EXERCISE TRAINING CAN ATTENUATE PREECLAMPSIA-LIKE SYMPTOMS IN AN ANIMAL MODEL.

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Abstract

Exercise training benefits on general health have been widely investigated and used as an alternative treatment in different pathological conditions. Since preeclampsia is a severe pregnancy-associated pathology for which no treatment is available, our aim was to investigate the protective role of exercise training on pregnancy outcome using a mouse model of the disease. To do so, we used transgenic female mice overexpressing human angiotensinogen, which develop preeclampsia when they are mated with human renin-overexpressing males. Females were placed in exercise cages 4 weeks prior to mating, and remained in these throughout gestation. Blood pressure was measured by telemetry, and proteinuria was quantified by ELISA. Placentas were assessed by histology and immunohistochemistry while vascular endothelial growth factor was measured by real-time PCR and immunoblot. Endothelial function was assessed in isolated mesenteric arteries. Conversely to sedentary transgenic females, mean arterial pressure was no longer different from non-transgenic mice at the end of gestation. Proteinuria, placental pathology and cardiac hypertrophy normally observed in sedentary transgenic mice were absent in trained mice. Impaired vascular reactivity was also significantly ameliorated. Furthermore, placentas from trained transgenic mice presented with normalized levels of vascular endothelial growth factor. To our knowledge, we are the first to clearly demonstrate that exercise training both before and during gestation can reduce preeclampsia symptoms in a mouse model of the disease. Consequently, women at risk for this pathology could benefit from exercise training to protect themselves and their future fetuses from this disease.

Keywords: exercise training, preeclampsia, mouse model, VEGF, placental markers

Introduction

It is now well-recognized that physical activity has a beneficial impact on general public health. More specifically, positive effects have been reported on many chronic and cardiovascular diseases, such as obesity, type 2 diabetes, chronic hypertension, and even certain types of cancer(1). Furthermore, it has also been suggested that physical activity may be beneficial to mother and fetus during pregnancy by reducing the risk of gestational hypertension, preeclampsia (PE) and gestational diabetes mellitus(2) as well as improving placental development(3). However, the number of studies is limited, and they are mostly not randomized(4). Furthermore, the criteria defining physical activity intensity fluctuate significantly from one study to another, and the number of women is often too low to draw reliable conclusions(4;5). Even if physical activity is already recommended by the American College of Gynecology and Obstetrics for healthy pregnancies(6), there is still a certain apprehension regarding the prescription of exercise training (ET) as a preventive treatment for women at risk(7).

Reducing the risk of PE with ET would represent important progress since, to date, no treatments are available for the pathology. Indeed, this disease, which affects 5-7% of all pregnancies, is the principal cause of maternal and fetal morbidity(8). PE is diagnosed by the onset of hypertension and proteinuria after 20 weeks of gestation and can deteriorate to maternal end organ damage and fetal and even maternal death(9). As symptoms resolve completely after delivery(10), it remains the only treatment for affected women and, therefore, is often associated with preterm delivery(9). Factors implicated in the development of PE are thought to be of various origins, believed to be a complex combination of genetic and environmental causes, although molecular

mechanisms underlying the pathology are still poorly understood(8;11;12). Consequently, pharmacological management of PE remains very complicated, especially since many anti-hypertensive drugs are teratogenic(13). ET is therefore an attractive means to protect mothers and fetuses.

Even though many cardiovascular benefits are associated with ET, it is still a popular belief that it may be deleterious to mothers and fetus during pregnancy(7). Indeed, it was suggested in some studies that physical activity might have adverse effects on pregnancy, such as decreases in fetal weight(14). However, as well reviewed by Clapp in 2006(3), many studies in the past decades have observed the exact opposite. Moreover, it was shown that if a decrease in birth weigh was observed, it was entirely due to a decrease in fat mass and not a result of a reduction in either skeletal or brain growth, nor in lean body mass(15). It was proposed that insufficient calorie intake in the mother's diet was involved in the decreased fetal weight observed in these studies. Moreover, it was demonstrated that ET improved placental perfusion by increasing villous area and placental vascular volume(15).

Rodent models mimicking PE have been characterized and are believed to be a good tool to study the disease. Indeed, mice gestation is similar to human pregnancy in many ways(16), and their genetic backgrounds, as well as their environment, can be managed without difficulty. Of particular interest, the decrease in BP response to pregnancy in mice is similar to that observed in humans near term(17;18). In addition, mice and humans have similar hemochorial placentation, for which alterations are crucial in development of the disease(19). Transgenic female mice overexpressing human angiotensinogen (hAng) spontaneously develop PE-like symptoms when

they are mated with human renin (hRN)-overexpressing males (20). Given that Ang cleavage by renin is species-specific, both single transgenic females and males are normotensive(21;22). However, as a consequence of this mating, there is overexpression of the utero-placental renin-angiotensin system and release of hRN by the placenta into the mother's circulation, which is thought to cause the PE-like symptoms in this model(20;23). Thus, these mice show an elevation in blood pressure during gestation, which resolves after delivery. In this model, transient hypertension is accompanied by proteinuria, cardiac hypertrophy and placental pathology, all distinctive symptoms of PE(20). It was also reported recently that fetoplacental vascular maturation and uteroplacental tissue remodelling are impaired in these mice, leading to fetal hypoxia and intrauterine growth restriction(24).

The impact of physical activity on human health and animal models has been widely studied in the past decade, but, to date, none have determined a cause-effect relationship between ET and the reduction of PE risk. Furthermore, the mechanisms by which physical activity may reduce PE symptoms have yet to be investigated. Hence, the aim of this study is to evaluate if ET will diminish PE symptoms in transgenic mice (female hAng X male hRN) that develop the disease when sedentary. Furthermore, given that endothelial dysfunction, which is often implicated in PE(25) has not yet been investigated in this model, this was also evaluated in our study.

Materials and Methods

An expanded Materials and Methods section is available in the online supplementary data.

Animals. hAng transgenic mice (204/1 line) were originally obtained from Dr. Curt D. Sigmund(21) of the University of Iowa, and, hRN transgenic mice (hRN8-12 line) were acquired from Riken BRC (Tsukuba, Japan) with the authorization of Dr. A. Fukamizu(26). Both transgenic lines were maintained in our animal facility by backcrossing with C57BL/6 mice (Charles River, St-Constant, QC, Canada). Mouse genotype was determined as described previously(21;27). The animals were kept on a 12-h light/dark cycle with water and standard laboratory chow (2018; Teklab Premier Laboratory Diets, Madison, WI) *ad libitum*. The mice in these experiments were 12-15 weeks of age, and their care met the standards set forth by the Canadian Council on Animal Care for experimental animals. All procedures were approved by the University Animal Care Committee of the CHUM Research Centre.

Similarly to what had been previously published (20), hAng females were bred with hRN male mice to produce PE-like symptoms in the females. Mice used for control purposes were all non-transgenic (NT) littermates.

ET. The mice were placed in cages with access to a running wheel 1 month prior to pregnancy, and remained in these throughout gestation. Indeed, studies have shown a significant increase in aerobic capacity with only 3 weeks of ET in a running wheel(28;29). ET was measured as each cage was connected to a computer and, thus, the number of revolutions was counted to confirm training status (Compte-tour5, Aquila, Boucherville, Qc, Canada). Running data were compiled and analysed.

Arterial pressure measurement. Arterial pressure (AP) and heart rate (HR) were directly measured by telemetry as done previously(30;31). After surgery recovery, baseline measurements were taken continuously for 3 days. The mice were then put in cages with access to an exercise wheel for 1 month. Animals that were kept sedentary were placed in the same type of cages but the exercise wheel was locked to prevent activity. After this one-month training period, male mice were introduced in the cages for timed-mating. Gestation was confirmed by the presence of a vaginal plug and was considered as day 1. Starting on this day, AP and HR were assessed every 2 days up to day 19 with measurements collected continuously until 48 h post-partum.

Proteinuria. Urine samples were collected before and on day 18 of gestation. Albumin and creatinine concentrations were quantified as described previously(30;31).

Tissue collection and histology. In separate time mating, on day 18 of gestation, the mice were anaesthetized with ketamine/xylazine (0.1ml/20g of mouse body weight of a 100mg/ml:20mg/ml solution). Blood was collected by heart punction and placed in chilled 1.5 ml tubes containing EDTA (EMD, Gibbstown, NJ, USA), or collected in microhematocrit tube that was subsequently centrifuged to measure hematocrit values. All pups were weighed and their tails were snipped and kept for genotyping. Kidneys, hearts, and placentas were collected, weighed, and snap frozen in liquid nitrogen or fixed until further assessed as previously published(30;31). In addition, specific markers (cytokeratin and Ki67) of placental development were assessed by immunohistochemistry. Mesenteric arteries were also collected for subsequent vessel studies. To

evaluate the impact of genotype and ET on placental and fetal growth, we calculated total placental and fetal mass by adding up the weight of all placentas and fetus for each litter.

Real-time PCR. Total RNA was extracted from placentas with Trizol (Invitrogen, Burlington, ON, Canada) according to the manufacturer's protocol. Removal of genomic DNA, as well as reverse transcription reaction and PCR were done using specific primers for VEGF and 18S as described in the online supplement. mRNA levels were expressed as values relative to 18S mRNA.

Western Blot. Protein samples were separated by electrophoresis and transferred on a nitrocellulose membrane. Proteins were detected with anti-VEGF (Abcam, Cambridge, MA) antiserum using ECL West Pico kits (Pierce, Rockford, IL). Total protein was subsequently measured by staining of the membrane with Amido Black (Sigma, St-Louis, MO, USA) and VEGF signal was normalized to total protein content of each sample.

Vessel studies. At 18 days of gestation, mesenteric resistance arteries were isolated and excised to assess vessel reactivity as described previously(31;31). Briefly, the vessels were double-cannulated between 2 glass micropipettes in a vessel chamber. Vessel reactivity was assessed according to 3 protocols and dose-response curves were charted: 1. Norepinephrine (NE, 10^{-9} to 10^{-5} M), 2. Acetylcholine (Ach, 10^{-9} to 10^{-4} M), 3. Sodium nitroprusside (SNP, 10^{-9} to 10^{-4} M).

Statistical analysis. All values are expressed as means ± SE except for placental pathology scores which are shown as median and 75th percentile. A p value of ≤ 0.05 was considered

significant. Two-way repeated measures ANOVA were performed to assess the impact of genotype and ET on AP, vascular reactivity and the albumin/creatinine ratio. Differences in tissue weights and ratios as well as in the number of pups were analyzed by 2-way ANOVA. These analyses were all followed by Tukey's post-hoc test when an interaction was detected. Statistical differences in placental pathology scores were computed by non-parametric 2-way ANOVA.

Results

Transgenic and NT mice showed the same pattern of exercise before and throughout gestation (Figure 1). All mice diminished their physical activity naturally when pregnant, and the amount of training decreased gradually during their gestation period. All mice almost completely stopped running 2 or 3 days before delivery.

Before gestation, we found no impact of ET or genotype on either mean AP (MAP; Figure 2) or proteinuria (Figure 3), as assessed by the albumin/creatinine ratio. However, as reported previously(20), sedentary hAng mice presented with hypertension (Figure 2) and proteinuria (Figure 3) by the end of gestation. Conversely, their exercise trained counterparts had normal proteinuria (Figure 3), and, although MAP was significantly elevated at day 17, it tended to be lower, as seen in Figure 2, it was no longer different from that of NT females at the end of gestation and was significantly lower from the sedentary hAng females. This might be a result of the normalization of impaired vascular reactivity, since we observed an increased vasodilatory response to SNP in trained hAng mice (Figure 4). Indeed, although a reduced response to SNP

was observed in sedentary hAng females compared to their NT littermates at the end of gestation, it was significantly enhanced in all trained mice (Figure 4).

In addition, placental pathology was apparent in samples from sedentary hAng mice (Table 1). It was characterized by a significant increase in hyalinization and loss of labyrinthine trophoblast structure which resulted in an elevated total placental pathology score (Table 1). Interestingly, this condition was completely normalized in trained mice, and accompanied by similar changes in VEGF. Indeed, although we noted a significant increment in both VEGF gene and protein expression in placentas from sedentary hAng mothers, this was completely normalized in their trained counterparts (Figure 5). In addition, we found that although both cytokeratin and Ki67 placental immunostaining were increased in our transgenic mothers, they were markedly reduced with exercise training (for representative images see Figure 1S and 2S). Furthermore, a slight increase in placental cytokeratin could also be detected in NT mothers. In line with these results, we observed an increased total fetal and placental mass in all trained mice, suggesting an ameliorated growth of both placentas and fetus (Table 2). Conversely, we did not detect any significant impact of either genotype or ET on litter size (Table 2).

As reported previously, hAng females suffered from cardiac hypertrophy at the end of gestation (Table 3). However, this was not seen in their trained counterparts. Interestingly, although we encountered no apparent renal pathology by histology, the kidneys-to-body-weight ratio in sedentary hAng mice tended to be higher ($p = 0.062$) at the end of gestation, and was significantly decreased in these mice by training (Table 3). However, no effect of training could be detected in NT mice.

Discussion

To our knowledge, we are the first to clearly demonstrate that ET can prevent PE symptoms. In our study, as reported previously(20), sedentary hAng mice developed PE-like symptoms spontaneously when they were mated with hRN males, and presented many hallmark features of the pathology, such as hypertension, proteinuria, impaired vascular reactivity, placental alterations and cardiac hypertrophy. Our data indicates that ET completely normalized most of these symptoms. The only exception was MAP which rose significantly with gestation but was no longer different from that of NT and sedentary transgenic mice at the end of gestation. This is of importance as a lower MAP increase, accompanied with the abolishment of other PE symptoms could prevent serious complications of the disease, such as eclampsia or preterm birth. Hence, there is no doubt that ET before and during gestation is an excellent preventive means against this pathology which, as yet, does not have any treatment apart from preterm delivery.

The amount of training observed in both NT and hAng mice correspond to what was previously reported in the literature for control rodents having access to a free wheel(32;33). Cardiovascular benefits associated to voluntary exercise training in rodents have already been reported, such as increased VO2max, diminished blood pressure as well as diminished adverse vessel remodelling (34-37), and are associated to similar improvements observed with exercise programs in humans(38). Therefore, our data suggests that the beneficial effects of ET described in our mouse model could be representative of what could be observed in pregnant women.

It is well-recognized that ET is an effective way of preventing and treating hypertension in non-pregnant individuals(39) and rats(36;37). Hence, the decrease in MAP observed in trained mice is in line with the literature. Endothelial dysfunction has been associated with hypertension and PE as the response to vasoconstrictors and vasodilators is reported to be impaired in both these conditions(40-42). Therefore, the normalization of impaired vascular reactivity in trained hAng mice certainly contributed to the MAP decline at the end of gestation. Indeed, improvement of endothelial function by ET in animals with other cardiovascular disease has been documented previously(43), and enhancement of the endothelium-independent response to SNP has also been implicated(44). It has been postulated that ET might lower oxidative stress in the vascular endothelium by regulating antioxidant enzyme activity such as superoxide dismutase (SOD)-1 and SOD-3(45). Extracellular (ec) SOD is increased by ET and, thus, the reduction of extracellular oxidative stress could contribute to the modulation of nitric oxide (NO) bioavailability and effect(46). This might explain the restitution of the vasodilator response in trained preeclamptic mice but remains to be investigated.

In the present study, we observed higher VEGF expression in the placentas from sedentary hAng compared to NT mice, which were significantly diminished when the animals were trained. This is inline with the premise that there is an anti-angiogenic shift in the placenta towards the end of gestation as there is completion of vasculogenesis in this tissue(47). Indeed, this is typically associated with an increase in sFlt-1 expression in normal pregnancy. However, as placental development is impaired in this animal model, VEGF remains increased possibly as a compensatory mechanism. Moreover, since angiogenic factors are secreted after hypoxia(48), it appears that placentas from sedentary transgenic mice are not adequately perfused and,

consequently, express higher VEGF levels. Interestingly, similar changes were observed in placental immunostaining for both cytokeratin and Ki67 which identify trophoblast and proliferating cells respectively. Indeed, both of these markers were increased in placenta from our mouse model whereas these were both reduced with exercise training, although no completely normalized. We propose that the increase presence of these markers in the placentas from hAng supports the premise that there is an exaggerated and generalized cell proliferation which produces a loss of labyrinthine trophoblast structure as we observed by histology. The marked reduction observed with exercise training suggests that these elements are implicated in the normalization of placental development reported herein. Hence, the fact that the upregulation of VEGF and placental markers as well as placental pathology were eliminated/reduced by training is in line with the literature regarding the benefits of exercise training on placental perfusion and development in normal pregnancy(43;49). Indeed, it has been demonstrated that ET before and throughout pregnancy increases villous area and vascular volume in human placenta, strongly suggesting an improved placental perfusion and transport capacity(15). Substantiating this hypothesis, we have shown that all our trained mice have an increased total placental and fetal mass. Therefore, this suggests that previously reported effects of exercise training on placenta in normal pregnancy can also be observed in pathological pregnancies. We thus suggest that, ET restores angiogenic balance by rehabilitating placental development and thus eliminating hypoxia. Indeed, it is widely thought that maternal systemic symptoms might originate from the release of molecules in the hypoxic placenta(11;41;50).

Cardiac hypertrophy is one of the end-organ damage that can be caused by PE. As reported in the past(20), sedentary hAng mice showed a significant rise in their heart-to-body weight ratio

compared to NT mice. However, this feature of PE is no longer observed in trained transgenic mice. We suggest that cardiac hypertrophy in sedentary mice was a consequence of pressure overload due to hypertension. Furthermore, it is well-known that cardiac function is improved with ET(51). Hence, we postulate that ET, by reducing MAP can prevent cardiac hypertrophy and therefore improve cardiac function.

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The molecular mechanisms by which ET can protect against PE still require further investigation. This study, however, is an important advance in our understanding of the role of ET in the prevention of this disease. The attenuation of maternal symptoms might prevent preterm delivery and eclampsia, which could reduce maternal and fetal mortality and morbidity associated to with PE. Since no treatment, apart from delivery, is available to protect mothers and fetuses, such a preventive, non-pharmacological, approach is a significant step forward.

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Conflict of Interest

None declared.

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Figure legends

Figure 1. Average total daily running distance before and throughout gestation.

There was no difference in training patterns between hAng (black, N = 5) and NT (white, N = 4) mice. Both group trained more before than during gestation, and the amount of running decreased throughout gestation to finish by a voluntary rest period in the 2 or 3 days prior to delivery.

Figure 2. Effect of exercise training (ET) on mean arterial pressure (MAP) during gestation.

MAP was increased from day 17 until the end of gestation, in sedentary (white) hAng females (circles, N = 5) compared to NT mice (squares, N = 8), and returned to normal by 48 h post-partum. However, in trained (black) hAng mice (N = 8), the values were significantly lower compared to sedentary mothers, and were no longer significantly different from NT mice (N = 4) at the end of gestation. Values are expressed as means ± SE. MAP, mean arterial pressure; PP, post-partum. * $p \leq 0.05$, and † $p \leq 0.001$, significantly different from NT females, and ‖ $p = 0.01$, significantly different from trained mothers.

Figure 3. Effect of ET on proteinuria.

Sedentary (white) hAng mice (circles, N = 8) showed a significant increase in the albumin/creatinine ratio at the end of gestation compared to NT females (squares, N = 7). However, proteinuria was significantly decreased by training (black) in hAng mice (N = 7) and therefore was no longer different from NT mice (N = 4). Values are expressed as means ± SE.

*p ≤ 0.05 significantly different from NT females and † p ≤ 0.05 significantly different from trained mothers at day 18.

Figure 4. Mesenteric artery response to Ach and SNP.

Mesenteric arteries were collected and isolated at 18 days of gestation. We found that, although no significant difference could be observed in the response to Ach (A), significant effects of both genotype and ET could be seen with SNP (B) at the end of gestation. Indeed, hAng mice (circles, N = 9) showed a marked reduction in their response compared to NT females (squares, N = 14). However, ET (black) increased the effect of SNP compared to sedentary animals (white), completely normalizing hAng mice (N = 9), with no difference compared to NT mothers (N = 5). Values are expressed as means ± SE. * p ≤ 0.05 significantly different from NT mice; † p ≤ 0.005 significant effect of ET. Ach, acetylcholine; SNP, sodium nitroprusside

Figure 5. Placental VEGF expression. VEGF gene (A) and protein (B) expression were both significantly increased in sedentary hAng (black) mice compared to NT (white). This increase was normalized by training and was no more different from NT mothers. * p < 0.05 and † p ≤ 0.001, significantly different from trained animals; ‖ p≤ 0.05, and ‡ p ≤0.001, significantly different from NT mice. VEFG gene expression: N respectively = 14 and 12 for NT sedentary and trained mice and N= 14 and 12 for hAng sedentary and trained females. VEGF protein expression: N respectively = 7 and 8 for NT sedentary and trained mice and N= 10 and 9 for hAng sedentary and trained mothers.

Table 1. Effect of PE and ET on placental pathology.

			Placental pathology parameters											
	Mother's genotype	**N**	**Necrosis**		**Hyalinization**		**Microcalc**		**CIL**		**LLTS**		**Total**	
			Mdn	75%	Mdn	75%	Mdn	75%	Mdn	75%	Mdn	75%	Mdn	75%
Sedentary	NT	32	1§	2	0	1	0	0	0	1	0§	1	2	3
	hAng	24	1	1.3	1‡§	1	0	0	0	1	1*§	1	3†§	5
Trained	NT	22	0	0	1	1	0	0	1	1	1	1	2	3
	hAng	46	2‡	2	1	1	0	0	0†	0	0‡	0	3	3

Values are expressed as median and 75th percentile. *, p≤0.05, †, p≤0.005 and ‡ p≤0.001 statistically different from the NT mice; § p≤0.001 statistically different from trained mice. CIL, Cytotrophoblastic island loss, LLTS, Loss of labyrinthine trophoblast structure; Mdn, Median; Microcalc, Microcalcification.

Table 2. Litter characteristics.

	Mother's genotype	Total Placental mass (mg)	Total fetal mass (mg)	Pups/litter (average)	Non-viable fetus
Sedentary	NT	752.7 ± 77.4	5304.4 ± 598.6	8.6 ± 0.7	0.4 ± 0.2
	hAng	736.0 ± 75.0	5600.0 ± 678.4	7.4 ± 0.8	0.3 ± 0.2
Trained	NT	1004.0 ± 107.6*	6964.8 ± 940.3*	8.8 ± 0.7	0.7 ± 0.3
	hAng	958.9 ± 105.4*	7358.3 ± 451.1*	9.0 ± 0.6	0.0 ± 0.0

Values are expressed as mean ± SE. * $p \leq 0.05$ statistically different from sedentary mice;

Table 3. Effect of ET and genotype on female characteristics.

	Mother's genotype	N	Htc	BW (g) (Baseline)	BW (g) (end of gestation)	Heart/BW ratio	Kidney/BW ratio
Sedentary	NT	11	40.8 ± 2.4	22.0 ± 0.3	35.5 ± 0.8	6.57 ± 0.13	7.06 ± 0.14
	hAng	10	40.0 ± 3.5	22.2 ± 0.4	36.6 ± 0.9	7.22 ± 0.28‡*	7.61 ± 0.21†
Trained	NT	7	34.8 ± 3.5	21.7 ± 0.7	36.4 ± 1.6	6.93 ± 0.41	7.06 ± 0.14
	hAng	9	40.9 ± 3.3	22.4 ± 0.6	37.2 ± 1.5	6.47 ± 0.17	6.83 ± 0.13

Values are expressed as mean ± SE. * $p \leq 0.05$, † $p \leq 0.001$, significantly different from trained mice. ‡ $p \leq 0.05$, significantly different from NT mice. BW, Body weight; Htc, Hematocrit.

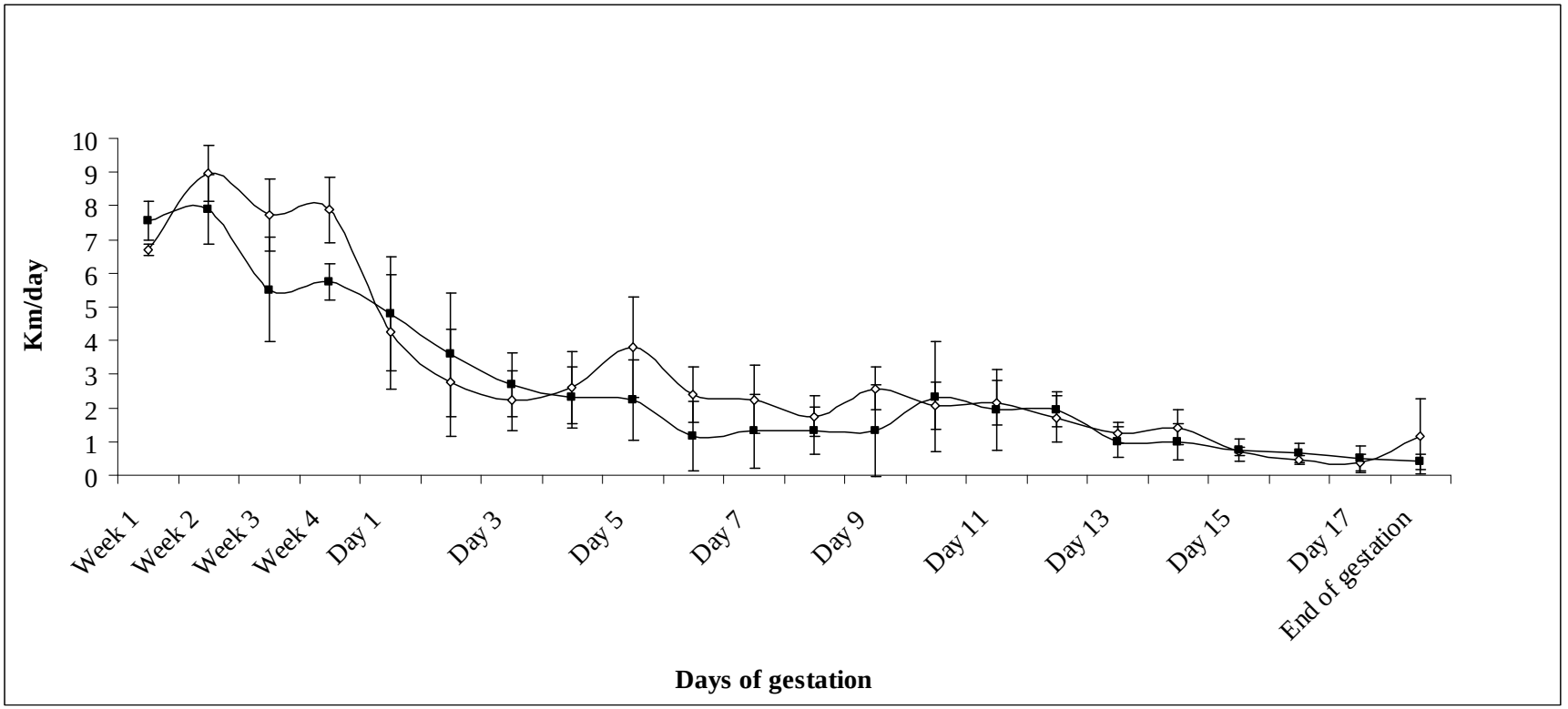

Km/day
10
9
8
7
6
5
4
3
2
1
0
Week 1
Week 2
Week 3
Week 4
Day 1
Day 3
Day 5
Day 7
Day 9
Day 11
Day 13
Day 15
Day 17
End of gestation
Days of gestation

Figure 1.

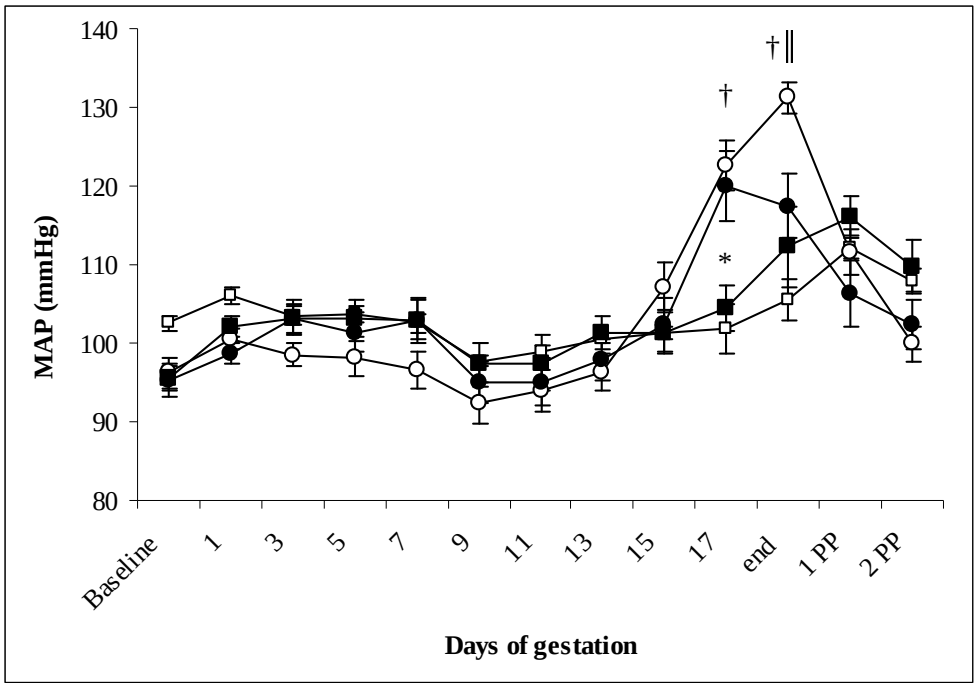

140
130
120
110
100
90
80
MAP (mmHg)
†‖
†
*
Baseline
1
3
5
7
9
11
13
15
17
end
1 PP
2 PP
Days of gestation

Figure 2.

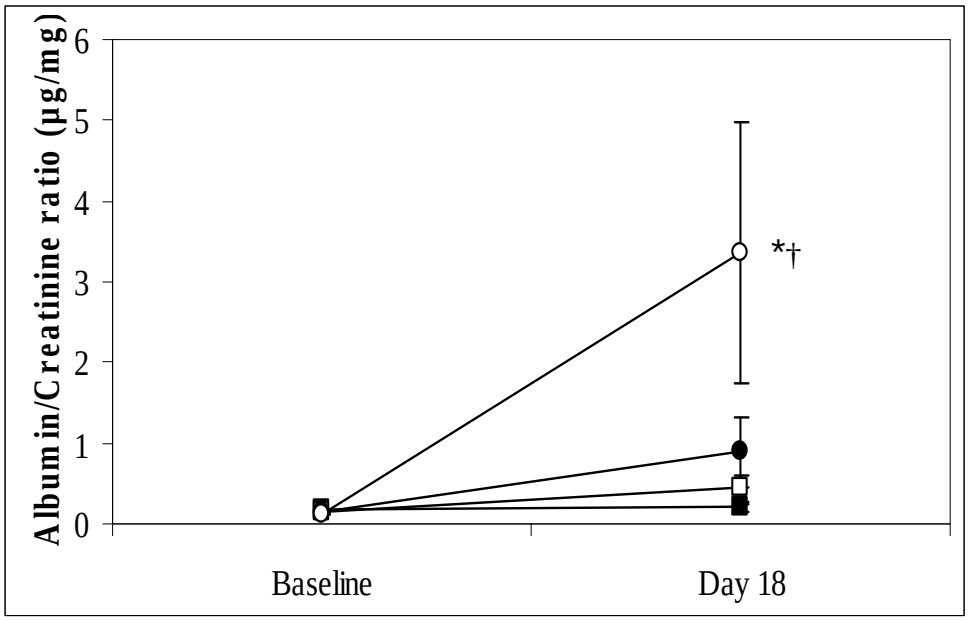


Figure 3.

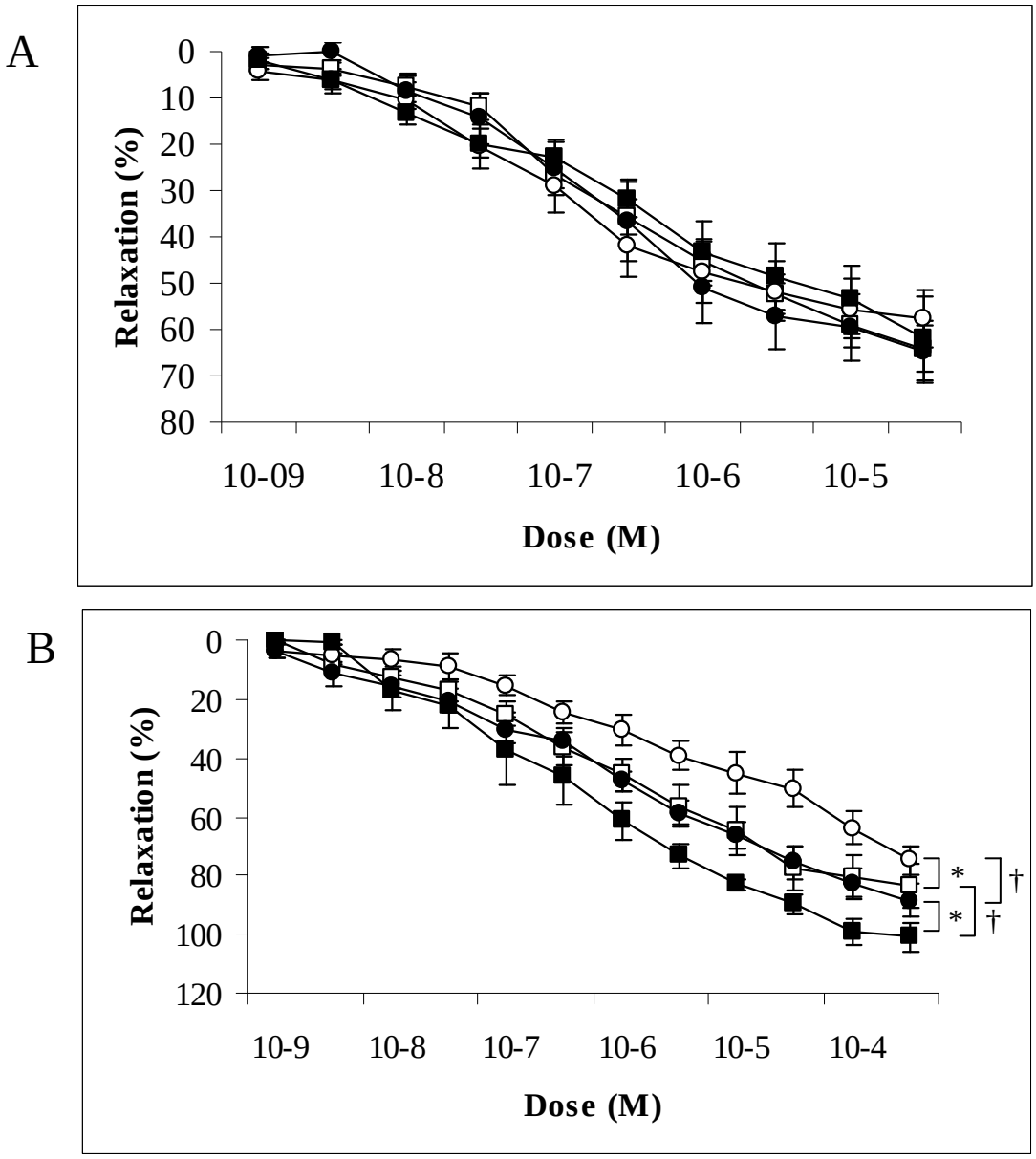
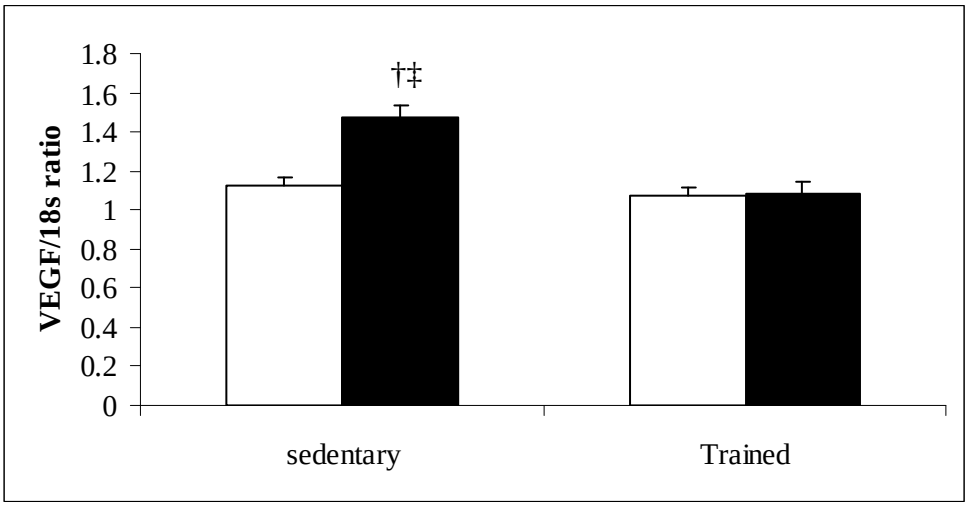

A
Relaxation (%)
0
10
20
30
40
50
60
70
80
10-09
10-8
10-7
10-6
10-5
Dose (M)
B
Relaxation (%)
0
20
40
60
80
100
120
10-9
10-8
10-7
10-6
10-5
10-4
Dose (M)
*
†
*
†

Figure 4.

A)


1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
VEGF/18s ratio
†‡
sedentary
Trained

B)


2
1.5
1
0.5
0
VEGF/protein level ratio
*‖
Sedentary
Trained

Figure 5