VASODILATOR EFFECTS OF ACETYLCHOLINE IN AN EXPERIMENTAL MODEL OF HEART FAILURE

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ABSTRACT

The purpose of the work presented here was to investigate endothelium-dependent relaxations in the rabbit coronary ligation model of heart failure. We investigated endothelium-dependent relaxations at the level of larger vessels (thoracic aorta and vena cava; left renal artery and left renal vein; lateral saphenous artery and lateral saphenous vein and finally central ear artery and marginal ear vein) in a model devised to mimic heart failure. The model presented here is the rabbit coronary ligation model in which myocardial infarction was produced in male New Zealand white rabbits (2.6kg-3.0kg) by ligation of the marginal branch of the left descending coronary artery. The development of chronic heart failure was allowed to proceed over eight weeks. Animals were killed by overdose with pentobarbitone sodium (i.v. injection). Arteries and veins were carefully removed with as little connective tissue as possible and placed in cold physiological salt solution (PSS). The arterial and venous rings were mounted in 10mL isolated organ baths, bathed in Krebs maintained at 37°C and gassed with 95% O_2 plus 5% CO_2. The rings were then placed under different resting tensions. Acetylcholine (ACh) was chosen as endothelium-dependent vasodilator. After initial application of tension, tissues were left to equilibrate for a 60 min period. Then all tissues were precontracted with noradrenaline (1μM) nearly ten minutes before initial application of vasodilator. This induced submaximal contraction in all vessels with the exception of the ear vein. When the noradrenaline-induced contraction reached a plateau, cumulative concentration-response curves (CCRC) to acetylcholine were obtained by increasing the concentration in half-log increments. The results led to two major conclusions with respect to the model. First, the relaxation responses to acetylcholine were not impaired. Second, the results of our experiments in this model of heart failure suggest that normal stimulation of endothelial NO is preserved in peripheral conduit and capacitance vessels.
MJIRI, Vol. 17, No. 4, 319-324, 2004.
Keywords: Heart failure, Endothelium-dependent relaxation, Acetylcholine.

INTRODUCTION

Chronic heart failure is a clinical syndrome characterised by the inability of the heart to provide adequate nutrient supply to metabolically active tissues.[1] Sudden cardiac death claims an estimated 350,000 lives per year in the United States and between 50,000 and 100,000 lives a year in the United Kingdom. There are numerous underlying diagnoses in pa-

tients suffering sudden cardiac death. In 75% of cases, the underlying pathology causing heart failure in patients with sudden cardiac death is coronary heart disease.[2] It has been described as a condition of generalised neurohumoral excitation, characterised by activation of the sympathetic nervous and renin-angiotensin systems, increases in plasma vasopressin concentration, and parasympathetic withdrawal.[3,4] Several animal models of human congestive heart failure (CHF) have been developed in attempts to reproduce these features to study the pathogenic mechanisms involved in this disease. The coronary artery occlusion model of heart failure in the rat has been extensively studied. The model has been validated by the measurement of hemodynamic variables.[5] Rapid ventricular pacing in the dog has been shown to fulfil the clinical, radiographic and hemodynamic definitions of congestive heart failure.[6] Cardiomyopathic male hamsters of the BIO TO-2 strain, a unique experimental model of CHF characterised by progressive myocytolytic necrosis of cardiac muscle are available in study of congestive heart failure.[7] Since in 75% of cases, the underlying pathology causing heart failure in patients with sudden cardiac death is coronary heart disease, this model produces a similar circumstance to coronary heart disease. In several studies, the response to acetylcholine, an endothelium-dependent vasodilator, was found to be markedly attenuated in patients and experimental animals with CHF examined *in vitro*.[8-12] In contrast to reports indicating endothelial dysfunction, there are many reports that show endothelial normal function in peripheral vessels in different animal models of heart failure.[13-16] Therefore, it was the aim of the present study to assess whether endothelium-dependent relaxations play an important role at the level of larger vessels in a model devised to mimic heart failure.
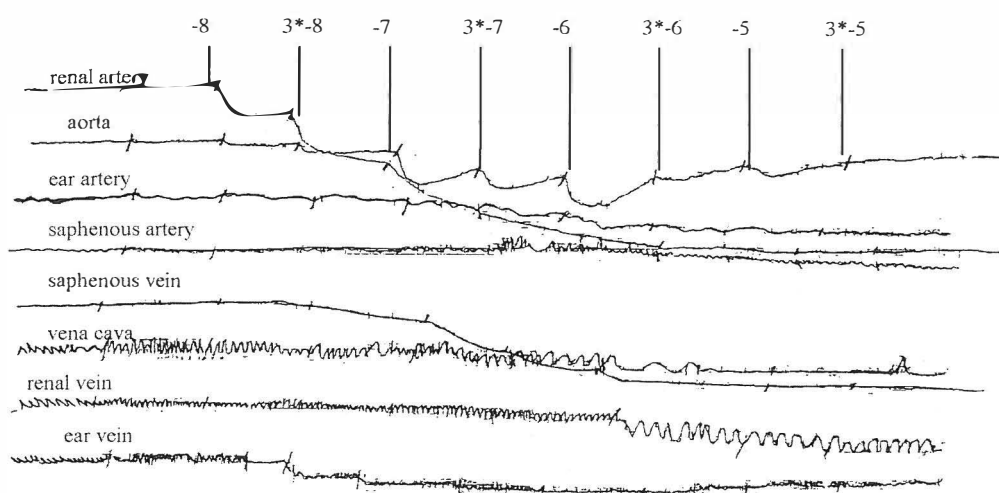
MATERIAL AND METHODS

The model was prepared by M. Hicks and co-workers in the Royal Infirmary, Glasgow. Myocardial infarction was produced in male New Zealand white rabbits (2.6kg-3.0kg) by ligation of the marginal branch of the left descending coronary artery. The development of chronic heart failure was allowed to proceed over eight weeks. Sham operated animals underwent a similar procedure but no ligation was performed. Animals were killed by overdose with pentobarbitone sodium (i.v. injection). Arteries and veins were carefully removed with as little connective tissue as possible and placed in cold physiological salt solution (PSS). Four pairs of arteries and veins (thoracic aorta and vena cava; left renal artery and left renal vein; lateral saphenous artery and lateral saphenous vein and finally central ear artery and marginal ear vein) of the sham operated with mean ejection fraction of (70.5±2.13) and coronary ligated rabbits with mean ejection fraction of (46.5±4.4), as determined by echocardiography, were studied. The arterial and venous

rings were mounted in 10mL isolated organ baths, bathed in Krebs maintained at 37°C and gassed with 95% O_2 plus 5% CO_2. Blood vessels were used immediately. The rings were then placed under different resting tensions which were determined by contraction to noradrenaline (NA, 1μM) from some preliminary experiments. Acetylcholine (ACh) was chosen as endothelium-dependent vasodilator. After initial application of tension tissues were left to equilibrate for a 60 min period. Then all tissues were precontracted with NA (1μM) nearly ten minutes before initial application of vasodilator. This induced submaximal contraction in all vessels with the exception of the ear vein. When the NA-induced contraction reached a plateau, cumulative concentration-response curves (CCRC) to ACh was obtained by increasing the concentration of the vasodilators in half-log increments. Relaxation to ACh in each concentration allowed five minutes to reach maximum. All concentrations are expressed as the final concentration in the organ bath fluid. The relaxation response to vasodilator ACh was expressed as percentage of the contraction generated by NA (1μM) against which it was tested. All data are given as mean±SEM. Significance was always accepted at the 0.05 level of probability.

RESULTS

Relaxation to ACh

Acetylcholine induced relaxations in all arteries and veins. Ear vein, saphenous vein and aorta were most sensitive in terms of threshold and concentration producing 50% maximum relaxation of tone. Saphenous vein and aorta produced the greatest maximum relaxations (78-90%). In renal artery and ear vein high concentrations of acetylcholine (>1μM) produced paradoxical contraction. We investigated the mechanism of this contraction in renal artery. Indomethacin (1μM), which blocks the production of prostaglandins, did not affect the contraction evoked by acetylcholine in renal artery. In the presence of L-NAME (100μM) acetylcholine produced poor relaxation but L-NAME failed to affect contraction produced by acetylcholine in high concentrations. Atropine, an antagonist of muscarinic receptors, blocked the contraction produced by acetylcholine. These results suggest that contraction to ACh in this artery is related to muscarinic receptors located in smooth muscle cells which are sensitive to high concentrations of ACh. Saphenous artery and ear artery had the poorest response to acetylcholine. In ear and renal veins the concentration response curves to ACh were clearly not monophasic, in the ear vein a rebound contraction occurred at high concentration of ACh and in the renal vein, in contrast there was a further relaxation phase at high concentration of ACh. In relaxation to acetylcholine, there was no difference between coronary ligated

-8   3*-8   -7   3*-7   -6   3*-6   -5   3*-5

renal artery

aorta

ear artery

saphenous artery

saphenous vein

vena cava

renal vein

ear vein

****

Base line after precontraction

1g (saphenous vein, renal artery and aorta)
0.5g (other preparations)

Fig. 1. Representative trace shows the cumulative relaxation responses to ACh in the rabbit isolated four pairs of arteries and veins: thoracic aorta and vena cava; left renal artery and left renal vein; lateral saphenous artery and lateral saphenous vein and finally central ear artery and marginal ear vein. All tissues were precontracted with NA (1μM). This induced submaximal contraction in all vessels with the exception of the ear vein. When the NA-induced contraction reached a plateau, cumulative concentration-response curves to ACh was obtained by increasing the concentration of the vasodilator in half-log increments.

Table I. Comparison of pIC_{50} and maximum relaxations in four pairs of arteries and veins in response to acetylcholine (ACh) of the coronary ligated and sham operated rabbits 8 weeks after operation.

Vessel	pIC_{50} (ACh) Sham	pIC_{50} (ACh) Ligated	Maximum relaxation% Sham	Maximum relaxation% Ligated
Aorta	7.44 ± 0.06	7.35 ± 0.09	76.7 ± 6.11	*91.97 ± 4.33
Vena cava	6.56 ± 0.3	6.93 ± 0.41	44.23 ± 9.59	59.39 ± 6.01
Renal artery	6.54 ± 0.67	7.09 ± 0.09	43.66 ± 6.05	55.02 ± 5.31
Renal vein	6.75 ± 0.37	7.13 ± 0.39	66.21 ± 6.72	69.55 ± 8.92
Saphenous artery	6.53 ± 0.74	6.13 ± 0.42	7.88 ± 1.35	20.79 ± 8.82
Saphenous vein	8.02 ± 0.11	7.89 ± 0.16	77.02 ± 6.8	90.43 ± 5.1
Ear artery	6.44 ± 0.18	6.33 ± 0.16	31.51 ± 2.85	38.08 ± 9.5
Ear vein	7.71 ± 0.23	7.89 ± 0.1	87.6 ± 7.54	84.15 ± 8.36

pIC_{50} is expressed as the -log of the IC_{50} (concentration producing 50% of the maximum relaxation attained within the range tested).
Maximum relaxation is expressed as % of NA (1μM) used for inducing tone. Data are expressed as mean ± s.e. mean (n=6). Statistical comparisons with controls were carried out using unpaired Student's t-test *$p < 0.05$.

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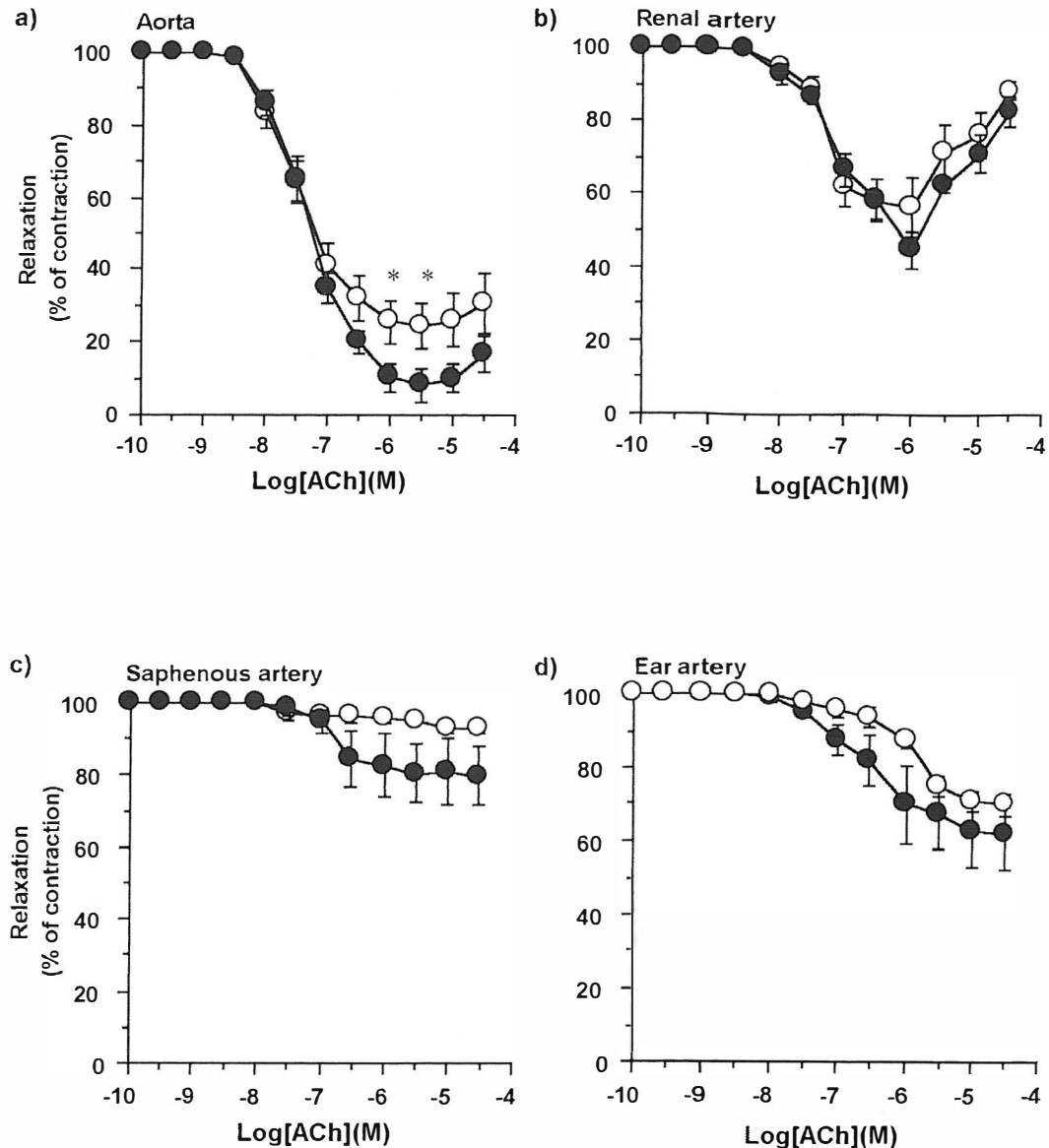
Fig. 2. Relaxation to ACh in the four isolated arteries of the sham operated (O) with mean ejection fraction of (70.5 ± 2.13) and coronary ligated rabbits (● ′) with mean ejection fraction of (46.5 ± 4.4) 8 weeks after operation. Results are expressed as % of maximum response to NA (1μM) used for inducing tone.
Each point represents mean±S.E. mean (n=6). Statistically significant differences are represented by * $p<0.05$, unpaired Student's t-test.
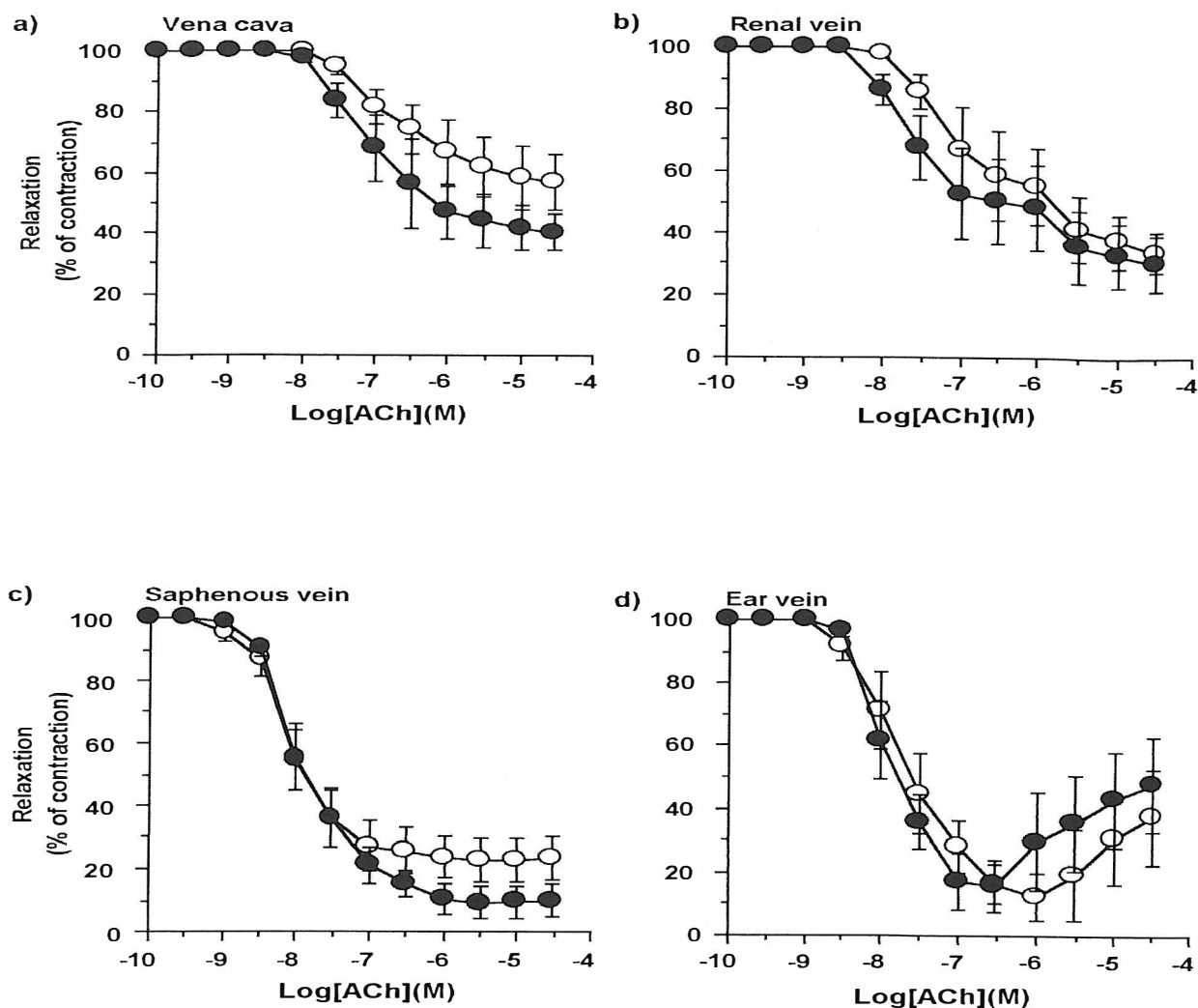
and sham operated rabbits 8 weeks after operation. (Table I, Figures 1,2,3).

DISCUSSION

The results of our present investigation led to major conclusion with respect to the model that the relaxation response to acetylcholine was not impaired. It is known that the endothelium is important in the control of vascular tone in both large and small vessels.[17] Our finding

in relaxations to acetylcholine are comparable to those observed in other models of heart failure in large vessels in isolated rings of dorsal pedal artery from dogs with pacing-induced heart failure and in the coronary and peripheral vessels in the same model of heart failure.[13,14] In another model very similar to our model, in basilar, mesenteric and renal arteries and iliac vein acetylcholine-induced relaxation was identical in control and rats with coronary ligation.[15]

Abnormalities of vasomotor tone are characteristic

Fig. 3. Relaxation to ACh in the four isolated veins of the sham operated (O) with mean ejection fraction of (70.5 ± 2.13) and coronary ligated rabbits (●) with mean ejection fraction of (46.5 ± 4.4) 8 weeks after operation. Results are expressed as % of maximum response to NA (1μM) used for inducing tone.
Each point represents mean ± S.E.mean (n=6).

of many cardiovascular diseases. Abnormalities of endothelium dependent vasodilation also have been shown in several cardiovascular diseases, such as hypertension or atherosclerosis. Cardiac output is reduced in heart failure and the stimulus for NO production by the peripheral circulation would be reduced and this depression represents one local mechanism of the vascular system to compensate for cardiac output. On the other hand congestive heart failure is associated with activation of the sympathetic nervous and renin-angiotensin systems. Sustained increase of local and circulating noradrenaline concentrations therefore apparently is associated with an increase in endothelial NO release, which could reflect a compensatory mechanism to counterbalance excessive peripheral catecholamine-depen-

dent vasoconstriction.[17] In agreement with this possibility it was reported that in the thoracic aorta and renal artery of cardiomyopathic hamsters relaxation response to acetylcholine was enhanced, demonstrating increased sensitivity of vascular smooth muscle to nitric oxide.[19]

Reports of endothelium function in heart failure yield conflicting results. One reason could be attributed to differences among different models of heart failure. In cardiomyopathic hamsters, indirect signs of haemodynamic changes such as lung weight were assessed and exact quantification of the stage of heart failure is not known. In chronic rapid ventricular pacing, hemodynamic responses, endocrine changes, and the clinical and pathological picture of heart failure are all consistent with naturally occurring heart failure.[6] The

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heart to body weight ratio was, however, significantly increased in rats with chronic heart failure induced by coronary ligation, as were plasma noradrenaline concentrations.[5] In the rabbit coronary ligated model we have found no significant neuroendocrine changes at 8 weeks. Since acetylcholine is the classic endothelium-dependent vasodilator, it was chosen as the reference endothelium-dependent vasodilator in our study and other models of heart failure. It was used to stimulate the release of endothelium-derived nitric oxide (NO).[17] According to reports arteries generally relax more in response to acetylcholine compared to veins.[20] They suggested that the endothelium has a much lower modulatory role in veins as compared with the arteries. Their study is not consistent with the current findings in some pairs of veins and arteries (saphenous vein and ear vein compared with corresponding arteries). In our study acetylcholine induced concentration-dependent dilations in all vessels examined with no difference of maximum relaxation or potency between coronary ligated and sham. Our data suggest normal stimulated nitric oxide release in systemic larger conduit and capacitance vessels in coronary ligated rabbits after 8 weeks.

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HISTOMORPHOMETRY OF TRABECULAR BONE OF CAUDAL VERTEBRAE DURING RAT PREGNANCY

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ABSTRACT

There are dramatic changes in skeletal physiology and metabolism to accommodate the mineral requirements of the developing fetus during pregnancy and milk production during lactation. In addition, there is substantial calcium transfer from the mother for redistribution to the fetus or infant. Recently changes in bone histomorphometry during human pregnancy have been associated also with the transformation of the cancellous architecture and the bone surface available for exchange. These changes are examined further in an animal model. Using twenty time mated Wistar rats in early and late pregnancy and eight virgin rats as a control fed ad libitum, the histomorphometry of cancellous bone was compared in undecalcified histological sections of caudal vertebrae. Pregnancy produced a rapid and substantial increase not only in the number of labelled sites but also in the mineralisation rate. There was an early stimulation of bone formation (which quadrupled at some sites) which was indicated by an increase in the skeletal uptake and spacing of double calcein labels in different sites including subperiosteal, epiphyseal plate and endosteal surface. However there was immediate generation of thicker more numerous and interconnected trabeculae. We concluded that in comparison with human pregnancy, there was more bone formation during rat pregnancy.
MJIRI, Vol. 17, No. 4, 325-329, 2004.

Keywords: Pregnancy, Histomorphometry, Caudal vertebrae, Calcein, Trabeculae.

INTRODUCTION

Substantial changes in mineral metabolism occur during pregnancy and lactation in both human and animals. Because of the altered amount of calcium required for fetal development and milk production, the maternal skeleton is particularly impacted. Maternal adaptation during pregnancy and lactation include increased intestinal absorption of calcium, increased blood flow, and increased mineral deposition and removal from the skeleton.[1-3] In rats, increased calcium retention during early

pregnancy is associated with bone growth and mass.[4] At the same time, while there are reports of changes in bone mass during lactation in certain long-lived mammals including man,[5] the gross skeletal changes have been most extensively documented in rodents, since these short-lived mammals have large litters and are known to be skeletally responsive.

In sheep, bone resorption rises at midterm (normal pregnancy takes 140d), at which time the rate of mineral transfer from the ewe to the fetus accelerates.[6] However, in human pregnancy, opinion concerning the extent to which the maternal skeleton is affected is divided.[7-11] In a related histomorphometric study of human cancellous bone by Purdie et al.[12] an unexpected loss of cancellous bone in early pregnancy due to increased resorption was apparently reversed by elevated bone formation in late

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pregnancy. This biphasic response has been supported by differences seen in biochemical markers.[13] On the other hand, there have been reports suggesting a relationship between osteoporosis and pregnancy.[14-17] As a result of this sequence of differential matrix attrition and apposition it has recently been suggested that not only is a significant proportion of maternal bone matrix turned over during pregnancy but also the structural character of the spongiosa is altered.[18] This example of significant mobilisation and rearrangement of trabeculae over a relatively short time period may have implications for bone biomechanical strength during reproduction and may explain pregnancy-associated fractures. It might also provide insight into the reversal of hypogonadal osteoporosis when reproductive life has ended. The aim of the present study is to use an animal model in which to establish the impact of pregnancy on maternal trabecular architecture.

MATERIAL AND METHODS

Twenty time mated Wistar rats were divided equally for comparison of the cancellous skeleton in early and late pregnancy and virgin rats as a control (Table I). The rats were housed at a temperature of 19-23°C and fed ad libitum, with light supplied from 6 a.m. to 6 p.m. The mated animals together with virgin controls were double labelled with calcein fluorochrome marker 5 d before killing (i.e. 6 d after mating) and again 1 d before killing (i.e. 10 d after mating). Each of the 10 rats in the early pregnancy group was given a single intraperitoneal injection of calcein solution (Sigma; 30 mg/kg body weight in 1 mL 0.15 NaCl containing 2 % $NaHCO_3$) at a dose of 1 mL of solution for each rat of 250 ± 10 g weight. The process was repeated in the 10 time-mated late pregnancy rats when the first injection was given 5 d before killing (i.e. 16 d after mating) and the second was given 1 d before killing (i.e. 20d after mating). The labeling schedule for the virgin rats was the same as that for the early pregnancy rats. All animals were killed using CO_2 gas. The 2nd caudal vertebrae were dissected and the intact specimens preserved in 70% ethanol and embedded undecalcified in methylmethacrylate.[19,20] Ten undecalcified serial sections (8μ) were cut from each on a Jung K heavy duty microtome (Reichert-Jung, Heidelberg). In this respect the bony processes provided natu-

ral markers enabling the location of the sections collected to be matched exactly in all groups. Sections were mounted unstained for calcein fluorescence analysis of the number of labelled sites and interlabel distance at equidistant places using an eyepiece graticule and a Zeiss epifluorescence microscope according to established procedure.[21]

RESULTS

In a pilot study performed at the outset, labelling with tetracycline was compared with calcein. When the two were administered as first and second labels the stronger fluorescence of the calcein tended to mask the weaker tetracycline band, such that it was preferable to use a single fluorochrome, calcein, for both labels (Figs. 1 & 2).

Mineralised bone matrix   1st band 2nd band

Fig. 1. Photomicrograph of calcein double labelled bone in a vertebral body. The distance mid-band was measured and divided by the time interval (Ultraviolet light, ×1600).
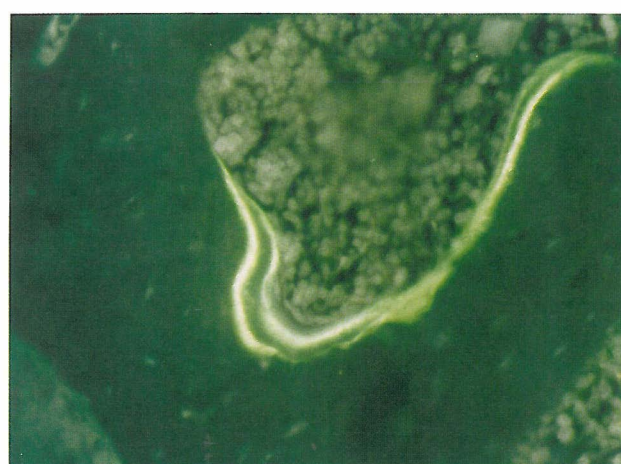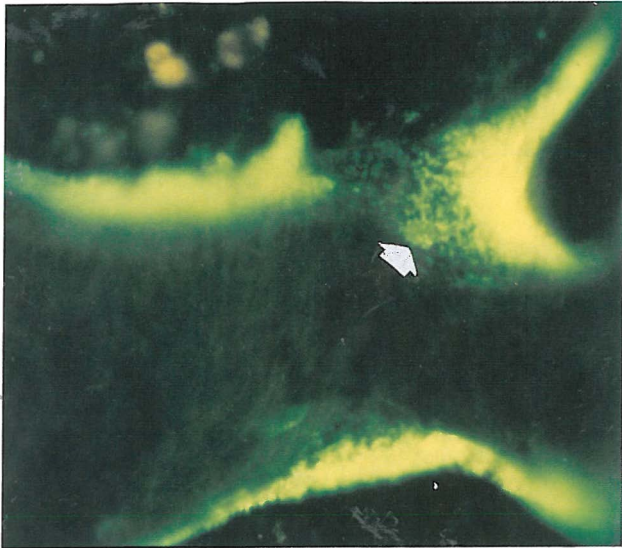

Fig. 2. Photomicrograph of calcein double-labelled bone in a caudal vertebral body (Ultraviolet light, ×1600).

Table I. Groups studied.

| Groups	n	Age (weeks)
Early pregnancy rats	10	11-12
Late pregnancy rats	10	14-15
Virgin rats	8	11-12

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Fig. 3. Photomicrograph showing the distribution of the calcein fluorochrome at the calcification front (Cf). Mineralised bone and osteoid tissue have autofluorescence of a dull green colour. Calcein staining at sites of bone formation is granular in character. An adjacent osteocyte (arrow) also fluoresces while other osteocytes do not (Ultraviolet light, ×1600).
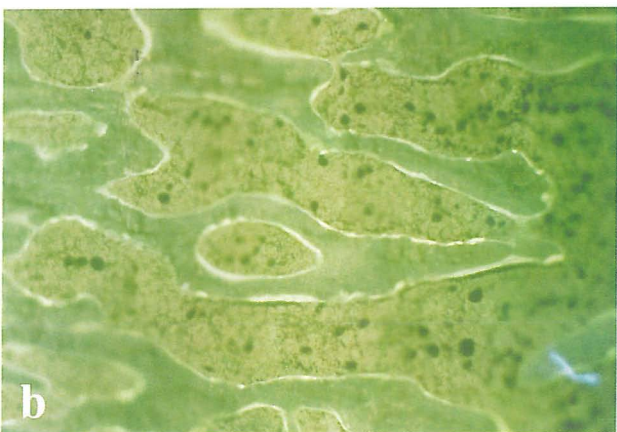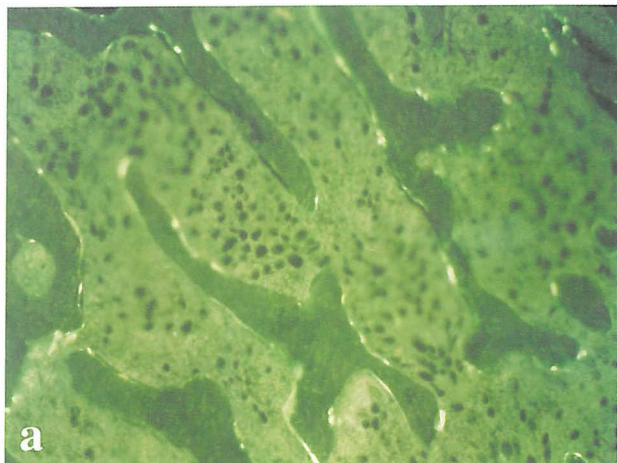
Fig. 4. Photomicrographs showing calcein labelled formation sites (arrowed) in the caudal vertebrae of a) virgin rats and their increase in number in b) rats in early pregnancy (Ultraviolet light, ×300).
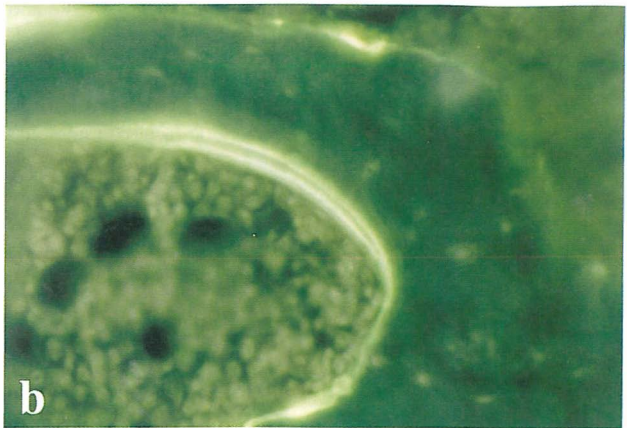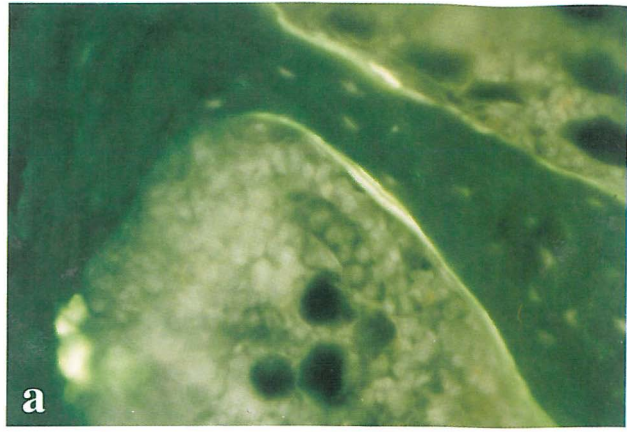
Fig. 5. Photomicrographs showing the increased bone formation rate at calcein labelled remodelling sites in the caudal vertebral body of a) a virgin rat when the labels were close and sometimes fused together and b) in early pregnancy when the bands were clearly separated and remained so in late pregnancy (Ultraviolet light , ×1600).

It was observed at the time that the calcification front was granular in nature and that like tetracycline, the calcein formed a fluorescent complex with mineral in the form of discrete calcified microspheres (Fig. 3). The results at cortical and cancellous sites of the caudal vertebrae are shown in Tables II and III and Fig. 4. In all areas the number of labelled sites rose with pregnancy. Labelled haversian systems were entirely absent from the caudal region of the virgin animals. Pregnancy produced a rapid and substantial increase not only in the number of labelled sites but also in the mineralisation rate. However, in the early pregnancy an early stimulation of bone formation was indicated by an increase in the skeletal uptake and spacing of double calcein labels (Fig. 5). This was expressed at all the cortical sites, including the previously inactive haversian canals where apposition rate was particularly affected, but this time the sharp rise to a plateau contrasted with a slower progressive response

Table II. Mean number/section of discrete calcein-labelled sites in different locations of the caudal vertebrae.

Area	Virgin rats (n=8)	Early pregnancy (n=10)	Late pregnancy (n=10)
Epiphyseal plate region	5 (1)*	8 (2)	10 (2)
Subperiosteal surface	4 (1)**	8 (2)	9 (3)
Cortical endosteal surface	4 (2)*	8 (1)	10 (3)
Trabecular surface	5 (2)*	9 (2)	8 (2)
Haversian canal	0	2 (1)	2 (2)

All results are given as mean ($\pm$S.D.). Significance of difference between nonpregnant age-matched control rats and between late and early pregnancy groups: *$p<0.05$; **$p<0.01$.

Table III. Comparison of bone mineralisation rate in caudal vertebral bodies.

Variables	Nonpregnant (n=8)	Early pregnancy (n=10)	Late pregnancy (n=10)
Subperiosteal (μm/day)	1.20 (0.10)***	3.41 (0.70)	3.43 (1.10)
Haversian canal (μm/day)	Absent	1.72 (0.40)	2.17 (0.70)
Epiphyseal plate (μm/day)	1.45 (0.10)**	2.00 (0.4)	1.75 (0.30)
Endosteal surface (μm/day)	1.40 (0.20)**	2.00 (0.20)	2.15 (0.40)
Trabecular bone (μm/day)	1.18 (0.10)	1.37 (0.30)	1.66 (0.20)*

All results are given as mean($\pm$S.D.). Significance of difference between nonpregnant age-matched control rats and between late and early pregnancy groups: *$p<0.05$; **$p<0.01$, ***$p<0.001$.

through gestation at cancellous surfaces. Activity at the epiphyseal plates was also stimulated by pregnancy.

Statistical analysis

Results were expressed as the mean$\pm$S.D. or mean$\pm$S.E. and the statistical significance of any differences between the groups was determined using the Minitab sortware package and the Minitab t test throughout.

DISCUSSION

The assessment of the trabecular architecture based upon morphological features in 2-dimensional images derived from thin sections of bone provides useful information about cancellous bone quality as well as its quantity. However, more striking than the change in cancellous bone mass in rat were the modulations in the trabecular architecture, confirming recent indications in human material. While it might argue that adult rats, unlike man, continue to grow, the improved connectivity

was not confined to the growth plate region but was apparently systemic. Moreover, the increased bone formation confirms the same observation made in human material[22] where the growth plates had long since closed. Changes during pregnancy had occured at all cancellous and cortical surfaces, as well as at the epiphyseal plate. This latter is in accord with changes observed in endochondral growth rates during pregnancy reported by others.[23] Similarly Miller et al.[24] found a significant increase in dentine appositional rate during pregnancy. In other studies, significant changes were reported in bone turnover, structure and mechanical properties during the first reproductive cycle in the rat which support the current study.[2,25] Consistent with previous reports, periosteal bone formation rates were increased in the end of pregnancy.[4] The relatively simple framework of thickened trabeculae may represent a stored source of calcium phosphate for the lactation period. Finally, there are similarities in the positive changes induced in the cancellous skeleton of the rat during a single pregnancy

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with those recently observed in man.[26] In both, the maternal skeleton responds by the proliferation of new sites of bone formation and a more complex spongiosa. In contrast with an initial phase of increased resorption and bone loss that is a feature of early human pregnancy no such phase was apparent in the rat. Instead, there was a rapid rise in bone formation which continued throughout gestation with no evidence of a preliminary loss of tissue. It is possible that an immediate resorption phase had occured earlier (i.e. within hours of conception) than the present sampling process (10 d after mating) and that the event had passed by the time that early pregnancy animals were examined.

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