependent relaxations between corpulent and lean 6 month male rats. Such a situation could arise if there was a non-random sampling of arterial rings used in the corpulent male rat group. If by pure chance, the arterial rings in the corpulent males all included more focal atherosclerotic lesions than that of a true population of corpulent males, we would expect a falsely depressed relaxation effect by acetylcholine.

b) Type II error where the null hypothesis (Ho) is false but not rejected, or the alternate hypothesis (H_1) is rejected when it should be accepted. This situation implies an error in the statistical analysis of endothelium dependent relaxation with the 9 month corpulent and lean male rats. Such an error would imply that we have failed to reject Ho which implies that Ho <u>may</u> be true but not that it is always true. It is possible that the true mean of the response to actylcholine for the 9 month lean male rat is higher than that which was measured. This might be due to a technical error causing excessive endothelial cell damage in the lean male group. However as all endothelium dependent relaxation curves were similar to each other except the relaxation curves of the 6 month corpulent males it is more likely that if an error occured it was a Type I error with this

group. Further studies with larger sample sizes are commencing to investigate the possibility of such an error occuring.

A second explanation would be an increase in Ach receptors on the 9 month old endothelial cell. However there was no increase in relaxation between 6 and 9 months with atropine present as a blocker in the male corpulent rats as there was with the male lean rats. This might reflect a decrease in the sensitivity of male lean muscarinic receptors to atropine at 9 months since there was little change in the sensitivity to Ach between 6 and 9 months for the lean male. There was no increase in Ach sensitivity between 6 and 9 months for corpulent or lean females and there was a decrease between 6 and 9 months for both groups of females with atropine as a blocker.

A third explanation might be that if there is endothelial dysfunction due to the hyperlipidemia, a concomitant change occured in the smooth muscle cells in the 9 month male corpulent rats to render them less contractable, and therefore full relaxation by a reduced amount of EDRF would be possible. In other words, we can possibly speculate that there is a true reduction in EDRF relaxation at 6 months, but at 9 months with the same level of EDRF, full relaxation can occur if there is a reduction in the smooth muscle contraction which would reduce the work needed to counteract the contraction. The results of this study show that the absolute force of contraction by Nor -7 log mol/l for the de-endothelialized ring in the corpulent male did not change from 6 months (2.21±0.445gm.) to 9 months (2.08±0.216gm.). There was however a great decrease in the force of contraction with Nor -7 log mol/l in rings with intact endothelium between 6 months (1.812±0.166gm.) and 9 months (1.16±0.166gm.) for corpulent males. This suggests that there might be a

critical level of contraction beyond which EDRF could not acheive full relaxation but this begs the question as to why the endothelium modulated the pre-contraction better in the 9 month than the 6-month old corpulent male. There is at the present no good answer for this except that in the 6 month corpulent male there was no failure of relaxation by Ach with NDGA present. It is possible but unlikely that in the 9 month corpulent male the pathway to EDRF was not through the lipoxygenase enzyme but rather through an ancillary path which was not affected by the biochemical changes in this animal. Thus in the 9 month corpulent male the path of formation for EDRF might have shifted to a pathway more resistant to atherosclerosis. There is however no reason to implicate another pathway for EDRF formation at this point since all other results in this study suggest that there is lipoxygenase but not prostaglandin participation in relaxation due to acetylcholine activation of muscarinic receptors in these animals.

One final result that argues against endothelial dysfunction in these animals with respect to relaxation by Ach is that the corpulent males at both 6 and 9 months of age have the highest values for absolute force of relaxation. If the atherosclerotic process attenuated either the production or the effects of EDRF the expectation would be for a reduced force of relaxation rather than the converse.

If further experiments with male corpulent rats confirm an early inhibition of EDRF with a spontaneous return to normal endothelium dependent relaxation at 9 months of age we speculate that this could be caused by several mechanisms.

1) Age - It is known that with aging endothelium dependent relaxation improves, while β agonist relaxation suffers a progressive inhibition[207]. Thus we should expect between 6 and 9 months a normal improvement in

endothelium dependent relaxation with a normal impairment in relaxation induced by β agonists. All our results agree with this save that in the corpulent males there was no dimunition in β agonist relaxation. This might be associated with atherosclerosis at the level of the smooth muscle.

2) Contracting Agent - One study has shown that noradrenalin through β receptors on endothelium cells can release EDRF[206]. Thus if the endothelium surface β receptors of the 9 month corpulent male rats increased their sensitivity to β agonists we would expect a reduced active tension with noradrenalin as a precontracting agent. Noradrenalin would not be expected to have a different effect on the smooth muscle cell; however through the algebraic sum of increased EDRF from the endothelium and the same force of contraction from the smooth muscle we would expect a reduced response in accordance with our results.

3) Fatty Acids - The VLDL molecules through the fatty acids they contain may themselves at 9 months facilitate the release of EDRF. This could be accomplished through two mechanisms:

a) A change in the ratio of phospholipid to cholesterol in the cell membrane. This would either permit easier access for Ach to reach it's muscarinic receptor, or it might amplify the work of these receptors.

b) A second possibility is that the fatty acids from the triglyceride moities may augment the EDRF formation from arachadonic acid in the cytoplasm by acting as the initial substrate for arachadonic acid formation. It is known that unsaturated fatty acids will produce endothelium dependent relaxation in both dog and rabbit aortas[59]. Unfortunatly, the fatty acid components of the triglyceride moities in the VLDL molecules of the rats have not as yet, been individually characterized.

4) Insulin - The young corpulent male rats exhibited a reactive hyperplasia of the β cells in the pancreas as compared to lean controls and female corpulent rats. This is reflected in a large increase in serum insulin levels with normal glucose levels and low glucagon concentrations. With age the insulin levels tend to increase to maintain a normal glucose level[208]. The associated actions of the increased serum insulin might act like thyroid hormone to enhance cellular reactions and thereby increase EDRF concentrations.

When endothelium dependent and independent relaxants are considered together further questions, not answers arise. The fact that a beta agonist acting through cAMP and Na nitrite acting through cGMP both impair relaxation in the LA/N-CP rats argues for the defect to be located in the final common pathway of relaxation distal to either cAMP or cGMP. Such a problem might be located in the myosin light chain phosphatase that is less able to split off the phosphate from the myosin heads to relax crossbridges. However ACH induced EDRF in the females is associated with full relaxation despite the fact that EDRF, like nitrites, acts through cGMP to induce relaxation. This indicates that if the defect in the muscle is in the generation of cGMP that there is more EDRF released, or the EDRF that is released is more active in the corpulent females than the lean females to generate the same amount of cGMP. If the defect is distal to cGMP then more cGMP would have to be released in the corpulent females to overcome the smooth muscle relaxation defect that accompanies the hyperlipidemic state.

Male corpulent animals at 6 months of age demonstrate impaired relaxation which is due to impaired muscle relaxation and might be due to an impairment of EDRF effect. Relaxation by 9 month corpulent males is

similar to lean males which is analagous to the female situation whereby corpulent females accomodate for a smooth muscle defect with resultant normal endothelium dependent relaxations. The mechanism in the males which allows increased relaxation by the endothelium despite a muscle problem is not answered from this study and will require further investigation. Future research in this area should focus on two areas: bioassay studies of EDRF and the endothelial basal controls of smooth muscle contraction.

Study Conclusions

The conclusions of this study are:

1) That male and female hyperlipidemic animals demonstrate impaired relaxation at the level of the smooth muscle cell. This is despite the lack of atherosclerotic lesions in the females and can only be due to the hyperlipidemia state as hypercholesterolemic animals do not demonstrate this[172].

2) That despite a smooth muscle cell relaxation problem corpulent females do not have impaired endothelium dependent relaxation. Further research must be done on the corpulent males to see if in addition to impaired smooth muscle relaxation there exists an associated impairment of endothelium dependent relaxation.

Clinical Implications of Endothelium Dependent Responses

Recent studies have changed the role of the endothelial cell from that of a passive sieve for blood constituents to that of an active modulator of several important physiological functions in the arterial circulation. Several key endothelial functions that are currently utilized to benefit

therapeutic manipulations are the anti-thrombotic and vasodilatory functions of the endothelial cell.

Research reveals that short periods of hypoxia induce morphologic and functional derangements to endothelial cells in vivo[190,191]. Furthermore, studies with in situ or reversed saphenous vein bypass grafts reveal extensive endothelial cell damage from handling alone. With time, morphologic healing of the endothelial monolayer progressed slowly back to normal, however endothelial cell biochemical changes were never normalized over a 6 week study period[192]. Other investigators have shown that endothelial cell destruction, and loss of fibrinolytic activity in autogenous arterialized vein grafts is directly proportional to the handling of the adventitial surface during it's preparation as a vein graft[193,194]. Recently it has been shown that endothelial seeding of small caliber prosthetic arterial grafts have enhanced patency as compared to prosthetic grafts without endothelium[195]. Such new insights as these reveal that we can not tolerate with impunity the loss of endothelial cell control over thrombosis during graft preparation when bypass grafts are used as the major blood conduit to critical end organs.

The other area that is currently undergoing investigation concerns endothelium dependent relaxation. In the past it has been well known that direct acetylcholine infusions to the pulmonary artery will transiently ameliorate chronic pulmonary artery hypertension[196]. Recent studies on endothelium dependent relaxation with human coronary arteries in vitro[197] and in vivo[198], demostrate that there is impaired relaxation in these arteries affected by atheroma. Coronary and systemic vascular atherosclerosis may predispose some patients to vasospasm[199,200] which may be associated with a defect in endothelium dependent relaxation[201].

Neurosurgical studies with sub-arachnoid hemorrhage have shown maximal endothelial cell loss in cerebral arteries associated with the onset of delayed vasospasm[202]. The loss of relaxation in cerebral arteries with spasm may be due to the loss of endothelium dependent relaxtion factor (known to be released by vasopressin[73]) counterbalancing an unknown vasoactive metabolite in the clotted blood around the artery[202].

Recent studies with cultured bovine endothelium cells seeded onto denuded canine coronary arteries demonstrate the release of EDRF from the seeded endothelium causing vasodilation of the coronary arteries[51]. Endothelium seeding of autogenous grafts may have future applications in vascular surgery.

Pharmacologic control of the endothelium dependent relaxation factor has great potential for future therapeutic strategies in patient management. Control of cerebral or coronary spasm could resolve a potentially lethal situation by correcting exaggerated vasoconstriction impairing blood delivery to critical tissues. Vascualar surgeons could benefit by control of distal runoff and graft thrombosis, which are apart from technical flaws, the main determinants of early graft failure[203,192].

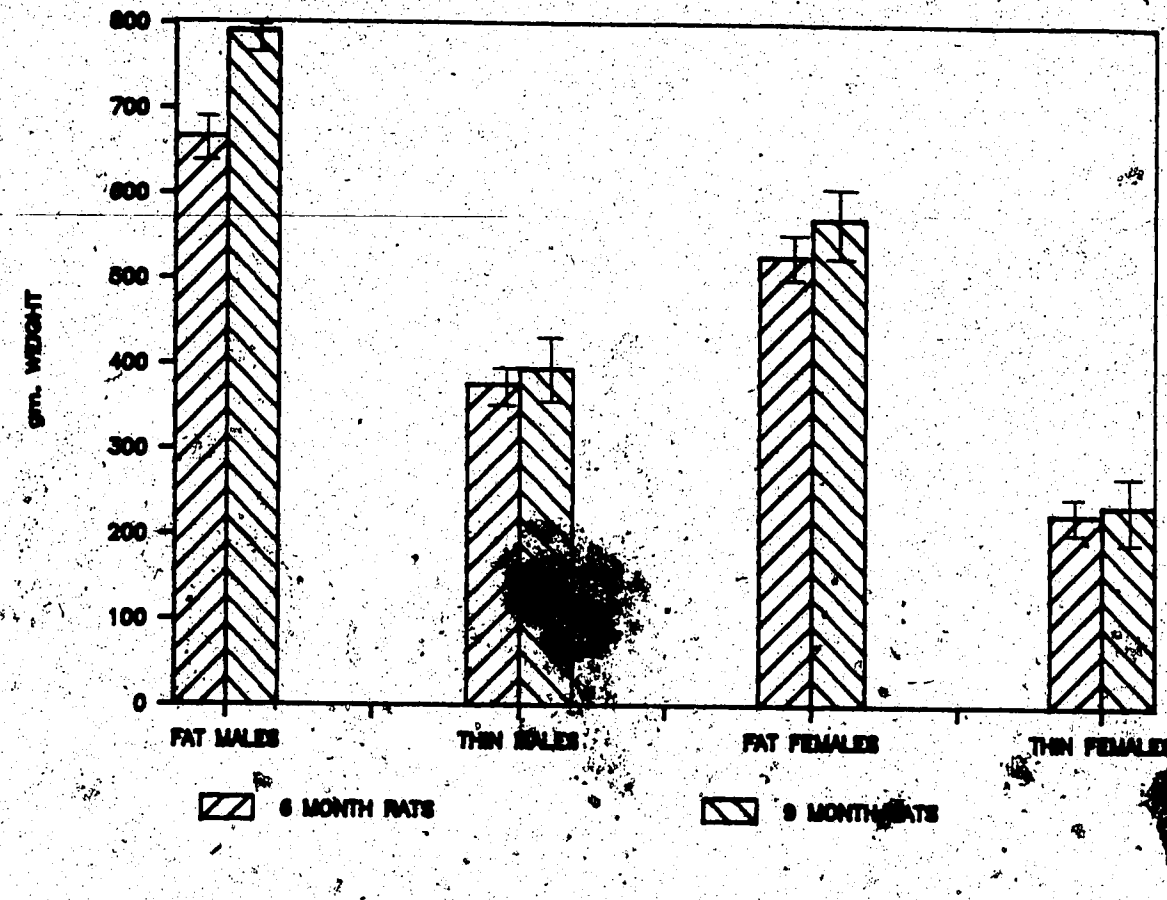
Shock studies of large arteries reveal that a decrease in hematocrit without a decrease in flow impairs normal endothelium dependent relaxations[204]. Microcirculatory studies to the level of third order arterioles (15-25μ) reveal that endothelium dependent relaxation occurs and may play a role in the regulation of microvascular tone[205]. It is interesting to speculate that hypovolemic or even compensated hemorraghic shock may induce reflex inappropriate microcirculatory vasoconstriction secondary to loss of EDRF leading to further levels of cellular shock.

Sepsis as well as hypoxia are known to damage endothelial cells[153,190] which might effect the barrier function. Generalized edema from sepsis or trauma is an incompletely understood phenomenon which might be associated with a failure of endothelial cell functions. Protection or pharmacologic control of either the endothelial cell or it's metabolites during shock might prevent the morbid sequellae which can follow severe sepsis or trauma. Our future ability to control the response of the endothelial cell may reshape several areas of clinical medicine as well as import financial relief to currently strained medical budgets.

XII Index of Figures

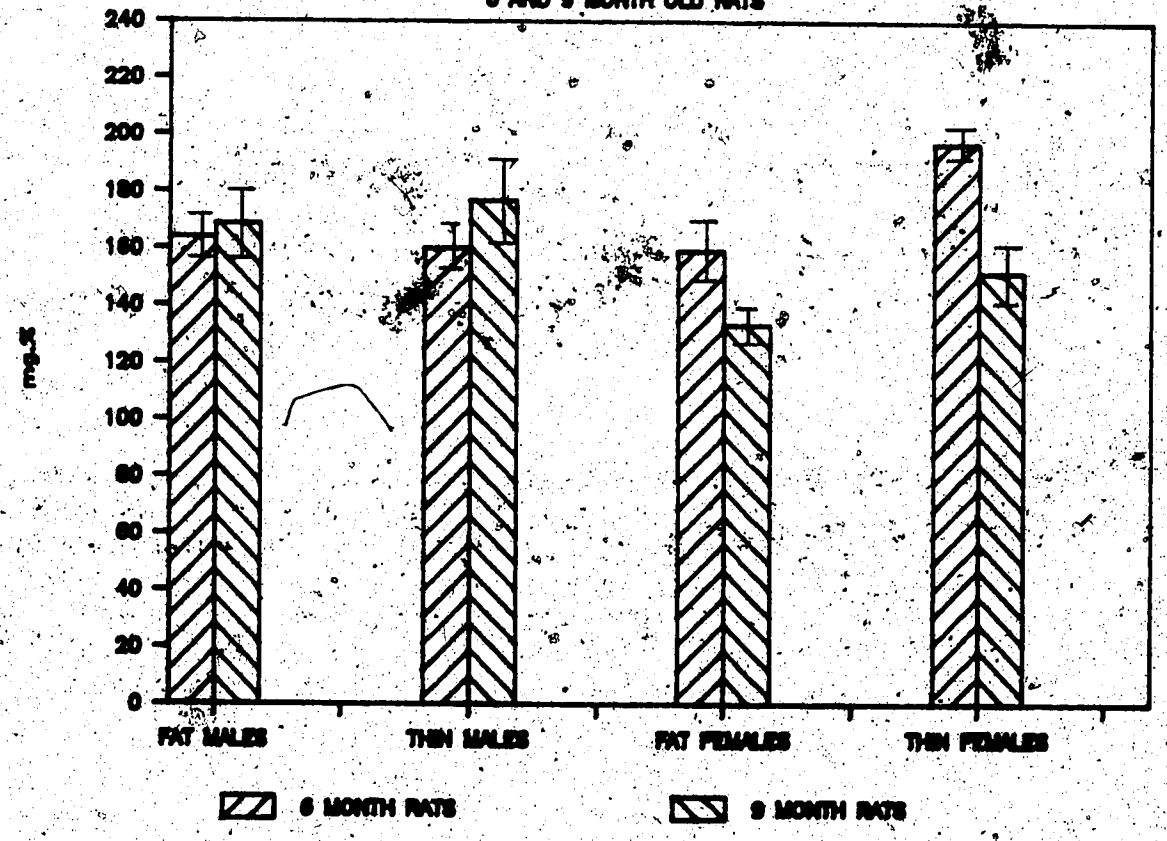
Note: The number of animals used for all groups of experiments is 4 (n=4).

WEIGHTS OF 6 AND 9 MONTH LA/N-CP RATS



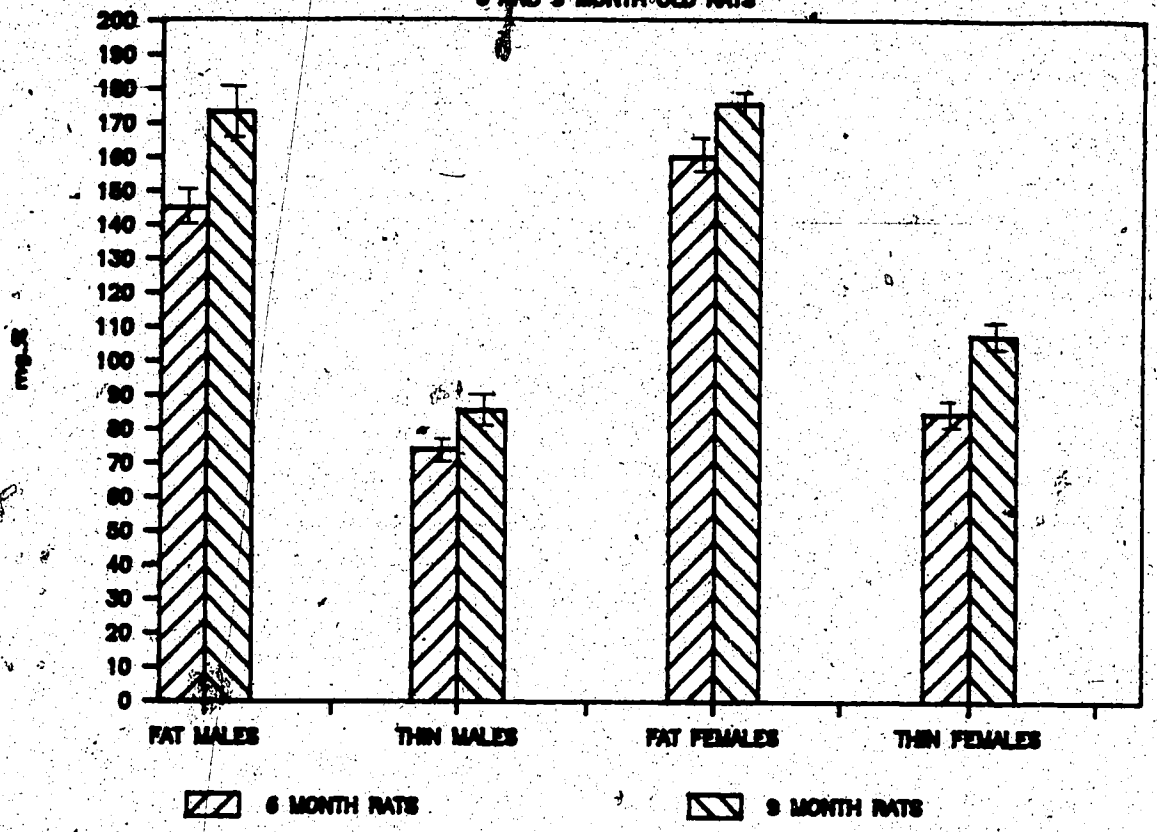
GLUCOSE LEVELS OF LA/N-CP RATS

6 AND 9 MONTH OLD RATS



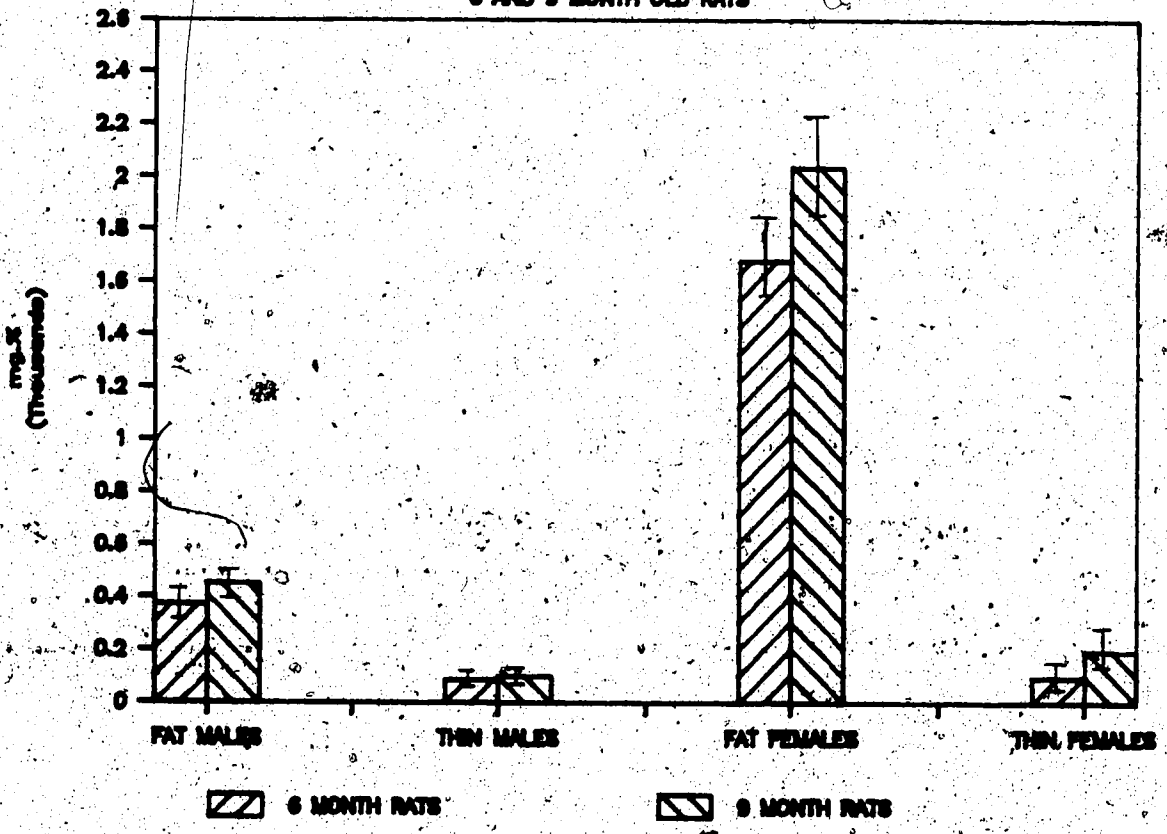
CHOLESTEROL LEVELS OF LA/N-CP RATS

6 AND 9 MONTH OLD RATS



TRIGLYCERIDE LEVELS OF LA/N-CP RATS

6 AND 9 MONTH OLD RATS



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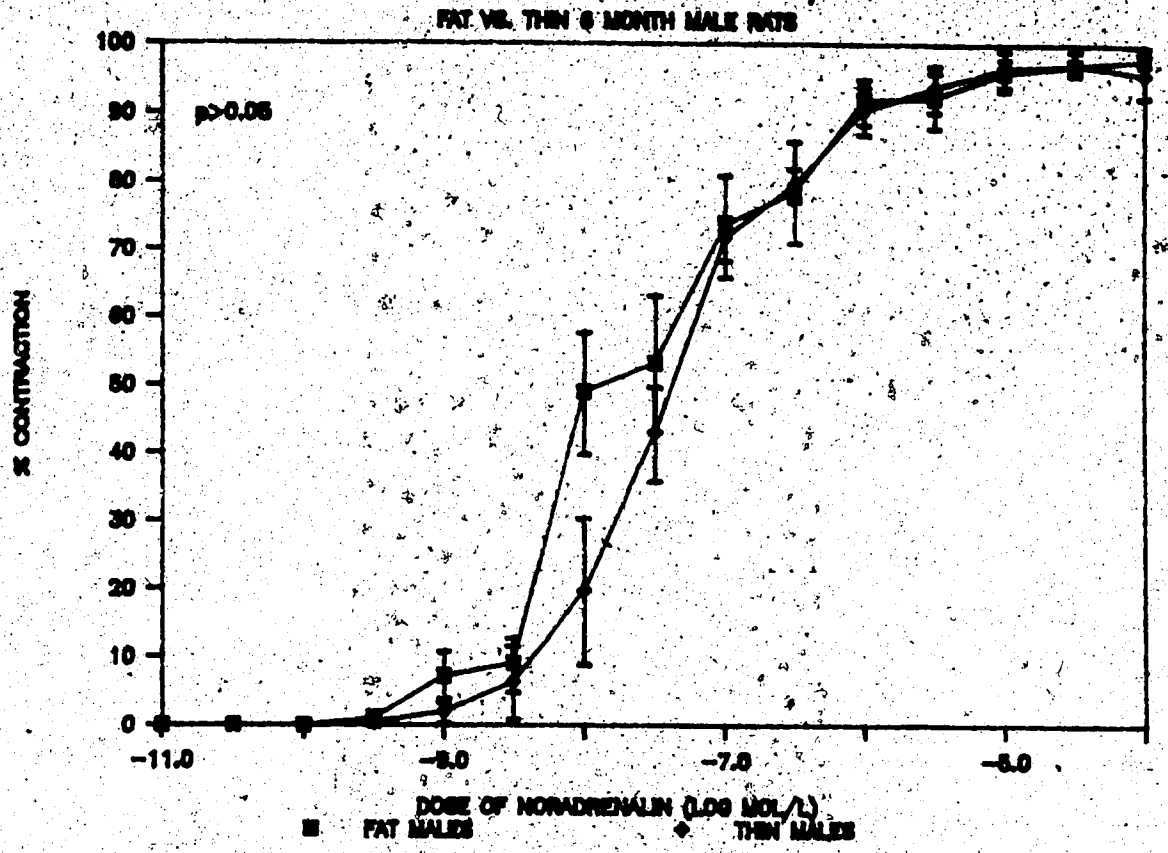

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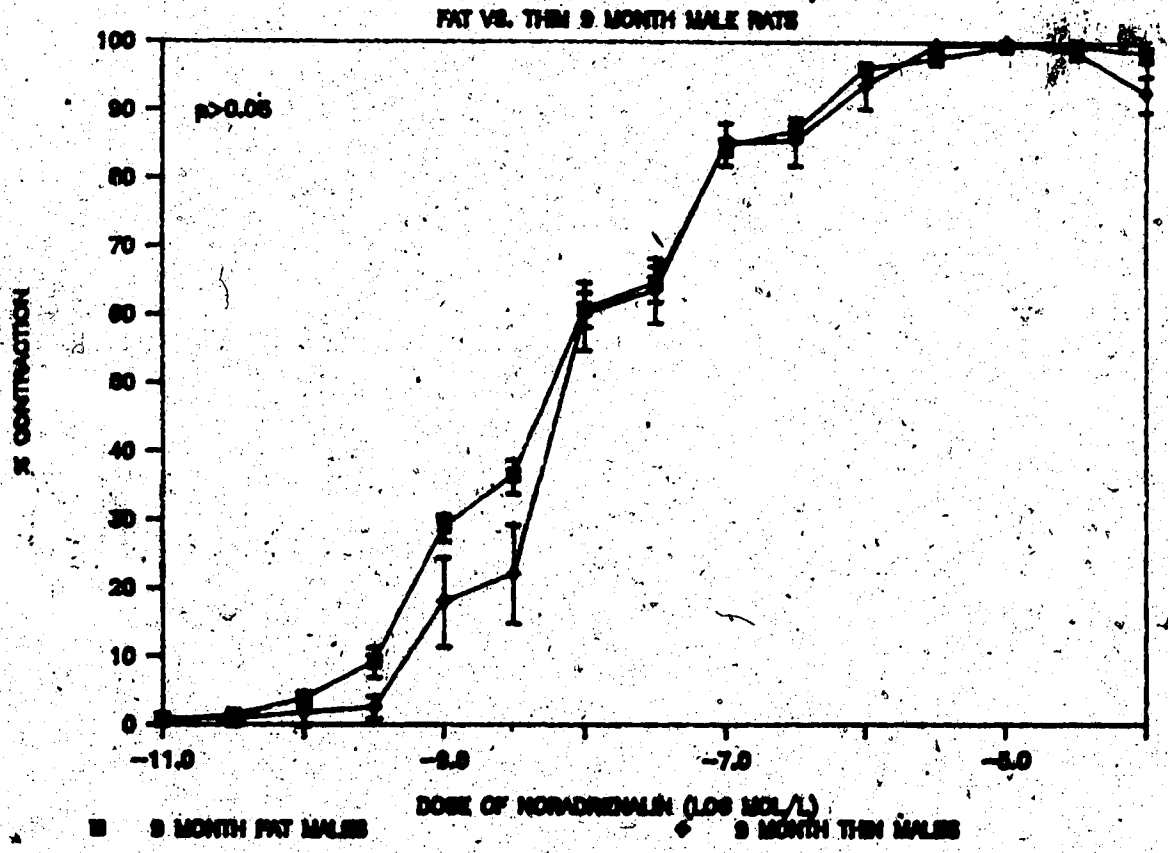

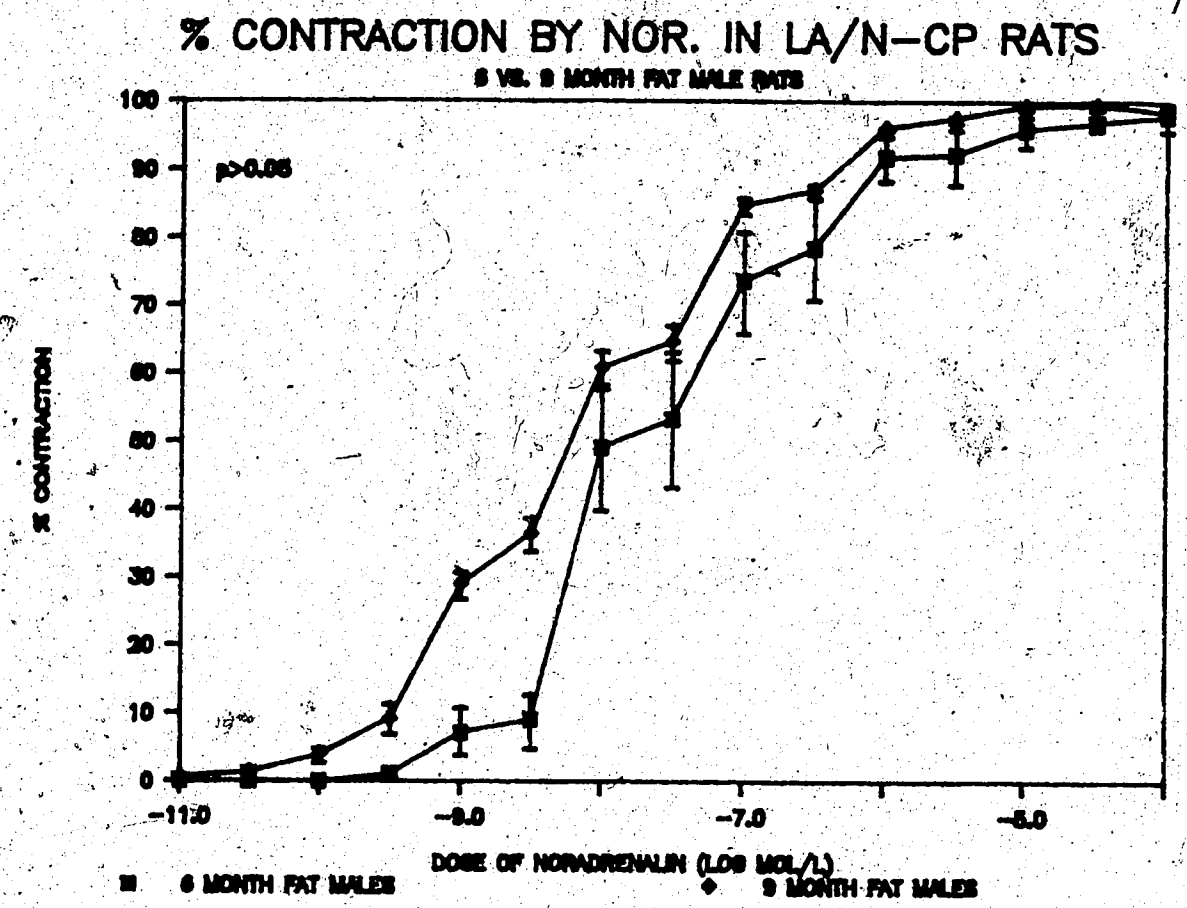
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6 VS. 9 MONTH FAT MALE RATS
p>0.05
% CONTRACTION
100
90
80
70
60
50
40
30
20
10
0
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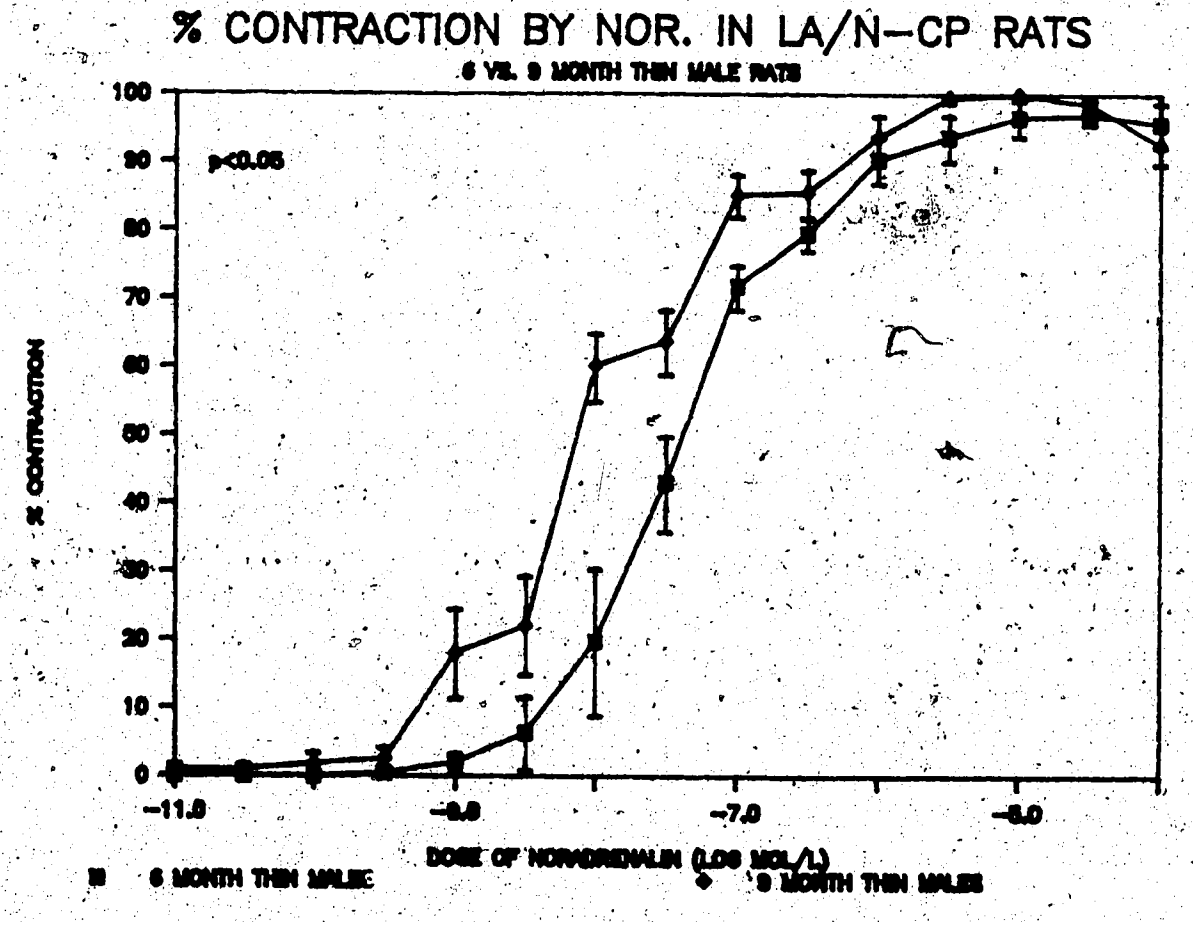
DOSE OF NORADRENALIN (LOG MOL/L)
6 MONTH FAT MALES
9 MONTH FAT MALES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-11.0
-9.0
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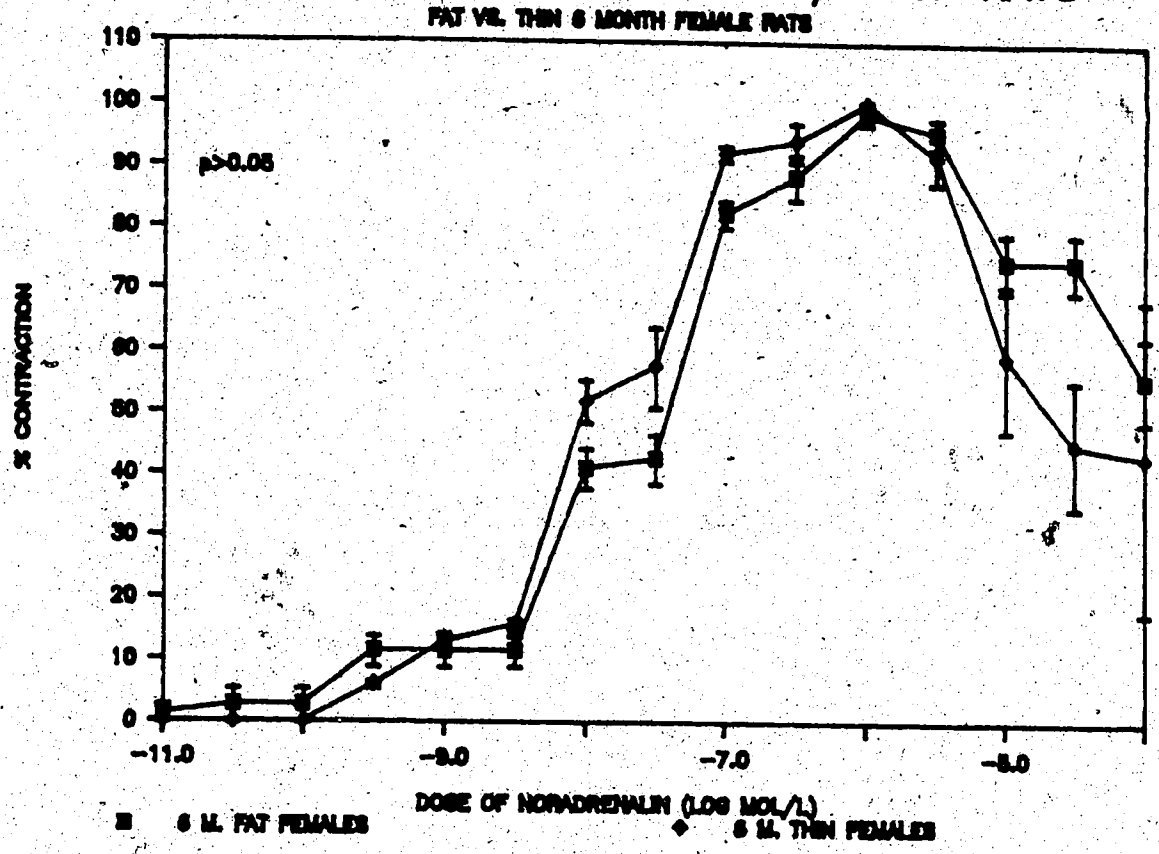
DOSE OF NORADRENALIN (LOG MOL/L)
6 MONTH THIN MALES
9 MONTH THIN MALES

% CONTRACTION BY NOR. IN LA/N-CP RATS



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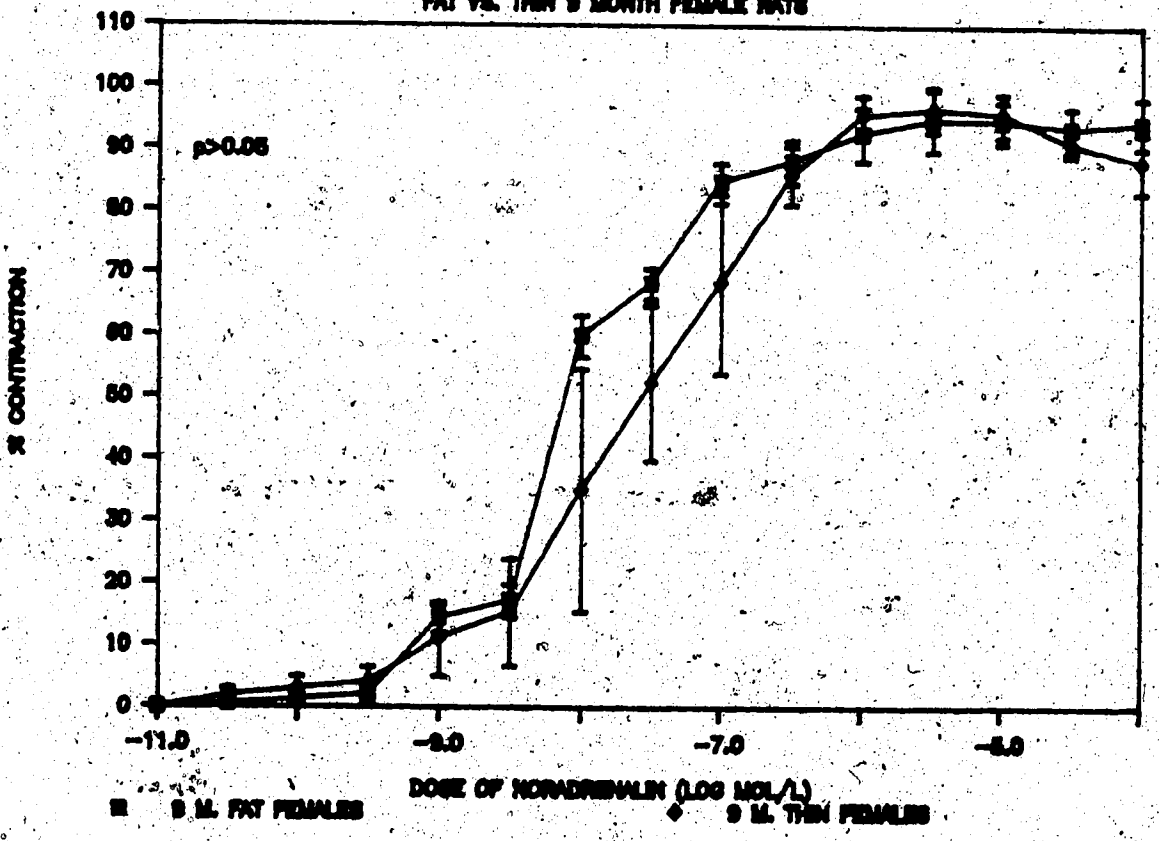

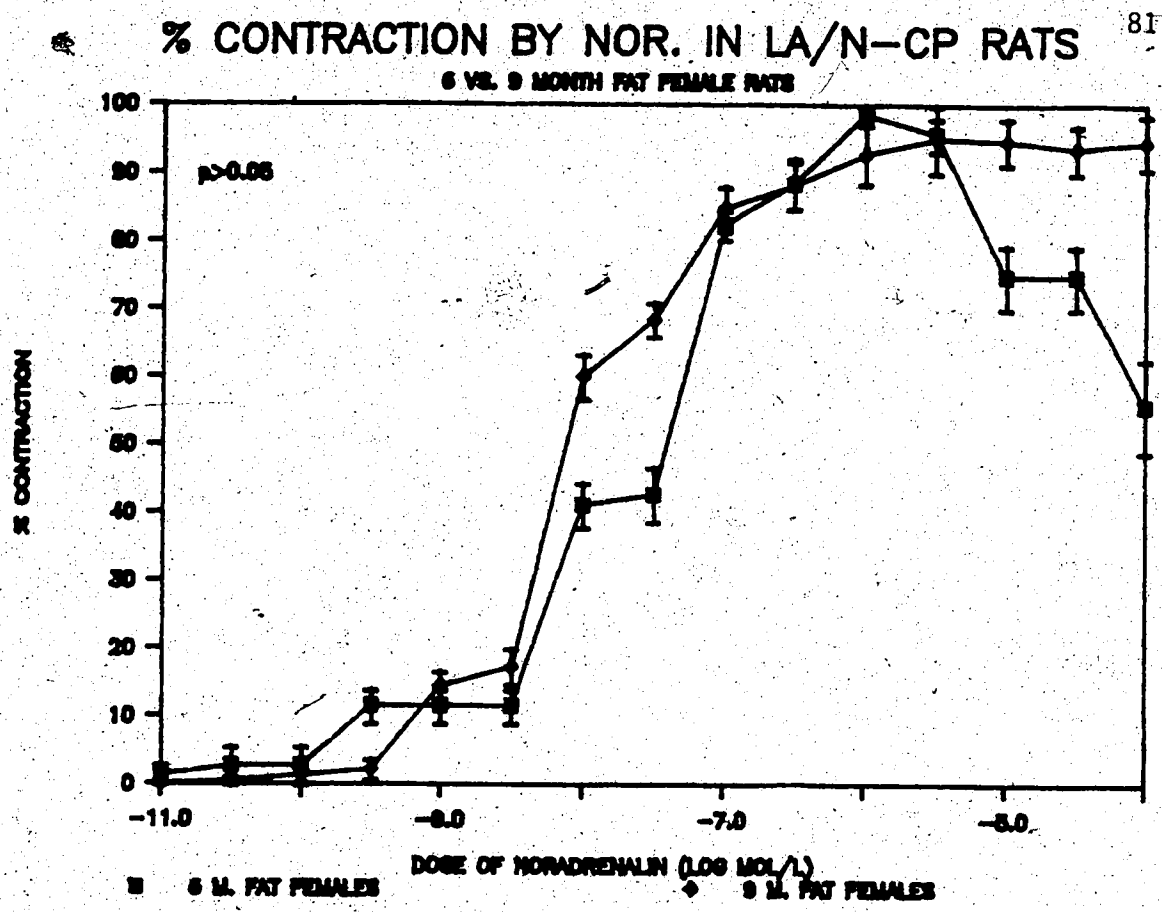
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% CONTRACTION
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10
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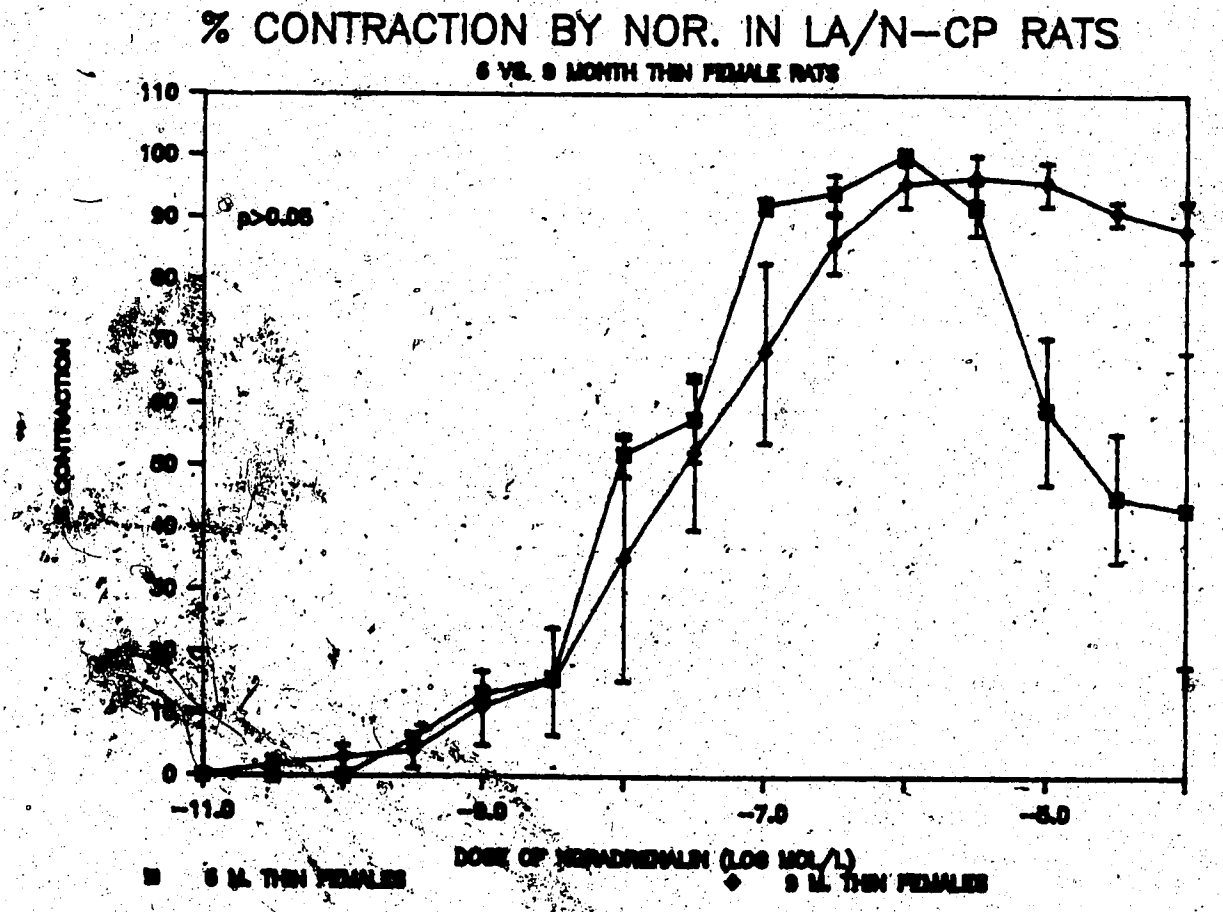
DOSE OF NORADRENALIN (LOG MOL/L)
6 M. FAT FEMALES
9 M. FAT FEMALES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6 VS. 9 MONTH THIN FEMALE RATS
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% CONTRACTION
110
100
90
0
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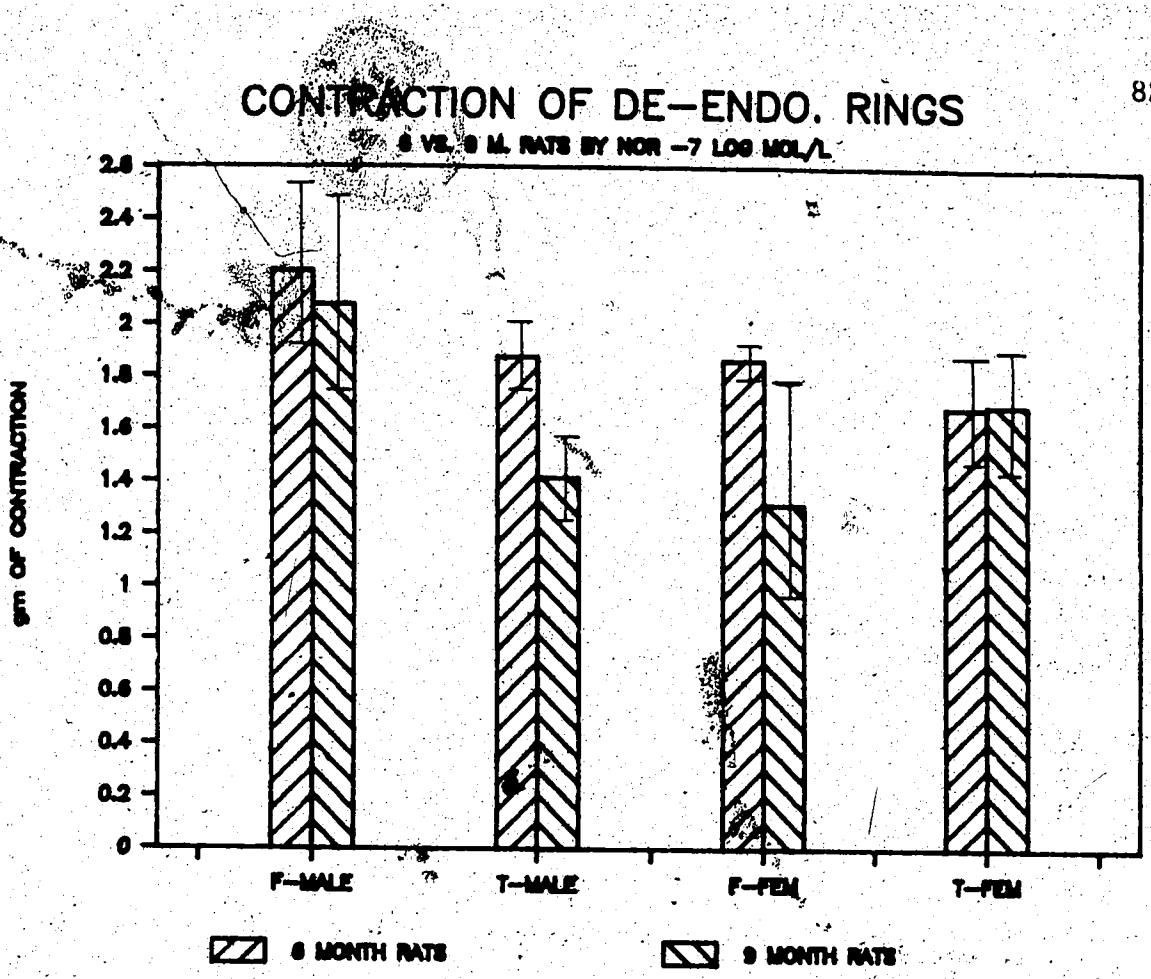
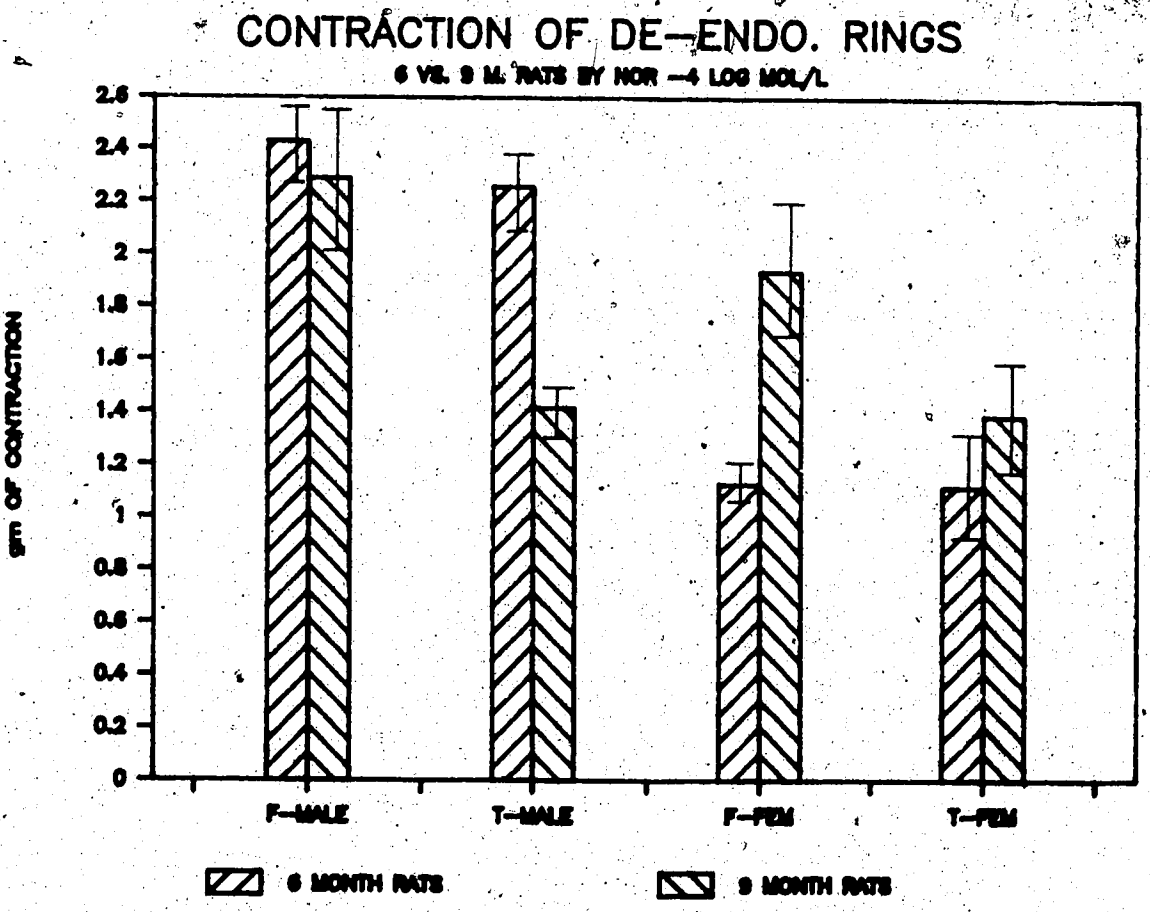
DOSE OF NORADRENALIN (LOG MOL/L)
6 M. THIN FEMALES
9 M. THIN FEMALES


CONTRACTION OF DE-ENDO. RINGS
6 VS. 9 M. RATS BY NOR -7 LOG MOL/L
gm OF CONTRACTION
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
F-MALE
T-MALE
F-FEM
T-FEM
6 MONTH RATS
9 MONTH RATS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gm OF CONTRACTION
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2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
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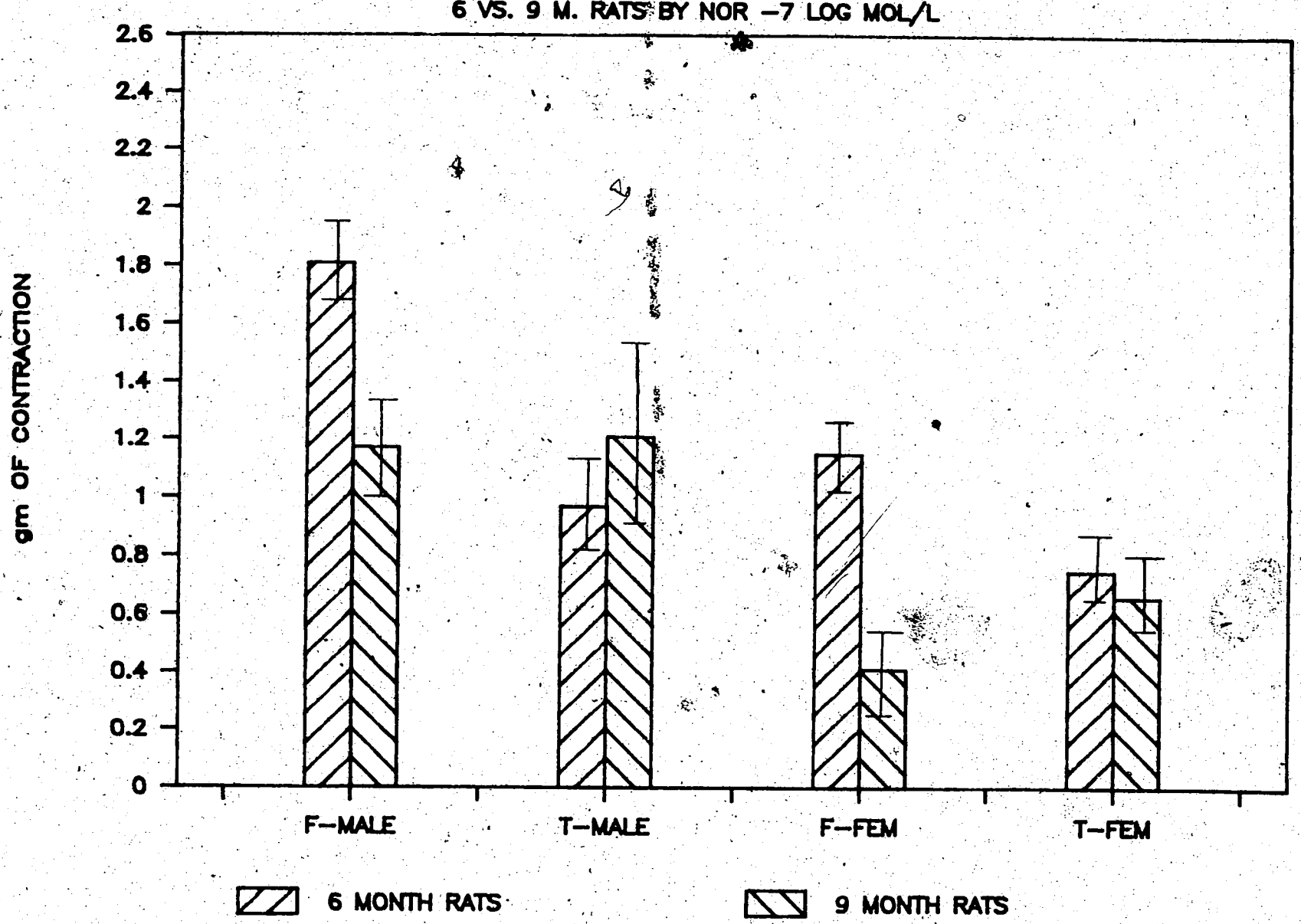
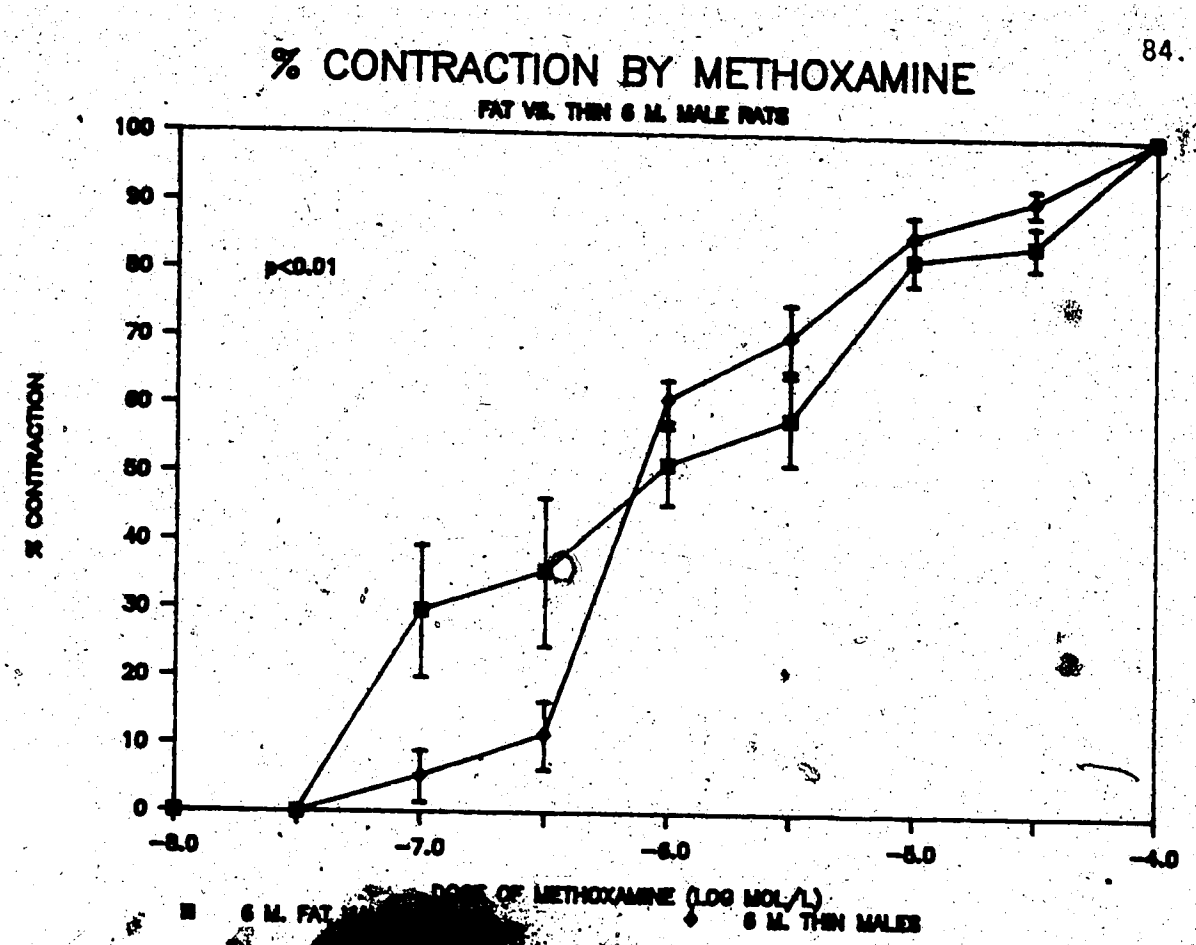
F-MALE
T-MALE
F-FEM
T-FEM
6 MONTH RATS
9 MONTH RATS

CONTRACTION OF ENDOTHELILIALIZED RINGS

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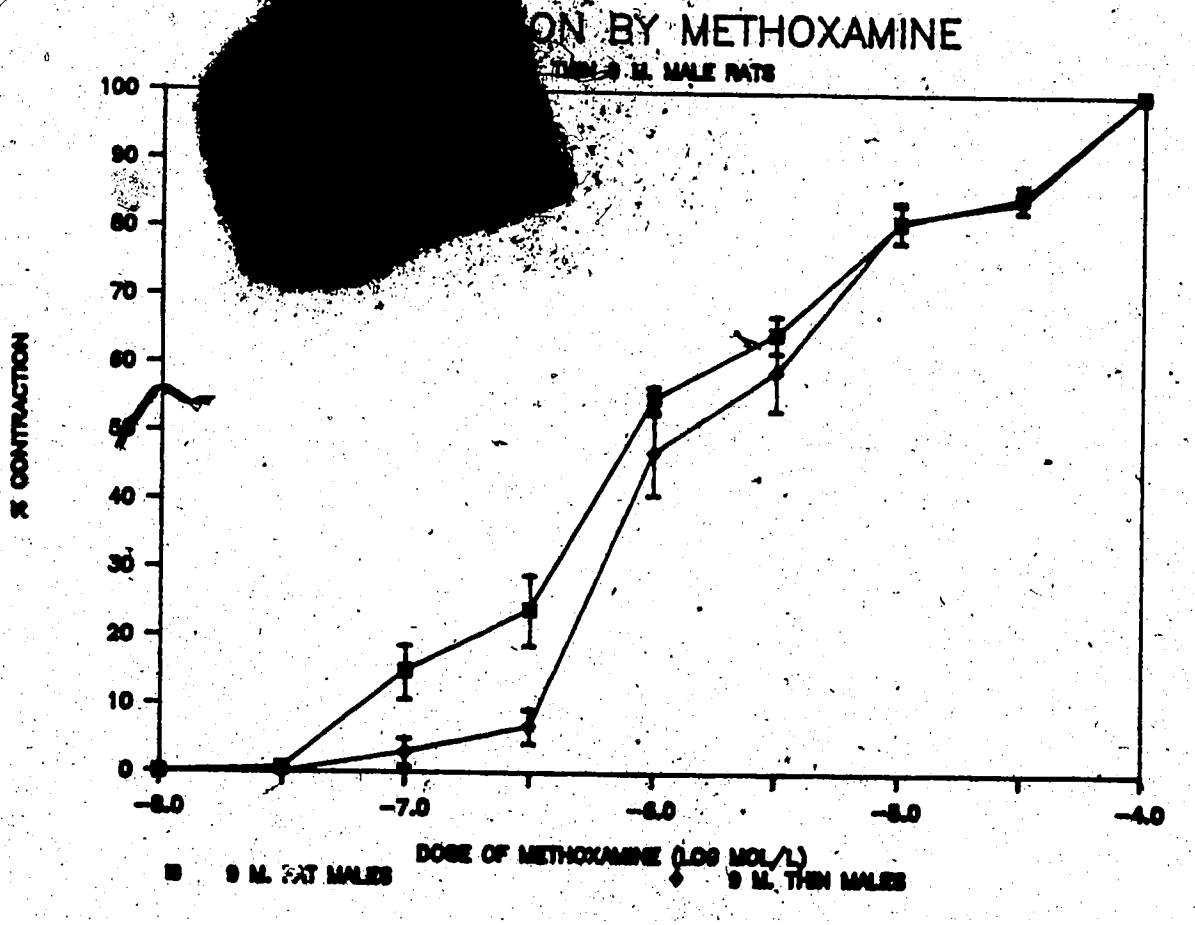
-4.0
DOSE OF METHOXAMINE (LOG MOL/L)
6 M. FAT
6 M. THIN MALES


ON BY METHOXAMINE
% CONTRACTION
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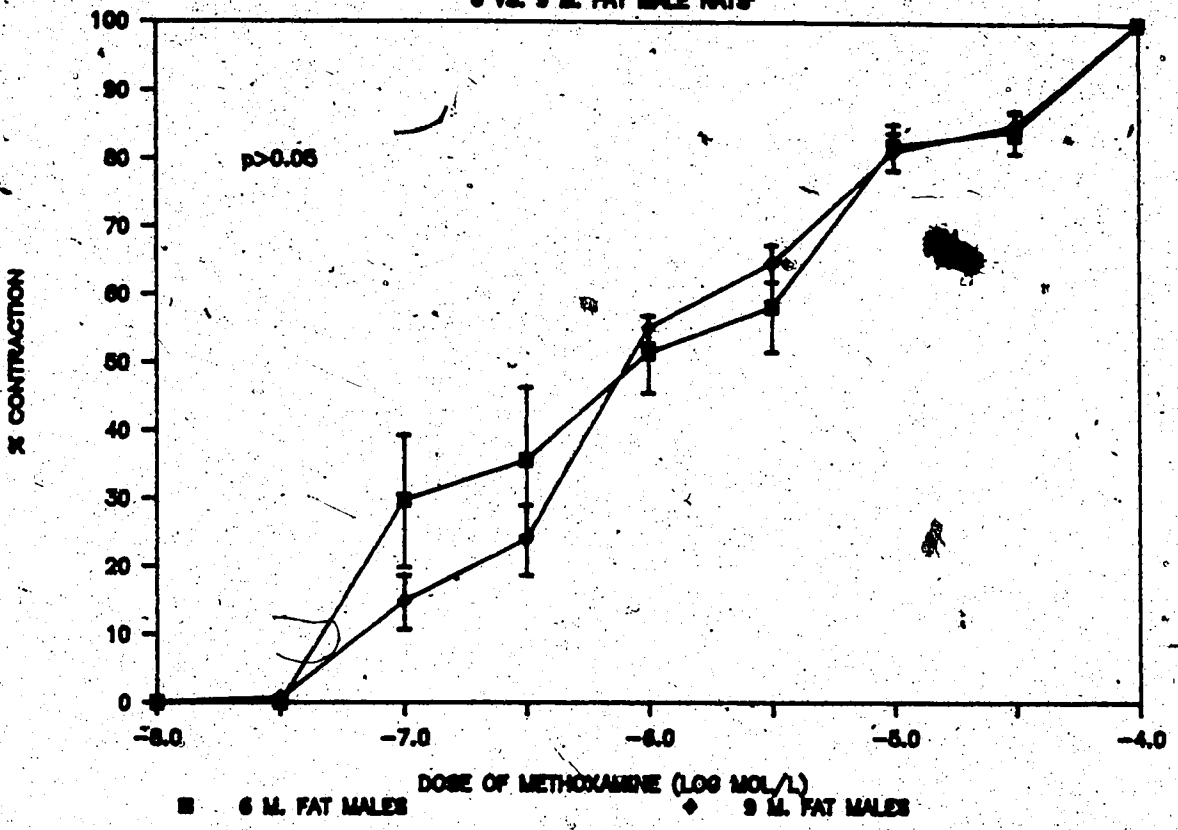
90
80
70
60
40
30
20
10
0
-8.0
-7.0
-6.0
-5.0
-4.0
DOSE OF METHOXAMINE (LOG MOL/L)
9 M. FAT MALES
9 M. THIN MALES

% CONTRACTION BY METHOXAMINE

6 VS. 9 M. FAT MALE RATS



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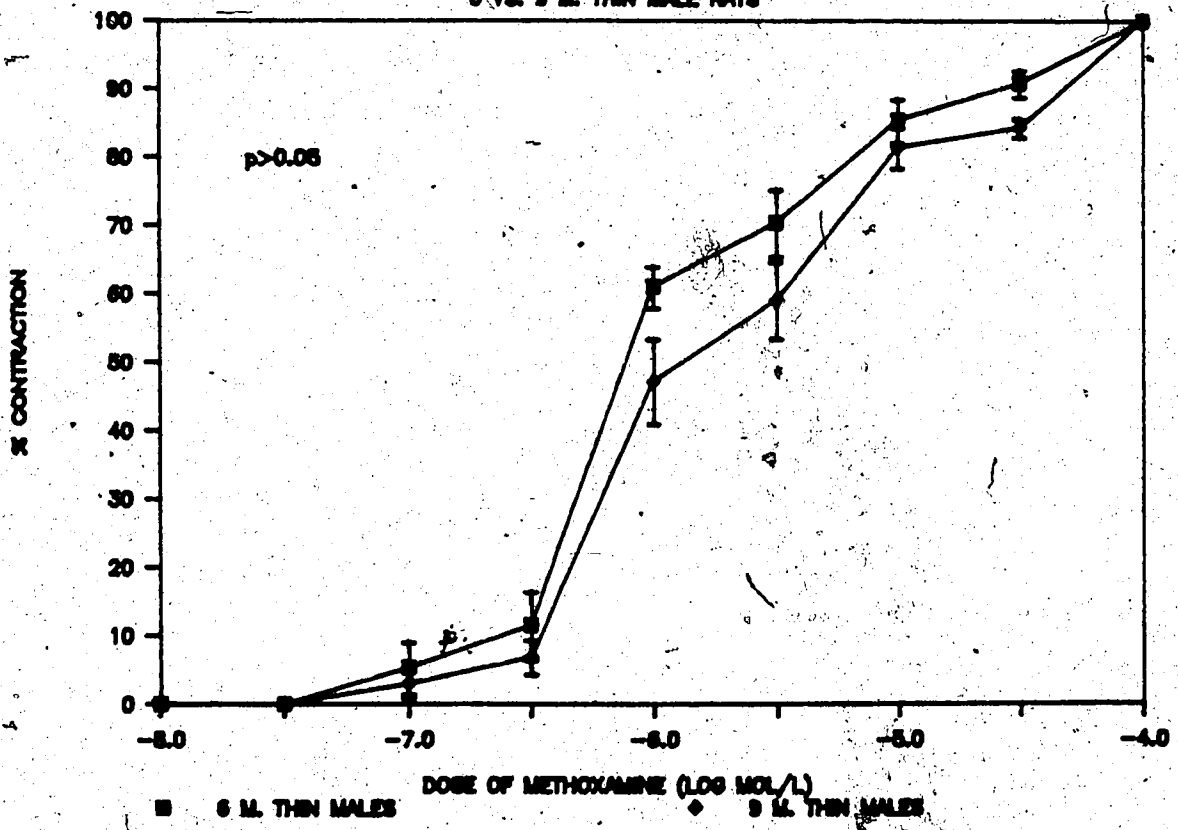

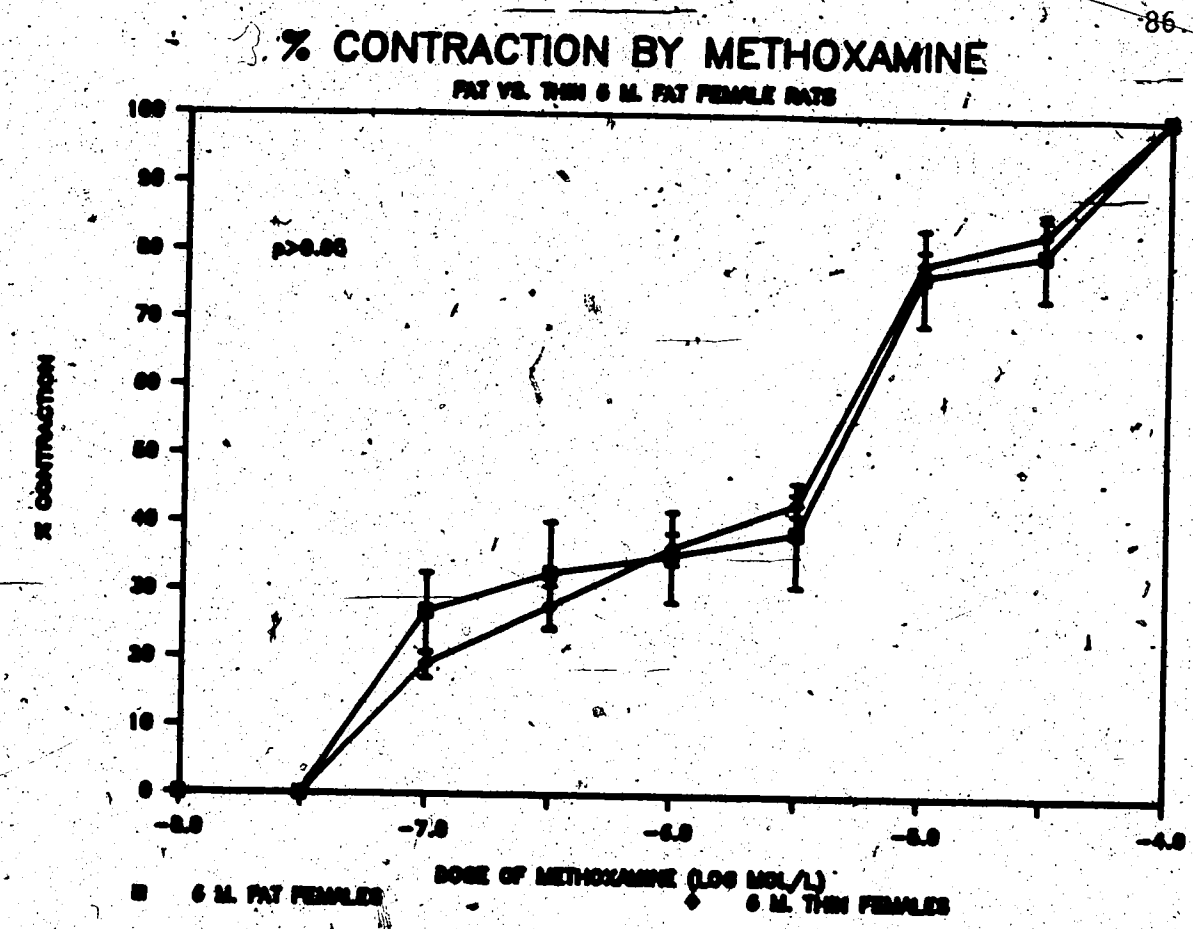
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FAT VS. THIN 6 M. FAT FEMALE RATS
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DOSE OF METHOXAMINE (LOG MOL/L)
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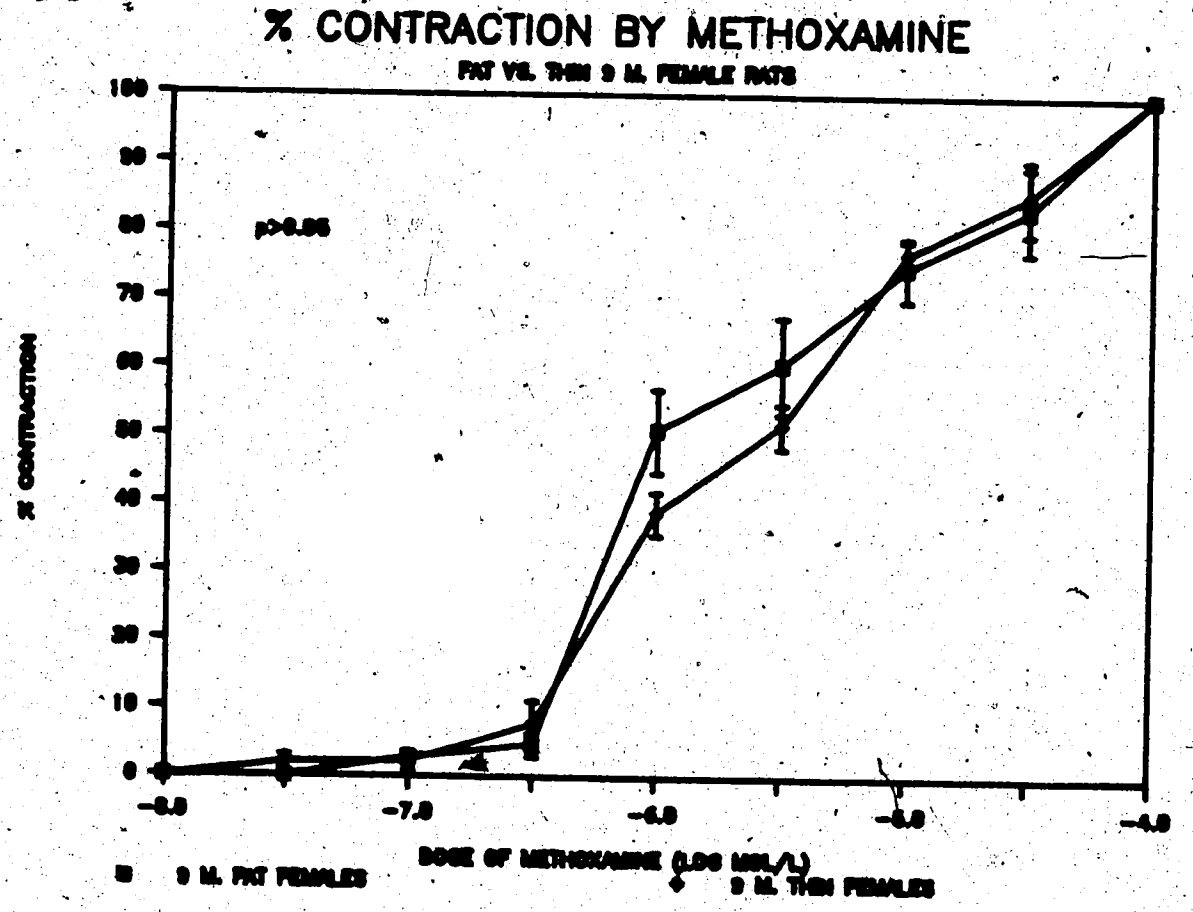
6 M. THIN FEMALES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FAT VS. THIN 9 M. FEMALE RATS
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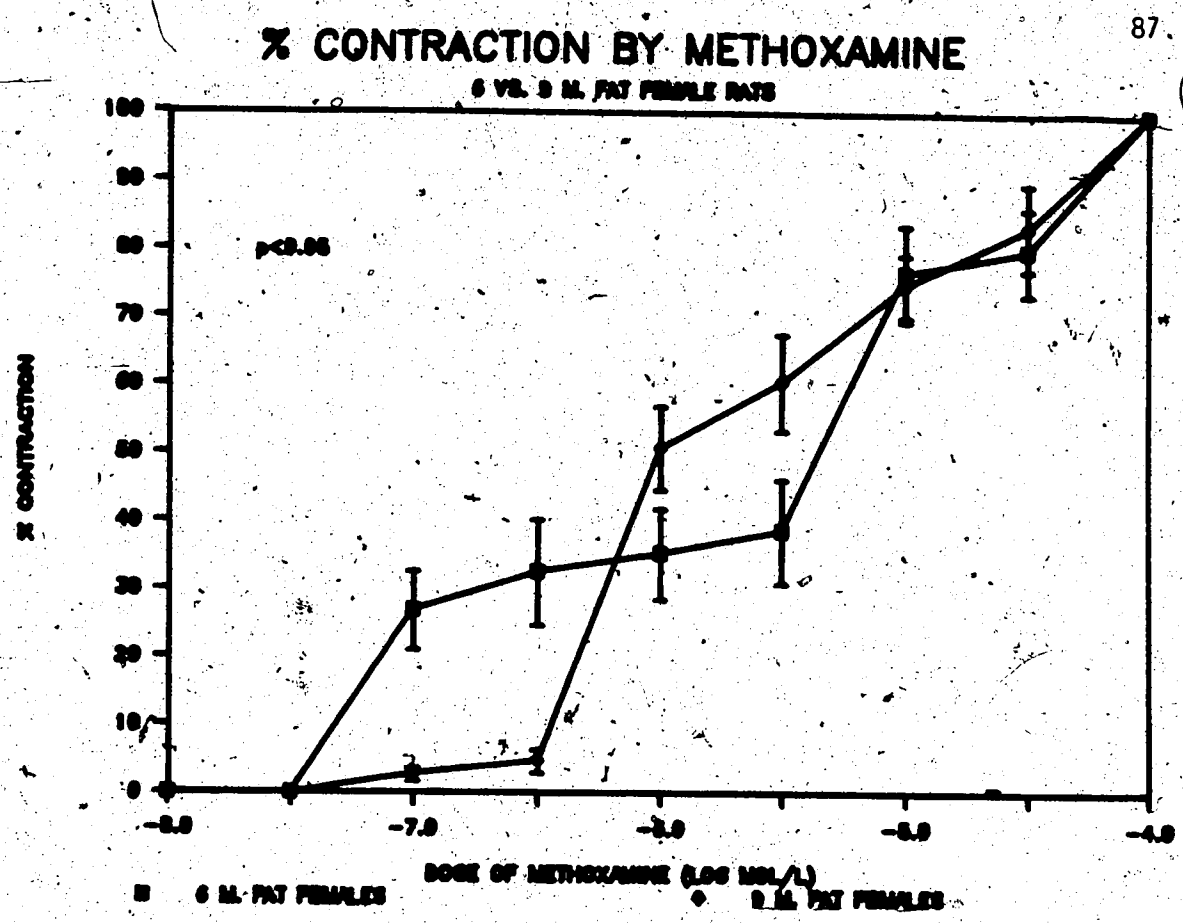
% CONTRACTION
DOSE OF METHOXAMINE (LOG MOL/L)
9 M. FAT FEMALES
9 M. THIN FEMALES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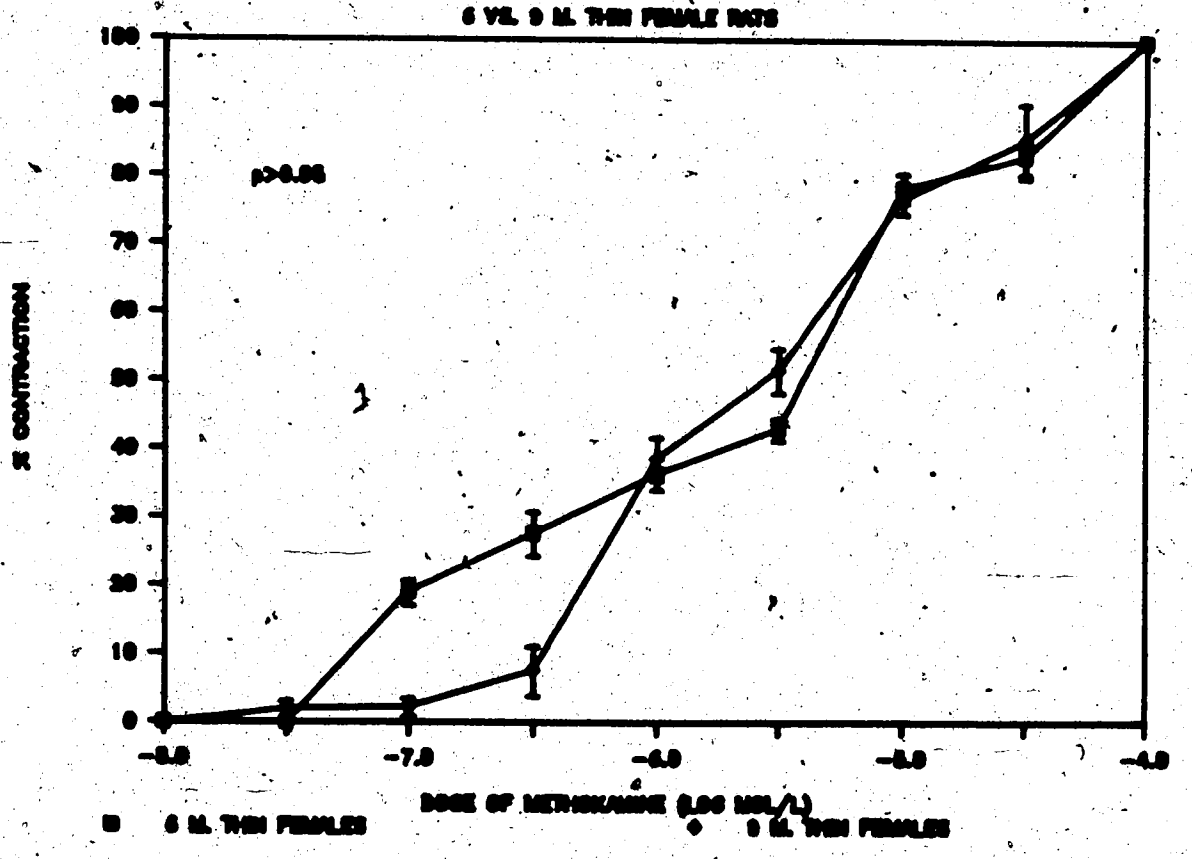
6 VS. 9 M. FAT FEMALE RATS
p<0.05
% CONTRACTION
DOSE OF METHOXAMINE (LOG MOL/L)
6 M. FAT FEMALES
9 M. FAT FEMALES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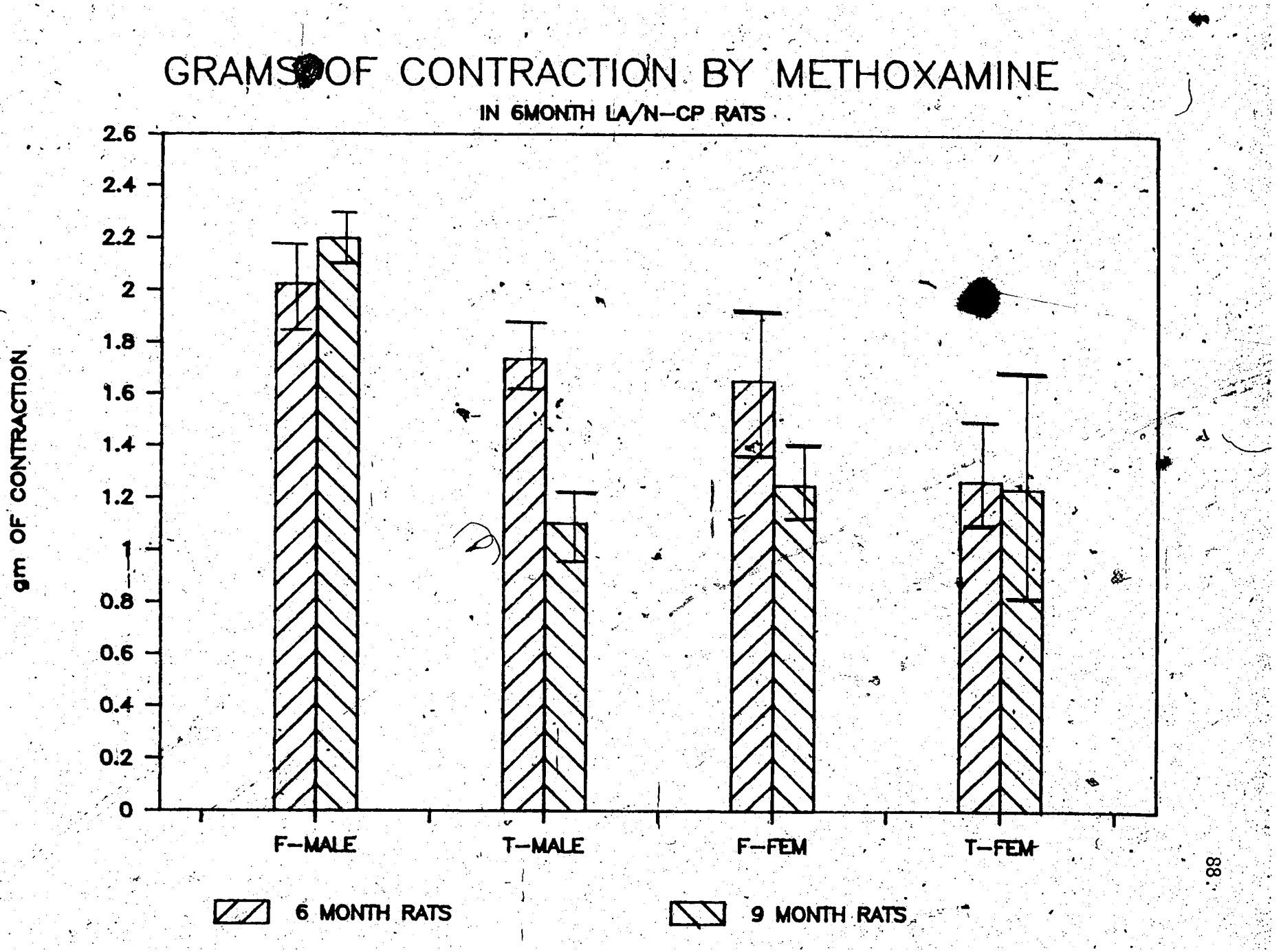
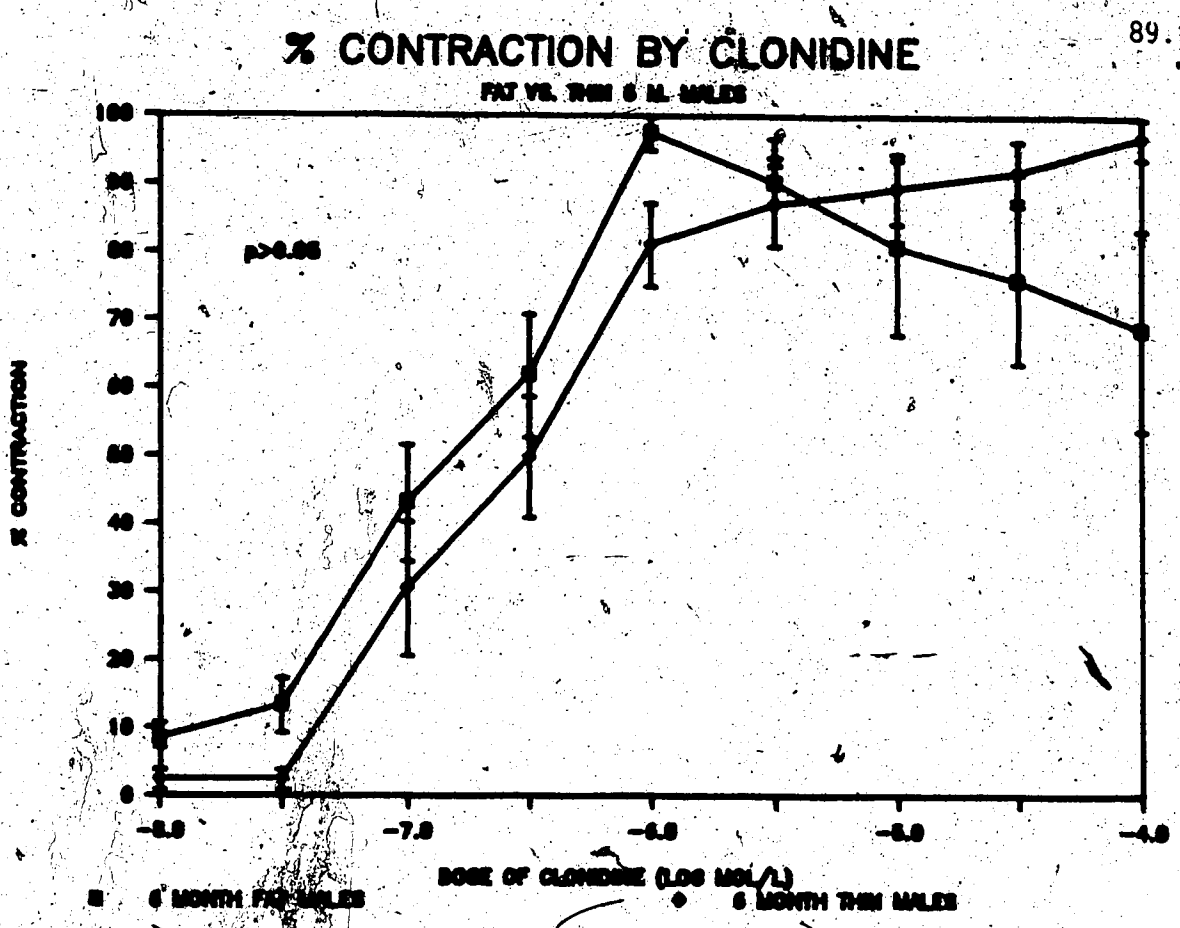
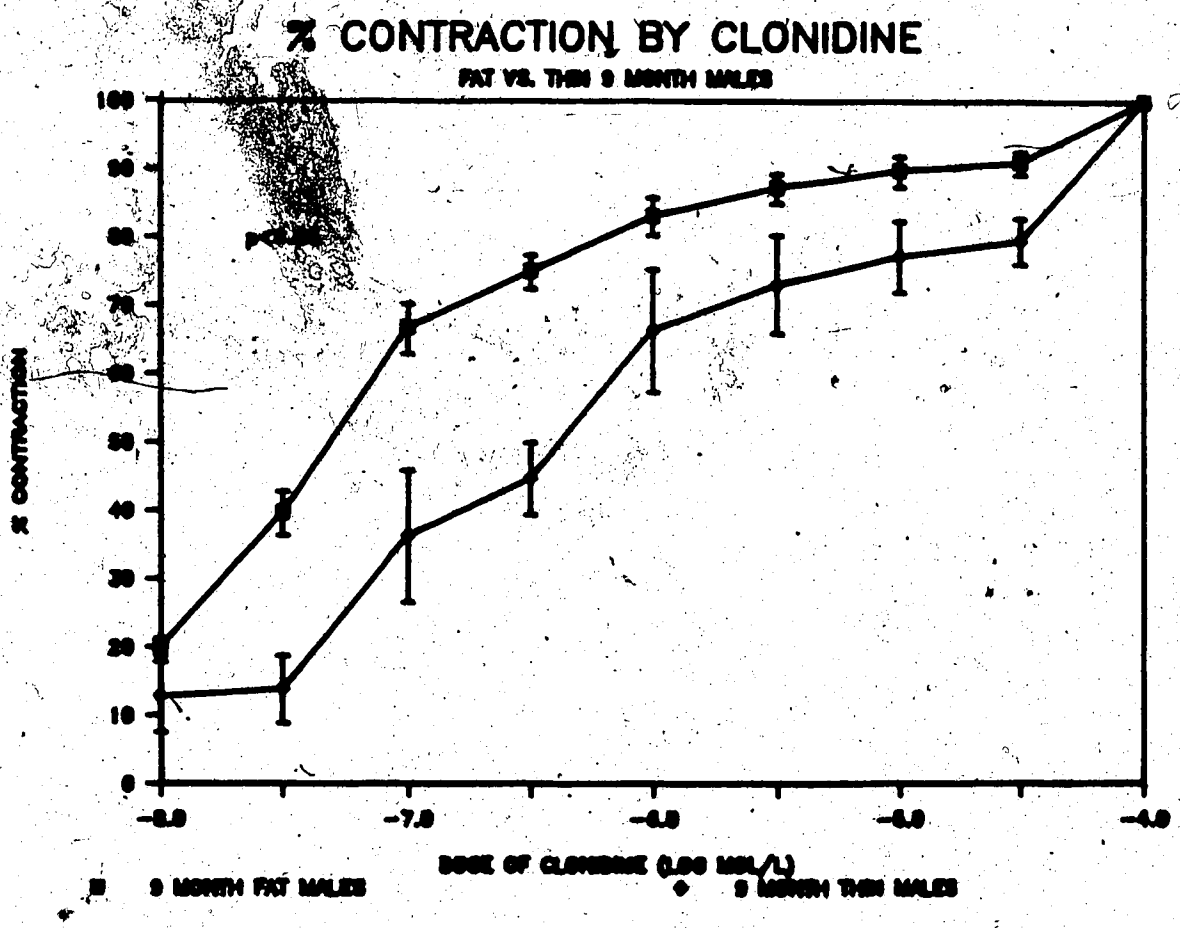
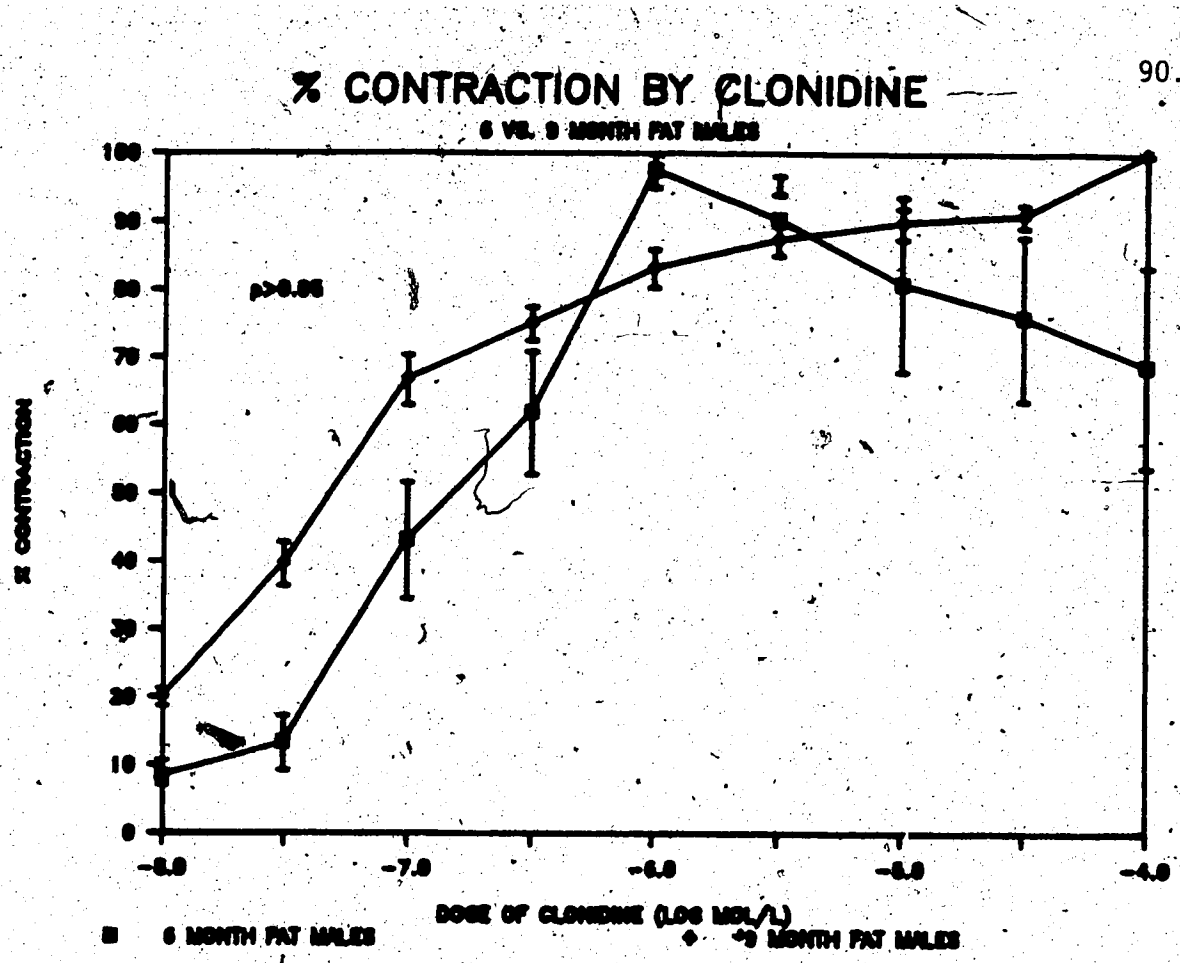
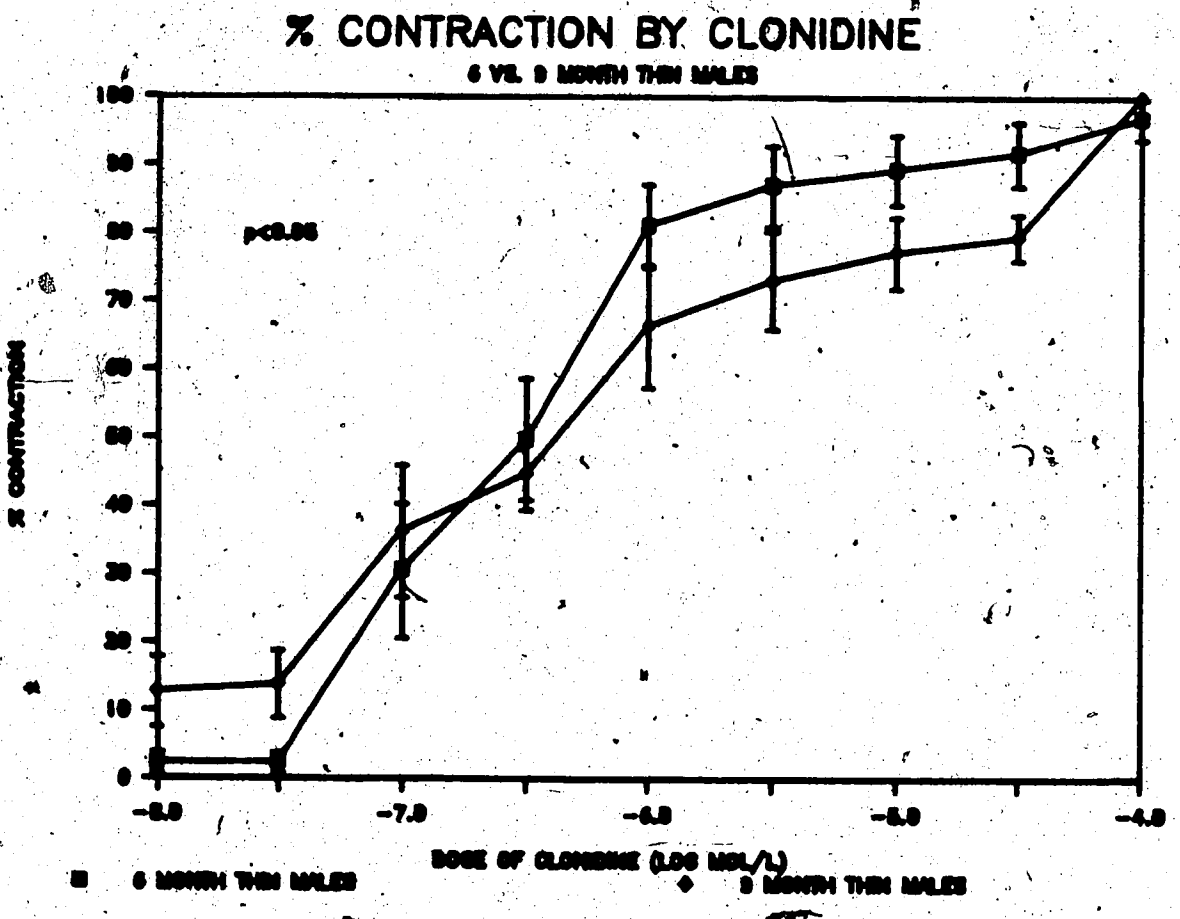
6 VS. 9 M. THIN FEMALE RATS
p>0.05
% CONTRACTION
DOSE OF METHOXAMINE (LOG MOL/L)
6 M. THIN FEMALES
9 M. THIN FEMALES

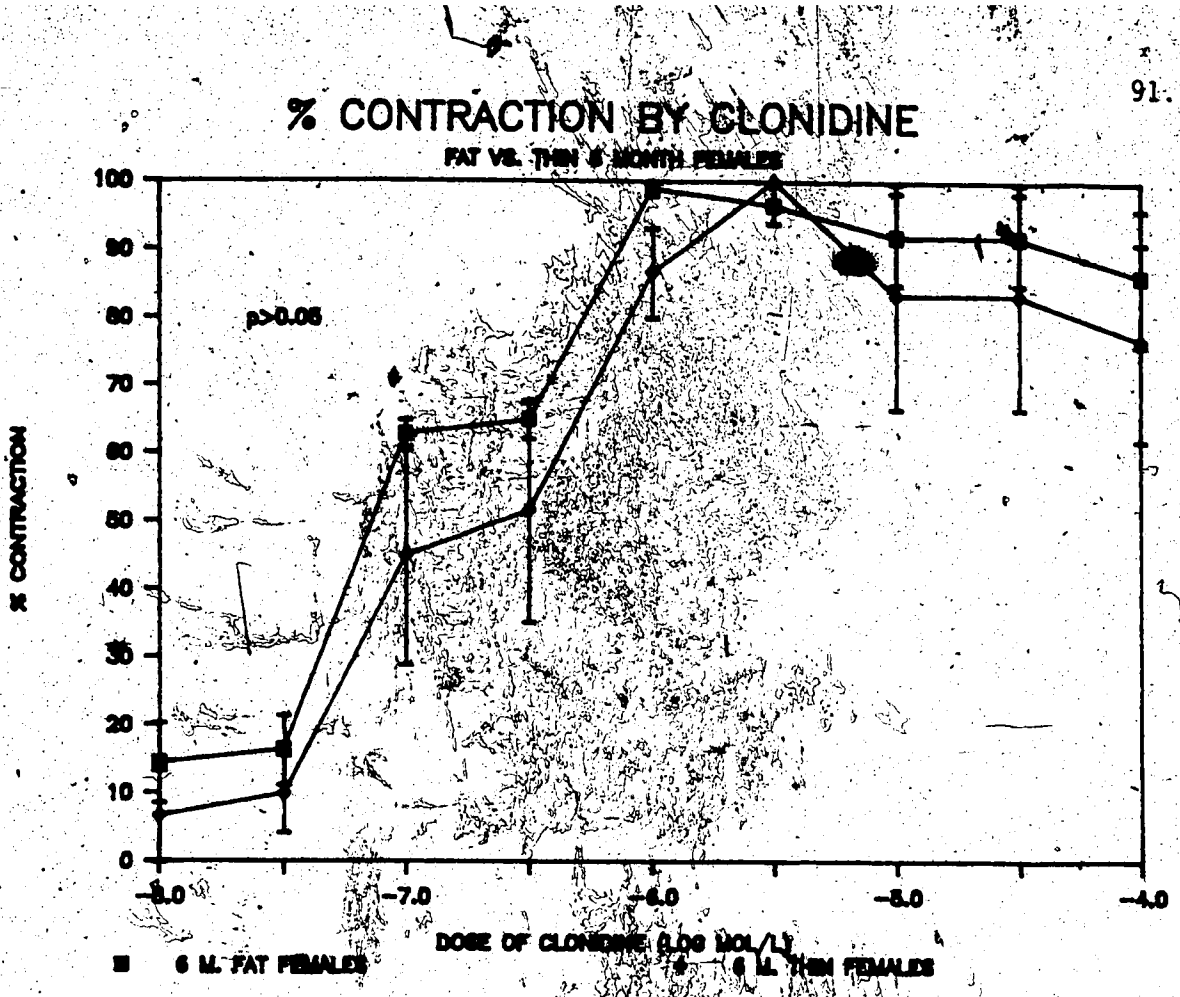

GRAMS OF CONTRACTION BY METHOXAMINE
IN 6MONTH LA/N-CP RATS
gm OF CONTRACTION
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
F-MALE
T-MALE
F-FEM
T-FEM
6 MONTH RATS
9 MONTH RATS

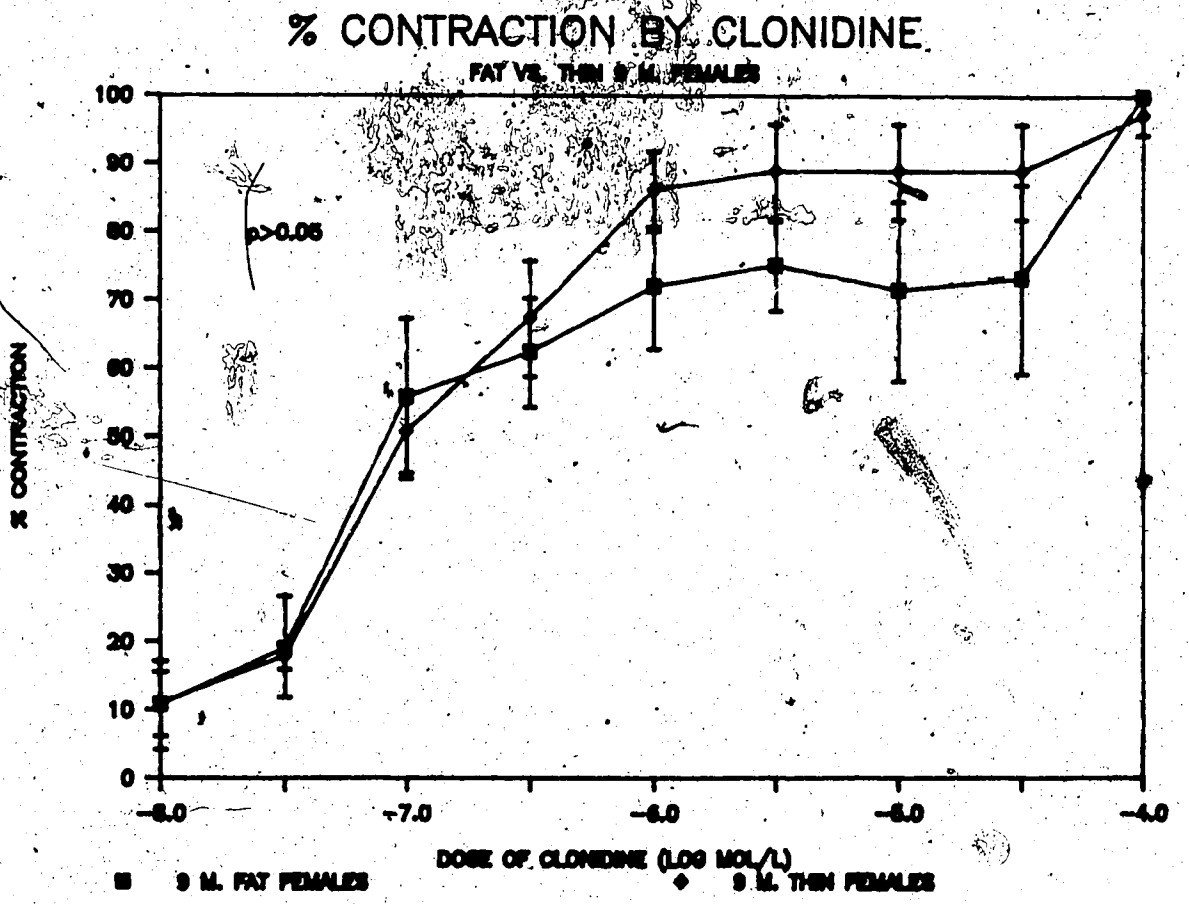

% CONTRACTION BY CLONIDINE
FAT VS. THIN 6 M. MALES
p>0.05
% CONTRACTION
DOSE OF CLONIDINE (LOG MOL/L)
6 MONTH FAT MALES
6 MONTH THIN MALES

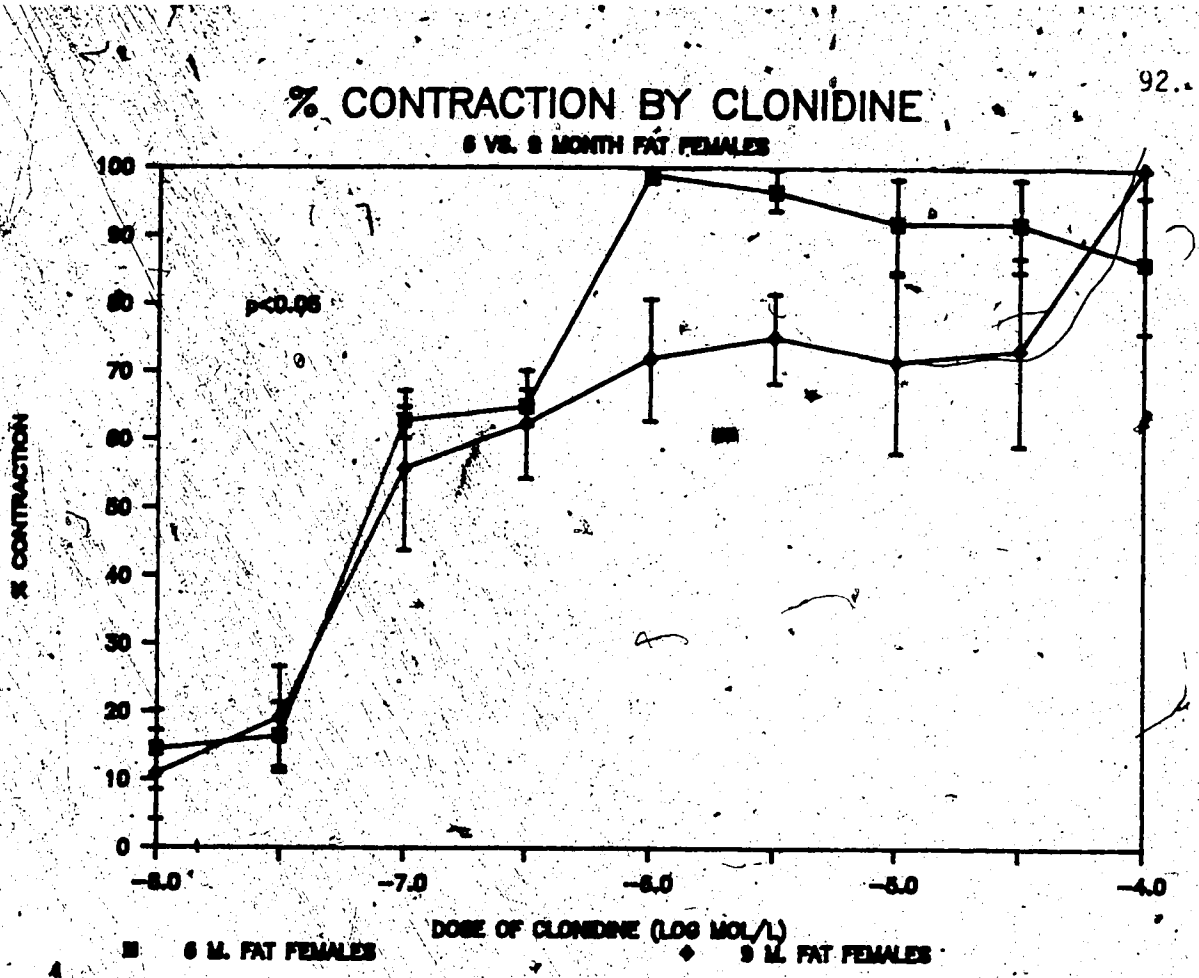

% CONTRACTION BY CLONIDINE
FAT VS. THIN 9 MONTH MALES
% CONTRACTION
DOSE OF CLONIDINE (LOG MOL/L)
9 MONTH FAT MALES
9 MONTH THIN MALES

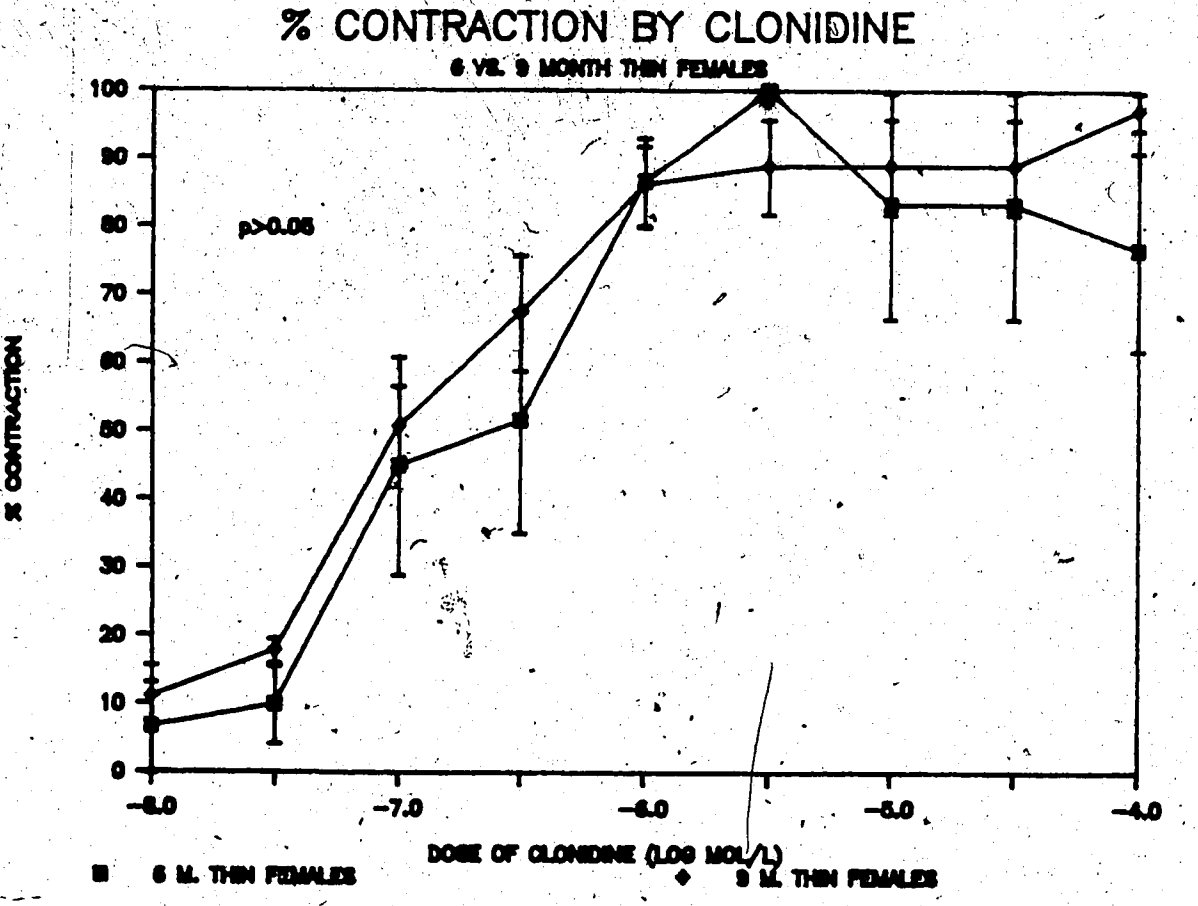

% CONTRACTION BY CLONIDINE
6 VS. 9 MONTH FAT MALES
p>0.05
% CONTRACTION
DOSE OF CLONIDINE (LOG MOL/L)
6 MONTH FAT MALES
9 MONTH FAT MALES

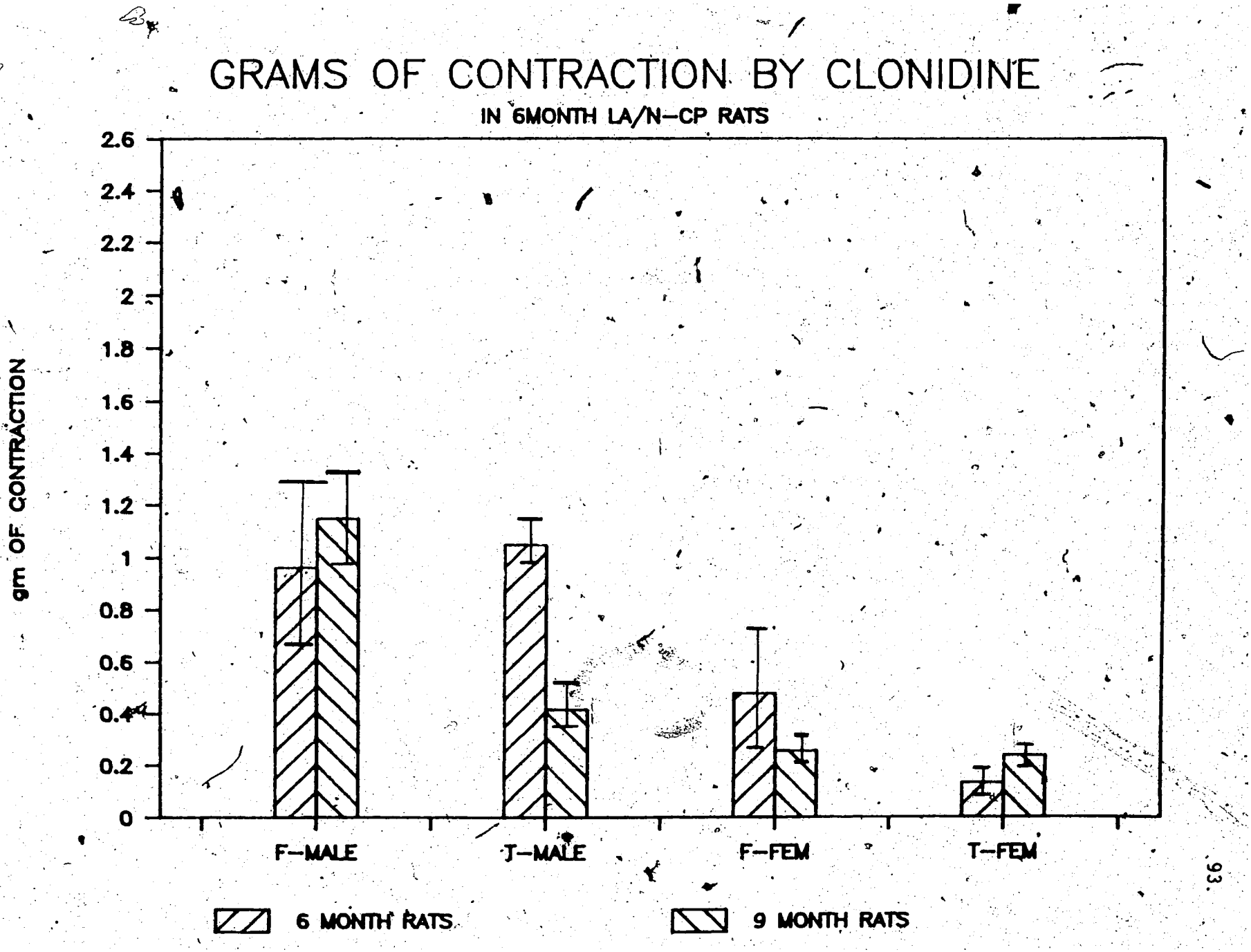

% CONTRACTION BY CLONIDINE
6 VS. 9 MONTH THIN MALES
p<0.05
% CONTRACTION
DOSE OF CLONIDINE (LOG MOL/L)
6 MONTH THIN MALES
9 MONTH THIN MALES


% CONTRACTION BY CLONIDINE
FAT VS. THIN 6 MONTH FEMALES
p>0.05
% CONTRACTION
100
90
80
70
60
50
40
30
20
10
0
-8.0
-7.0
-6.0
-5.0
-4.0
DOSE OF CLONIDINE (LOG MOL/L)
6 M. FAT FEMALES
6 M. THIN FEMALES


% CONTRACTION BY CLONIDINE
FAT VS. THIN 9 M. FEMALES
p>0.05
% CONTRACTION
100
90
80
70
60
50
40
30
20
10
0
-8.0
-7.0
-6.0
-5.0
-4.0
DOSE OF CLONIDINE (LOG MOL/L)
9 M. FAT FEMALES
9 M. THIN FEMALES

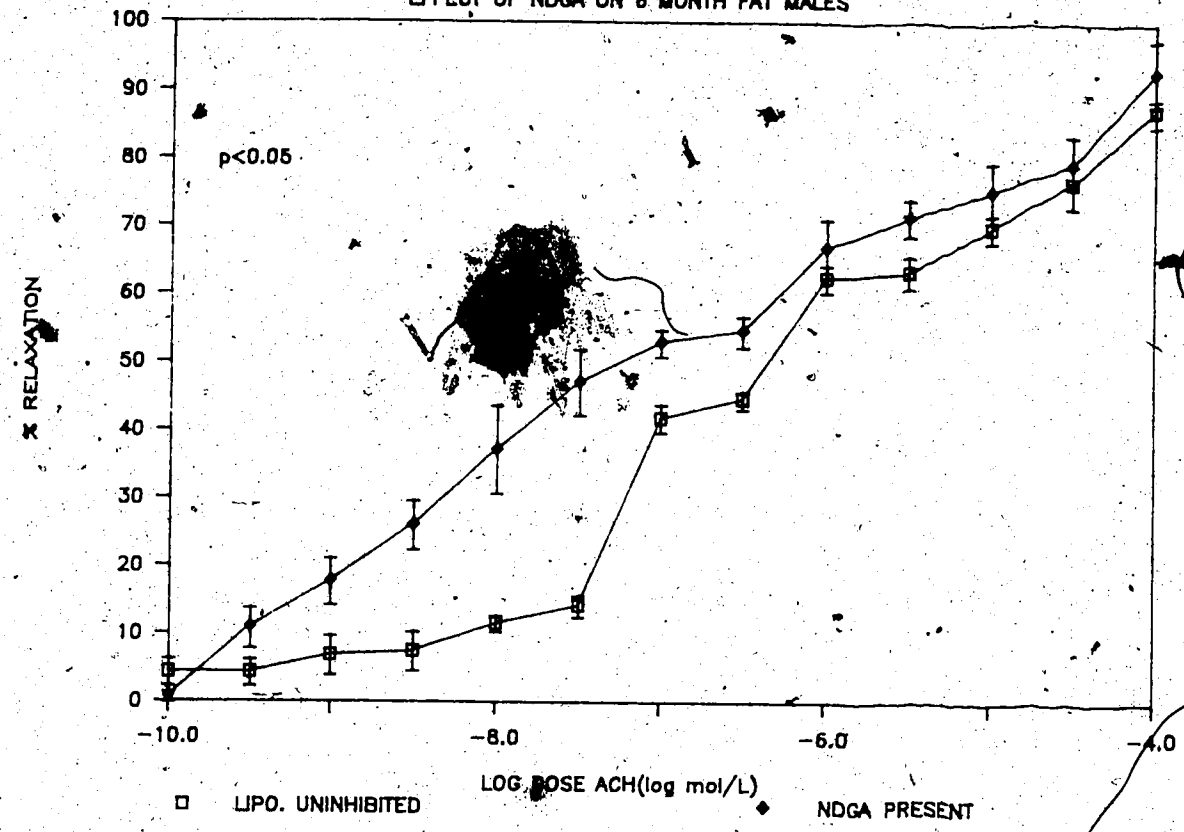

% CONTRACTION BY CLONIDINE
6 VS. 9 MONTH FAT FEMALES
p<0.05
% CONTRACTION
100
90
80
70
60
50
40
30
20
10
0
-8.0
-7.0
-6.0
-5.0
-4.0
DOSE OF CLONIDINE (LOG MOL/L)
6 M. FAT FEMALES
9 M. FAT FEMALES


% CONTRACTION BY CLONIDINE
6 VS. 9 MONTH THIN FEMALES
p>0.05
% CONTRACTION
100
90
80
70
60
50
40
30
20
10
0
-8.0
-7.0
-6.0
-5.0
-4.0
DOSE OF CLONIDINE (LOG MOL/L)
6 M. THIN FEMALES
9 M. THIN FEMALES


GRAMS OF CONTRACTION BY CLONIDINE
IN 6MONTH LA/N-CP RATS
gm OF CONTRACTION
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
F-MALE
T-MALE
F-FEM
T-FEM
6 MONTH RATS
9 MONTH RATS

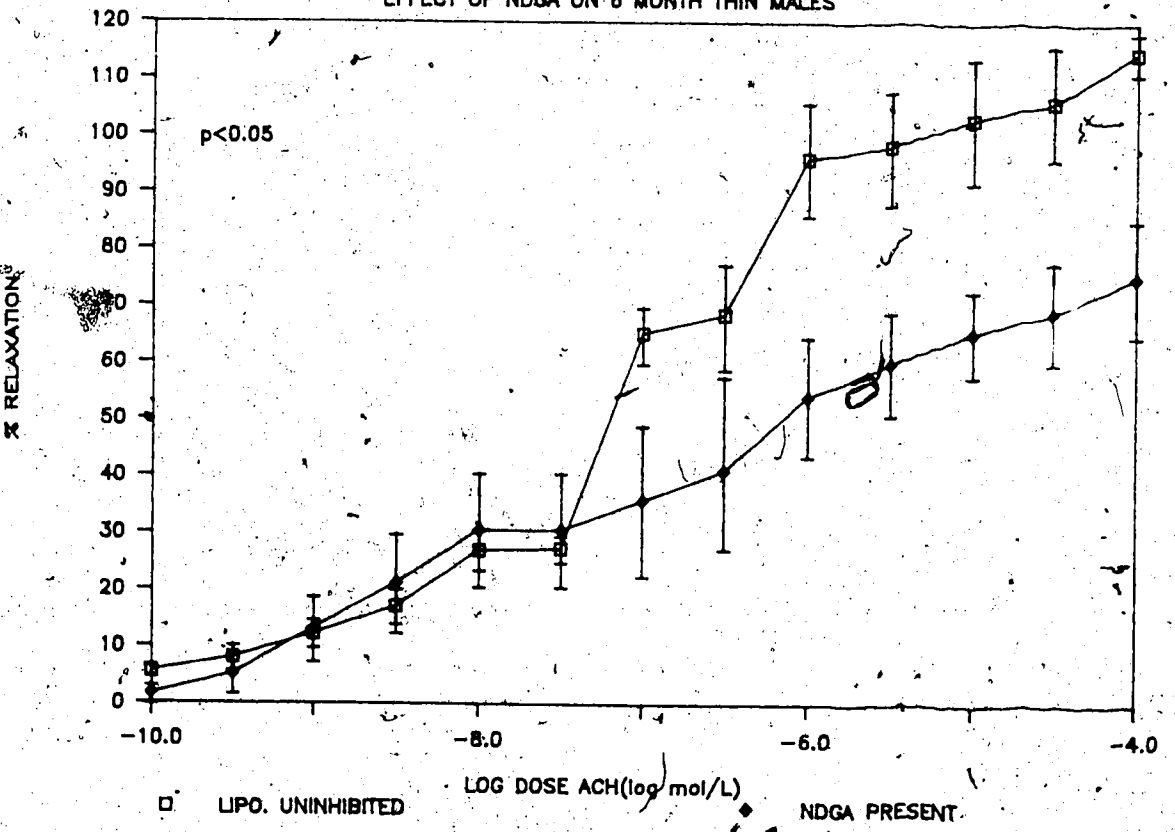
% RELAXATION BY ACH IN LA/N-CP RATS

EFFECT OF NDGA ON 6 MONTH FAT MALES



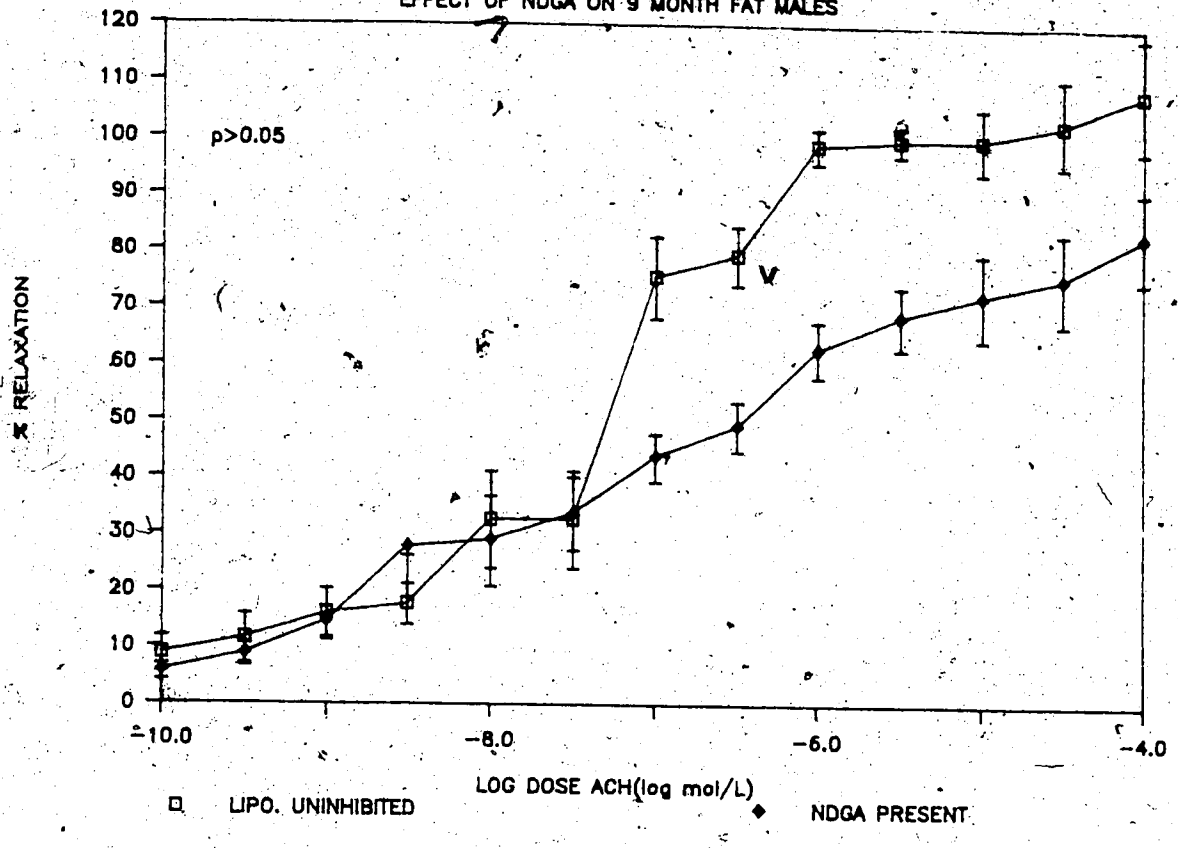
% RELAXATION BY ACH IN LA/N-CP RATS

EFFECT OF NDGA ON 6 MONTH THIN MALES



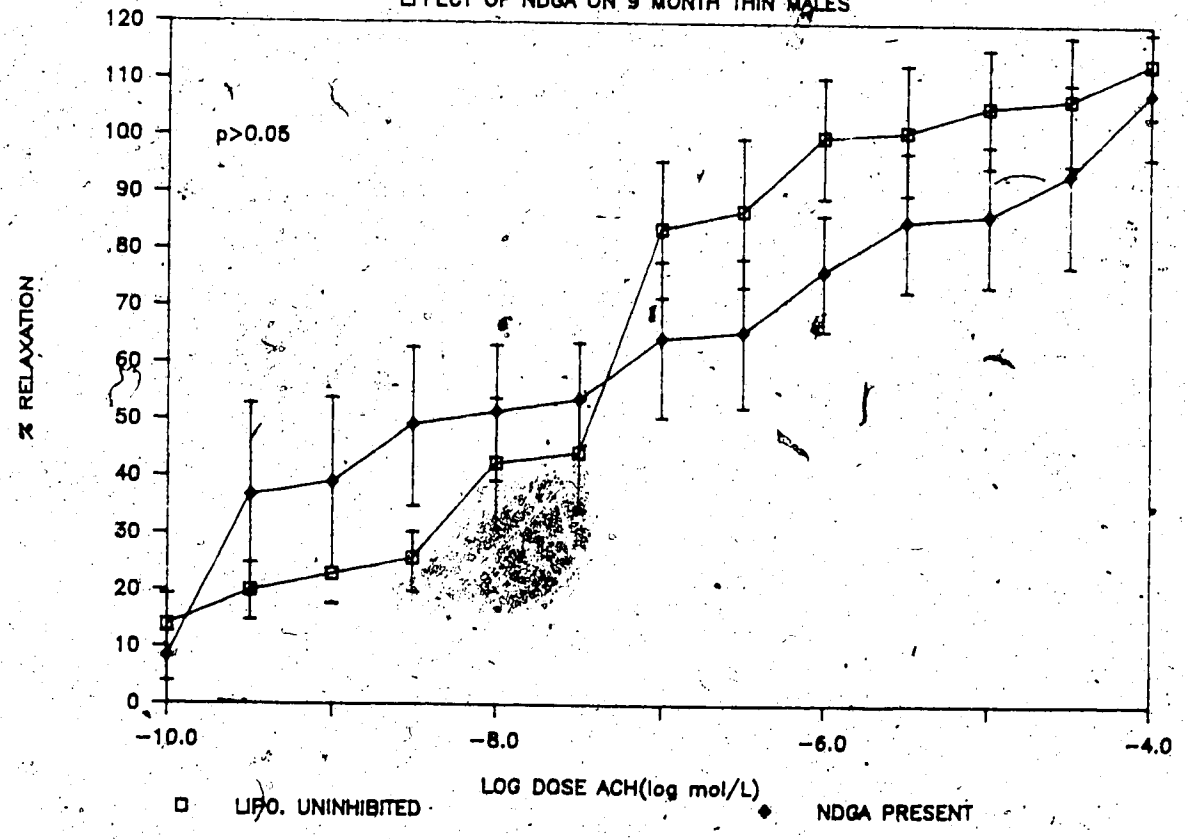
% RELAXATION BY ACH IN LA/N-CP RATS

EFFECT OF NDGA ON 9 MONTH FAT MALES

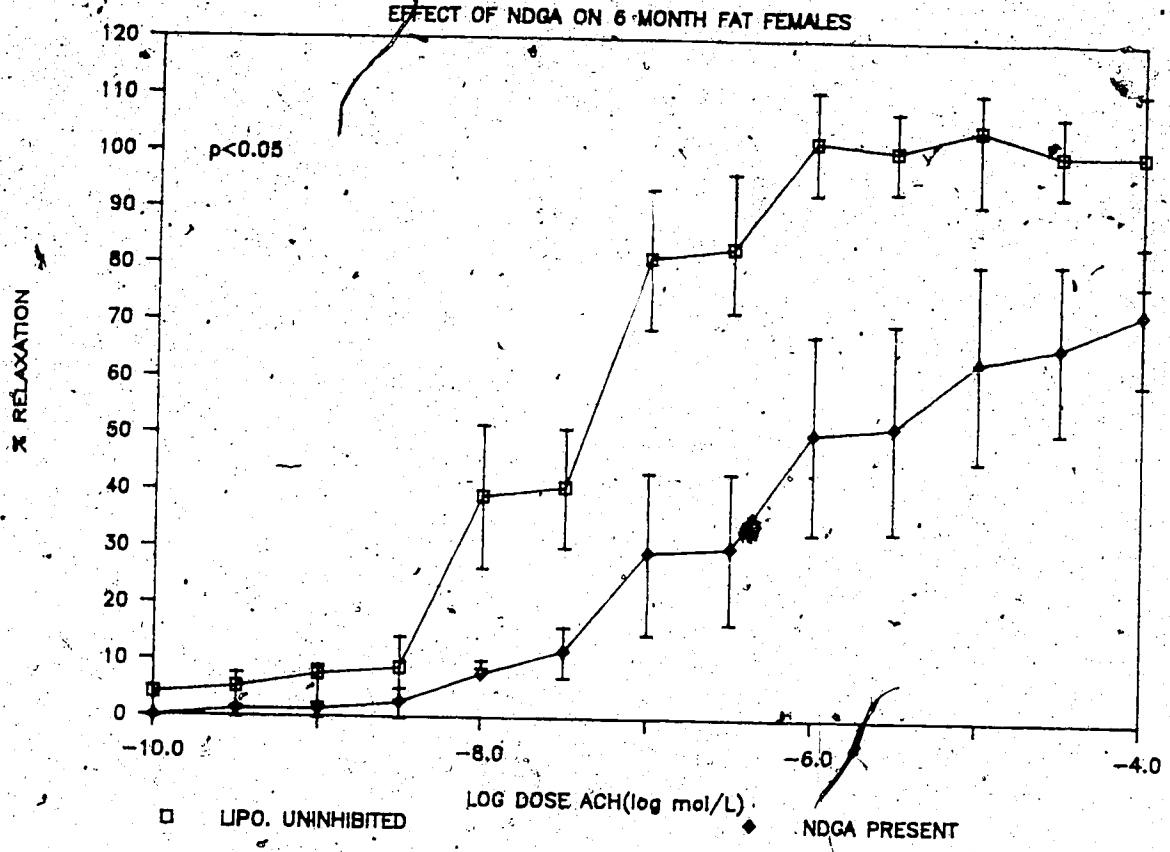


% RELAXATION BY ACH IN LA/N-CP RATS

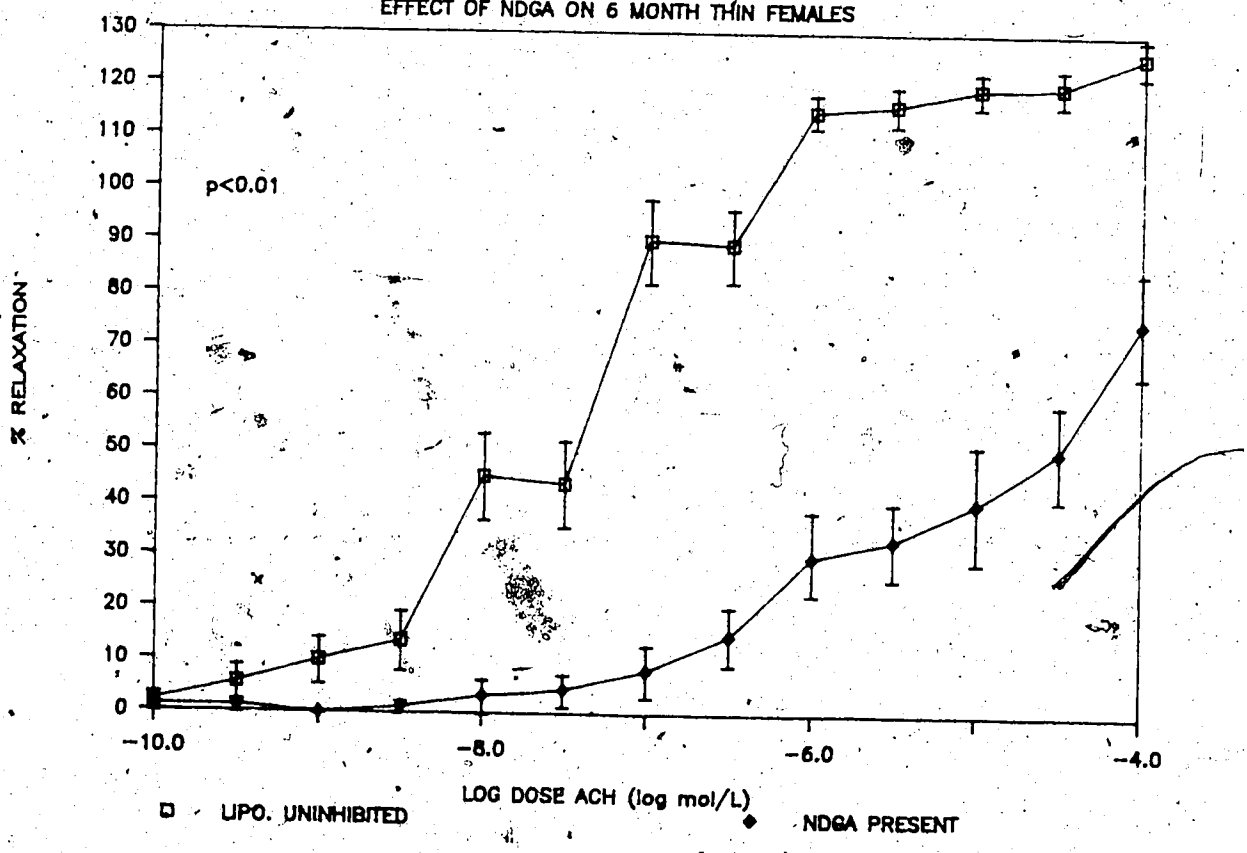
EFFECT OF NDGA ON 9 MONTH THIN MALES



PER CENT RELAXATION BY ACETYLCHOLINE

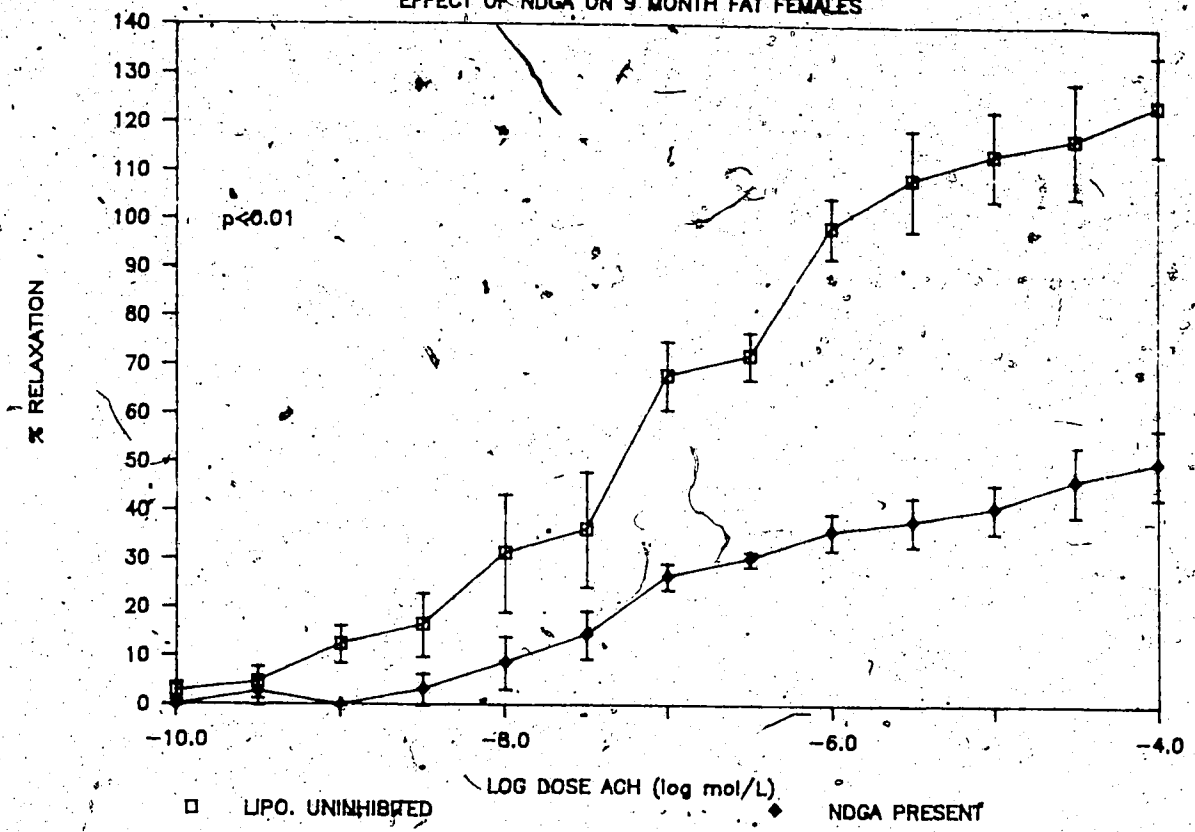


% RELAXATION BY ACH. IN LA/N-CP RATS



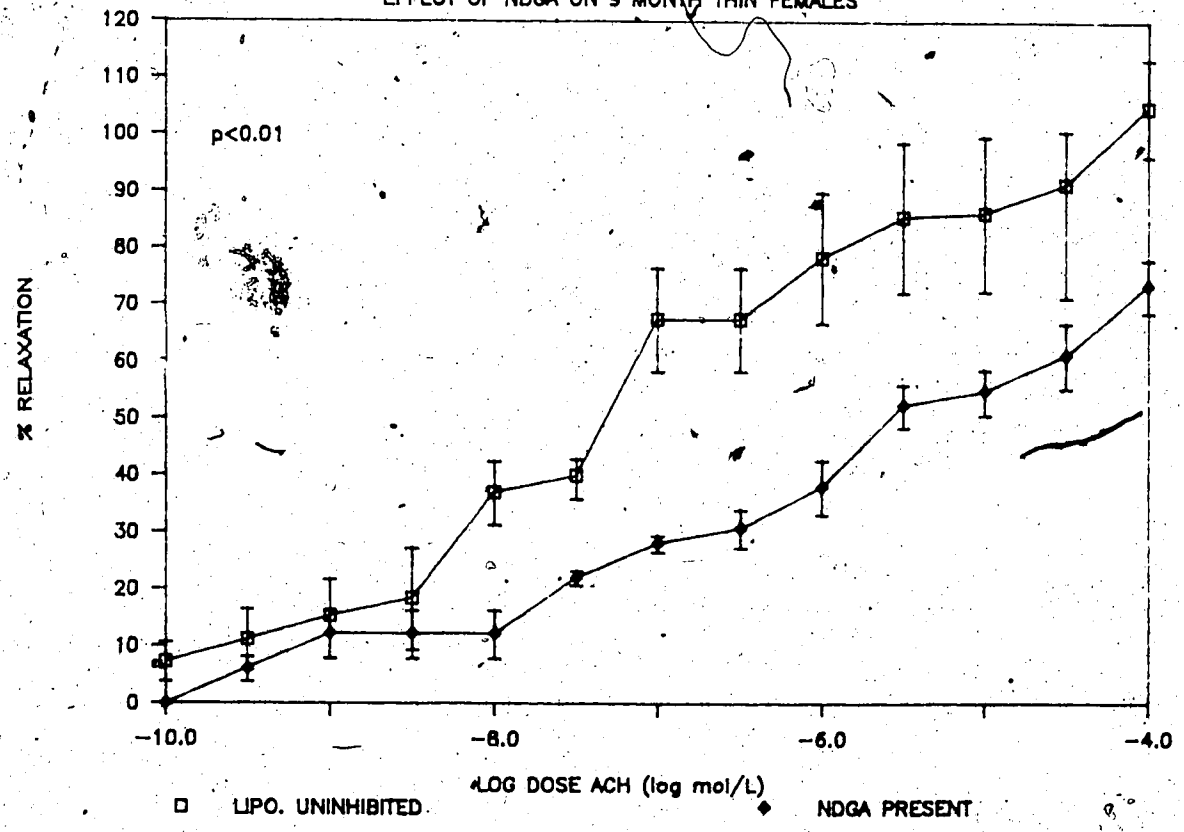
% RELAXATION BY ACH. IN LA/N-CP RATS

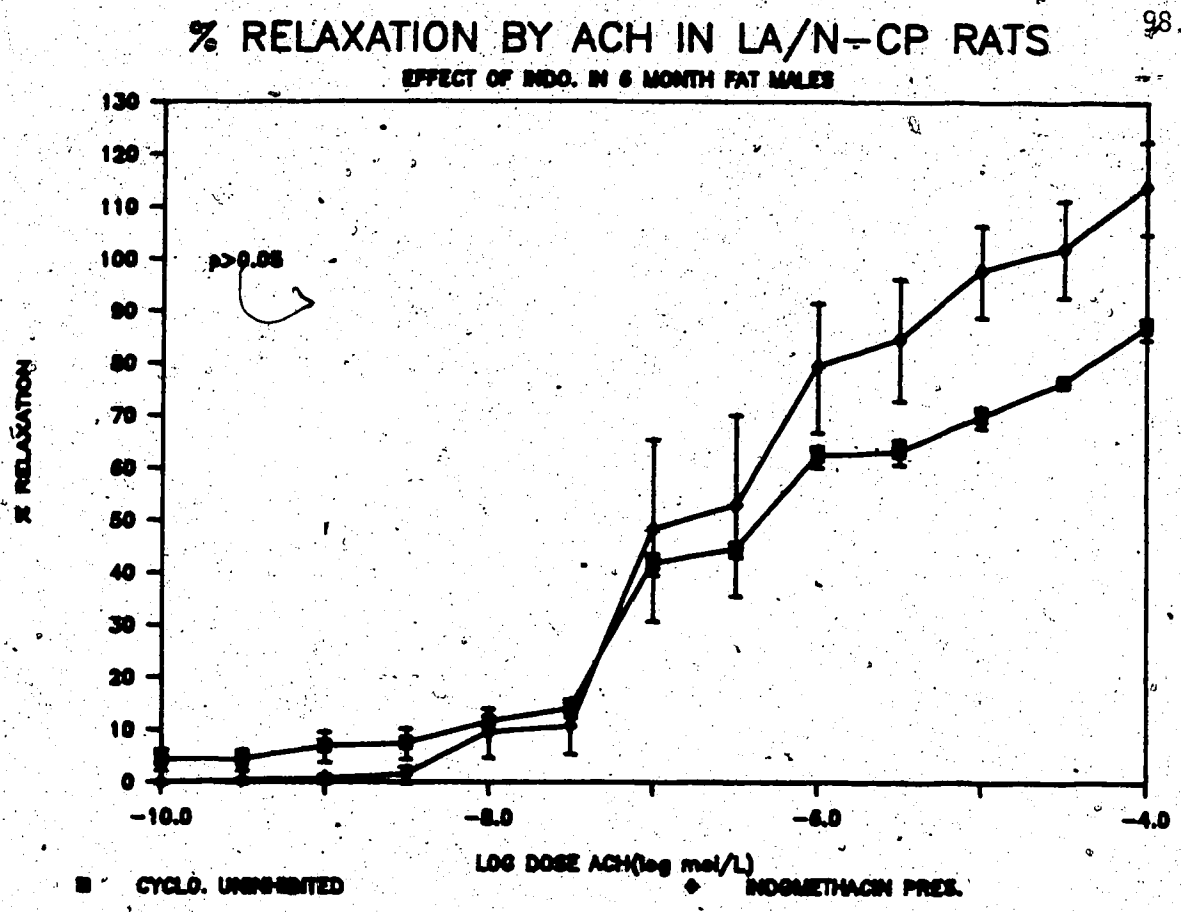
EFFECT OF NDGA ON 9 MONTH FAT FEMALES

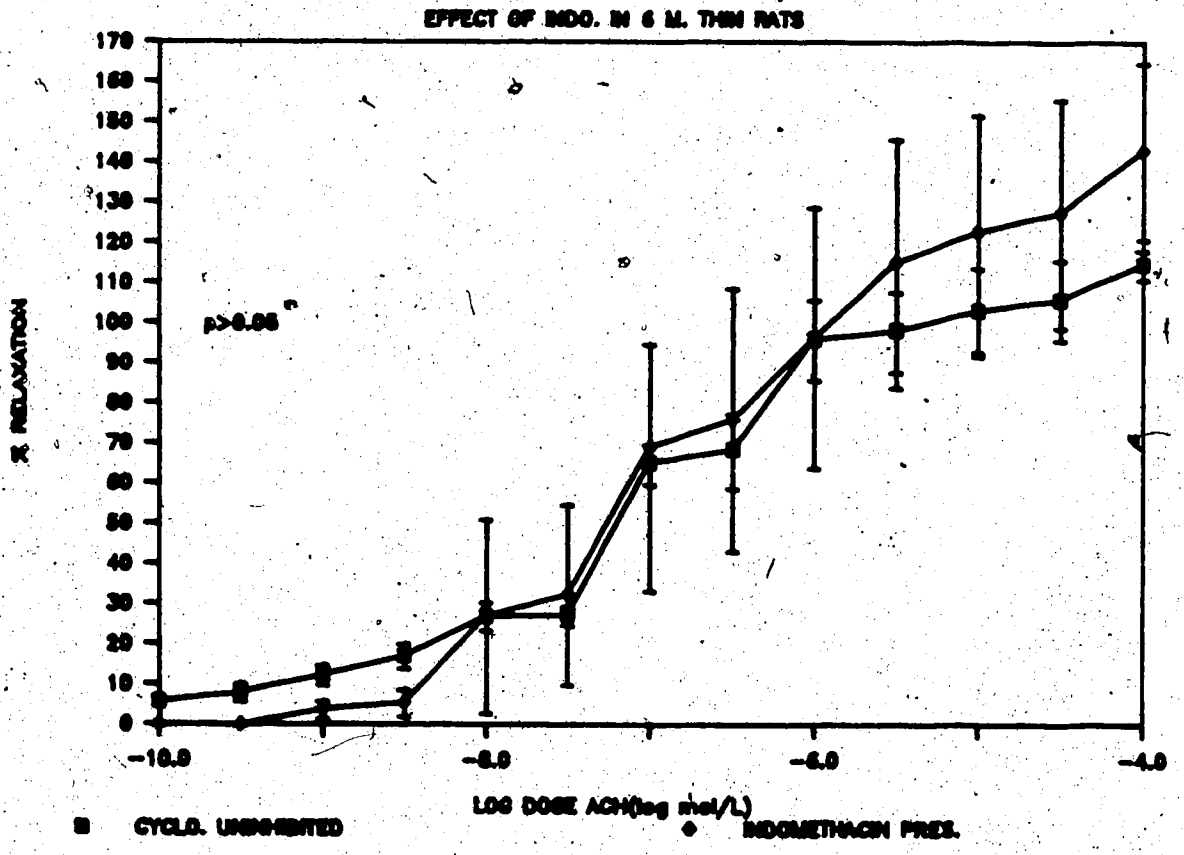


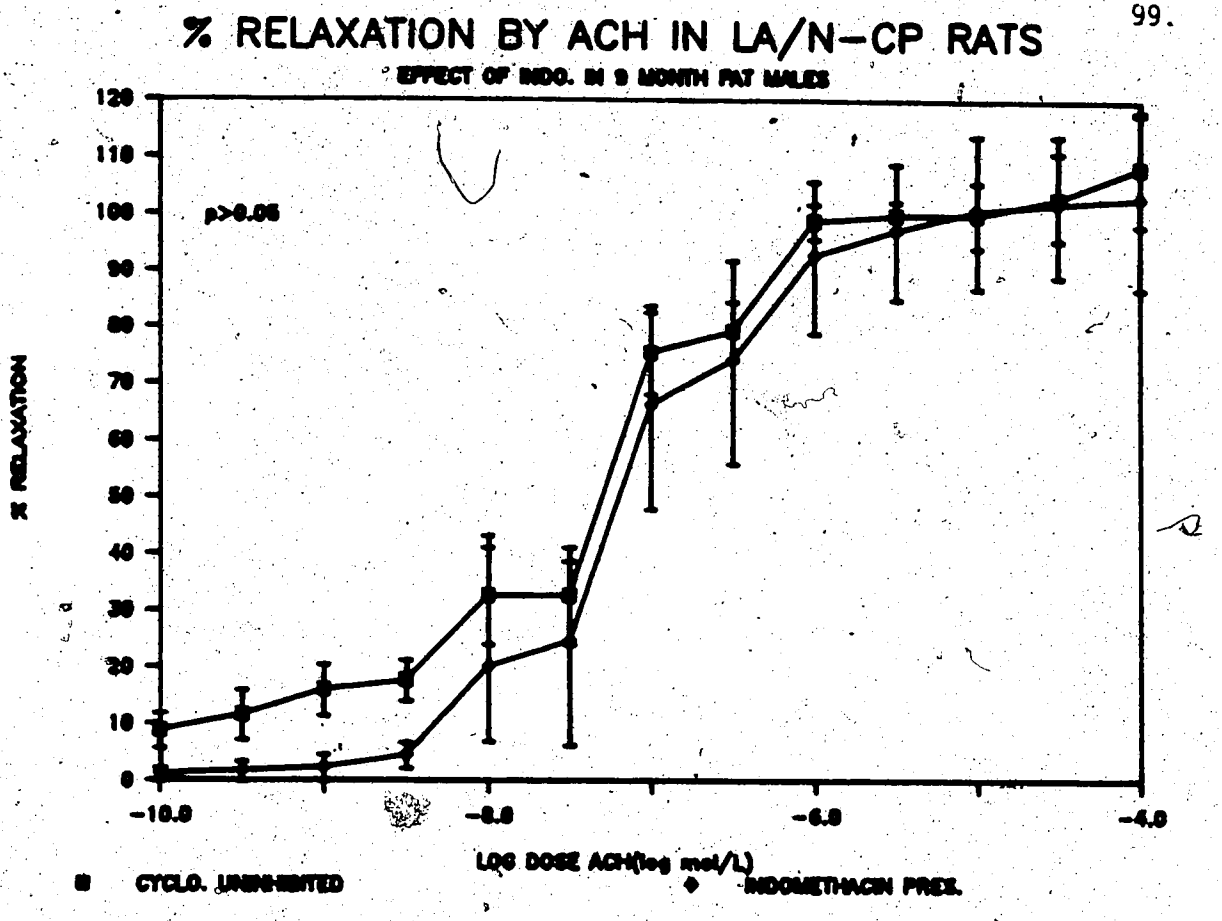
% RELAXATION BY ACH. IN LA/N-CP RATS

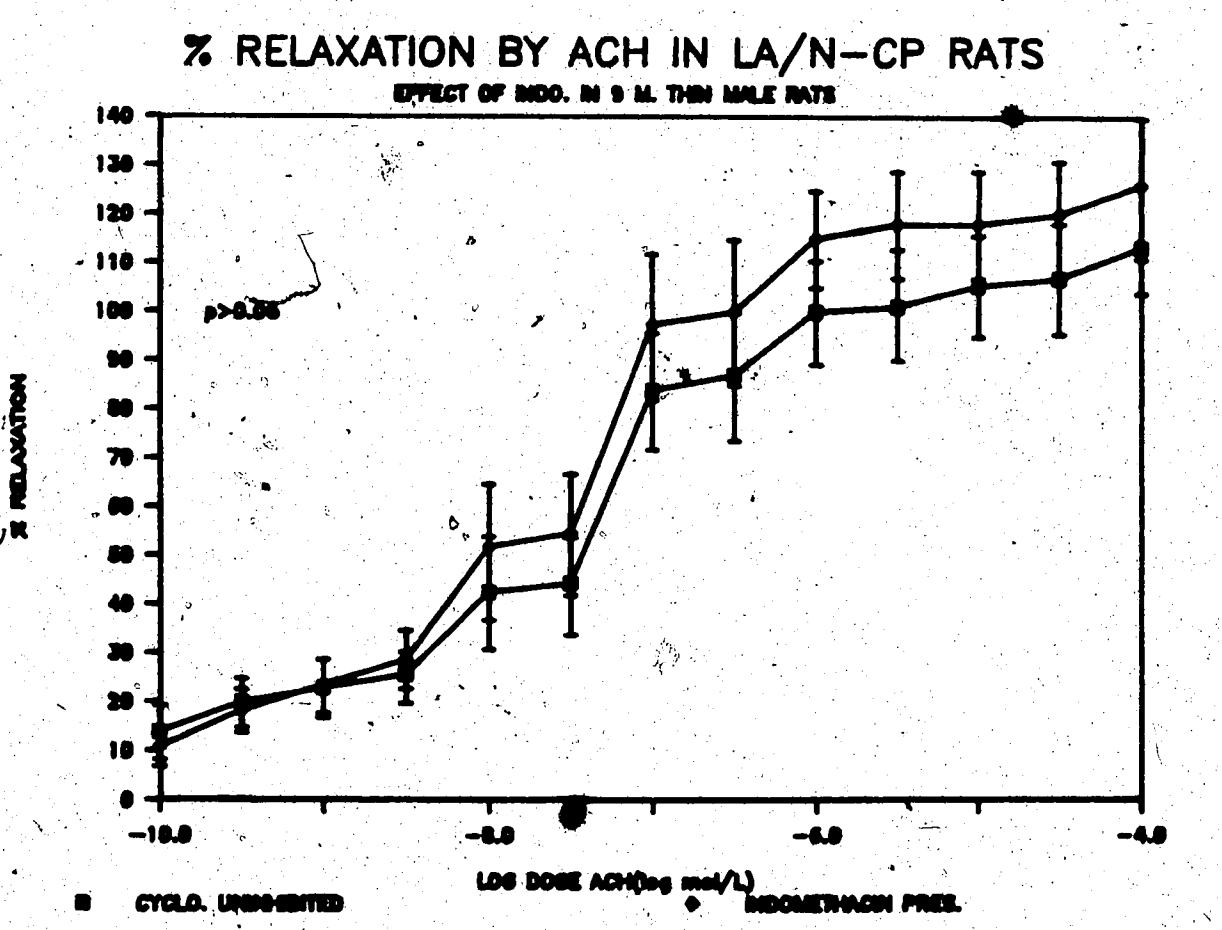
EFFECT OF NDGA ON 9 MONTH THIN FEMALES



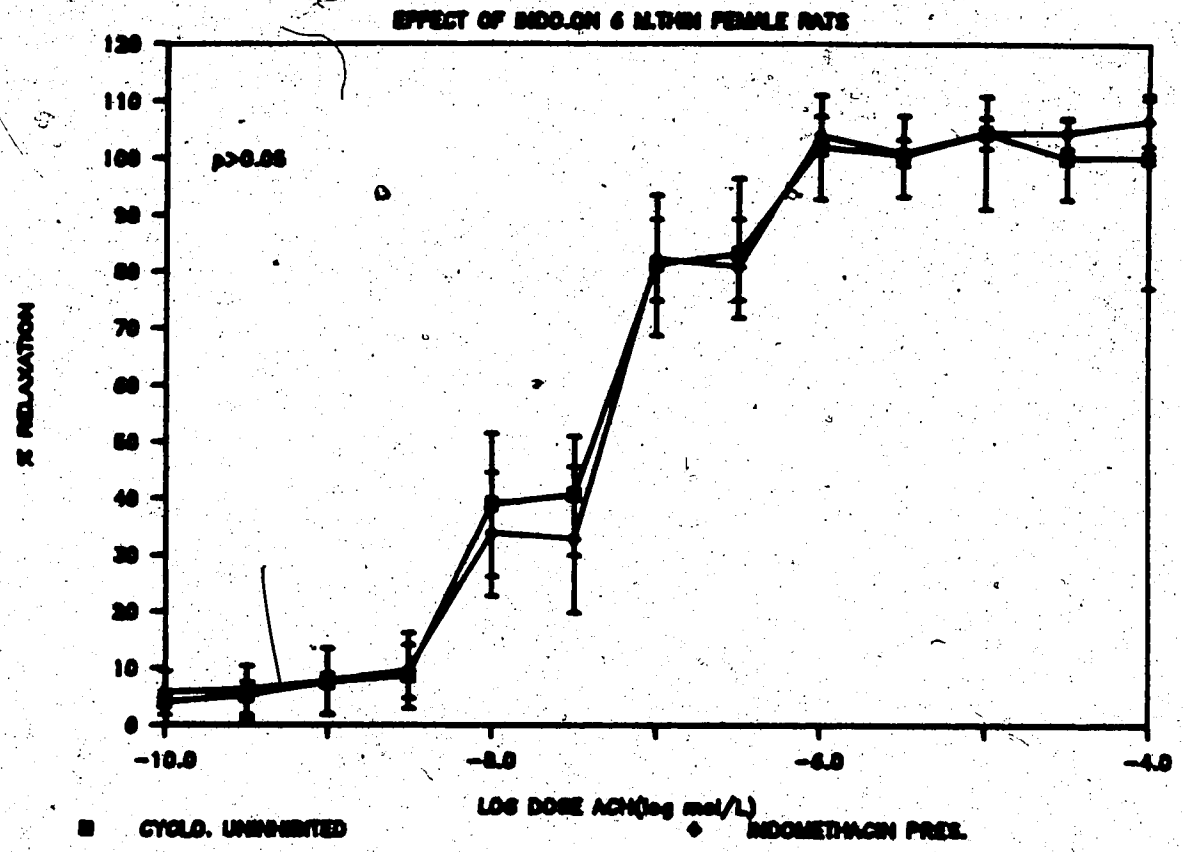

% RELAXATION BY ACH IN LA/N-CP RATS
EFFECT OF INDO. IN 6 MONTH FAT MALES
% RELAXATION
p>0.05
LOG DOSE ACH(log mol/L)
CYCLO. UNINHIBITED
INDOMETHACIN PRES.


% RELAXATION BY ACH IN LA/N-CP RATS
EFFECT OF INDO. IN 6 M. THIN RATS
% RELAXATION
p>0.05
LOG DOSE ACH(log mol/L)
CYCLO. UNINHIBITED
INDOMETHACIN PRES.

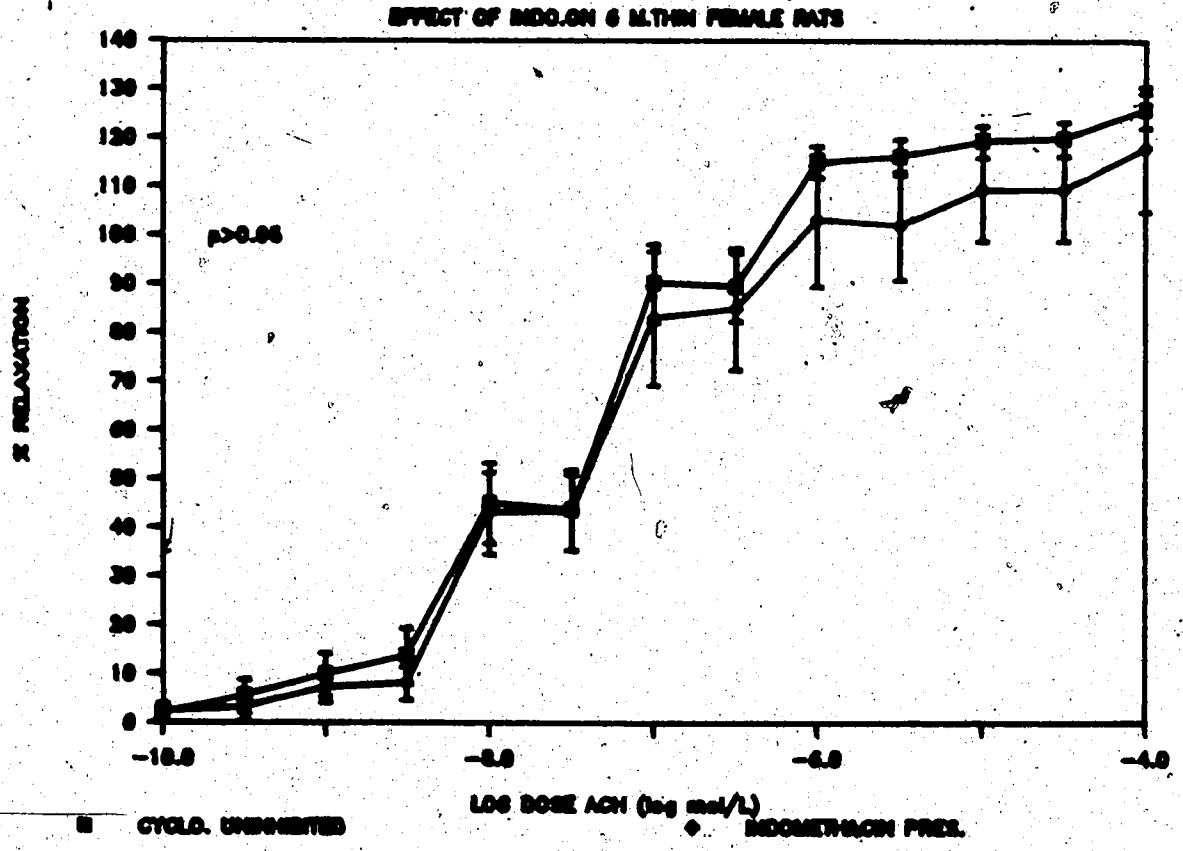

% RELAXATION BY ACH IN LA/N-CP RATS
EFFECT OF INDO. IN 9 MONTH FAT MALES
p>0.05
% RELAXATION
LOG DOSE ACH(log mol/L)
CYCLO. UNINHIBITED
INDOMETHACIN PREX.

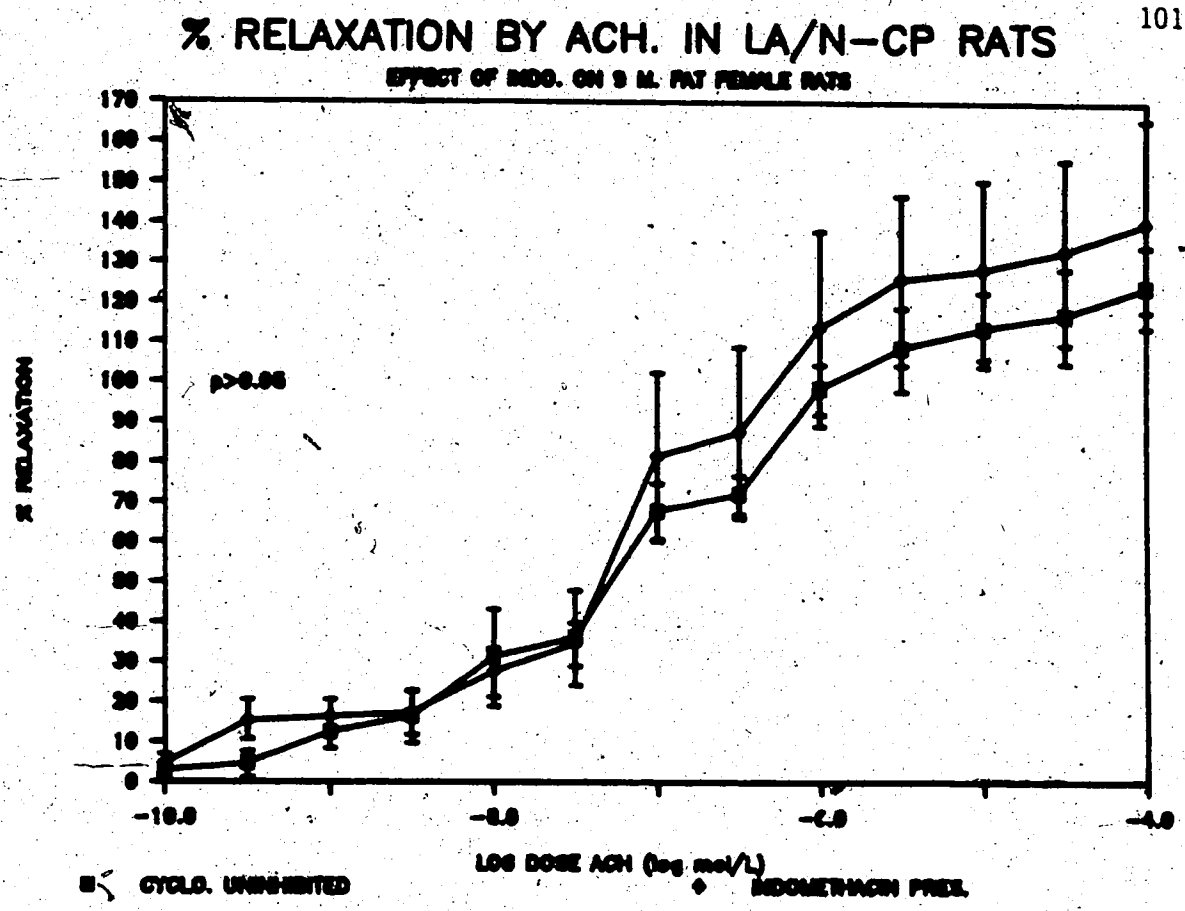

% RELAXATION BY ACH IN LA/N-CP RATS
EFFECT OF INDO. IN 9 M. THIN MALE RATS
% RELAXATION
LOG DOSE ACH(log mol/L)
CYCLO. UNINHIBITED
INDOMETHACIN PREX.

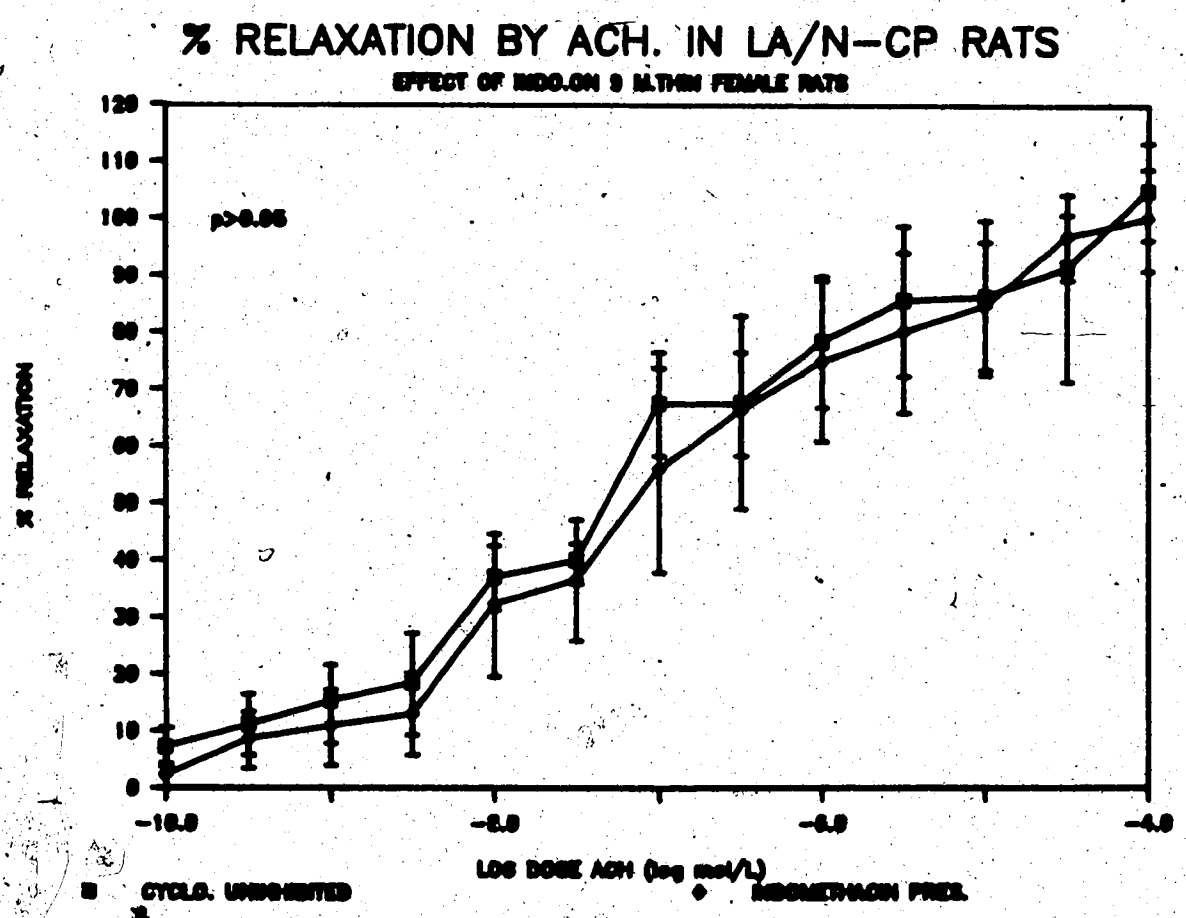
PER CENT RELAXATION OF FAT FEMALE RATS



% RELAXATION BY ACH. IN LA/N-CP RATS

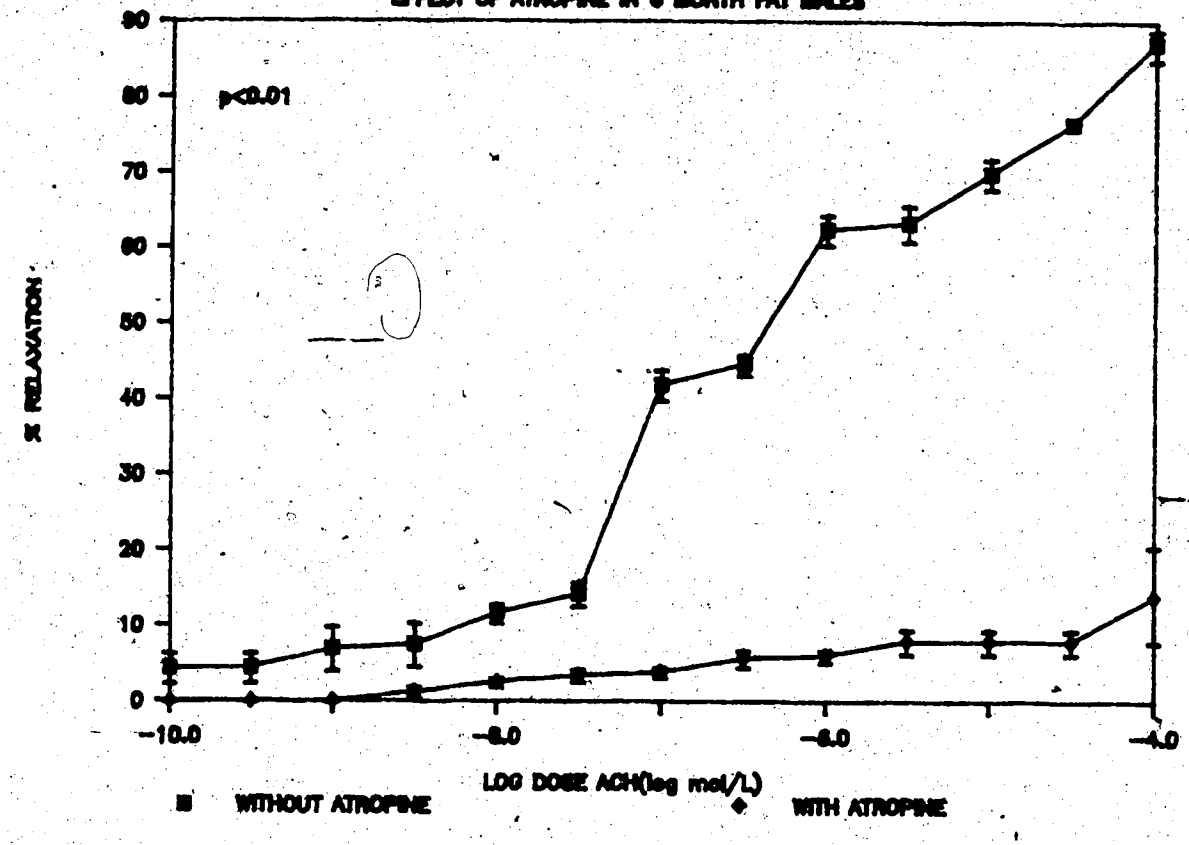



% RELAXATION BY ACH. IN LA/N-CP RATS
EFFECT OF INDO. ON 9 M. FAT FEMALE RATS
% RELAXATION
p>0.05
LOG DOSE ACH (log mol/L)
CYCLO. UNINHIBITED
INDOMETHACIN PREV.


% RELAXATION BY ACH. IN LA/N-CP RATS
EFFECT OF INDO.ON 9 M.THIN FEMALE RATS
% RELAXATION
p>0.05
LOG DOSE ACH (log mol/L)
CYCLO. UNINHIBITED
INDOMETHACIN PREV.

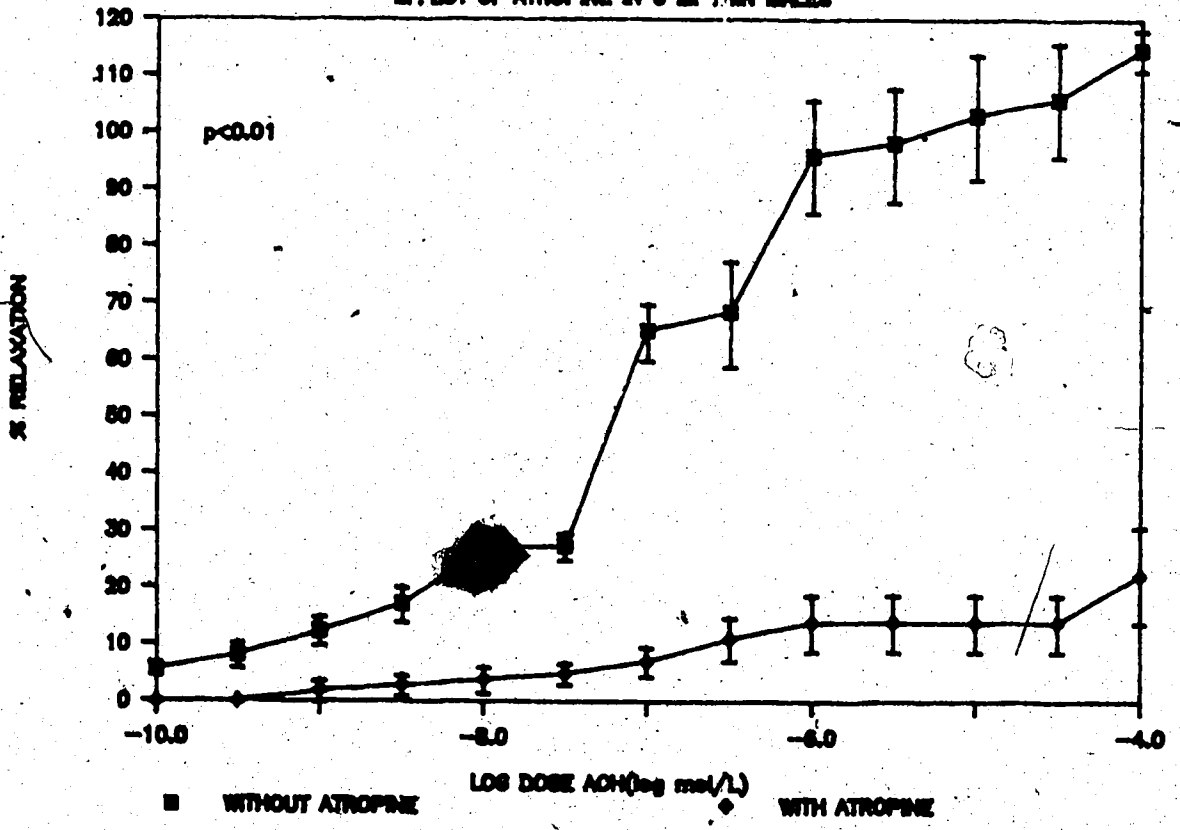
% RELAXATION BY ACH IN LA/N-CP RATS

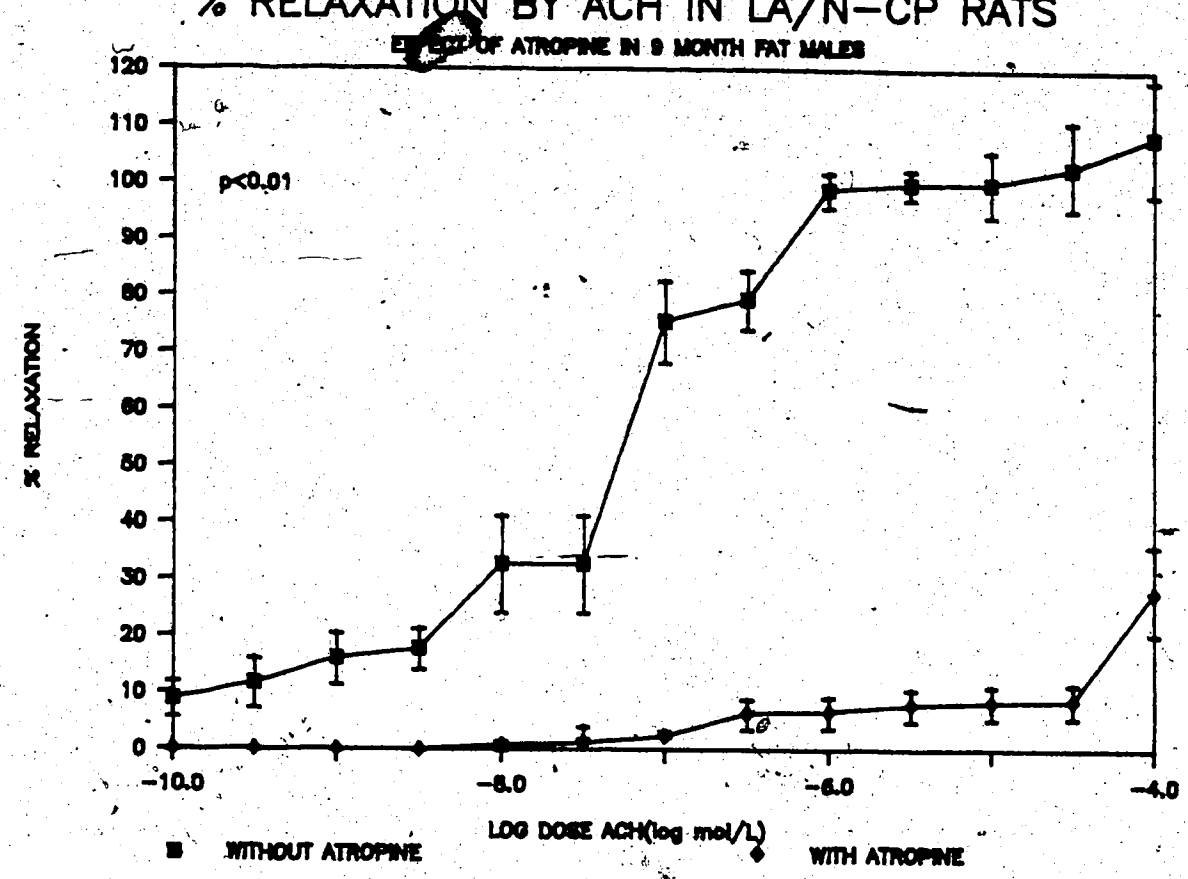
EFFECT OF ATROPINE IN 6 MONTH FAT MALES

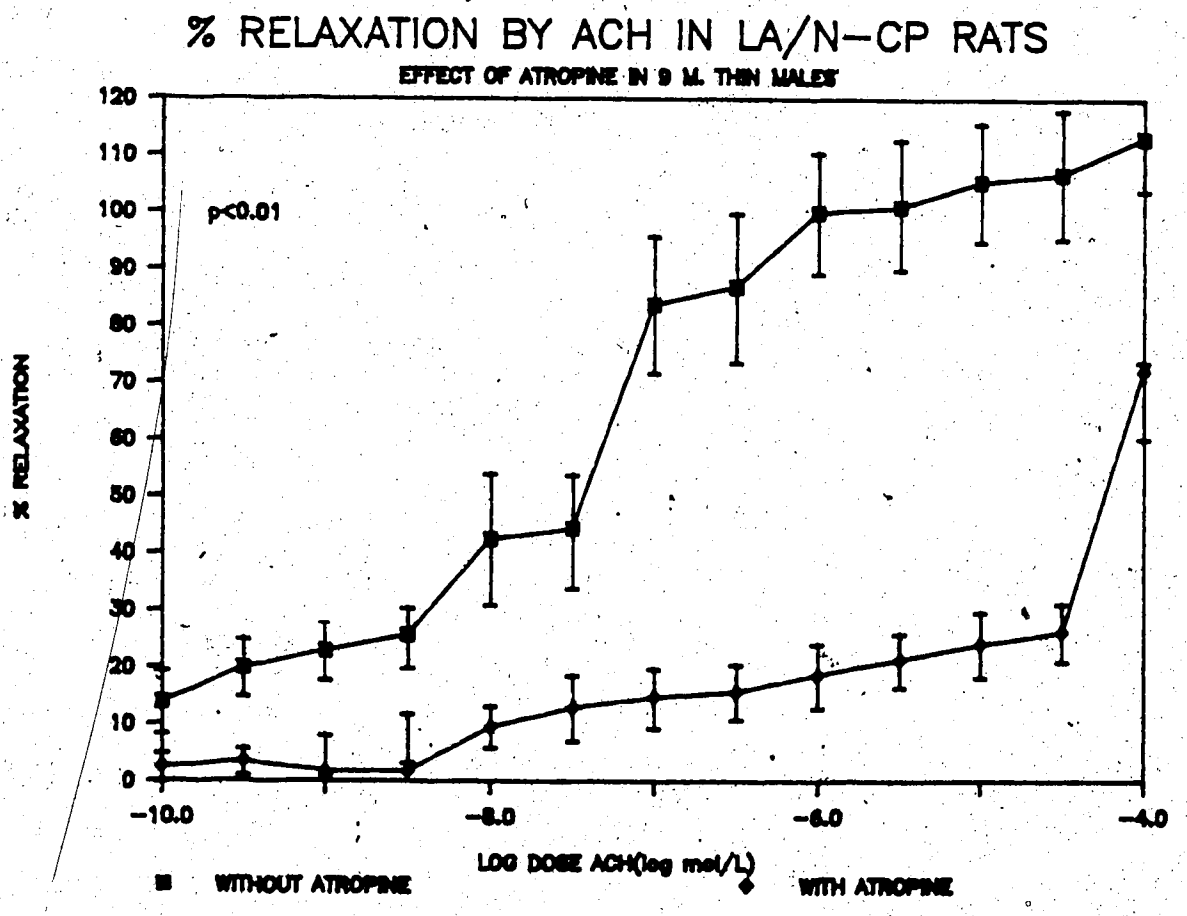


% RELAXATION BY ACH IN LA/N-CP RATS

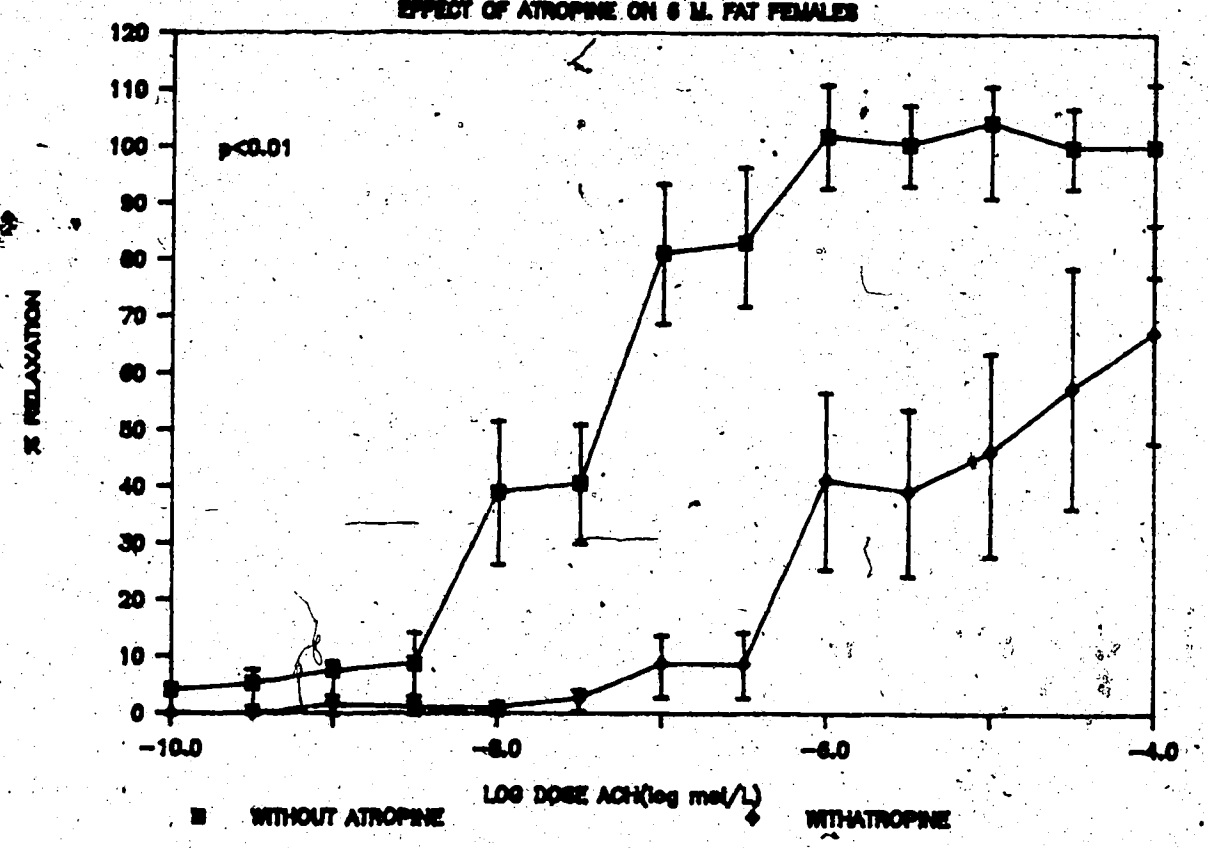
EFFECT OF ATROPINE IN 6 M. THIN MALES



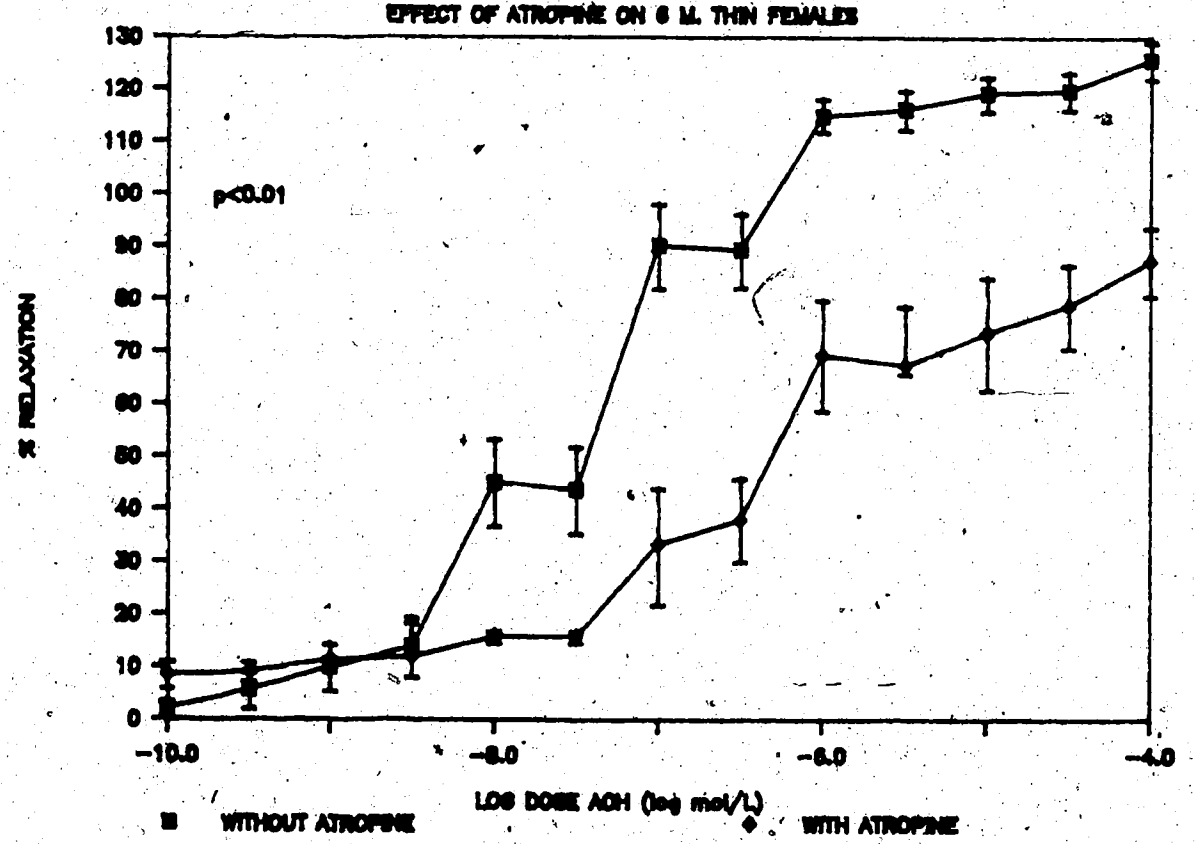

% RELAXATION BY ACH IN LA/N-CP RATS
EFFECT OF ATROPINE IN 9 MONTH FAT MALES
p<0.01
% RELAXATION
LOG DOSE ACH(log mol/L)
WITHOUT ATROPINE
WITH ATROPINE

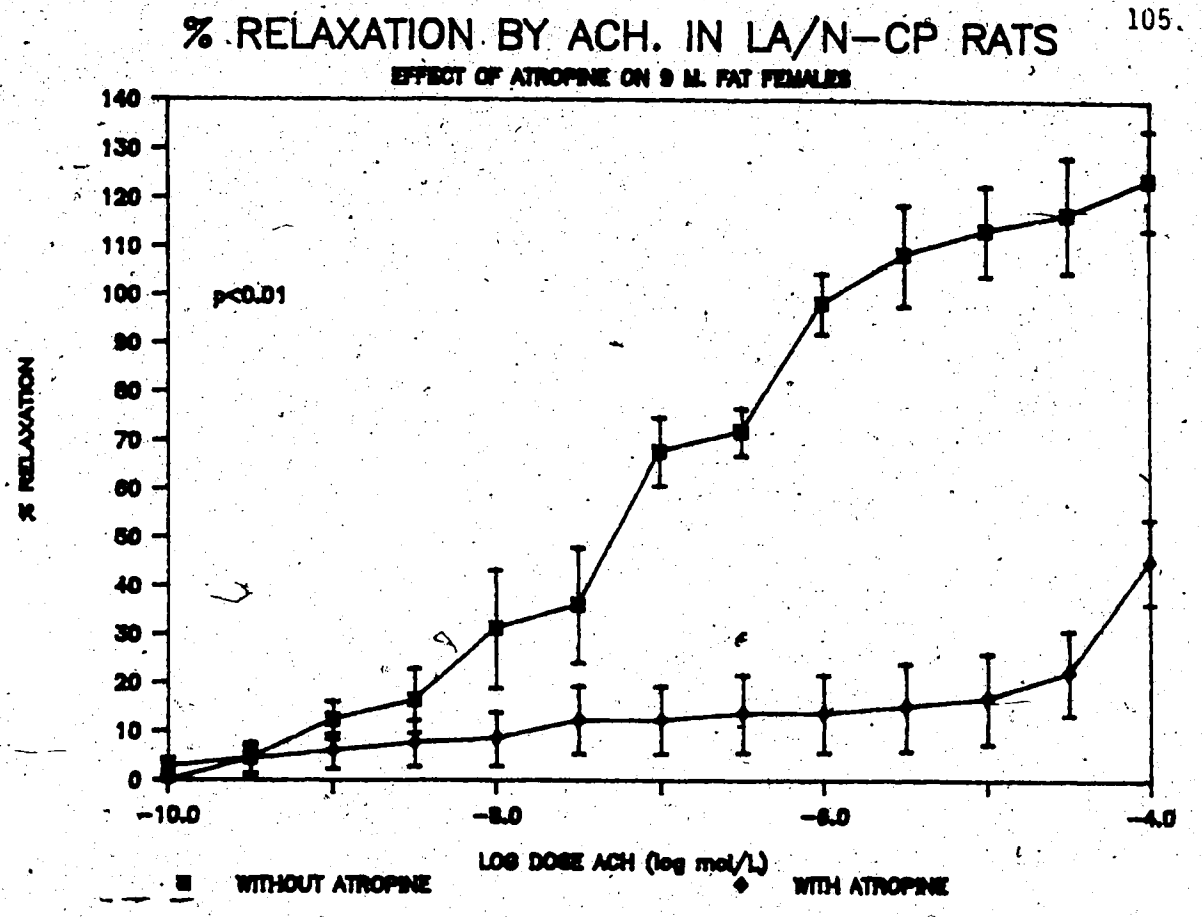

% RELAXATION BY ACH IN LA/N-CP RATS
EFFECT OF ATROPINE IN 9 M. THIN MALES
p<0.01
% RELAXATION
LOG DOSE ACH(log mol/L)
WITHOUT ATROPINE
WITH ATROPINE

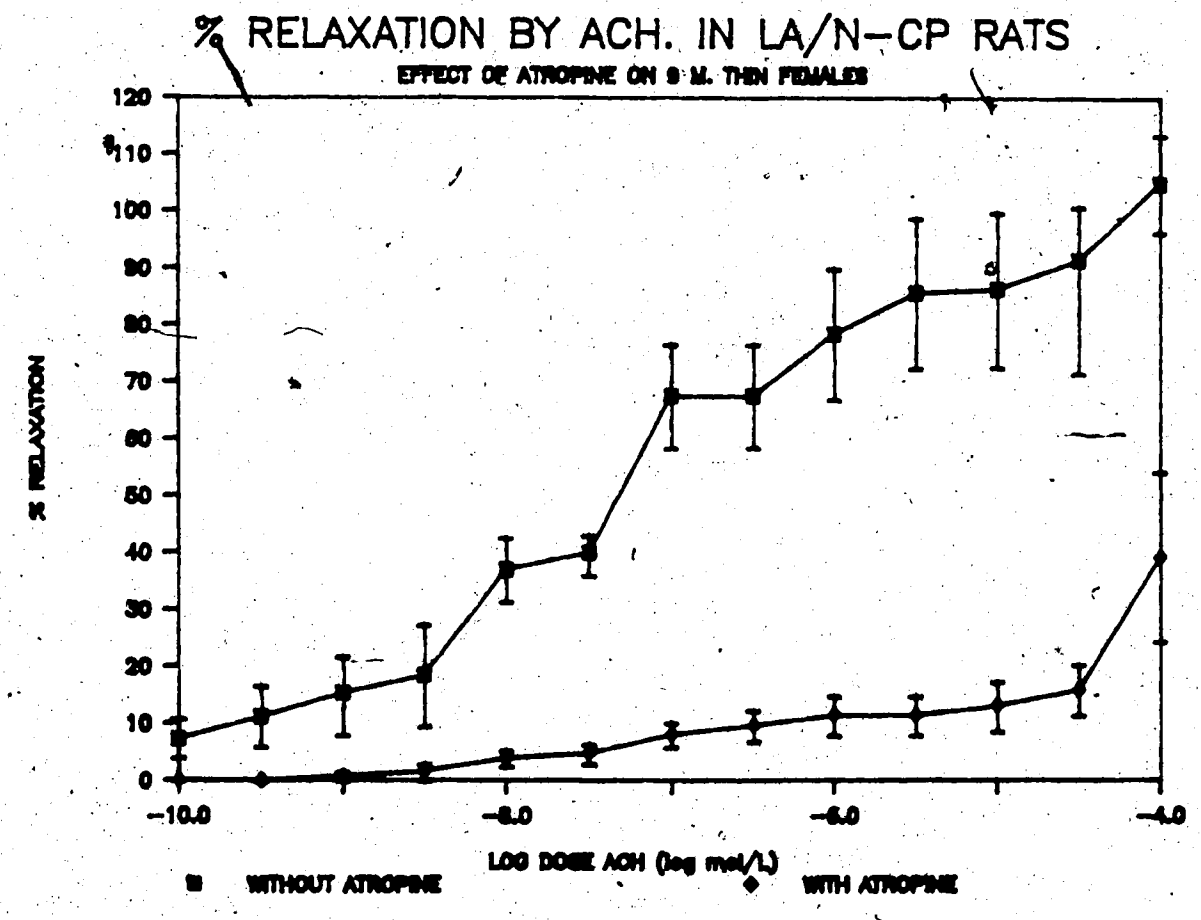
% RELAXATION BY ACH IN LA/N-CP RATS

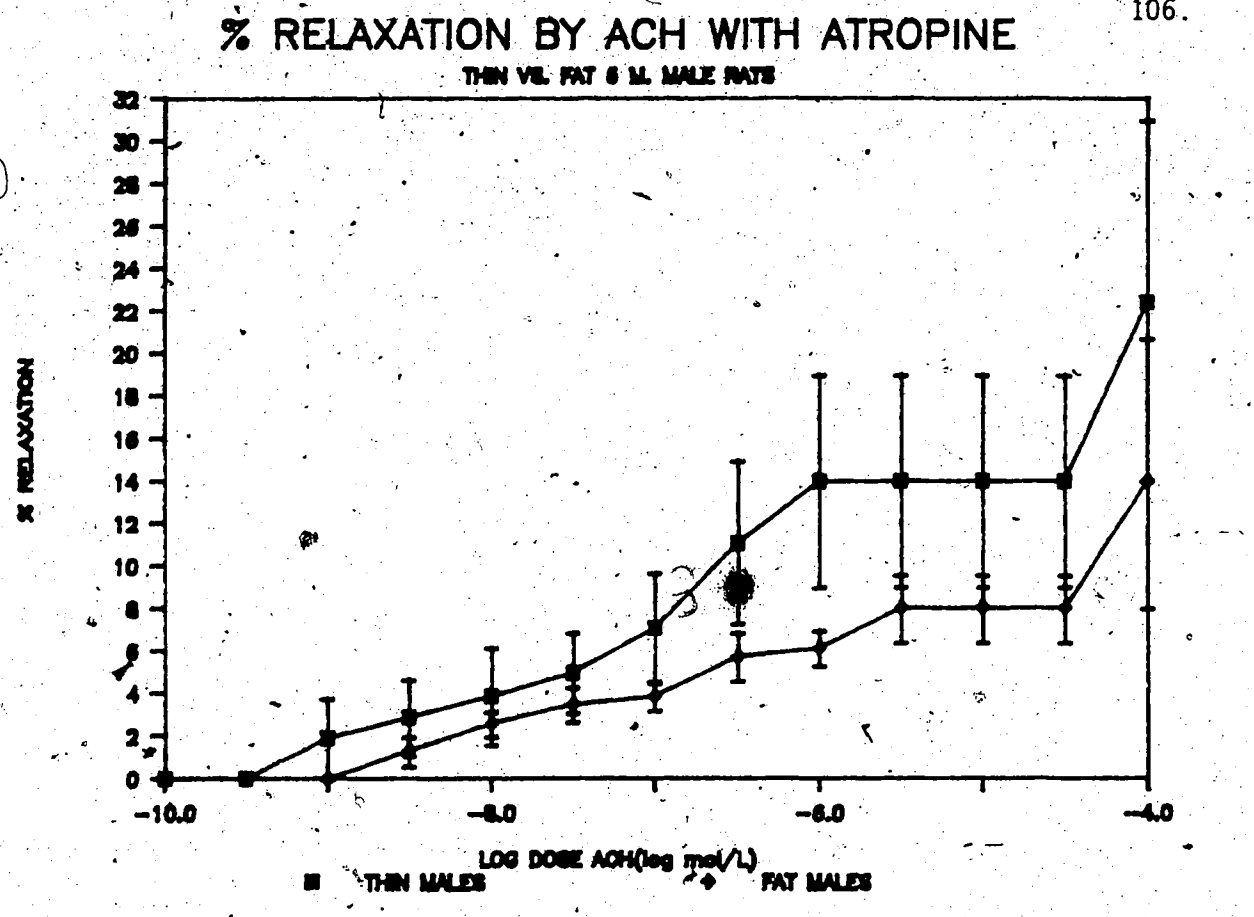


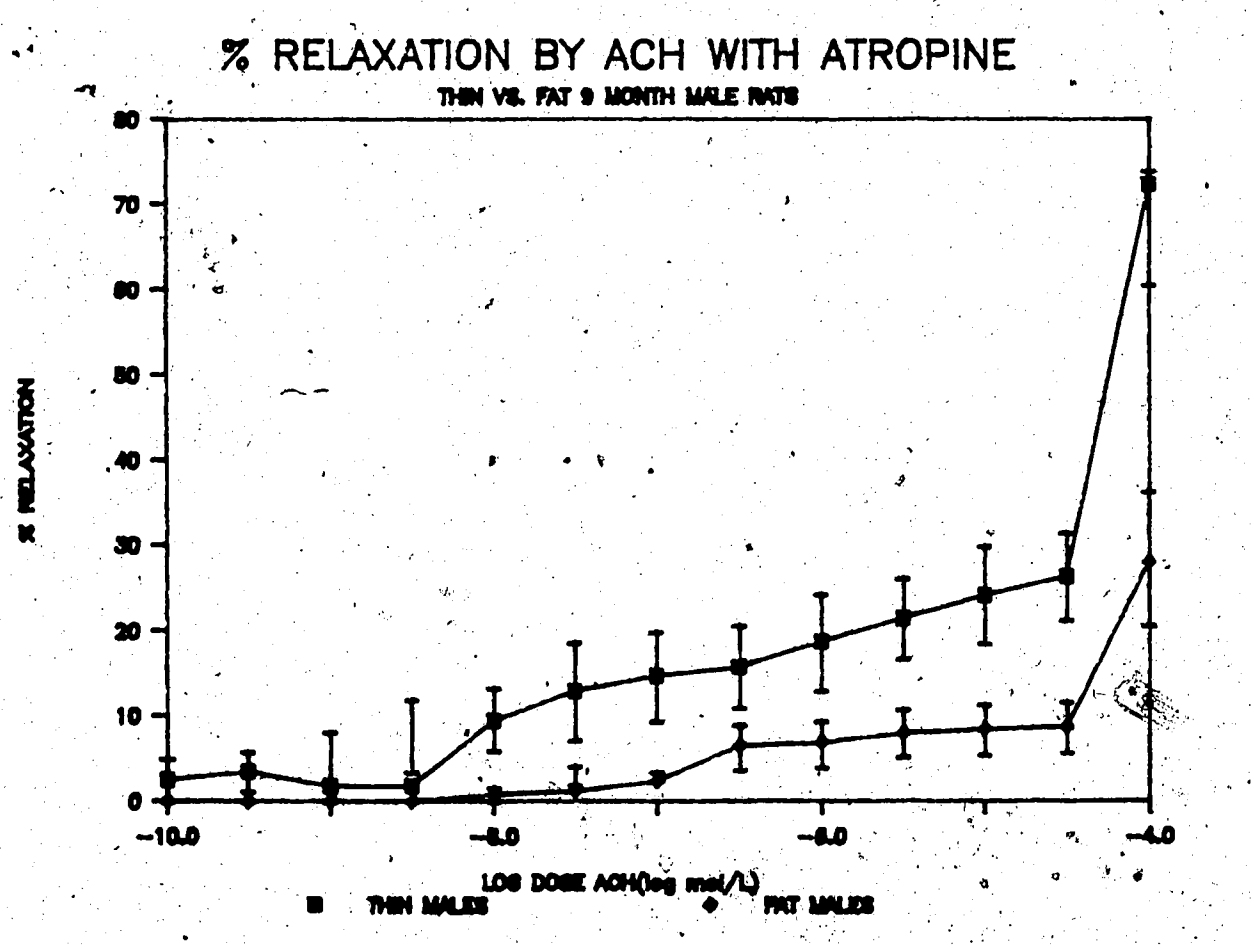
% RELAXATION BY ACH. IN LA/N-CP RATS

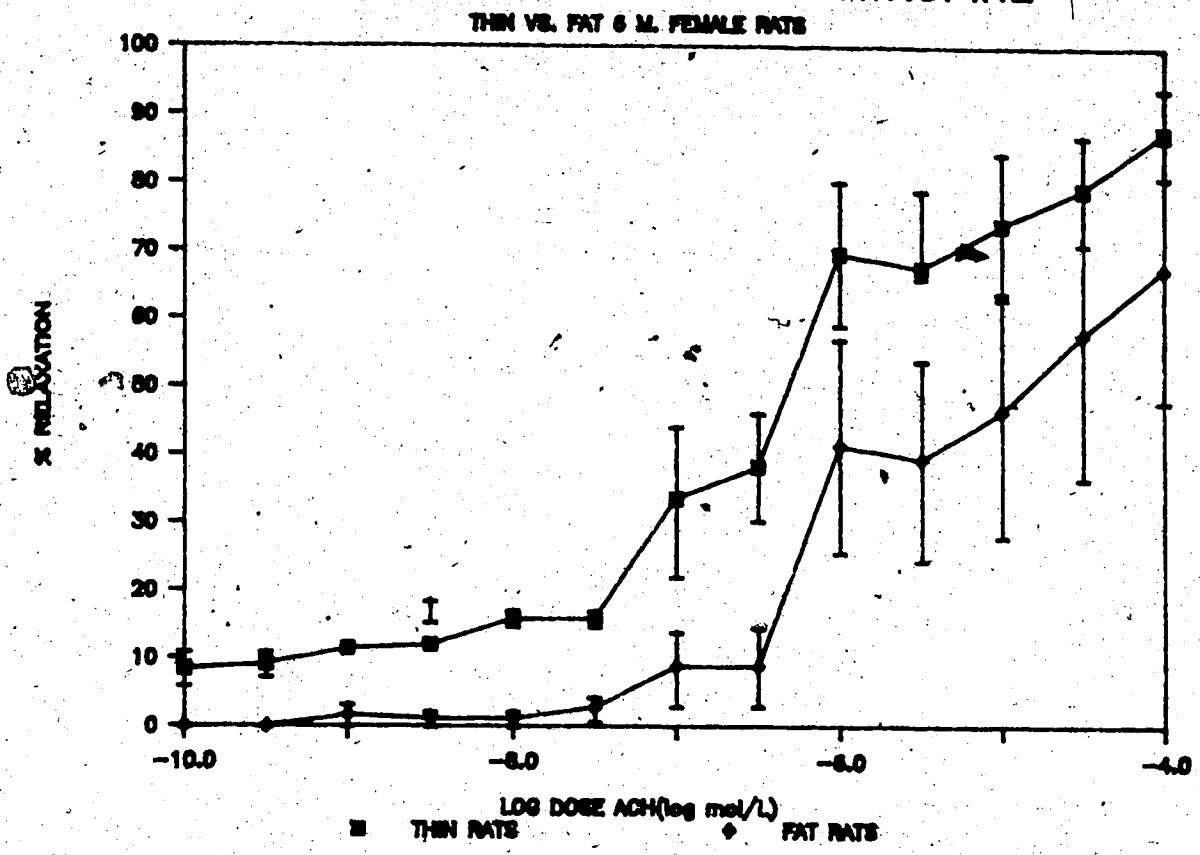


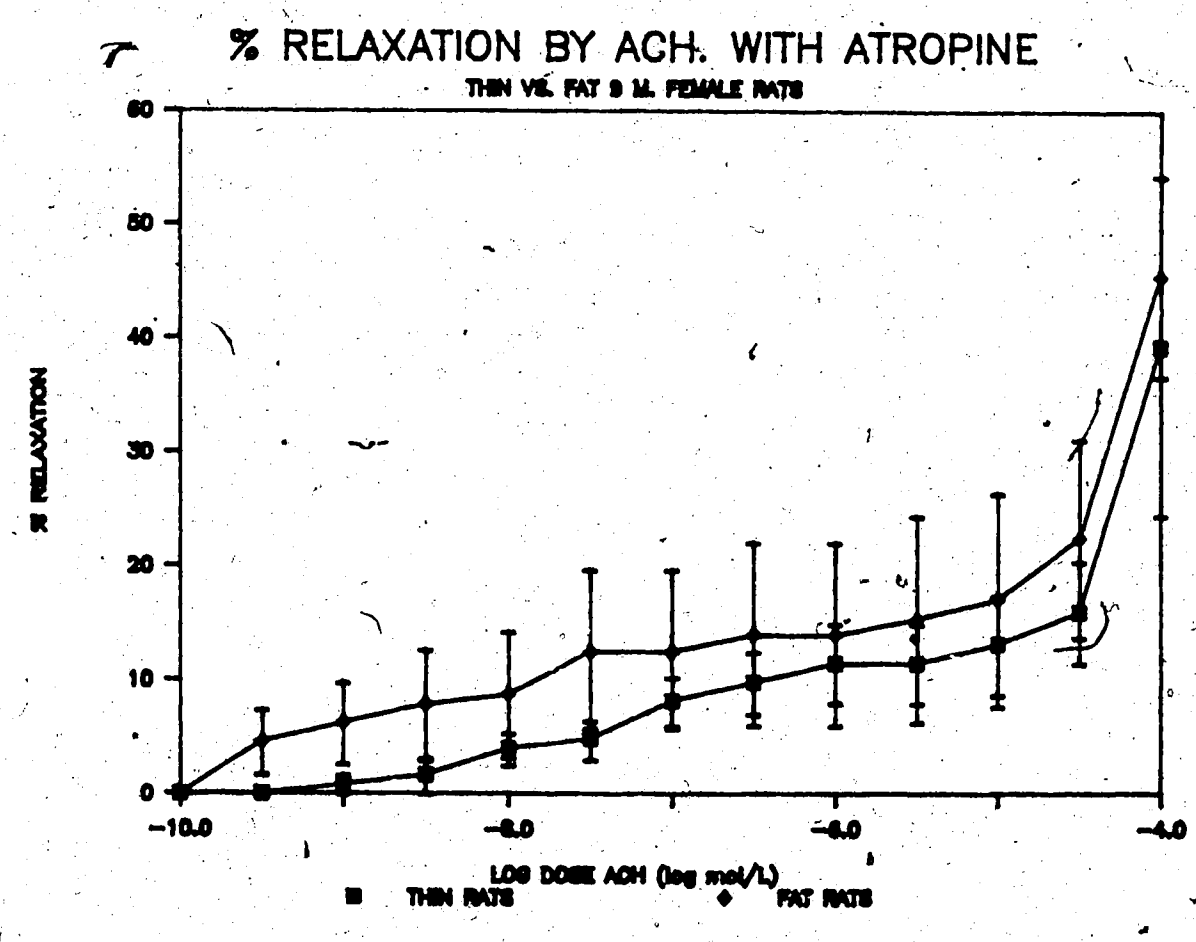

% RELAXATION BY ACH. IN LA/N-CP RATS
EFFECT OF ATROPINE ON 9 M. FAT FEMALES
% RELAXATION
p<0.01
LOG DOSE ACH (log mol/L)
WITHOUT ATROPINE
WITH ATROPINE

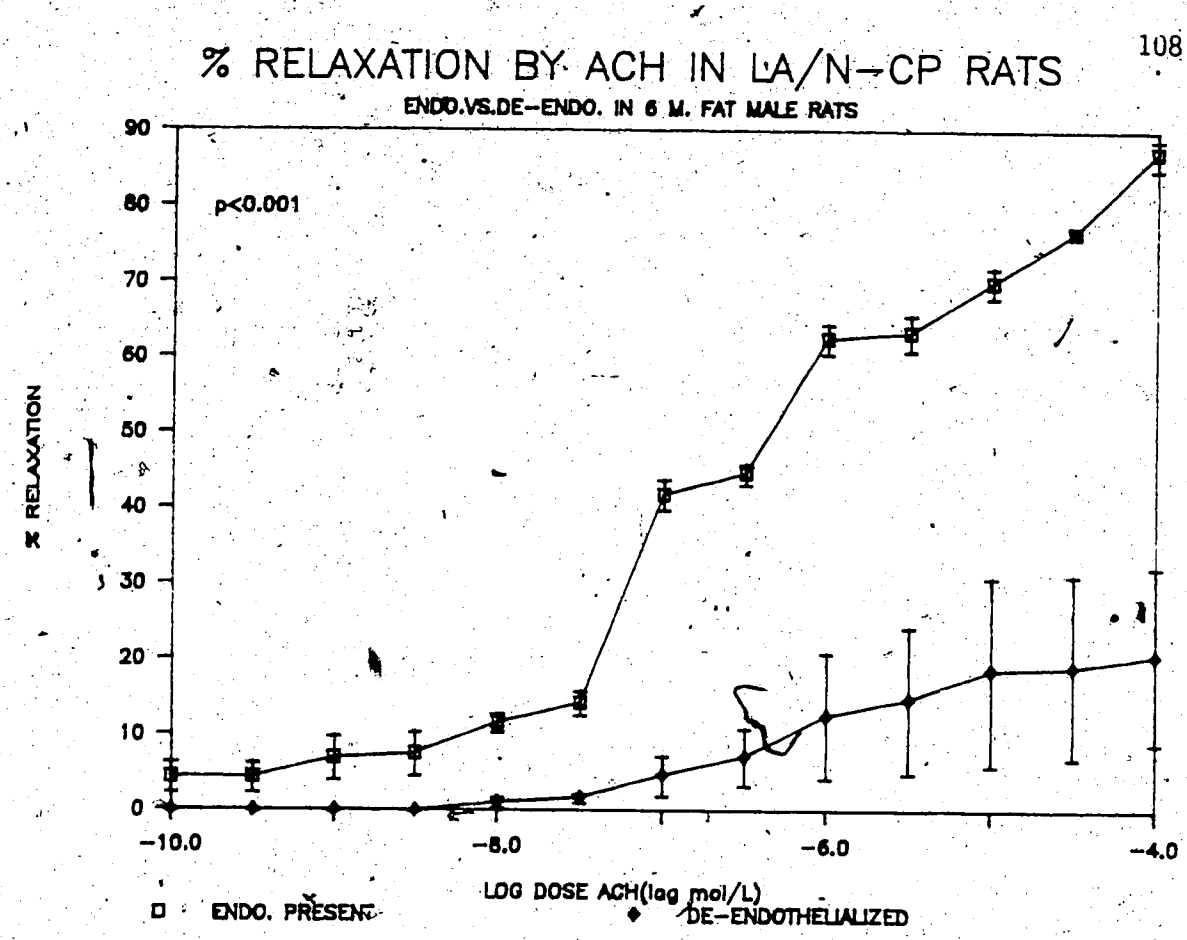

% RELAXATION BY ACH. IN LA/N-CP RATS
EFFECT OF ATROPINE ON 9 M. THIN FEMALES
% RELAXATION
LOG DOSE ACH (log mol/L)
WITHOUT ATROPINE
WITH ATROPINE

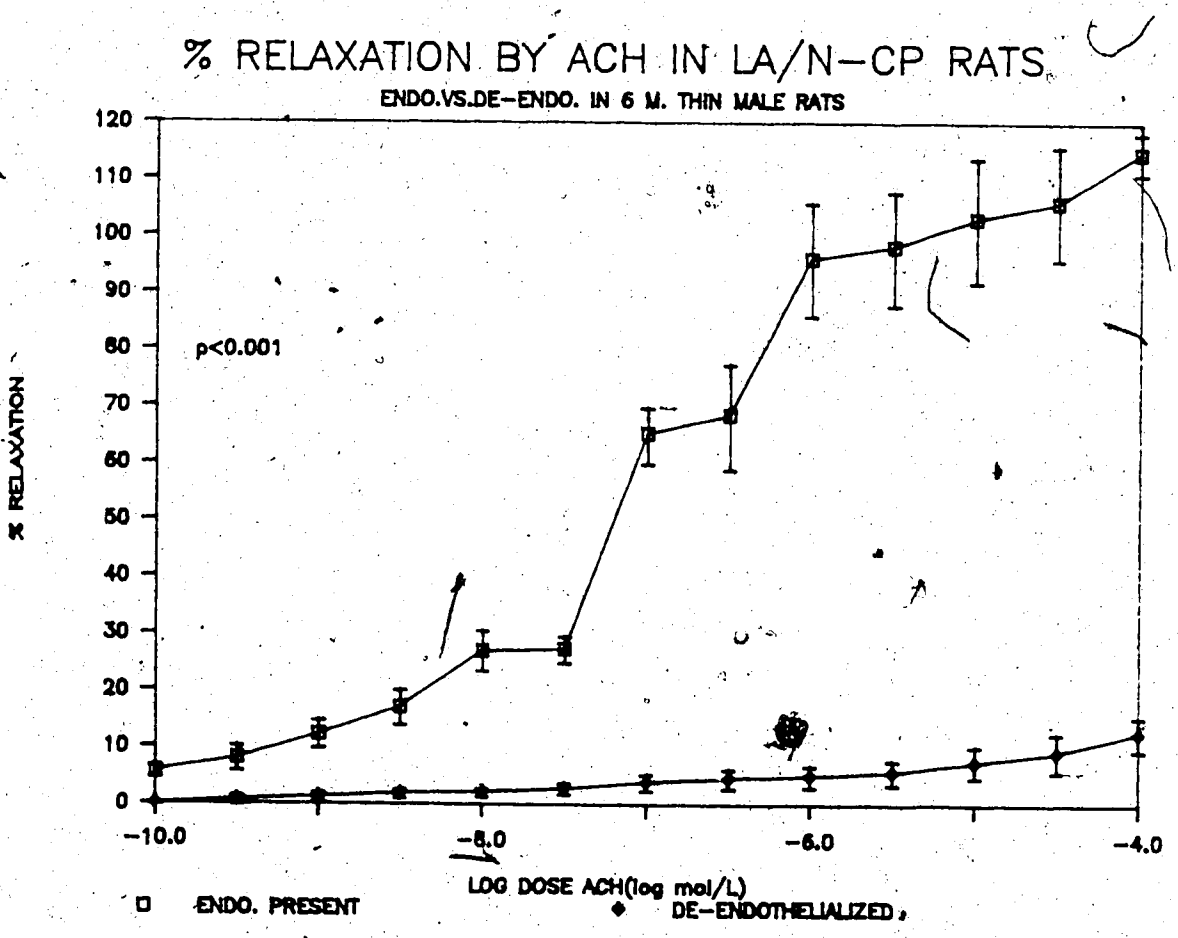

% RELAXATION BY ACH WITH ATROPINE
THIN VS. FAT 6 M. MALE RATS
% RELAXATION
LOG DOSE ACH(log mol/L)
-10.0
-8.0
-6.0
-4.0
THIN MALES
FAT MALES

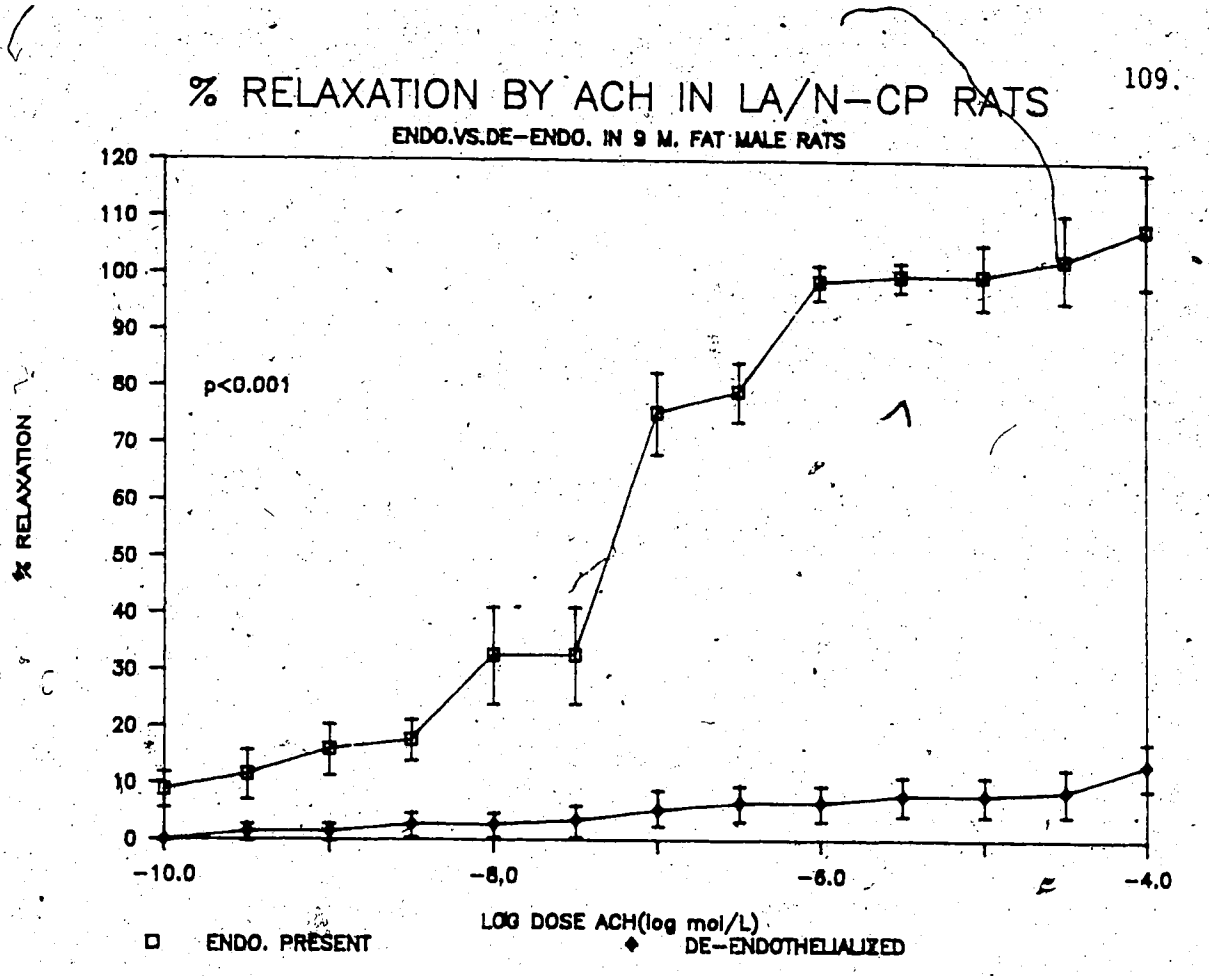

% RELAXATION BY ACH WITH ATROPINE
THIN VS. FAT 9 MONTH MALE RATS
% RELAXATION
LOG DOSE ACH(log mol/L)
-10.0
-8.0
-6.0
-4.0
THIN MALES
FAT MALES

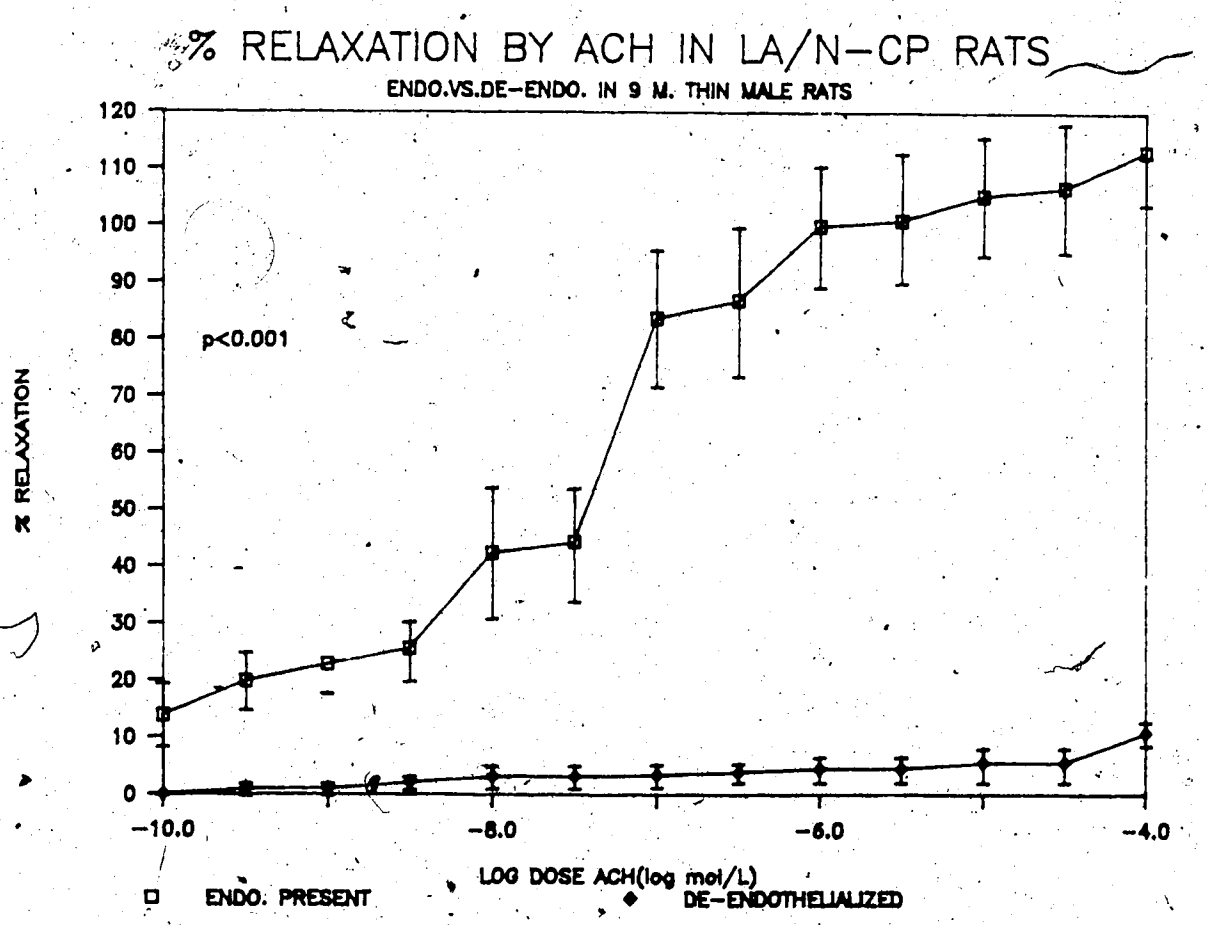

% RELAXATION BY ACH WITH ATROPINE
THIN VS. FAT 6 M. FEMALE RATS
% RELAXATION
100
90
80
70
60
50
40
30
20
10
0
-10.0
-8.0
-6.0
-4.0
LOG DOSE ACH(log mol/L)
THIN RATS
FAT RATS


% RELAXATION BY ACH. WITH ATROPINE
THIN VS. FAT 9 M. FEMALE RATS
% RELAXATION
60
50
40
30
20
10
0
-10.0
-8.0
-6.0
-4.0
LOG DOSE ACH (log mol/L)
THIN RATS
FAT RATS

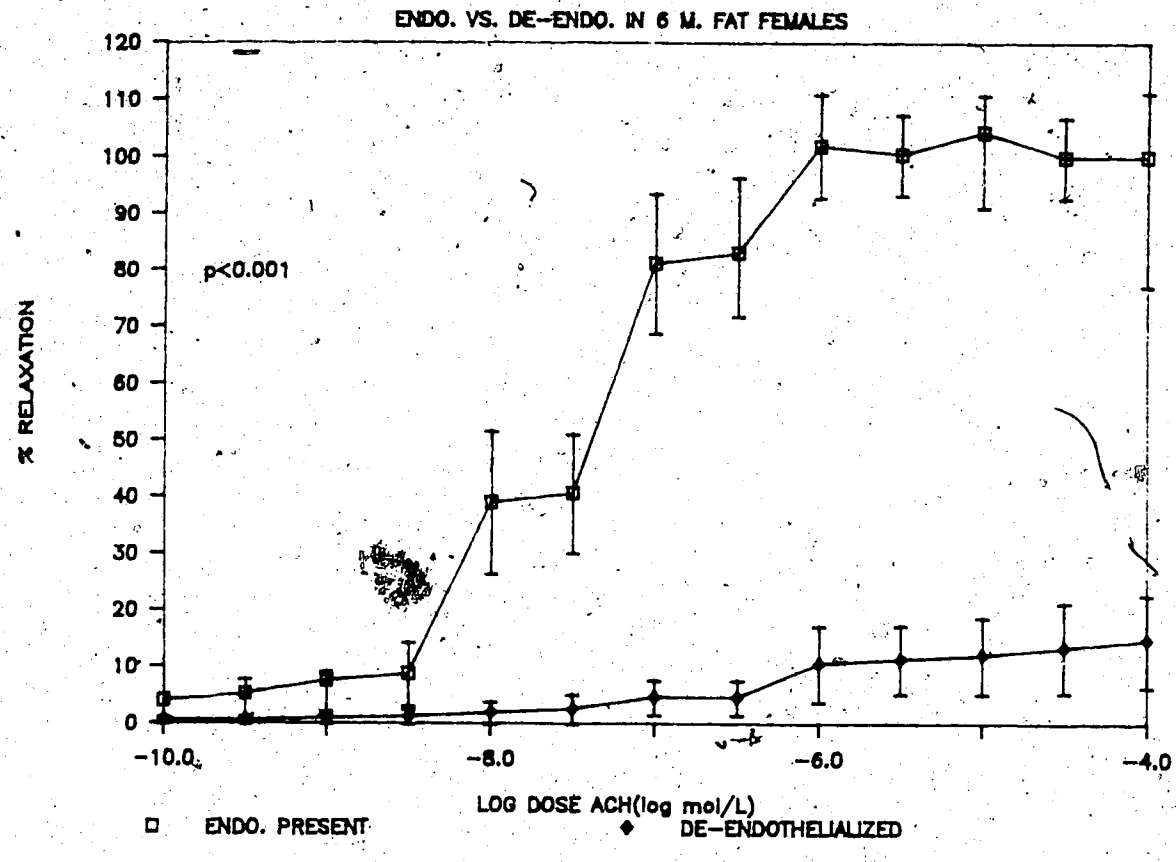

% RELAXATION BY ACH IN LA/N-CP RATS
ENDO.VS.DE-ENDO. IN 6 M. FAT MALE RATS
p<0.001
% RELAXATION
90
80
70
60
50
40
30
20
10
0
-10.0
-8.0
-6.0
-4.0
LOG DOSE ACH(log mol/L)
ENDO. PRESENT
DE-ENDOTHELIALIZED


% RELAXATION BY ACH IN LA/N-CP RATS
ENDO.VS.DE-ENDO. IN 6 M. THIN MALE RATS
p<0.001
% RELAXATION
120
110
100
90
80
70
60
50
40
30
20
10
0
-10.0
-8.0
-6.0
-4.0
LOG DOSE ACH(log mol/L)
ENDO. PRESENT
DE-ENDOTHELIALIZED

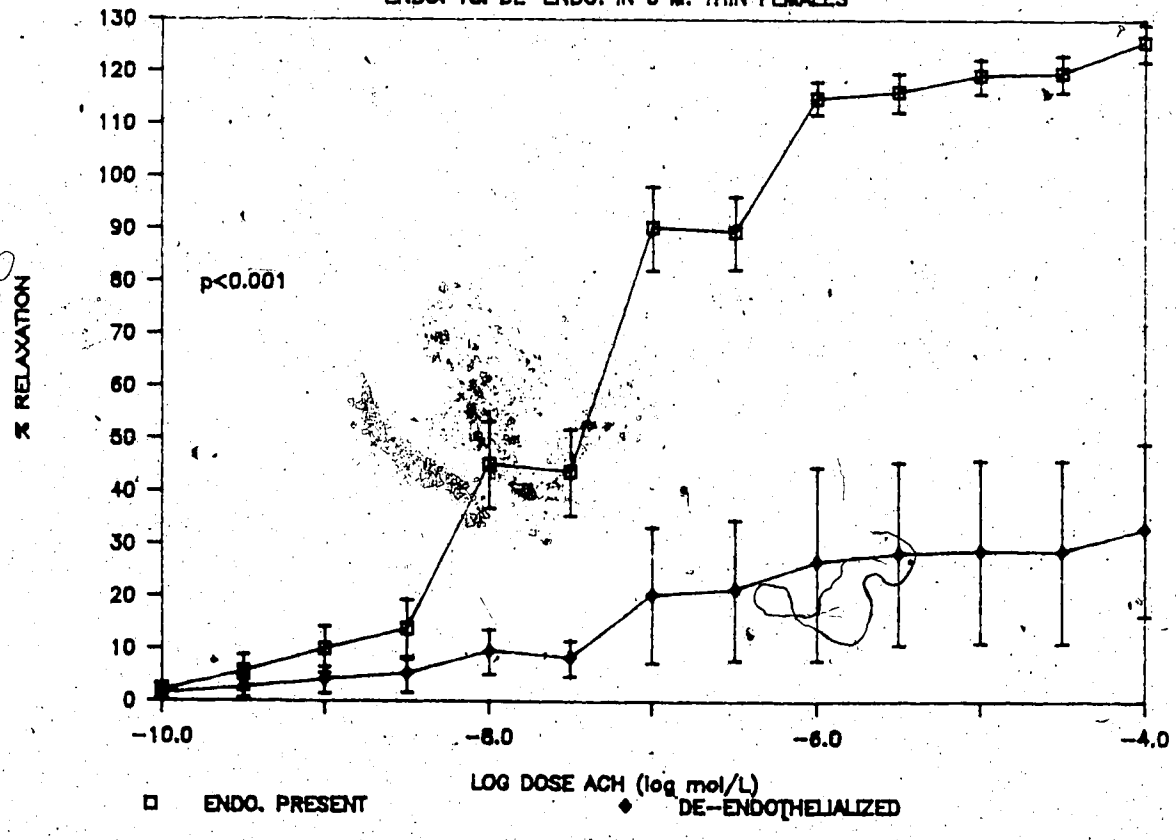

% RELAXATION BY ACH IN LA/N-CP RATS
ENDO.VS.DE-ENDO. IN 9 M. FAT MALE RATS
% RELAXATION
p<0.001
120
110
100
90
80
70
60
50
40
30
20
10
0
-10.0
-8.0
-6.0
-4.0
LOG DOSE ACH(log mol/L)
ENDO. PRESENT
DE-ENDOTHELIALIZED


% RELAXATION BY ACH IN LA/N-CP RATS
ENDO.VS.DE-ENDO. IN 9 M. THIN MALE RATS
% RELAXATION
p<0.001
120
110
100
90
80
70
60
50
40
30
20
10
0
-10.0
-8.0
-6.0
-4.0
LOG DOSE ACH(log mol/L)
ENDO. PRESENT
DE-ENDOTHELIALIZED

PER CENT RELAXATION OF FAT FEMALE RATS

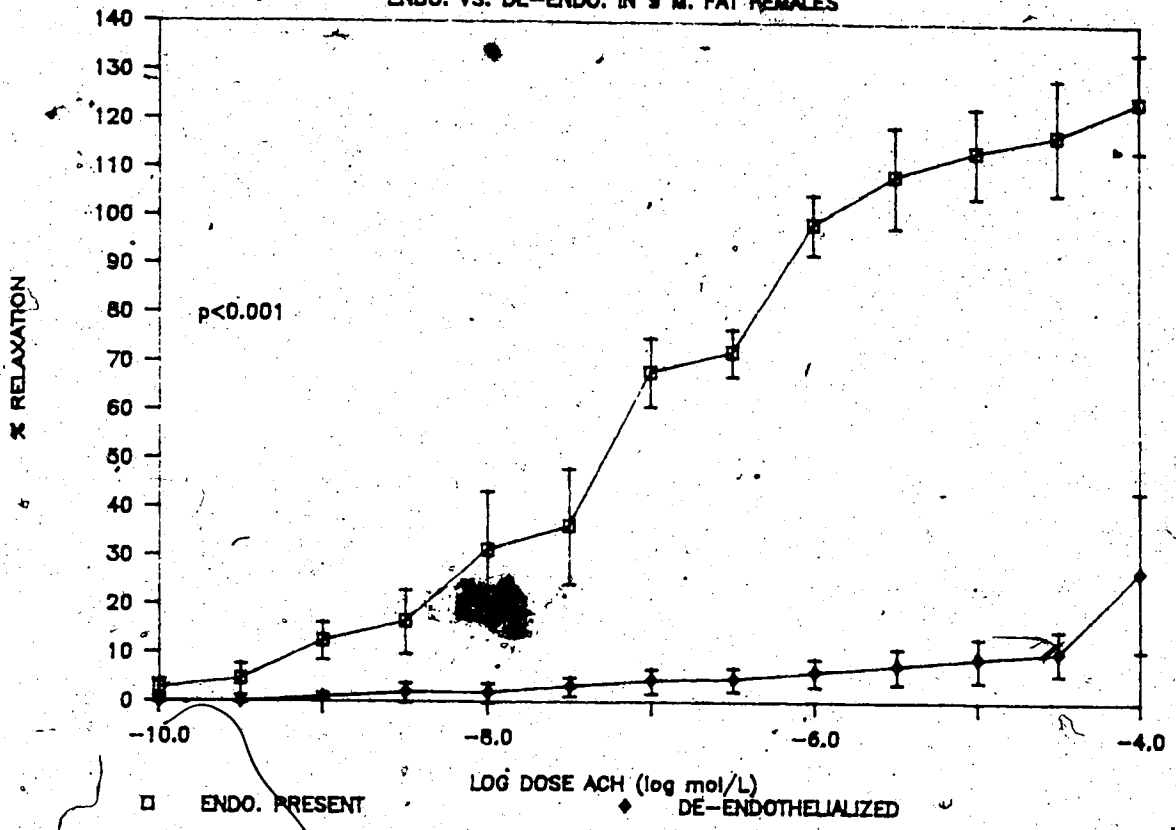


% RELAXATION BY ACH. IN LA/N-CP RATS



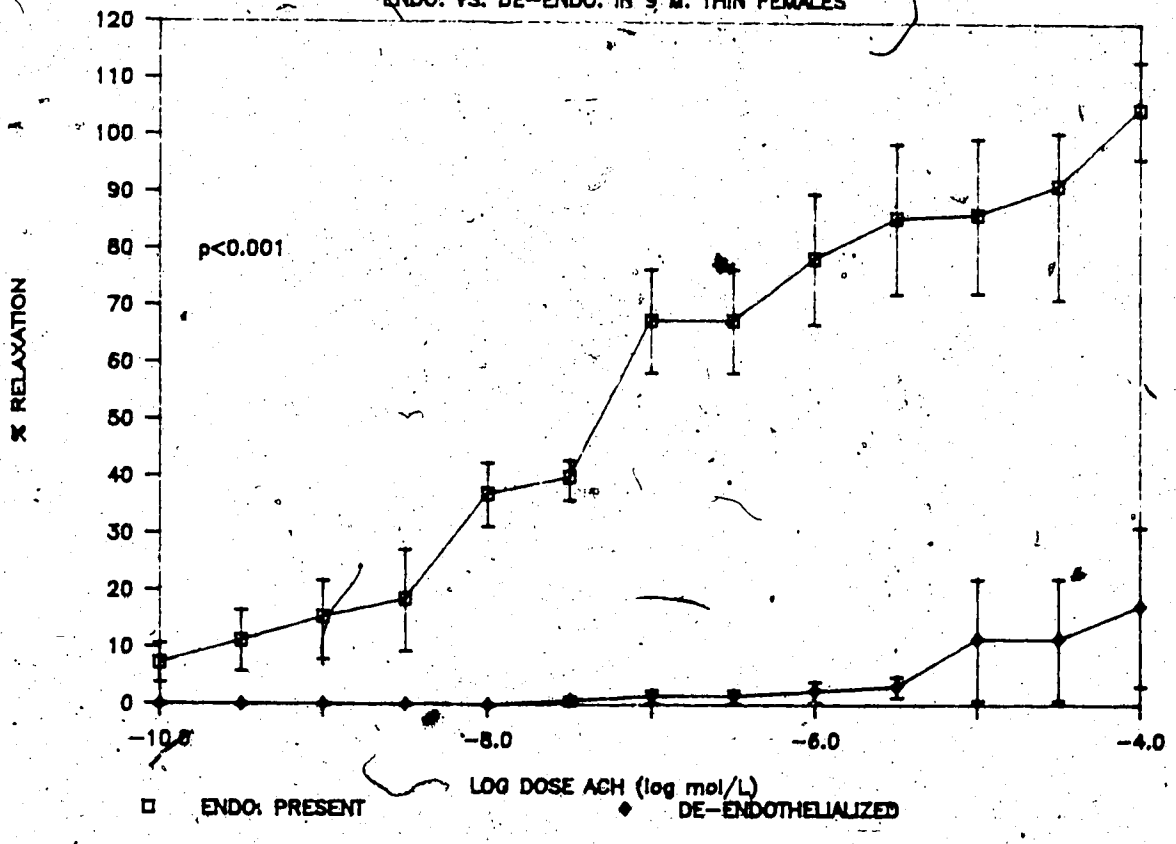
% RELAXATION BY ACH. IN LA/N-CP RATS

ENDO. VS. DE-ENDO. IN 9 M. FAT FEMALES



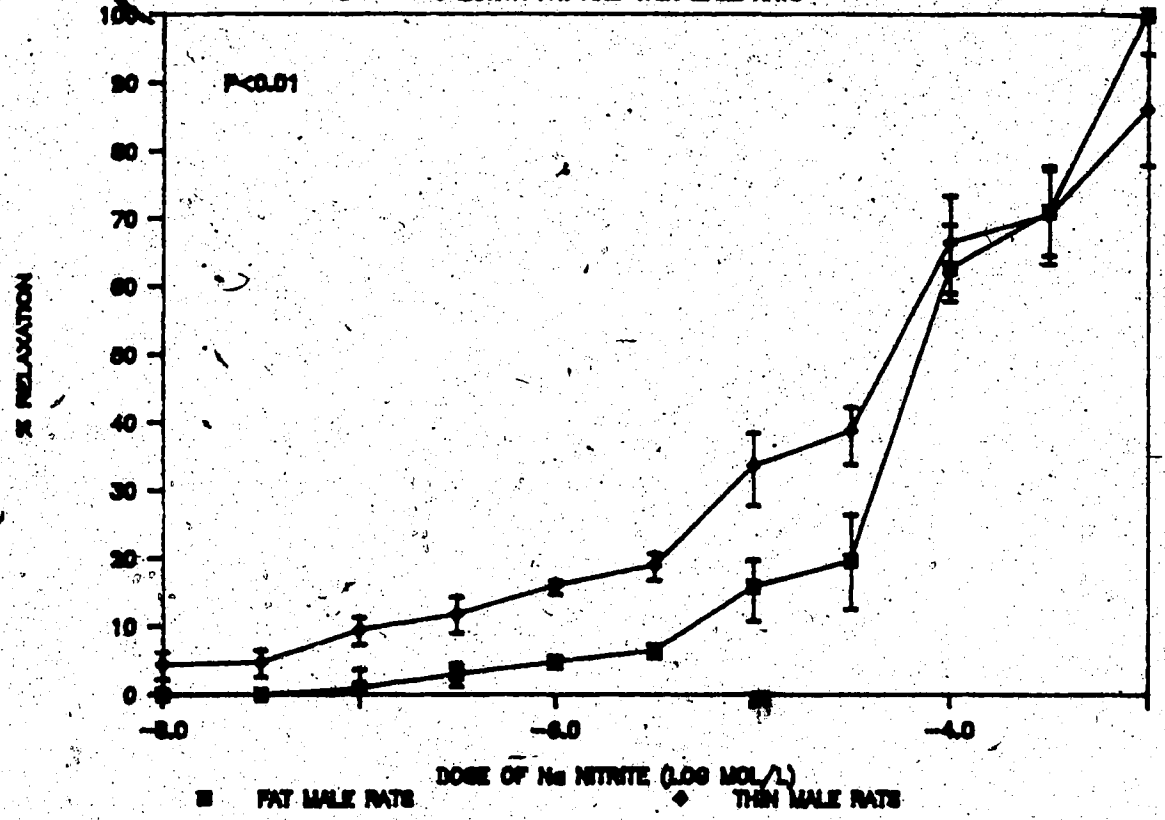
% RELAXATION BY ACH. IN LA/N-CP RATS

ENDO. VS. DE-ENDO. IN 9 M. THIN FEMALES



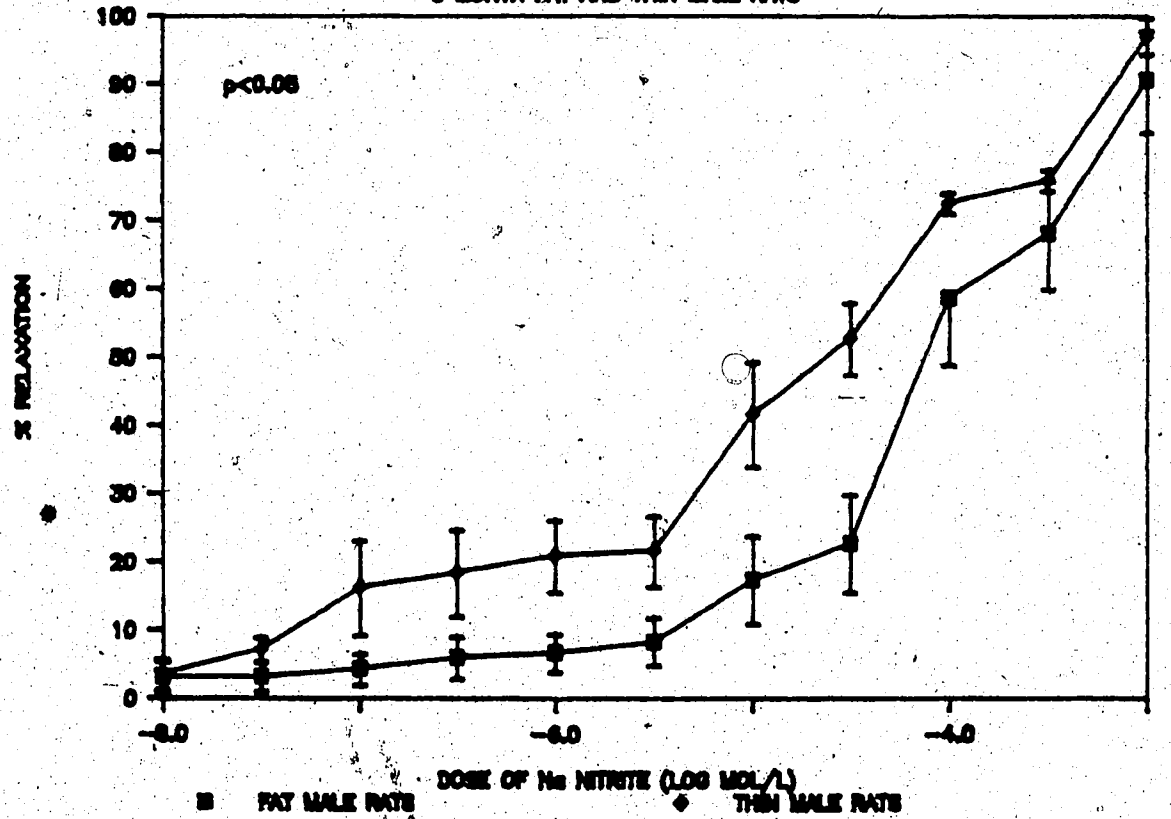
ENDO.PRESENT – RELAXATION BY Na NITRITE

9 MONTH FAT AND THIN MALE RATS



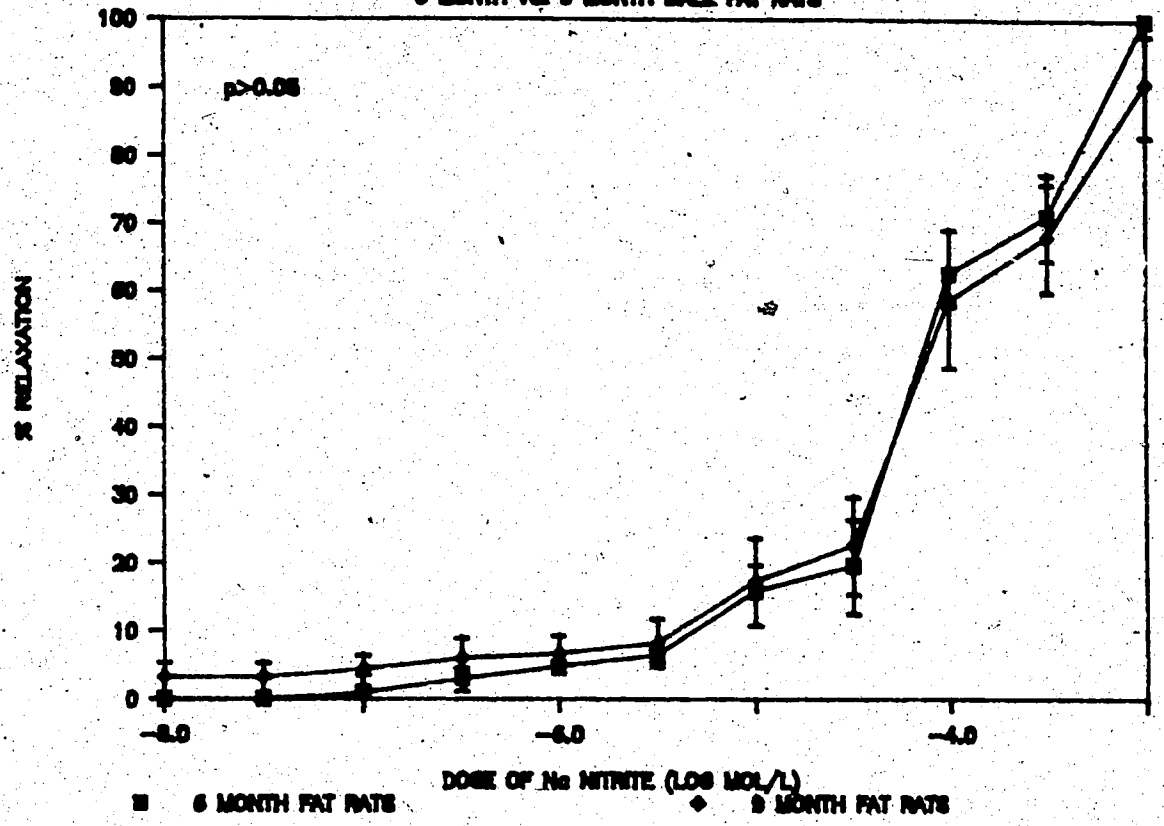
ENDO.PRESENT – RELAXATION BY Na NITRITE

9 MONTH FAT AND THIN MALE RATS



ENDO.PRESENT – RELAXATION BY Na NITRITE

6 MONTH VS. 9 MONTH MALE FAT RATS



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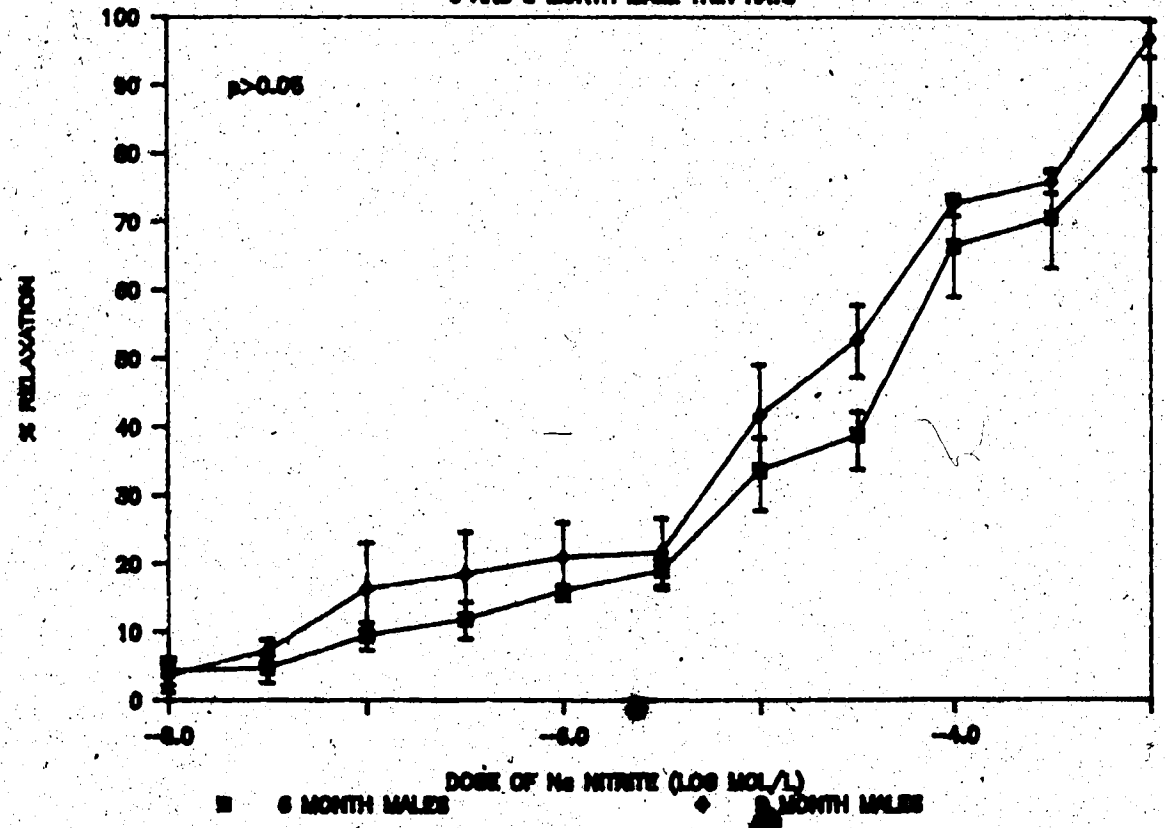
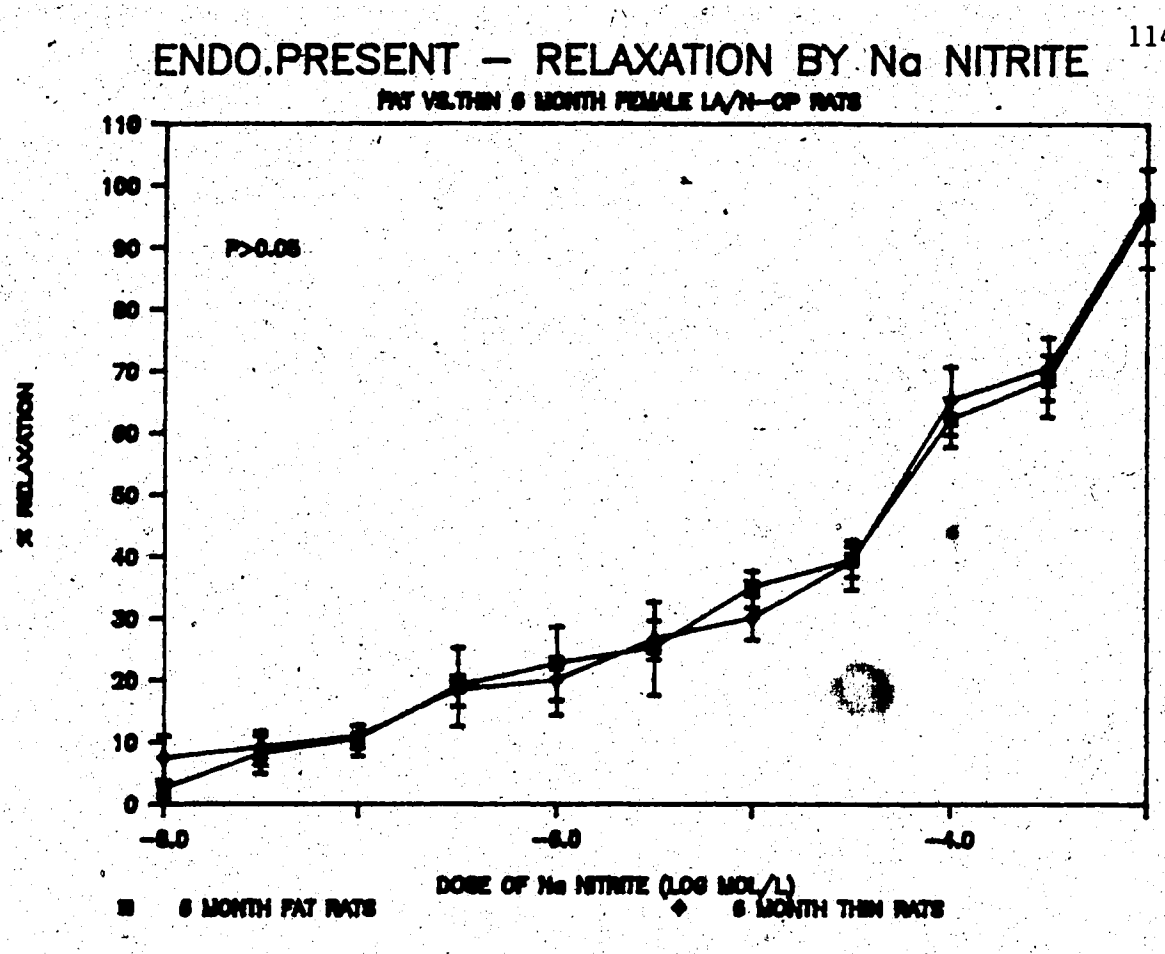
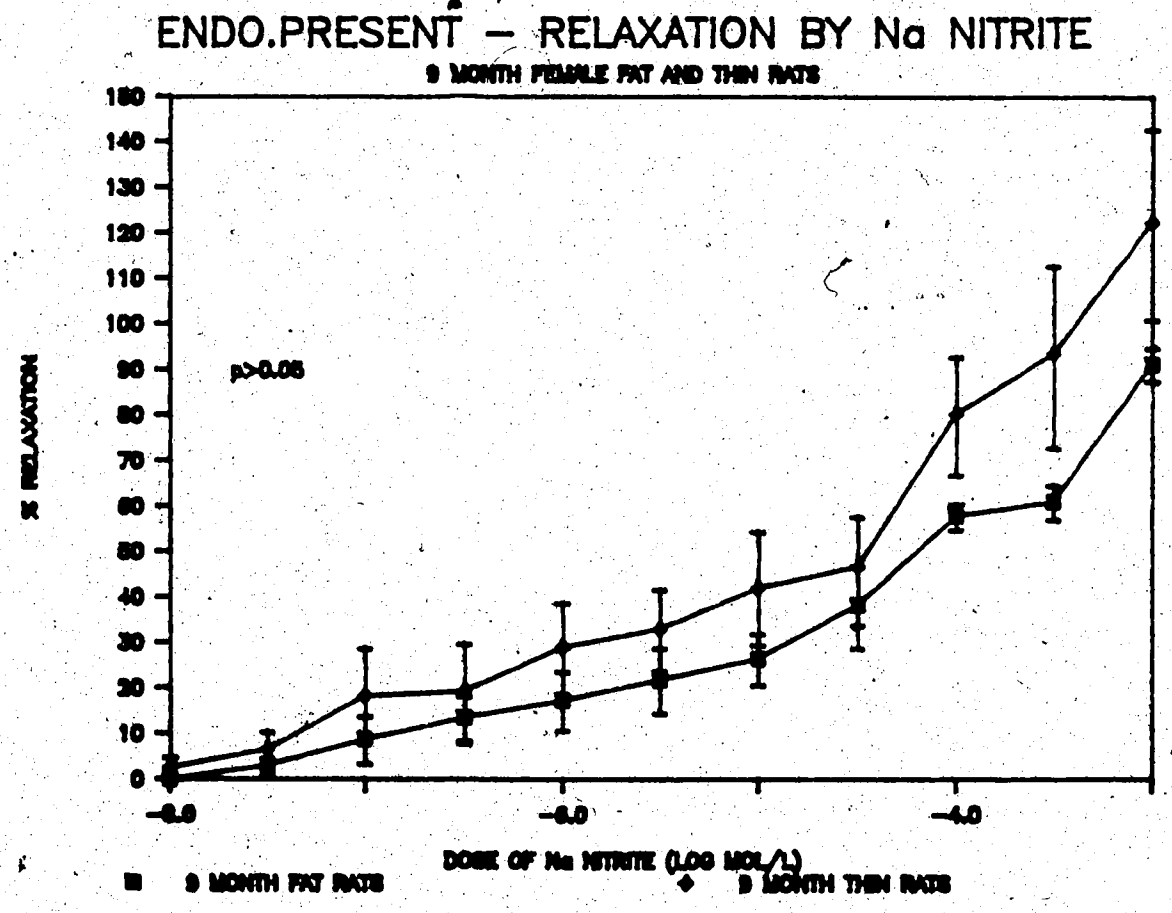
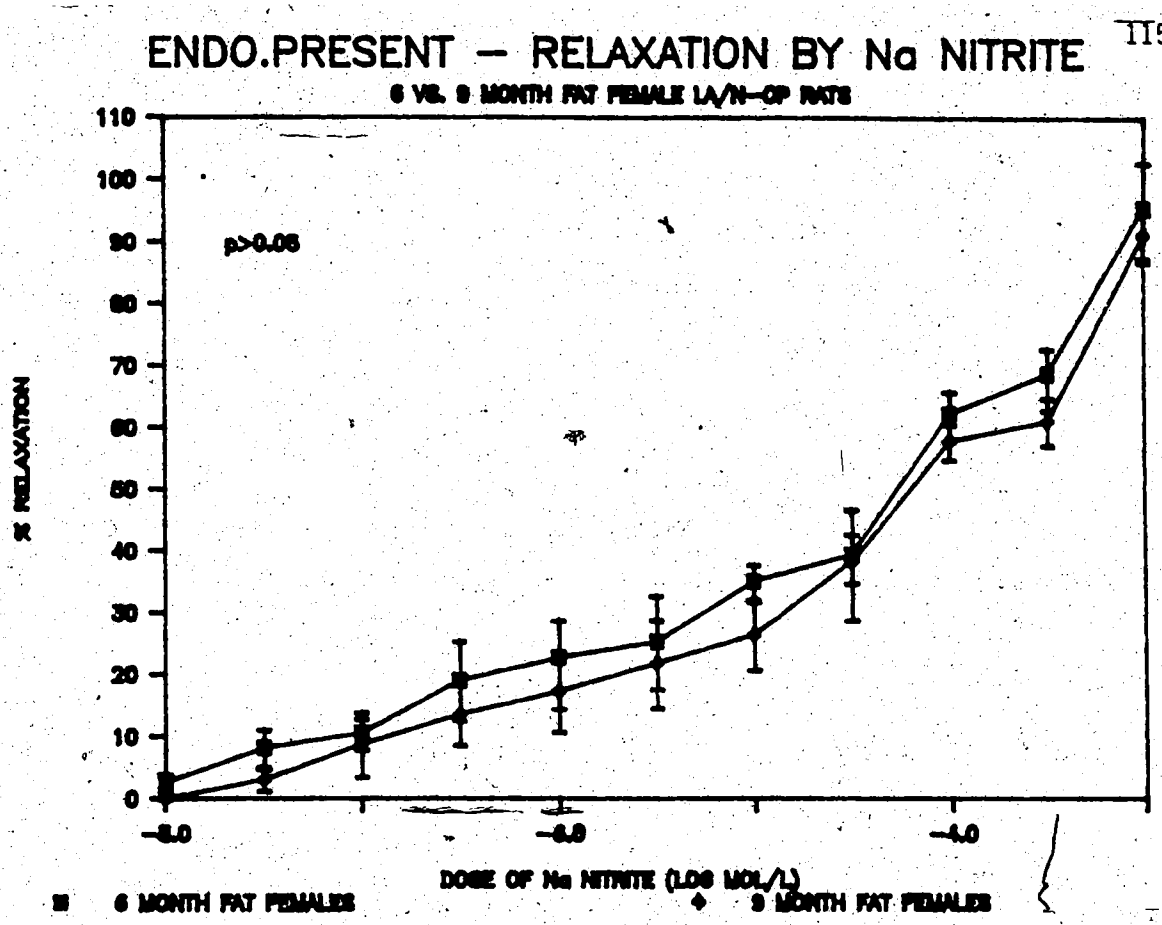
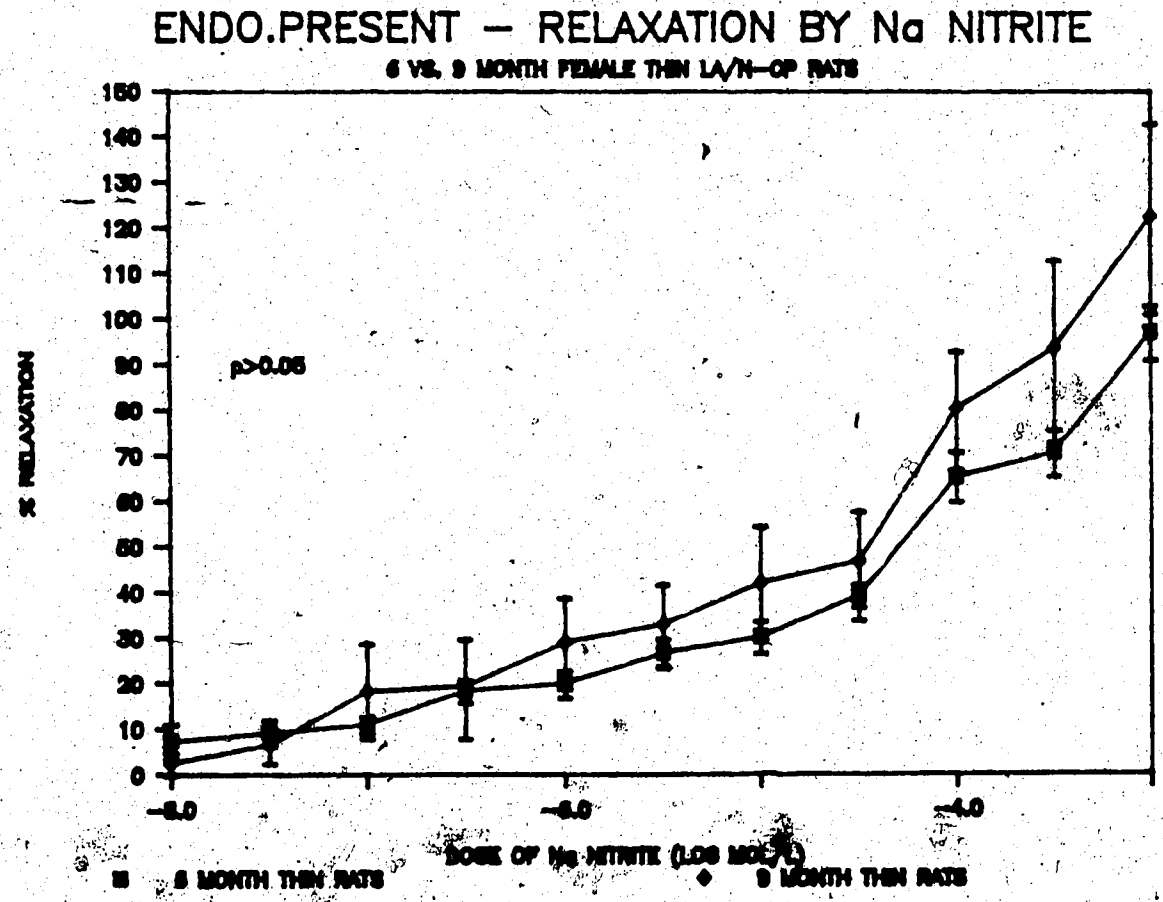
6 AND 9 MONTH MALE THIN RATS

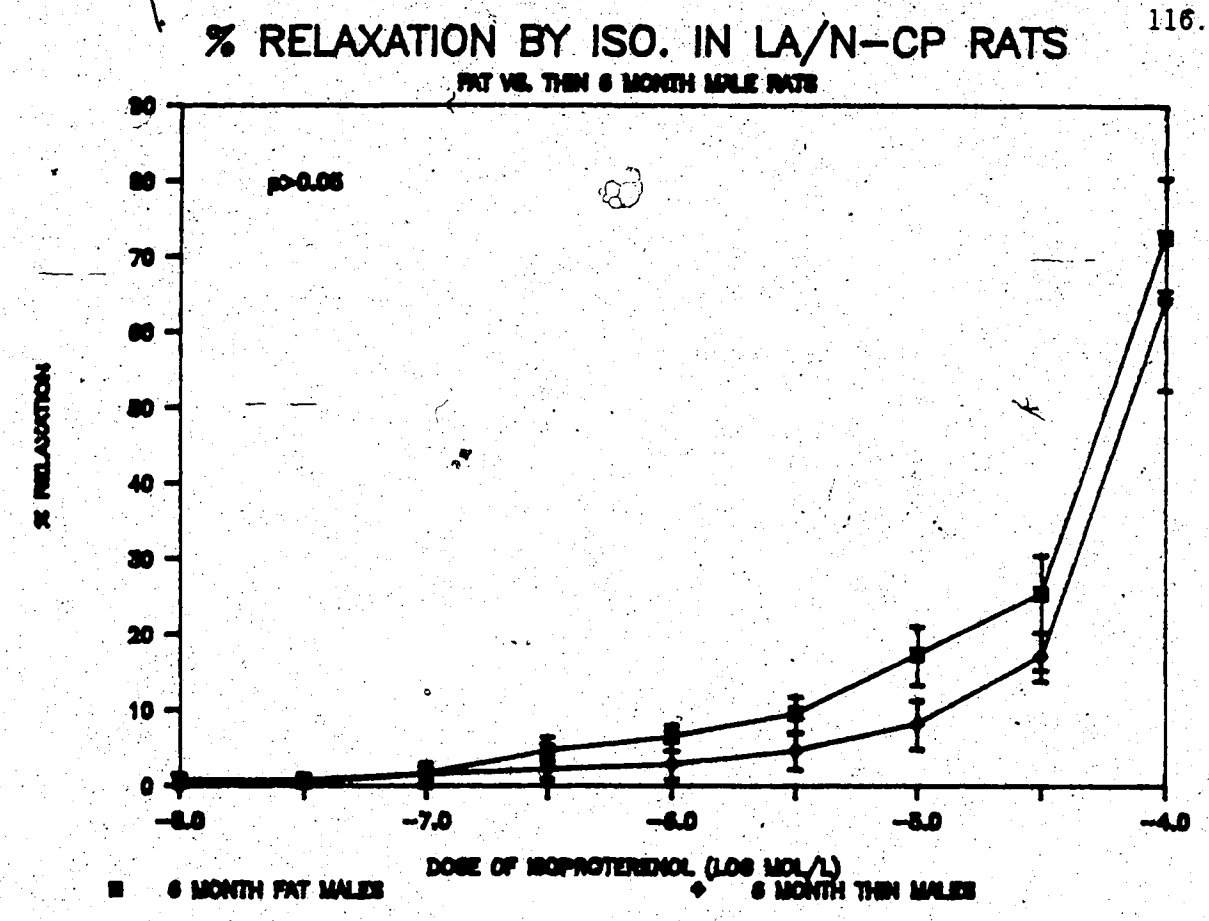


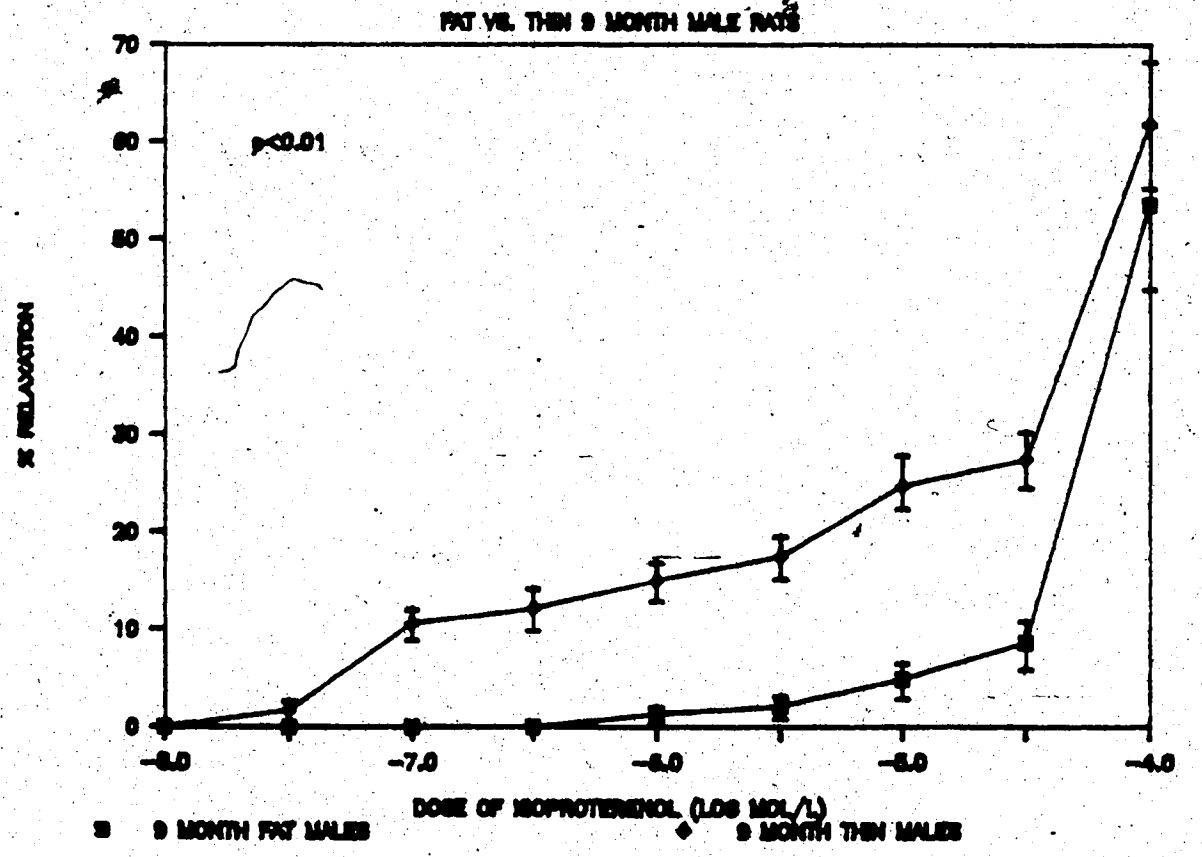

ENDO.PRESENT – RELAXATION BY Na NITRITE
FAT VS.THIN 6 MONTH FEMALE LA/N-OP RATS
% RELAXATION
P>0.05
DOSE OF Na NITRITE (LOG MOL/L)
6 MONTH FAT RATS
6 MONTH THIN RATS

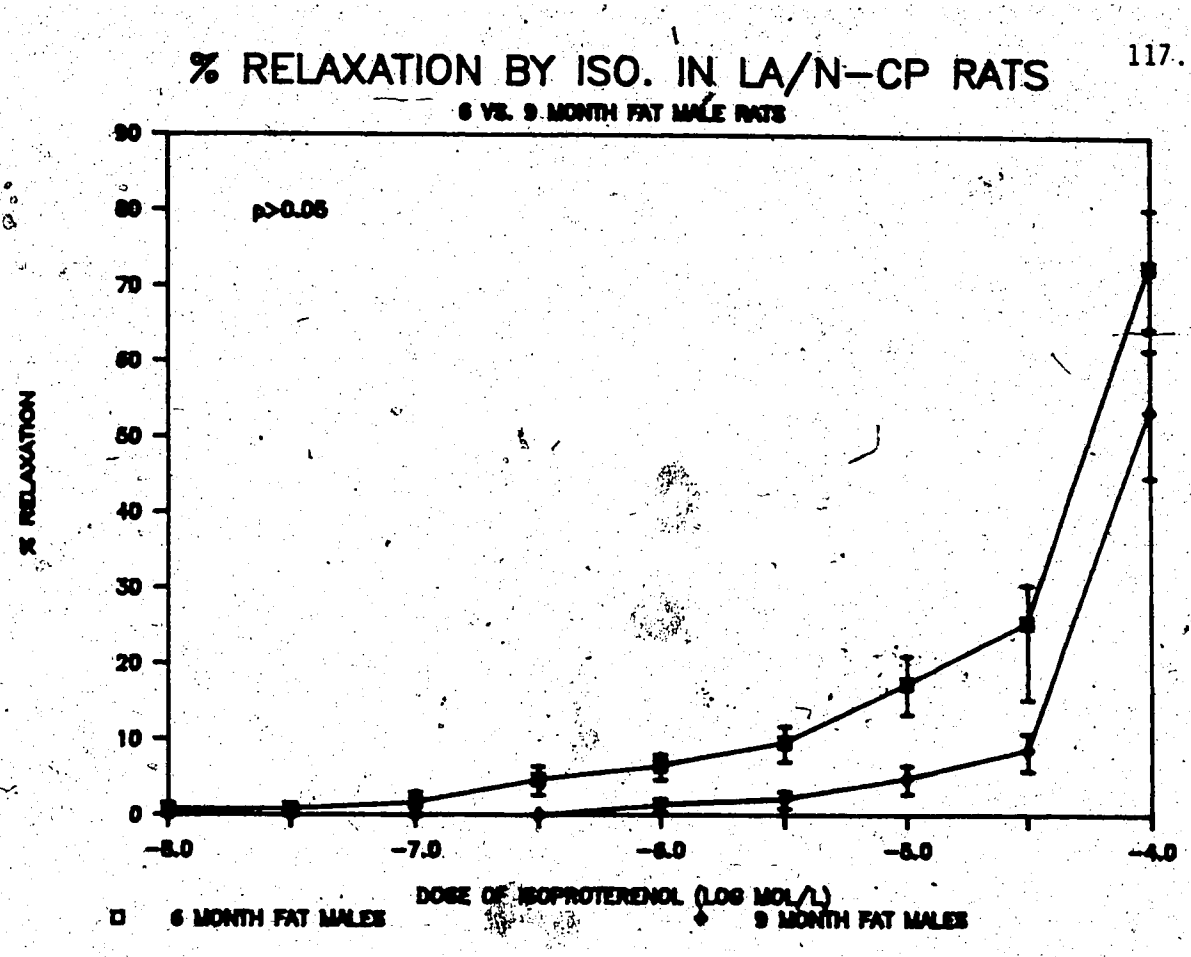

ENDO.PRESENT – RELAXATION BY Na NITRITE
9 MONTH FEMALE FAT AND THIN RATS
% RELAXATION
p>0.05
DOSE OF Na NITRITE (LOG MOL/L)
9 MONTH FAT RATS
9 MONTH THIN RATS

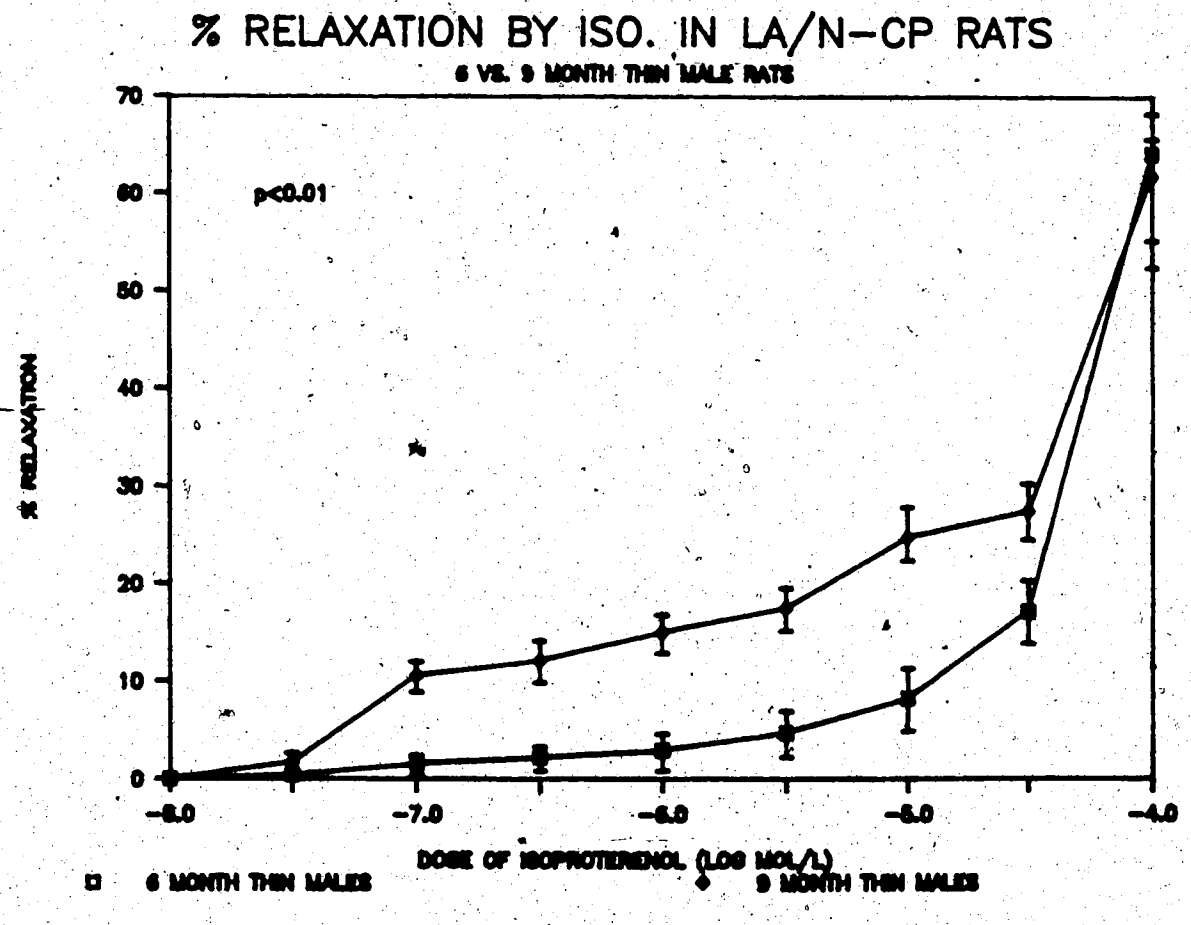

ENDO.PRESENT – RELAXATION BY Na NITRITE
6 VS. 9 MONTH FAT FEMALE LA/N–OP RATS
p>0.05
% RELAXATION
DOSE OF Na NITRITE (LOG MOL/L)
6 MONTH FAT FEMALES
9 MONTH FAT FEMALES

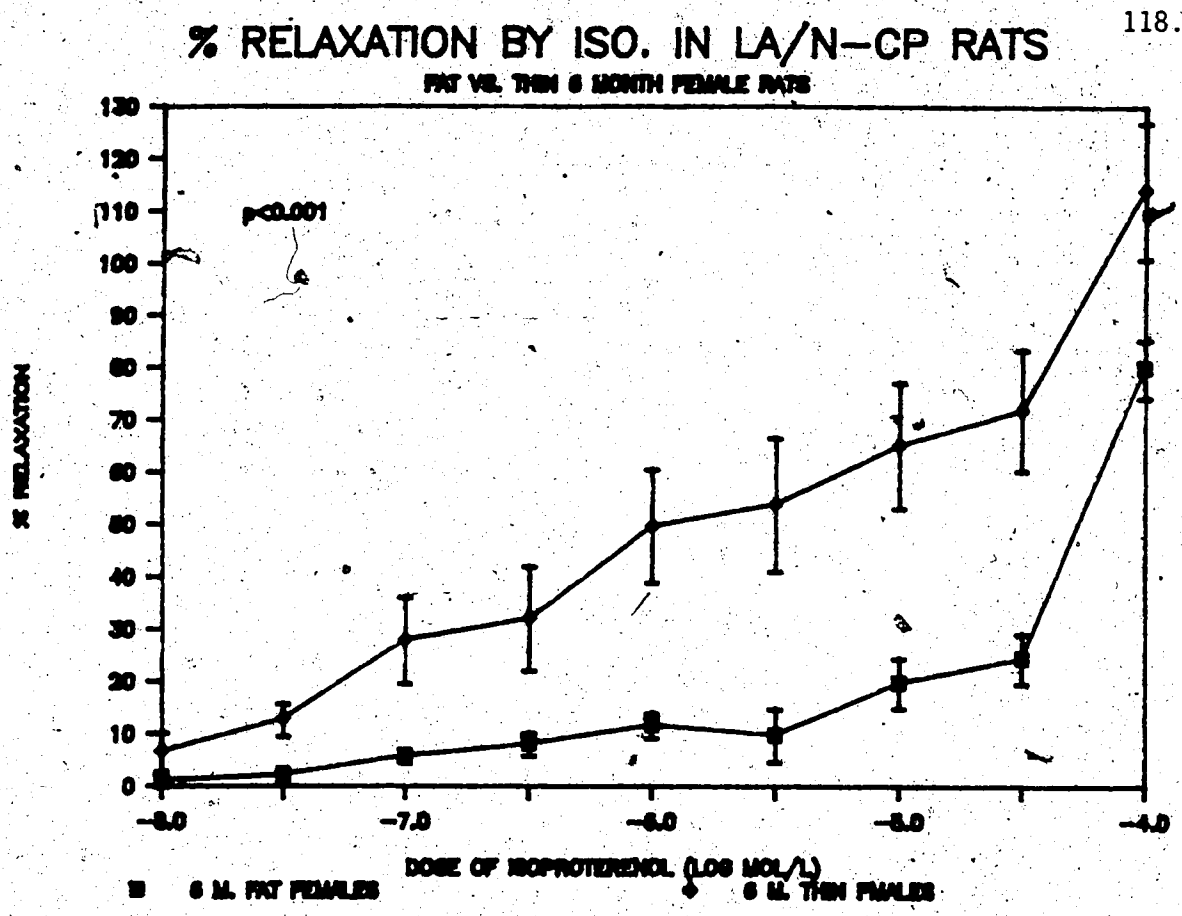

ENDO.PRESENT – RELAXATION BY Na NITRITE
6 VS. 9 MONTH FEMALE THIN LA/N–OP RATS
p>0.05
% RELAXATION
6 MONTH THIN RATS
9 MONTH THIN RATS

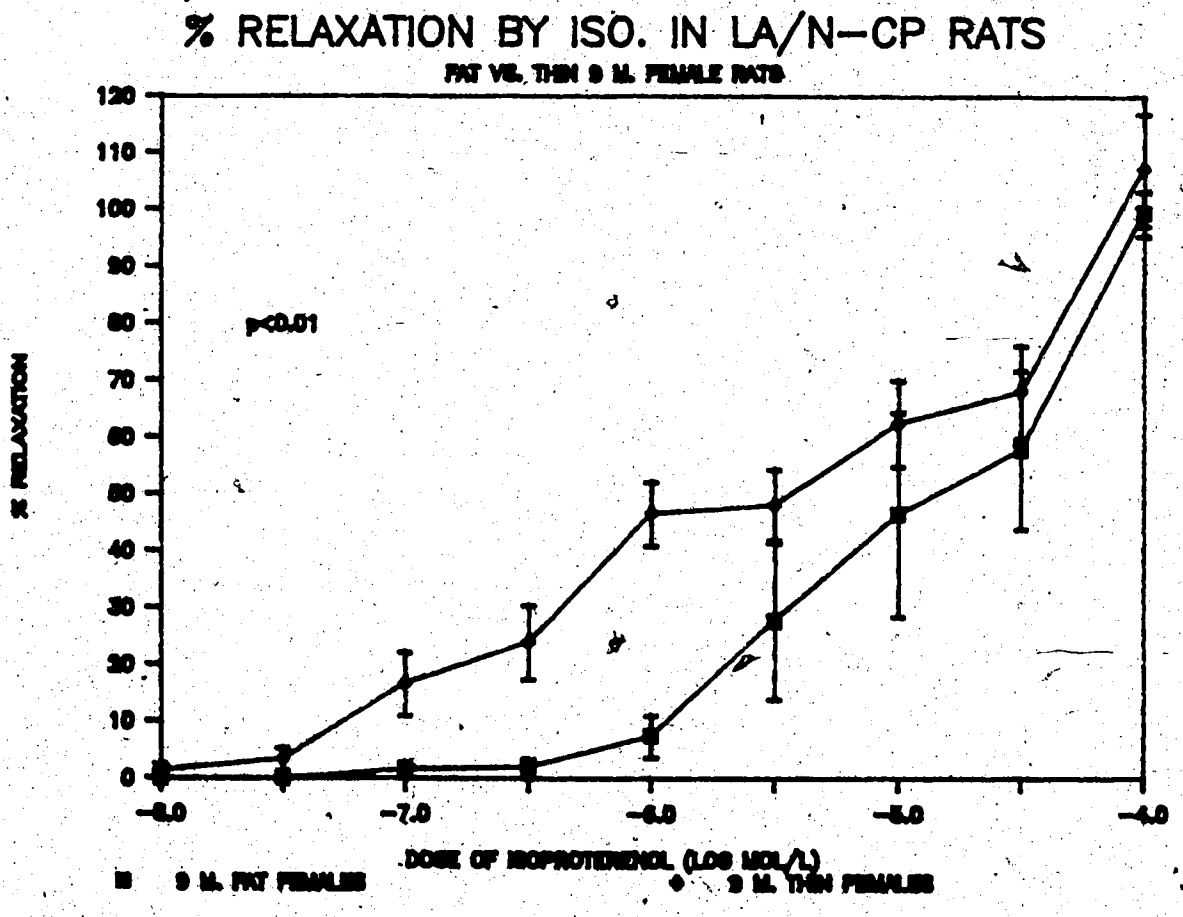

% RELAXATION BY ISO. IN LA/N-CP RATS
FAT VS. THIN 6 MONTH MALE RATS
p>0.05
% RELAXATION
DOSE OF ISOPROTERENOL (LOG MOL/L)
6 MONTH FAT MALES
6 MONTH THIN MALES

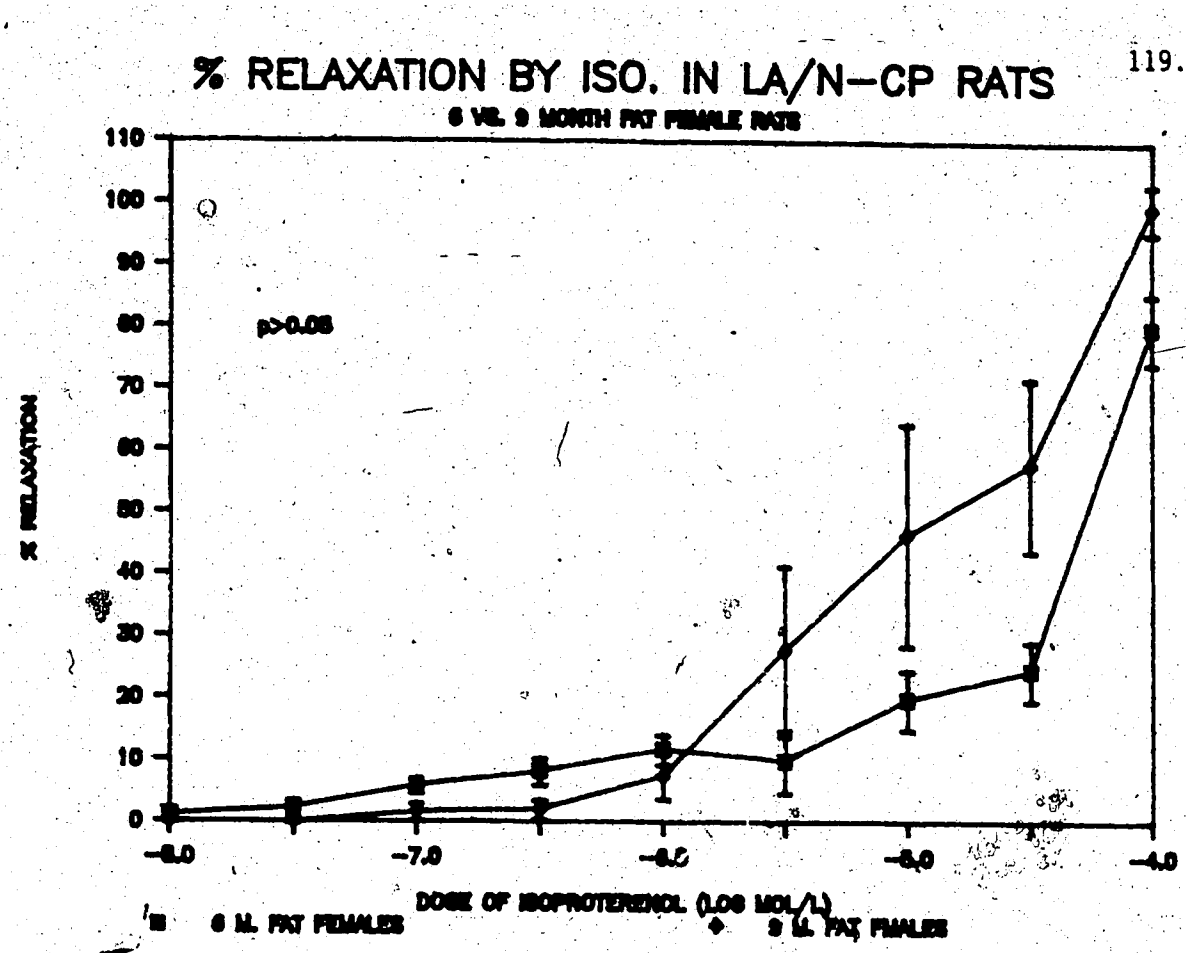

% RELAXATION BY ISO. IN LA/N-CP RATS
FAT VS. THIN 9 MONTH MALE RATS
p<0.01
% RELAXATION
DOSE OF ISOPROTERENOL (LOG MOL/L)
9 MONTH FAT MALES
9 MONTH THIN MALES

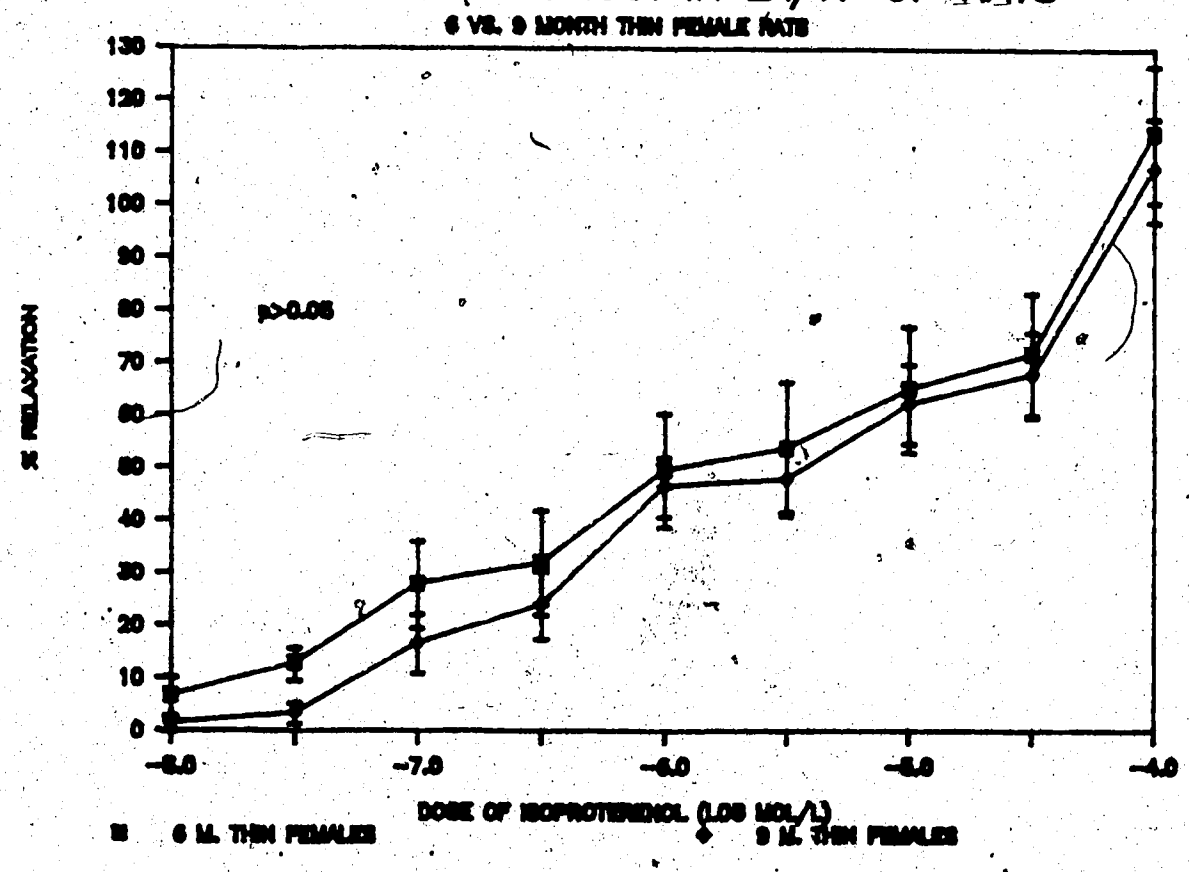

% RELAXATION BY ISO. IN LA/N-CP RATS
6 VS. 9 MONTH FAT MALE RATS
p>0.05
% RELAXATION
90
80
70
60
50
40
30
20
10
0
-8.0
-7.0
-6.0
-5.0
-4.0
DOSE OF ISOPROTERENOL (LOG MOL/L)
6 MONTH FAT MALES
9 MONTH FAT MALES

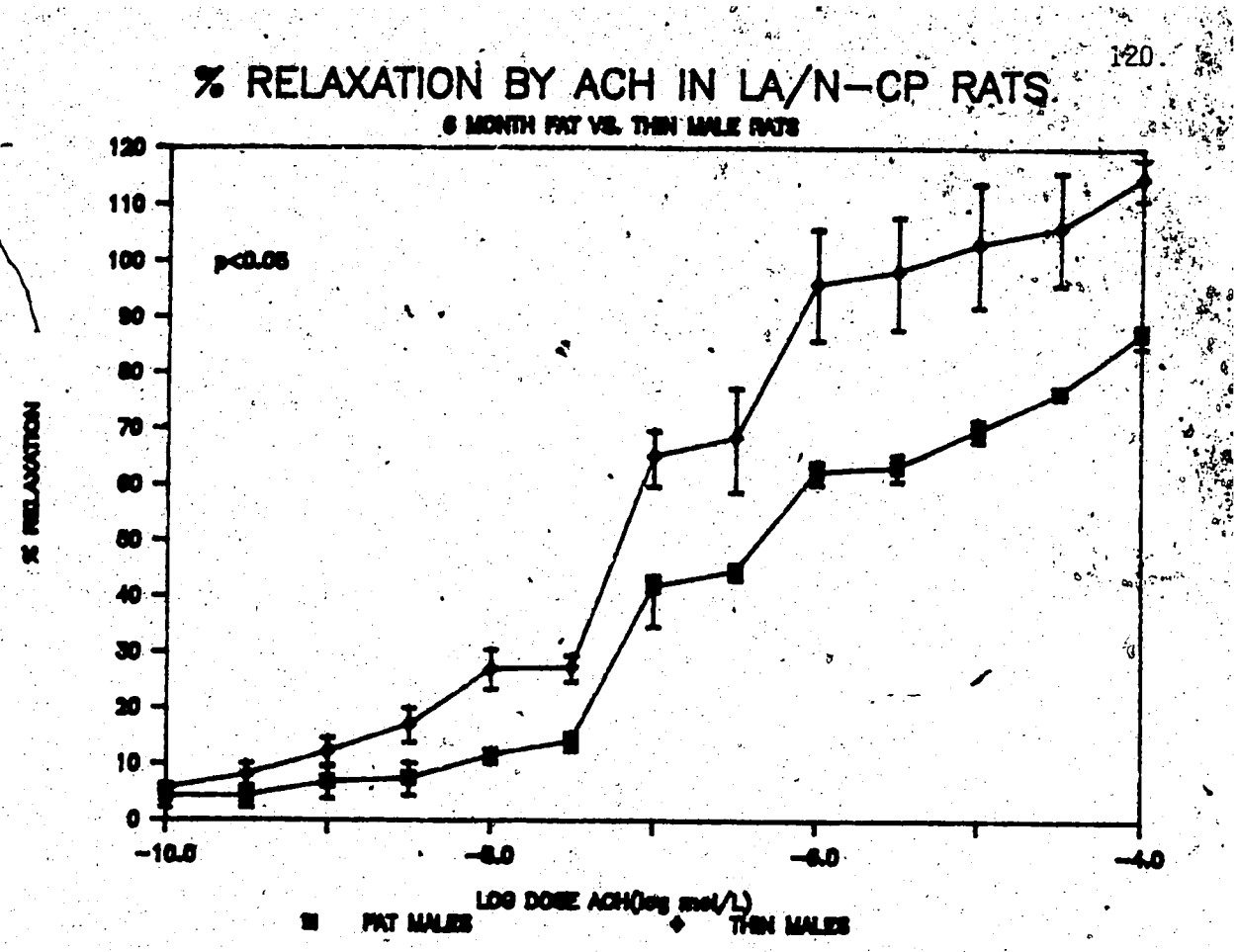

% RELAXATION BY ISO. IN LA/N-CP RATS
6 VS. 9 MONTH THIN MALE RATS
p<0.01
% RELAXATION
70
60
50
40
30
20
10
0
-8.0
-7.0
-6.0
-5.0
-4.0
DOSE OF ISOPROTERENOL (LOG MOL/L)
6 MONTH THIN MALES
9 MONTH THIN MALES

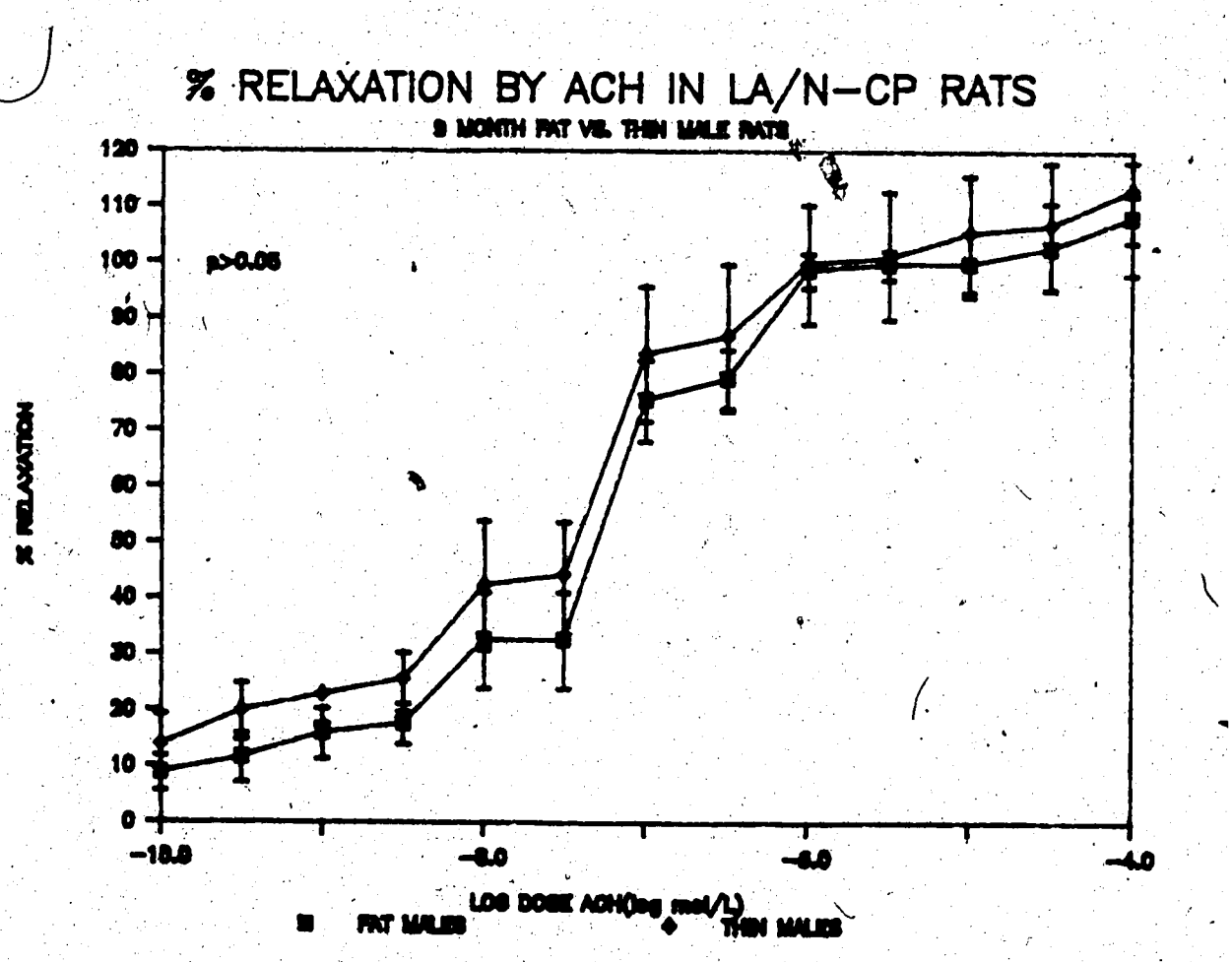

% RELAXATION BY ISO. IN LA/N-CP RATS
FAT VS. THIN 6 MONTH FEMALE RATS
p<0.001
% RELAXATION
DOSE OF ISOPROTERENOL (LOG MOL/L)
6 M. FAT FEMALES
6 M. THIN FEMALES


% RELAXATION BY ISO. IN LA/N-CP RATS
FAT VS. THIN 9 M. FEMALE RATS
p<0.01
% RELAXATION
DOSE OF ISOPROTERENOL (LOG MOL/L)
9 M. FAT FEMALES
9 M. THIN FEMALES

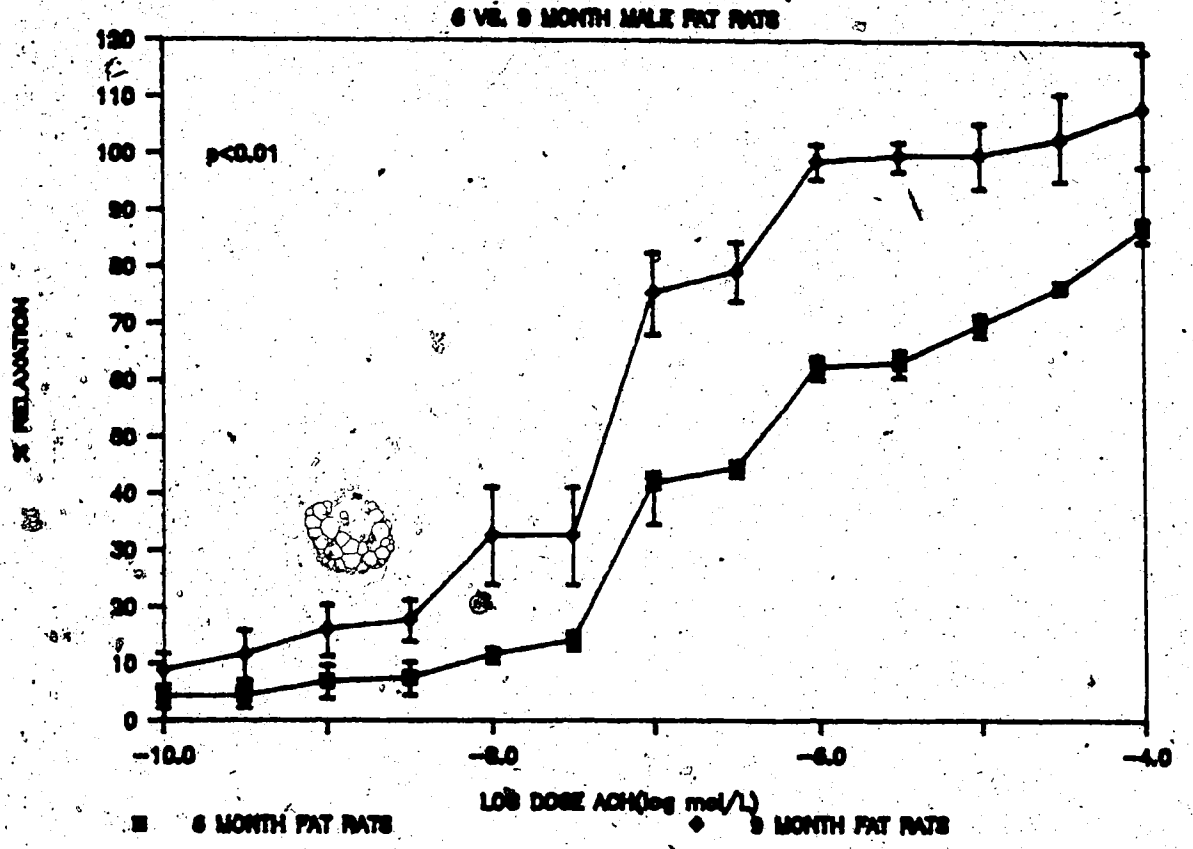

% RELAXATION BY ISO. IN LA/N-CP RATS
6 VS. 9 MONTH FAT FEMALE RATS
p>0.05
% RELAXATION
DOSE OF ISOPROTERENOL (LOG MOL/L)
6 M. FAT FEMALES
9 M. FAT FEMALES


% RELAXATION BY ISO. IN LA/N-CP RATS
6 VS. 9 MONTH THIN FEMALE RATS
p>0.05
% RELAXATION
DOSE OF ISOPROTERENOL (LOG MOL/L)
6 M. THIN FEMALES
9 M. THIN FEMALES

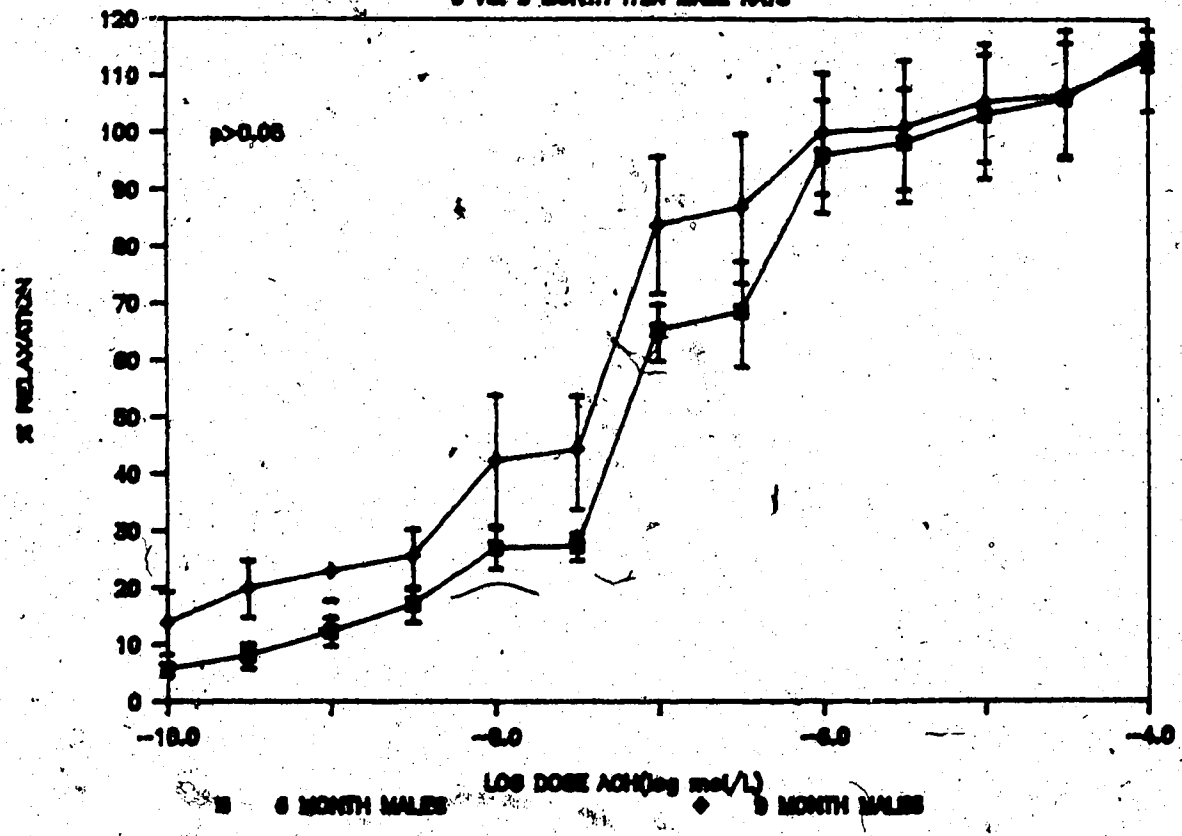

% RELAXATION BY ACH IN LA/N-CP RATS.
6 MONTH FAT VS. THIN MALE RATS
p<0.05
% RELAXATION
LOG DOSE ACH(log mol/L)
FAT MALES
THIN MALES


% RELAXATION BY ACH IN LA/N-CP RATS
6 MONTH FAT VS. THIN MALE RATS
p>0.05
% RELAXATION
LOG DOSE ACH(log mol/L)
FAT MALES
THIN MALES

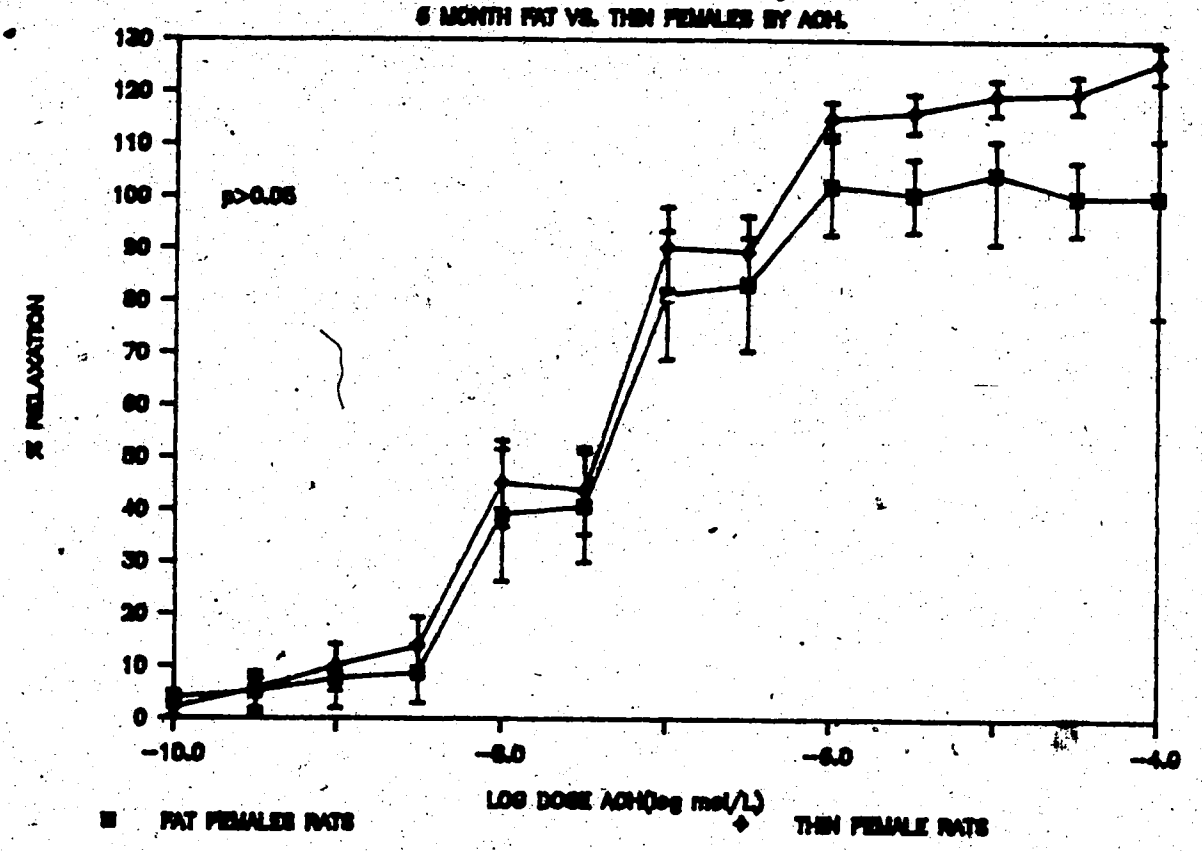
% RELAXATION BY ACH. IN LA/N-CP RATS



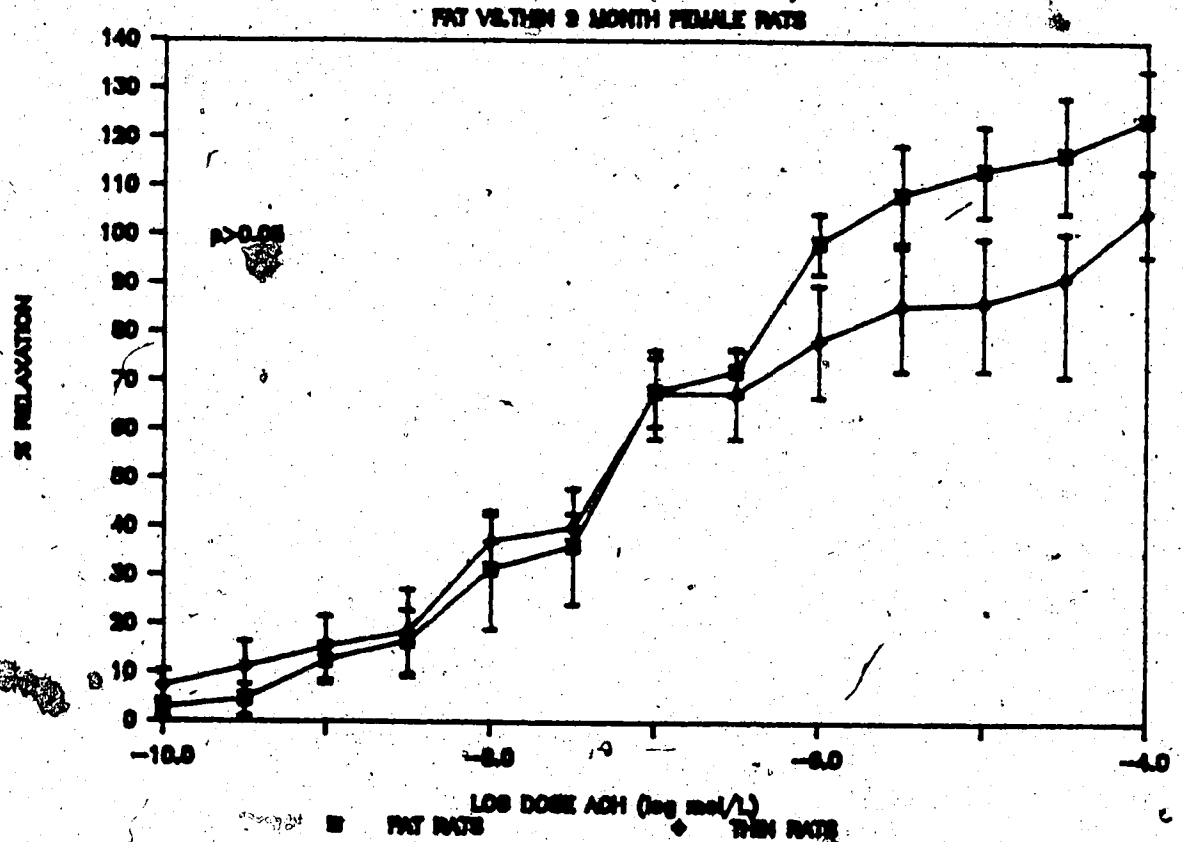
% RELAXATION BY ACH IN LA/N-CP RATS



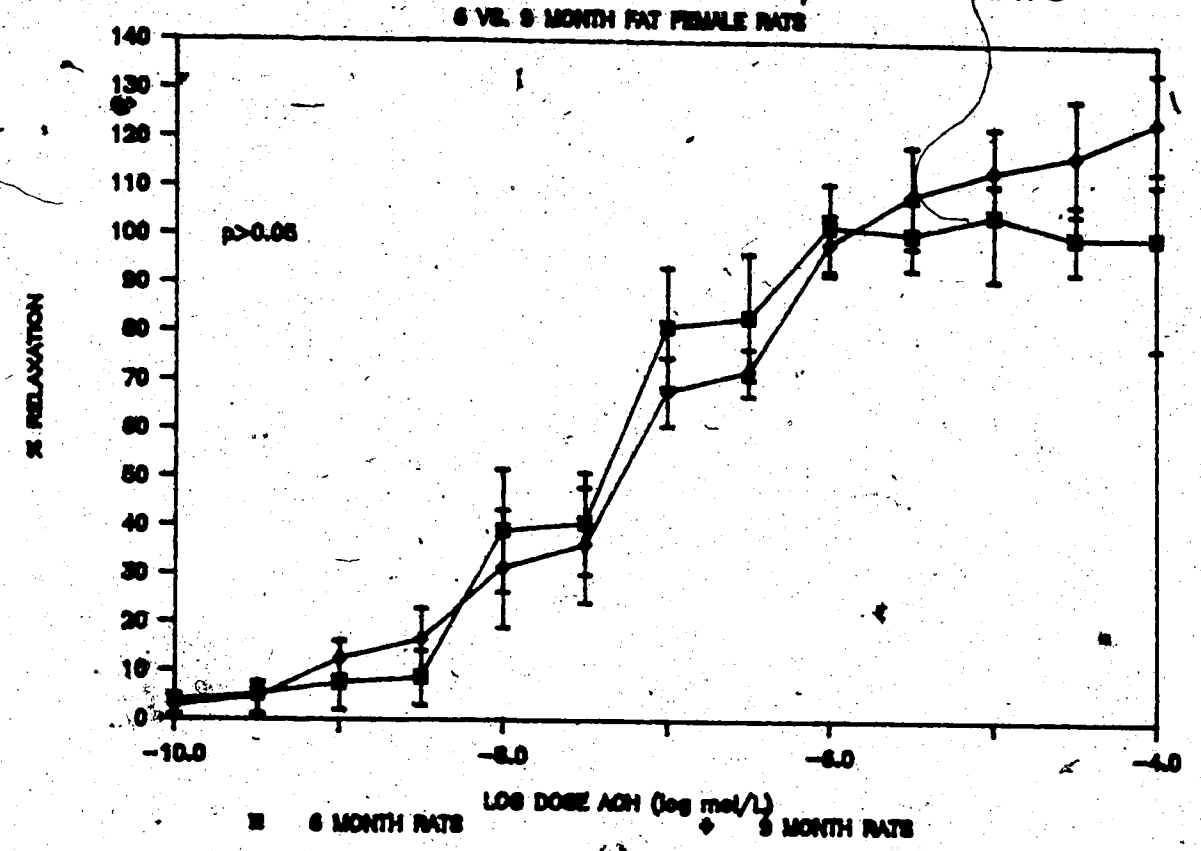
PER CENT RELAXATION OF FAT FEMALE RATS



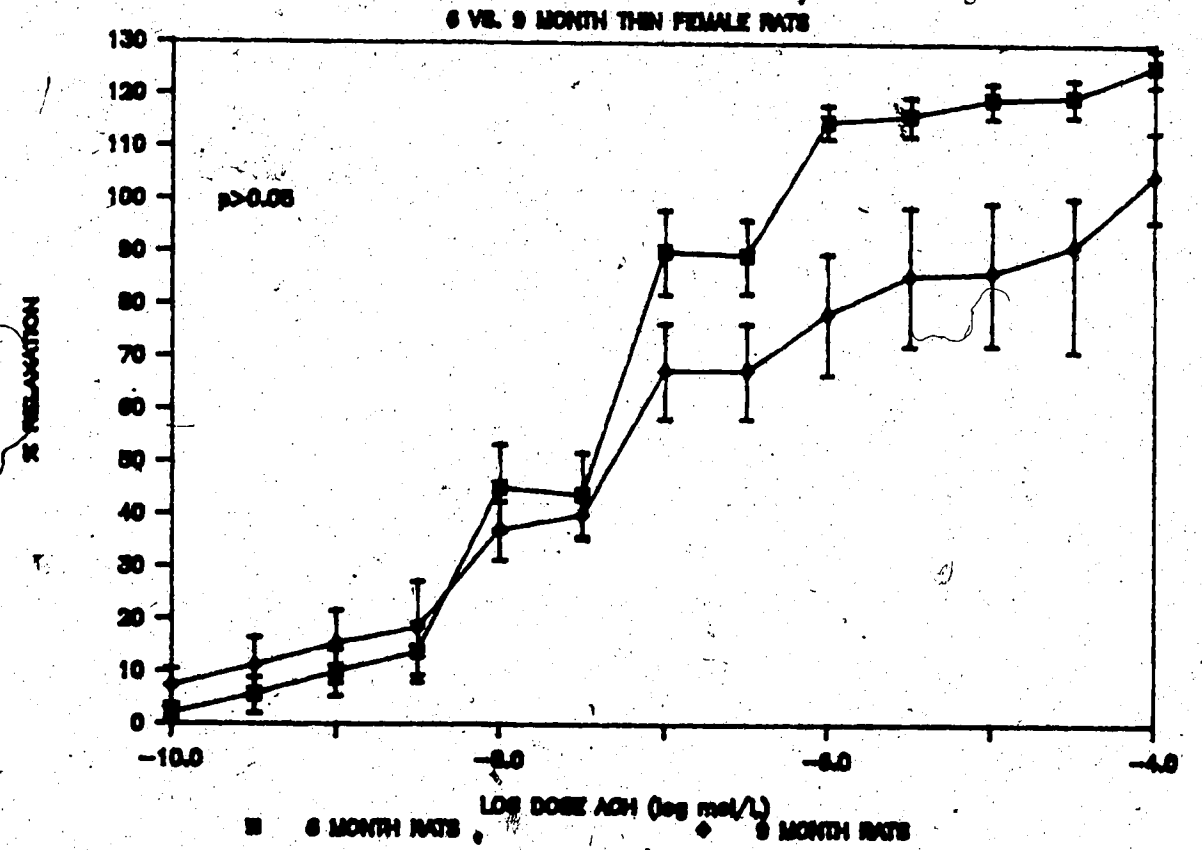
% RELAXATION BY ACH. IN LA/N-CP RATS



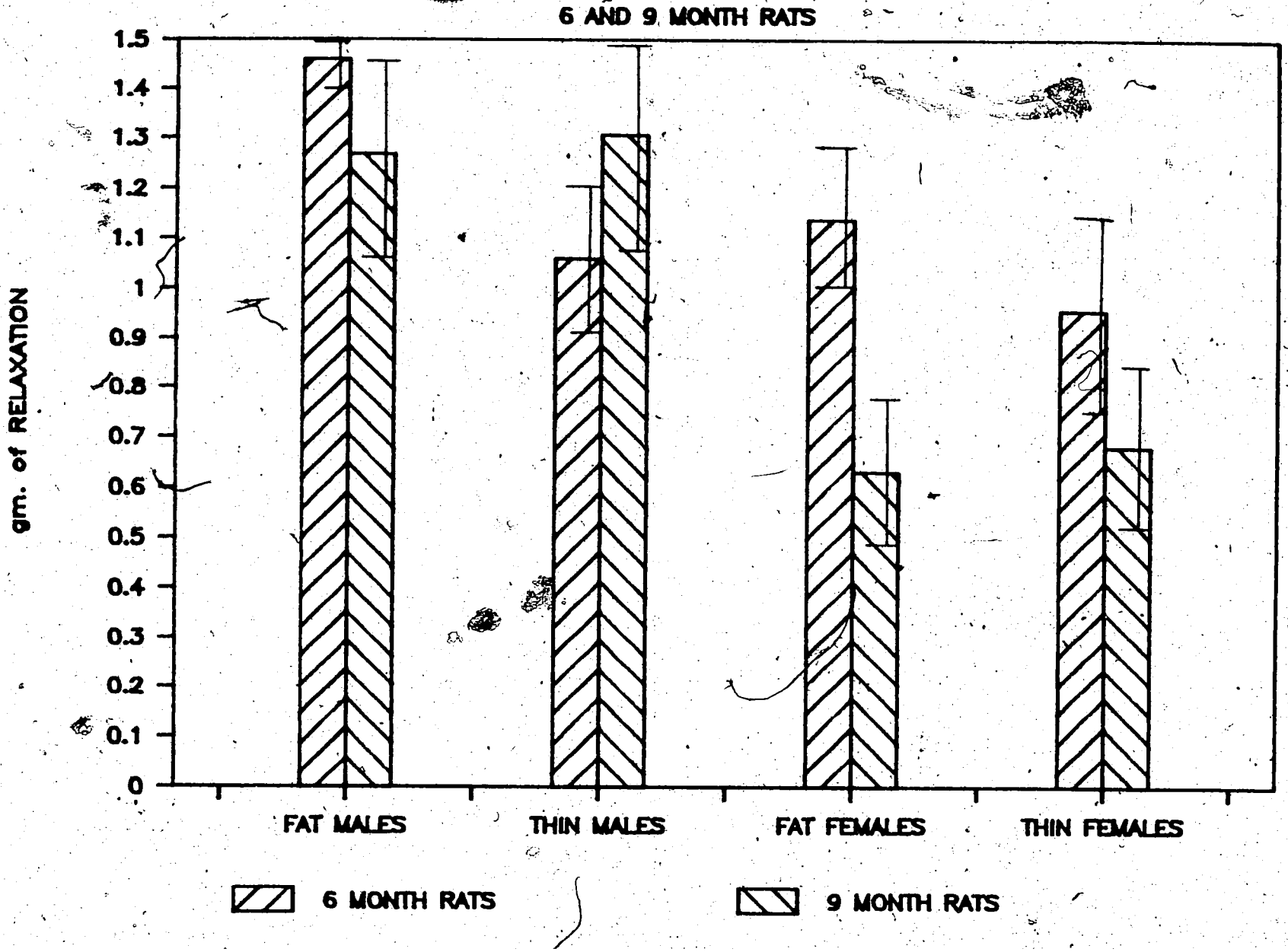
% RELAXATION BY ACH. IN LA/N-CP RATS



% RELAXATION BY ACH. IN LA/N-CP RATS



RELAXATION OF ARTERIAL RINGS BY ACH.—4



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