ORIGINAL ARTICLE

Unaltered Long-Term Maternal Endothelin System in a Gestational Hypertensive Rat Model

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ABSTRACT

INTRODUCTION: Cardiovascular diseases (CVDs) are two to four times more likely to affect women with a history of hypertensive disorders of pregnancy (HDPs). One of the etiologies of CVDs is endothelial dysfunction, which results from an imbalance in the production of endothelin-1 (ET-1) and nitric oxide (NO). Although blood pressure (BP) is normalized postpartum, we hypothesize that a transient increase in BP during HDPs may cause ongoing endothelial dysfunction. We aimed to discover the impact of HDPs in the development of cardiovascular diseases after long-term postpartum changes in endothelin-A receptor (ETAR) and endothelin-B receptor (ETBR) expression and the concentration of ET-1 and NO. **MATERIALS AND METHOD:** Twenty-four female Sprague-Dawley (SD) rats were assigned into four groups (n=6), including two treatment and two control groups. All rats were sacrificed on Day 30 postpartum. The NO and ET-1 concentrations were determined by ELISA and the ETAR and ETBR expression of the mesenteric arteries were measured by immunohistochemistry studies. **RESULTS:** The mean concentrations of ET-1 and NO were not significantly different in all groups. There was no significant difference in the mean immunoreactivity of ETAR and ETBR percentages area in the tunica intima and media in all groups. **CONCLUSION:** No evidence demonstrates significant changes in the endothelin system of the resistance arteries at the proteomic level in the long-term duration following HDP. Further investigation of its potential chronic effect warrants a deeper analysis at the molecular and ultrastructural levels.

Keywords
Hypertension, pregnant, endothelin, endothelial dysfunction, postpartum

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INTRODUCTION

Cardiovascular diseases (CVDs) continue to be the leading cause of mortality worldwide. Globally, it was estimated that there would be 19.05 million CVD-related deaths in 2020, an increase of 18.71% from 2010.[1] In women, a history of hypertensive disorders of pregnancy (HDPs) is a significant but typically overlooked risk factor for CVDs.[2] The term "HDPs" is used broadly to describe pregnancy-related high blood pressure (BP). It is defined as a condition in which the pregnant mother has been diagnosed with high BP (≥140/90 mmHg) for at least two or more within a six-hour interval.[3] The various HDPs are determined by the time of the mother's hypertension diagnosis. Among these include pre-existing hypertension, gestational hypertension, pre-eclampsia, and eclampsia.[4] HDPs typically affect 8–10% of all expectant mothers and are a major cause of significant fetal and maternal morbidity and mortality.[5] In the current practice, there is no definitive treatment for HDPs. Placenta delivery is believed to be the only measure to reduce BP and its associated complications.[6] For the majority of women, BP usually returns to normal in the first week of the postpartum period.[7] Despite the fairly rapid normalization of blood pressure, these women still have a two- to four-fold greater risk of having CVDs in later life.[8] The majority of them were linked to cardiovascular problems like cerebral vascular accidents, peripheral vascular diseases, and ischaemic heart diseases.[9] The endothelin system plays an important role in the pathogenesis of endothelial dysfunction.[10] The primary vasoactive substances, endothelin-1 (ET-1) and nitric oxide (NO), which are parts

of the endothelin system, are crucial in controlling BP. ET-1 is synthesized in two secretory pathways, constitutive and regulated, to contribute to vasomotor tone and can affect it in both directions. It interacts with ETAR and ETBR on the underlying vascular smooth muscles (VSMCs) to cause vasoconstriction. It can also cause vasodilation by interacting with endothelial ETBR and leading to the release of a potent vasodilator, NO.[11] A balance between ET-1 and NO bioavailability is crucial to maintain cardiovascular homeostasis. ET-1 has been reported to be higher in hypertensive individuals, making it a possible biomarker of endothelial dysfunction.[12] It has been reported that there was a significant reduction of serum NO after induction of Nω-Nitro-L-Arginine Methyl Ester Hydrochloride (L-NAME) in animal models.[13,14]

In a separate study, less ETBR staining was observed in the endothelium of the renal artery than in the VSMCs.[15] This finding was supported by a more recent study that demonstrated decreased endothelial ETBR expression in the reduced uterine perfusion pressure (RUPP) animal model, indicating its involvement in the central mechanism of hypertensive pregnancy.[16] However, their study found no difference in ETAR staining in all vascular layers between the RUPP and control groups. Numerous animal models for HDPs have been developed based on the proposed mechanisms that are thought to be involved in the pathophysiology of HDPs. It is generally agreed that due to the complexity and heterogeneity of the disease, a single model is unlikely to cover all aspects of its pathogenesis.

Instead, each model may represent a particular disease process or set of pathways. Some of these studies focus on the alterations in vessel physiology, mechanics, biochemistry, and structure that result from HDPs. However, the majority of current HDP models do not account for the disease's long-term consequences.[17,18,19] A study used the same drug, L-NAME, to mimic preeclampsia in mice and looked at the long-term effects on maternal cardiovascular health. This model did not appear to show adverse effects on long-term cardiovascular risk after insult by preeclampsia because all preeclamptic cardiovascular indices, including circulating ET-1 levels, were resolved by 10 weeks postpartum.[20] Another animal model of preeclampsia was used, using the RUPP technique, and the results showed that impaired vascular function persisted at three months postpartum in both the mesenteric and aortic vessels, suggesting that the vasculature did not fully recover from the insult of pre-eclampsia and remained at increased risk for cardiovascular diseases in later life.[21] There was a human study that found a NO/ET-1 imbalance, suggesting that endothelial dysfunction rather than BP normalisation persisted for up to three months postpartum and possibly accounted for the increased CVD risk in women with a history of HDPs.[22] In the current study, the idea that persistent endothelial dysfunction exists was tested by giving a drug that blocks nitric oxide synthase (NOS) in pregnant rats. This was done to simulate hypertension in pregnancy and test the hypothesis that persistent endothelial dysfunction exists. We aimed to determine the levels of ETAR and ETBR expression as well as the plasma concentrations of ET-1 and NO during the long-term postpartum effect of HDPs.

MATERIAL AND METHODS

Animals

24 female Sprague-Dawley (SD) rats (weighing 180–200 g) aged five to six weeks were used in this study. Each rat was kept in a polypropylene cage, which had a 12-hour light/dark cycle and free access to food and water. The experimental protocols were approved by the Animal Care and Use Committee, International Islamic University Malaysia (IIUM/IACUC-2019 (17)-1). The animals were acclimatized for two weeks prior to the study.

Nω-Nitro-L-Arginine Methyl Ester Hydrochloride (L-NAME)

Five grams of L-NAME powder were supplied by Sigma Aldrich (United States). This powder's solubility in water was 50 mg/ml, and its L-NAME concentration was 50 mg/ml. L-NAME was administered to the rats subcutaneously at a dose of 125 mg/kg/day.

Experimental Design and Induction of Hypertension

The rats were randomly divided into four groups, and each group had six rats (Figure 1). The groups were the control-non-pregnant group (C), control-pregnant group (P), non-

pregnant + L-NAME group (CL), and pregnant + L-NAME group (PL). The pregnant groups (P and PL) were mated with a male SD rat overnight. On the next day, the vaginal smear was viewed under a light microscope. If spermatozoa were present, it was considered a positive pregnancy (Day 0 of gestation). The subcutaneous injection of L-NAME at 125 mg/kg/day was administered to the treatment groups (CL and PL) beginning on day 10 of pregnancy and continuing until the day the foetus was delivered (Day 21 or Day 22 of gestation).[23]

Determination of Blood Pressure

The CODA non-invasive blood pressure equipment (Kent Scientific Corporation, USA) was used to measure a series of mean arterial pressures (MAP) in the conscious state using the pre-warmed tail plethysmography method at baseline (pre-pregnancy), Day 14 of gestation, and then weekly until the day of sacrifice. Blood pressure readings were taken, and the data was analyzed as per standard protocol.[24]

Samples Collection and Processing

At Day 30 postpartum, the rats were administered an intraperitoneal injection of the anaesthetic drugs Xylazine (5 mg/kg) and Ketamine (50 mg/kg). The abdominal cavity was opened and the whole rat's digestive tract was identified with the attached mesentery. Subsequently, the mesenteric arteries were harvested as described in the previous study.[25] For the immunohistochemistry study, the samples were fixed with 10% neutral buffered formalin (NBF) for 24 hours and proceeded to make a formalin-fixed paraffin-embedded (FFPE) block.

Blood Samples Collection

Blood samples (1 ml each) were collected through retroorbital sinus puncture while the animal was being anaesthetized to measure plasma ET-1 and NO. Both were collected in EDTA tubes. To obtain plasma ET-1, the blood samples were centrifuged at 1600 x g for 15 minutes at 0 °C. Then, the supernatant was transferred into a plastic tube and stored at -70°C. For plasma NO, the blood samples were centrifuged for 30 minutes at a speed

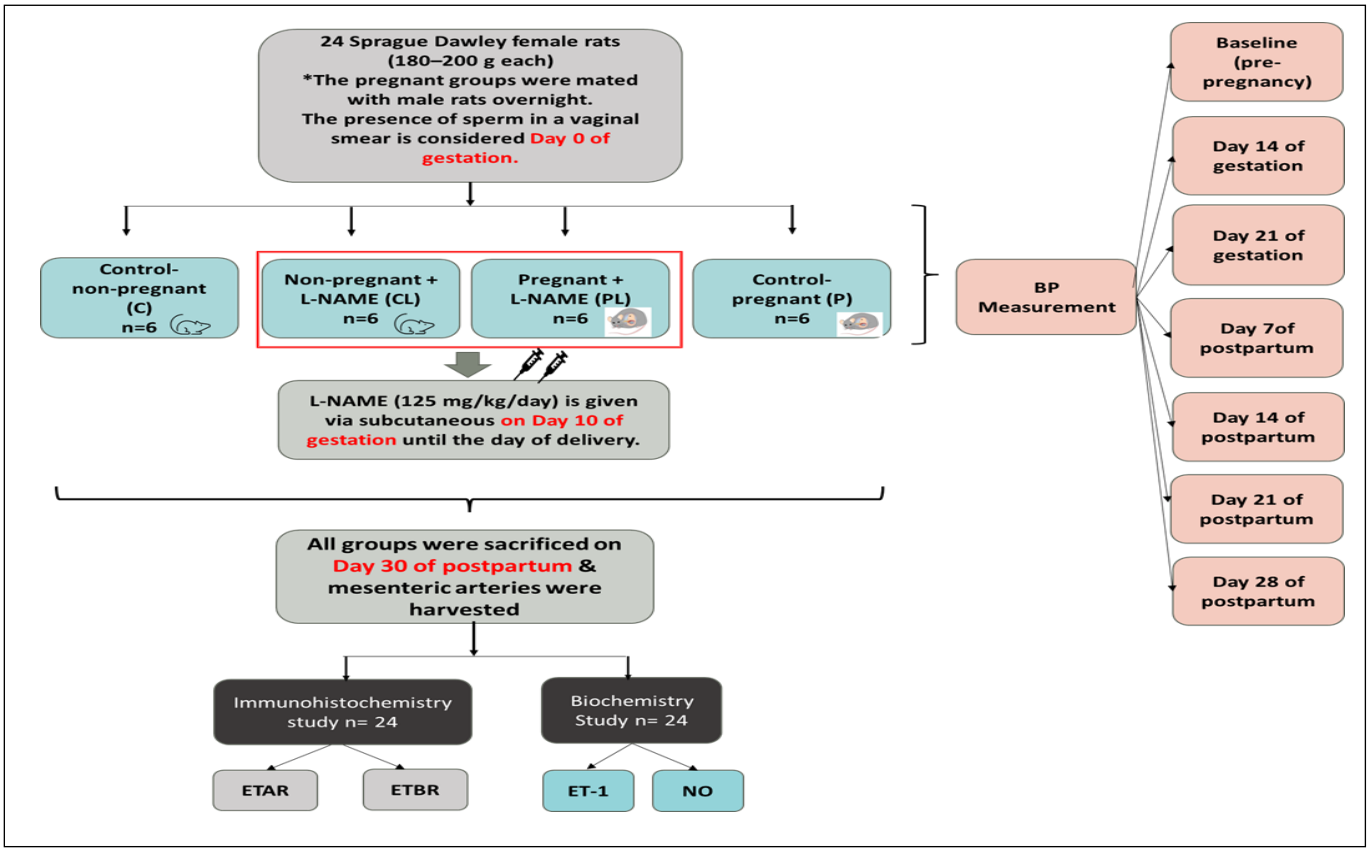

Figure 1: The animal grouping and experimental workflow

of 3000 rpm. The supernatant was collected and stored at -80°C before analysis was done.

Enzyme-linked Immunosorbent Assay (ELISA) Determination of Plasma ET-1 and NO

Plasma concentration of ET-1 was quantitatively detected using Abcam's ET-1 in vitro ELISA kit (ab133030) and plasma concentration of NO using a double-antibody sandwich ELISA utilizing the rat NO ELISA kit, QY-E10941 (Qayee-Bio, Shanghai). The tests were performed according to the protocol of the manufacturer. The amount of ET-1 and NO sample that was acquired on the plate is directly proportional to the colour density. The microplate's optical density (OD) absorbance was measured at 450 nm. Plots were made between the average net OD for each standard and its ET-1 and NO concentrations, respectively. Through each point, an approximately straight line was drawn. Interpolation can be used to calculate the concentration of the unknowns. The concentration of ET-1 was expressed as pg/mL, while that of NO was expressed as ng/mL. The detection limit of the assay for plasma ET-1 was between 1.1 and 35.9 pg/mL, while that of NO was 12.5-800 ng/mL.

Immunohistochemistry (IHC) Study

In this study, Anti- Endothelin A Receptor (ETAR antibody) [UMB-8-37-1] ab178454 (Abcam, United States) and a rabbit anti-endothelin Receptor B affinity-purified polyclonal antibody (ETBR) (AB3284-50UL) (Millipore Corp., USA) were used. For both antibodies, we used Rabbit-specific HRP/DAB Detection IHC Detection Kit-micro-polymer-ab236469 (Abcam, United States). The mesenteric arteries were cut into 4 μm-thick sections using a semi-motorized rotatory microtome (Leica RM2245, USA). The tissue ribbon was then laid out in a water bath at 45 °C and retrieved using a silane-coated glass. Briefly, the tissue sections were deparaffinized and hydrated in graded ethanol concentrations using an autostainer machine (Leica ST5010, United States). The procedure was divided into pre-staining, primary antibody, secondary antibody, and lastly, counter-staining. These steps are applied to both antibodies, and the differences were only the antibody dilution and the use of the DAKO EnVision FLEX+ Rabbit (LINKER). The human placenta served as the positive control. The samples were placed into a target retrieval solution (EDTA pH 9, and placed in the pressure cooker (Biocare Medical, United States). The next steps were followed according to manufacturer guidelines. In the primary antibody step, specimens were added with optimally diluted primary antibodies, as for ETAR (1:100) and ETBR (1:500), respectively. Both antibodies were incubated overnight (16-18 hours) in a 4°C chiller. The specimens were added to DAKO EnVision FLEX+ Rabbit (LINKER) (Dako, USA) for signal amplification of primary rabbit antibodies in combination with the EnVision FLEX visualization system. They were incubated for 15 minutes. This step was only for ETBR antibodies and was skipped for ETAR antibodies. The following steps were secondary antibody and counterstaining steps were followed accordingly.

Immunoreactivity Analysis

A light microscope was used to examine the slides after they had been cover-slipped with a DPX mounting medium. Next, all images were captured digitally for analysis. The immunoreactivity (IR) of ETAR and ETBR was analyzed using ImageJ-win64 (National Institute of Health). The total area was determined by the total number of pixels in the region of interest (ROI). Then, ETAR-IR and ETBR-IR areas were determined by measuring brown pixels on the tunica intima and media within ROI. Finally, the mean immunoreactivity area was presented as the percentage of the ETAR-IR over the total area and ETBR-IR over the total area.[16,26]

Statistical Test

All the data sets were analyzed using the one-way analysis of variance (ANOVA) test (IBM SPSS Statistics 22). The data were presented as means and standard deviations (SD). Differences were statistically significant when $p<0.05$.

RESULTS

High blood pressure was successfully induced in the treatment groups (CL and PL groups) by subcutaneous injection of L-NAME at day 14 of gestation and maintained throughout the pregnancy (MAP ≥ 107 mm Hg). After cessation of L-NAME at delivery, the MAP of

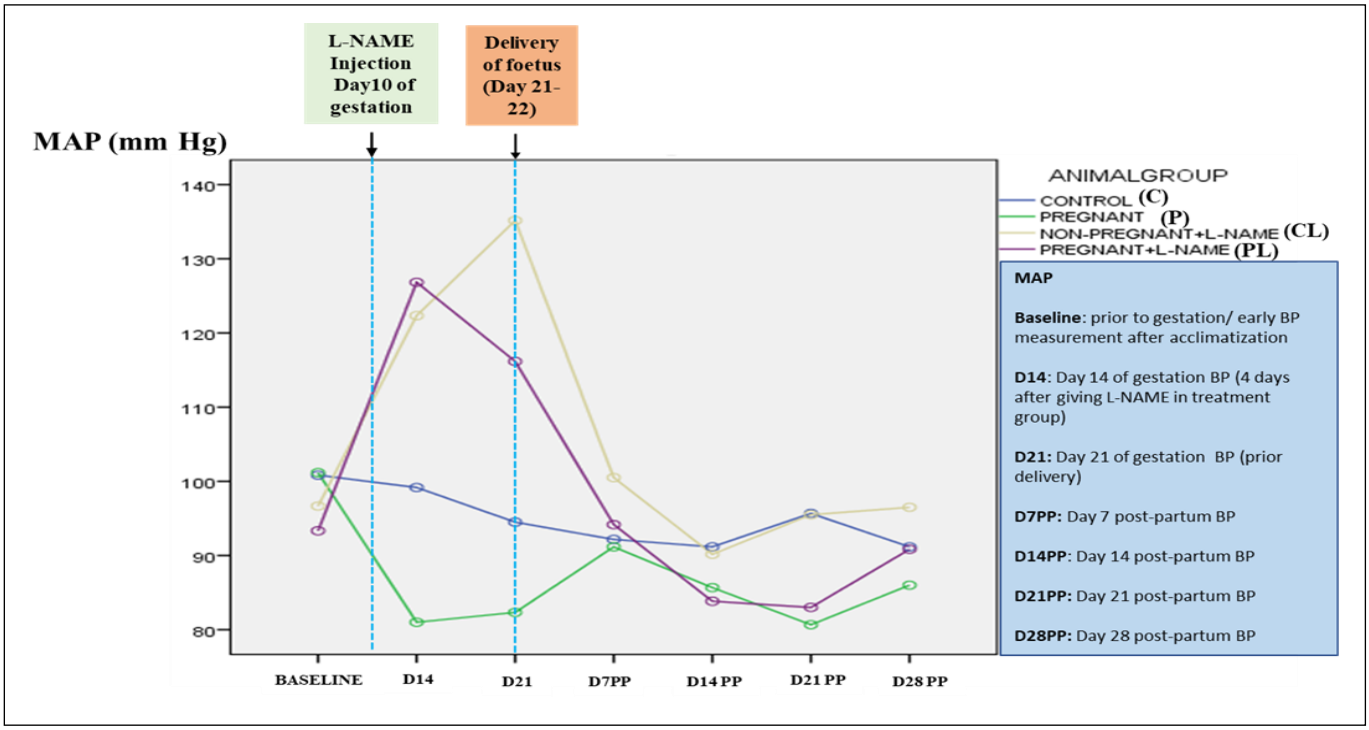

Figure 2: The graph shows the mean MAP for all groups

treatment groups returned to normal levels at day 7 postpartum (Figure 2), thus mimicking the normal return of blood pressure in postpartum mothers, and this persisted until the end of the study.

Biochemistry

Endothelin-1(ET-1)

The study found that the mean concentration of ET-1 was 7.24 ±1.3 pg/mL in the C group, 6.16±1.0 pg/mL in the P group, 6.61±0.4 pg/mL in the CL group, and 5.89 ±1.3 pg/mL in the PL group. However, there was no significant difference in the mean concentration of ET-1 in all groups, p>0.05 (Figure 3a).

Nitric Oxide (NO)

The mean NO level concentration for the C group was 78.68 ±5.7ng/mL, the P group was 77.77±7.2 ng/mL, the CL group was 82.62±5.0 ng/mL, and the PL group was 78.86±5.6 ng/mL. However, there was no significant difference in the mean concentration of NO in all groups (Figure 3b).

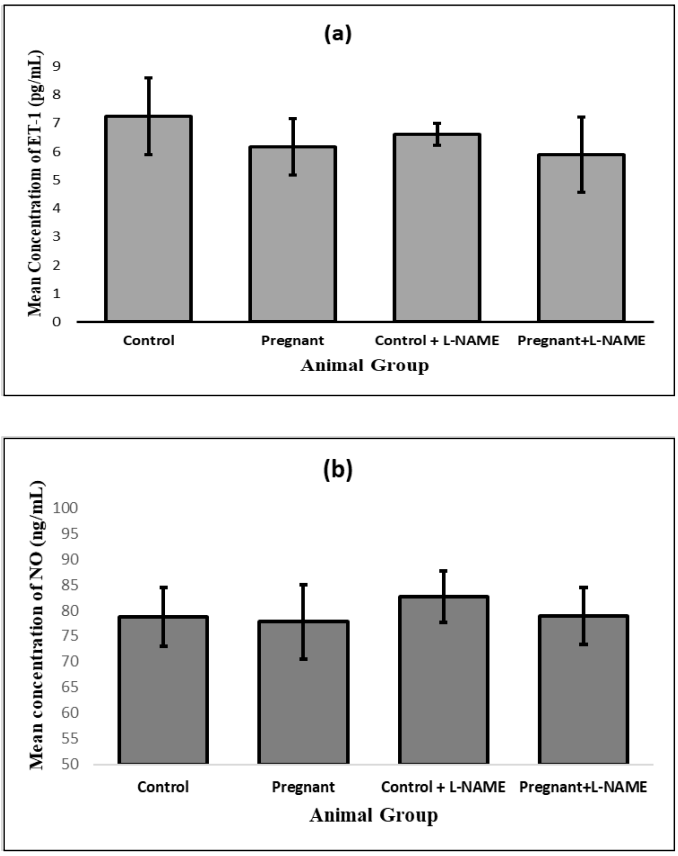

Figure 3: The bar graph shows the mean concentration of a) ET-1 and b) NO in all groups with error bars: ± 1 SD

Immunohistochemistry Study

Expression of ETAR

The study shows that the mean ETAR immunoreactivity percentage area in the media layer for the C group (0.46±0.26), P group (0.40±0.18), CL group (0.24±0.05) and PL group (0.23±0.12). However, there was no significant difference in the mean ETAR immunoreactivity percentage area for media in all groups (Figure 4).

Expression of ETBR

The study shows that the mean ETBR immunoreactivity percentage area in intima for the C group (0.16±0.08), P group (0.21±0.13), CL group (0.10±0.03) and PL group (0.13±0.06). Meanwhile, the mean ETBR immunoreactivity percentage area in media for the C group (0.25± 0.12), P group (0.28±0.15), CL group (0.18±0.08) and PL group (0.17±0.09). However, there was no significant difference in the mean ETBR immunoreactivity percentage area for intima and media in all groups (Figure 4).

DISCUSSION

In this study, we successfully induced hypertension in pregnant rats by administering L-NAME at 125 mg/kg/day beginning on day 10 of gestation (second trimester). This established gestational hypertension, a condition in which high BP is detected after 20 weeks of pregnancy.[4] The cessation of the treatment with L-NAME at delivery (Day 20 or 21 of gestation) mimics the delivery of placenta in women with HDPs. We found that the mean MAP of the treatment groups (CL and PL) returned to a normal level at Day 7 postpartum. This is supported by the fact that most women with HDPs return to normal BP within one week postpartum[7], but they still have an increased two - to fourfold risk of developing CVDs later in life.[8]

We chose day 30 of the postpartum period, which is equivalent to 2.5 human years, as the study period to find out the best time to look for long-term changes in the endothelin system that could cause persistent endothelial dysfunction and lead to the development of CVD in women after HDPs.[27] L-NAME was used to inhibit NO

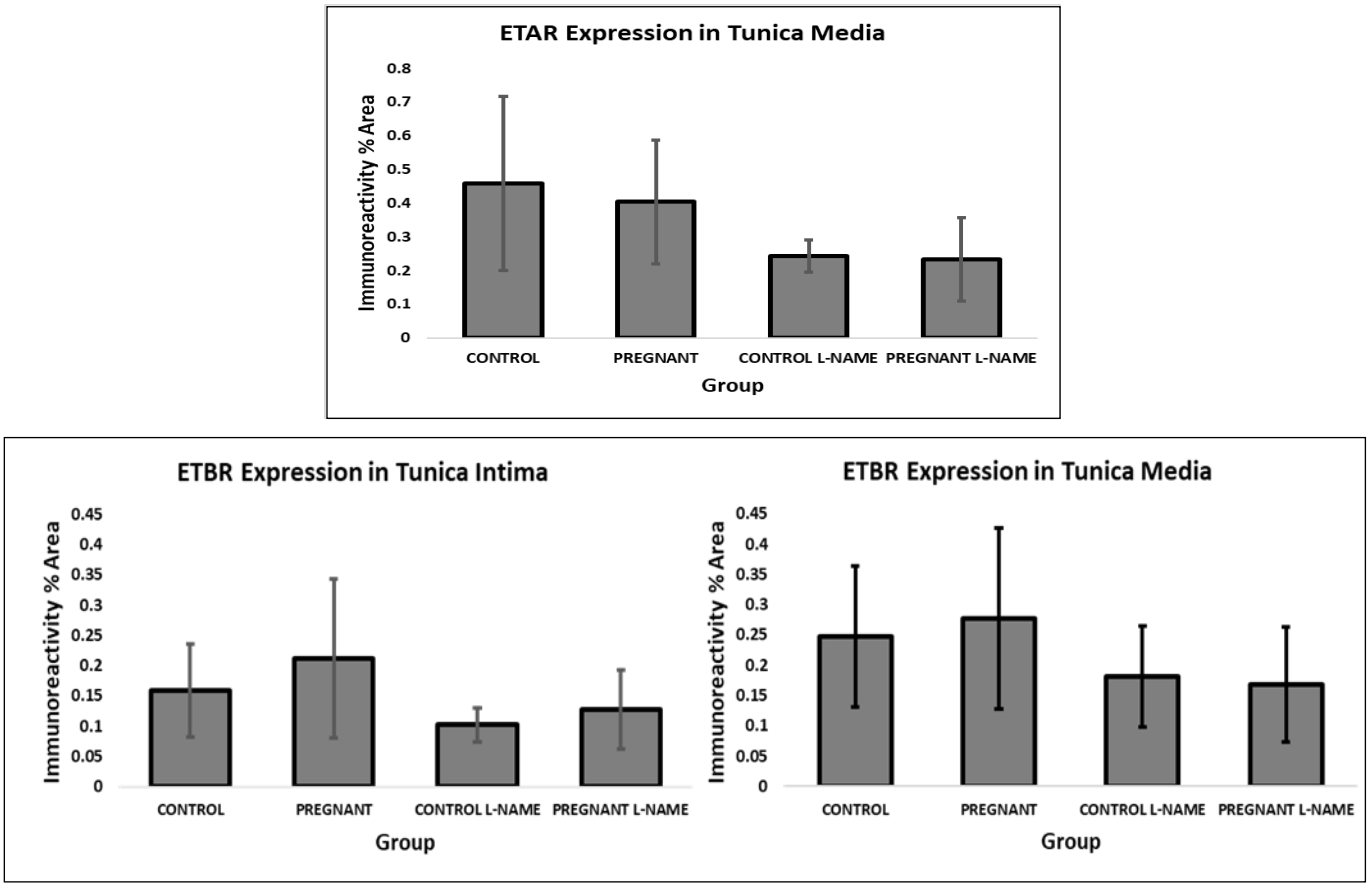

Figure 4: The bar graph shows the mean immunoreactivity percentage area for ETAR and ETBR with error bars: ± 1 SD

production by blocking the enzyme nitric oxide synthase (NOS). Reduction of NO resulting in vasoconstriction and subsequently leading to high BP. We found that discontinuation of L-NAME for 30 days may result in recovery of ET-1 and NO levels, allowing vasodilation and lowering blood pressure. We found there was no significant difference in the mean concentrations of ET-1 and NO between the control and treatment groups at day 30 postpartum. It might be because all the biochemicals returned to normal levels after 30 days of cessation of treatment. These findings were supported by a recent study reporting that the MAP of the mice receiving L-NAME during pregnancy returned to normal levels after discontinuation of treatment at 1 week postpartum, and this level was maintained up to 10 weeks postpartum, as was the case with the circulating ET-1 levels.[20]

However, these findings were opposite to the findings in human studies, in which they had proven that there was persistent endothelial dysfunction with hypertension and an increase in inflammatory biomarkers up to more than 20 years postpartum.[28,29] They believed that persistent endothelial dysfunction in at-risk women was due to low-grade chronic inflammation. In contrast to the previous animal study, no biomarkers of the endothelin system were measured in this one.

ET-1 exerts its vasoconstrictor effect by binding with ETAR and, to some extent, with ETBR in the vascular smooth muscles (VSMCs), with additional dual effects by binding with endothelial ETBR and causing the release of NO. The decrease in vasoconstriction effect of ET-1 in normal pregnancy on ETAR and ETBR in VSMCs was associated with a decrease in expression of ETBR mRNA and protein and also decreased immunostaining of ETAR and ETBR in VSMCs.[30] In our study, we found that there were no significant differences in the immunoreactivity staining of ETAR and ETBR at the mesenteric vascular layers between the control and treatment groups, suggesting a possible connection with the circulating levels of ET-1 and NO returning to baseline levels. The significance of ET-1 receptor subtypes in maintaining vascular homeostasis is currently unknown, but it might be related to gene regulation, transcription, and the production of ET-1 and NO. The ratios of ETAR and ETBR on vascular smooth muscle cells are dependent on the type of vascular bed, and the level of ETBR expression on smooth muscles could be elevated in vascular pathologies.[31]

In the current practice, the mothers are being monitored during clinic follow-up until six weeks postpartum.[32] There is an overlooked window period that makes women vulnerable to cardiovascular disease if they do not receive prompt intervention or long-term surveillance following the puerperium phase. This has become alarming because this population is still at higher risk for hypertension in prehypertensive patients[33] and other cardiovascular diseases.[8] A pilot study of postpartum sequelae of the HDP concluded that a tenth of pregnant women with the hypertensive disease had persistent hypertension six weeks after delivery, whereas a high proportion of hypertensive and normotensive pregnant women had blood pressure levels within prehypertensive ranges by six weeks postpartum.[33] Thus, it is crucial to reconsider the extension of the monitoring and follow-up period for patients, especially those with HDP.

There is still more to learn about the links between HDPs and CVD, and it is also possible that epigenetic inheritance is responsible for the increased maternal risk of CVD over the long term. Endothelial dysfunction, poor placentation, and organ dysfunction during pregnancy may all be exacerbated by a preexisting genetic susceptibility to cardiovascular disease, which in turn may increase the risk of preeclampsia.[34,35] Therefore, pregnancy may provide a window of opportunity to screen for vascular dysfunction and evaluate the individual and familial risk factors for cardiovascular disease.

CONCLUSION

There are no abnormal changes at the proteomic level in the endothelin system of resistance arteries over the study period in the gestational hypertensive rat model. Longitudinal studies of the effects of HDP on the progression of CVD require deeper molecular and ultrastructural analysis of the vessels.

CONFLICT OF INTEREST

The researchers affirm that there were no financial or commercial affiliations that might be seen as a potential conflict of interest throughout the research's execution.

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