

Original Article

Effects of low molecular heparin sodium on HPA and MMP-9 expression in tissues and serum of hypertensive pregnant rats

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Abstract: Pregnancy induced hypertension (PIH) is one pregnant related disease. Heparanase (HPA) and matrix metalloproteinase-9 (MMP-9) are important degradation enzymes of extracellular matrix. This study established PIH rat model, on which low molecular heparin sodium was applied to analyze its effect on HPA and MMP-9 expression. SPF grade healthy Wistar rats were generated for PIH model using nitro L arginine methyl ester (L-NAME). Low molecular heparin sodium was intraperitoneally injected in experimental group, in parallel with normal pregnant rats in control group. Average artery pressure and urea protein levels were measured. Expression of HPA and MMP-9 in serum and placental tissues were measured by ELISA and IHA method, respectively. The correlation between HPA, MMP-9 and PIH was analyzed. Experimental group had lower artery pressure and urea protein levels than model group but higher than blank control group ($P<0.05$). Serum and placental levels of HPA and MMP-9 were higher in experimental group compared to model group and were lower than blank control group ($P<0.05$). HPA was positively correlated with MMP-9 expression ($P<0.05$). Low molecular heparin sodium can decrease average artery pressure and urea protein level in PIH rats, and elevated serum and tissue levels of HPA and MMP-9 in PIH rats, thus exerting certain treatment effects.

Keywords: Low molecular heparin sodium, pregnancy induced hypertension, matrix metalloproteinase-9

Introduction

Pregnancy induces hypertension (PIH) is one pregnant complication, and severely compromises both maternal and fetal health, making it one major reason for death of pregnant women and perinatal [1]. Recent studies showed the correlation between PIH pathogenesis and ischemia of placental trophoblast layer, injury of vascular endothelial and functional dysregulation. In PIH patients, major pathological features under the microscope include dysfunction of trophoblast, leading to lower depth of endometrial infiltration as limited to spiral arterioles at decidual segment. Meanwhile, remodeling of uterus spiral arterioles is significantly weakened, causing physiological remodeling dysfunction, eventually leading to hypo-perfusion of placental. All these processes are critical steps for pathogenesis and progression of PIH [2-5]. Heparanase (HPA) is one degrading enzyme for extracellular matrix. Previous study showed the important role of HPA in embryo

implantation, infiltration of trophoblast, and placental angiogenesis [6]. Matrix metalloproteinase-9 (MMP-9) can be secreted from trophoblast, endothelial cells, mesenchymal tissues and certain tumors [7]. Study attributed MMP hydrolysis of extracellular matrix as one critical important factor for trophoblast invasion [8]. Low molecular heparin sodium can decrease protein urea excretion rate in PIH patients, and has certain preventive and treatment effects of PIH-related renal damage. This study thus established a rat PIH model, on which low molecular sodium was used for intervention, followed by the assay of serum/tissue expression of HPA and MMP-9, in an attempt to analyze the effect of low molecular heparin sodium on their expressions and related mechanisms.

Methods

Experimental animal

Healthy SPF grade Wistar rats (30 males, 60 females, aging between 12-14 weeks, body

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Table 1. Average artery blood pressure and urea protein level

Group	N	Average artery pressure (mmHg)	Urea protein (g/l)
Experimental	20	108.35±3.21* [#]	1.51±0.12* [#]
Model	20	134.75±4.24 [#]	2.28±0.17 [#]
Blank control	20	90.85±3.04	0.26±0.05

Note: *P<0.05 compared to model group; [#]P<0.05 compared to blank control group.

Table 2. Rat serum HPA and MMP-9 levels (ng/ml)

Group	N	HPA	MMP-9
Experimental	20	4.21±0.11* [#]	4.43±0.13* [#]
Model	20	1.25±0.24 [#]	1.57±0.32 [#]
Blank control	20	9.05±0.04	8.06±0.03

Note: *P<0.05 compared to model group; [#]P<0.05 compared to blank control group.

weight 320±20 g) were provided by Laboratory Animal Center, Jinan University (Certificate No. SCXK-2002-001) with food and water provided ad libitum.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of First Affiliated Hospital of Jinan University.

Drugs and instruments

Nitro L arginine methyl ester (L-NAME) was purchased from Sigma (US). Low molecular heparin sodium injection solution, ELISA kit for HPA and MMP-9, rabbit anti-rat HPA polyclonal antibody, rabbit anti-rat MMP-9 polyclonal antibody, ready-to-use streptavidin-biotin-peroxidase complex (SABC) kit, DAB development kit, citric acid antigen retrieval buffer were provided by Boster (China).

PIH model generation

All Wistar rats were housed in 2 males vs. 1 female. Vaginal secretion smear was collected on each morning. First day of pregnancy was identified as occurrence of sperm. Pregnant rats were randomly divided into 3 groups (N=20 each).

Model group: 250 mg/kg/d L-NAME was injected subcutaneously at 8 am since P14, for 7 consecutive days to generate PIH model.

Experimental group: 250 mg/kg/d L-NAME was injected subcutaneously at 8 am since P14, for 7 consecutive days, plus daily subcutaneously injection of 600 IU/kg low molecular heparin sodium at 4 pm.

Blank control group: Normal pregnant rats received equal volume of 0.9% NaCl solution at 4 pm each day for 7 consecutive days.

All rats were measured for tail artery pressure at P21, and were collected from urea protein in 24 h. Rats were then sacrificed by cervical dislocation.

Sample collection

Rats were fixed for the head by clamping neck skins. Hairs around the eye were removed, with compression on both sides of rat neck. A capillary was inserted vertically into fundus ocular venous plexus. Blood was collected via capillary into EP tubes for cold storage.

Rat placental tissues were collected at 4°C to prepare 0.5 cm×0.5 cm pieces. Tissue samples were fixed in neutral buffered formalin, for observing histopathological change under microscope following HE staining. Some tissues were kept at -80°C for further experiments.

Serum HPA and MMP-9 levels by ELISA

Venous blood samples collected from fundus ocular were centrifuged to collect the supernatant. Standard sample were serially diluted in fold-decrease concentration gradient following the manual instruction. 120 µl standard samples in the test kit were added into one EP tube, in combined with 120 µl dilution buffer to prepare 1200 ng/L concentration. Such standard samples were drawn for 120 µl in mixing with 120 µl dilution buffer to prepare 600 ng/L concentrations. All 5 standard concentrations were prepared using this method following the manual instruction of test kit for each factor in triplicates. Samples were added following the manual instruction. The plate was sealed for 37°C incubation for 30 min. The membrane was carefully removed with discarding all liquids. Washing buffer was added into each well for 30 s incubation. After washing for 5 times, liquids were discarded. Chromogenic substrates A and B were sequentially added (50 µl each), fol-

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Table 3. Placental HPA and MMP-9 expression

Group	N	HPA expression strength			MMP-9 expression strength				
		-	++	+++	Positive (%)	-	++	+++	Positive (%)
Experimental	20	10	9	1	50*#	11	8	1	45*#
Model	20	16	4	0	20#	15	5	0	25#
Blank control	20	3	7	10	85	4	7	9	80

Note: *P<0.05 compared to model group; #P<0.05 compared to blank control group.

lowed by 37°C dark incubation for 15 min. The test plate was then mixed with 50 µl quenching buffer as the color change. Using blank control well as the reference, absorbance (A) values at 450 nm wavelength were measured by a micro-plate reader after 30 min pre-warm.

Placental HPA and MMP-9 expression by immunohistochemistry (IHC) method

Tissues were fixed in formalin, dehydrated, embedded in paraffin and sectioned. Tissues slices were processed by heat-retrieval, and were blocked by H₂O₂, followed by normal goat serum blocking. 50 µl primary antibody was added for 1 h incubation, followed by 50 µl secondary antibody incubation (10 min). The color was developed, followed by quenching, counter-staining, differentiation and mounting.

Judgement criteria: HPA and MMP-9 positive expression was deduced as brown-yellow granules in cytoplasm, with higher staining strength than non-specific background staining. Using positive tissues slices and PBS as positive and negative controls, 10 fields were randomly selected under 400× magnification. 100 cells were counted in each field. Negative (-) was deduced as less than 5% positive cells, weak positive (+) was deduced as 5%~25% positive cells, moderate positive (++) was identified with 25%~50% positive cells, whilst strong positive (+++) was deduced as more than 50% positive cells.

Data processing

SPSS17.0 software was used to process all data, of which enumeration data were tested by chi-square method, whilst measurement data were analyzed by analysis of variance (ANOVA) and were presented as mean ± standard deviation (SD). A statistical significance was defined when P<0.05.

Results

Average artery blood pressure and urea protein level

We measured averaged artery pressure and urea protein levels in all groups of rats. Results showed lower averaged artery pressure

and urea protein levels in experimental group compared to model group, but higher than blank control group (P<0.05, **Table 1**).

Serum HPA and MMP-9 levels in all rats

We further measured serum levels of HPA and MMP-9 in all groups of rats. Results found that compared to blank control group, model rats had significantly depressed serum HPA and MMP-9 levels (P<0.05). After using low molecular heparin sodium, experimental rats had elevated serum HPA and MMP-9 levels, although still lower than blank control group (P<0.05, **Table 2**).

Placental expression of HPA and MMP-9

In rat placental tissues, expression of HPA and MMP-9 was measured. Results showed 20% HPA positive rate in model group, plus 25% MMP-9 positive rate, both of which were significantly lower than those in blank control group (P<0.05). After intervention using low molecular heparin sodium, positive rates of HPA and MMP-9 were 50% and 45%, respectively. Both of those were higher than model group although still lower than blank control group (P<0.05, **Table 3; Figure 1**).

Correlation analysis between HPA and MMP-9

A correlation analysis between HPA and MMP-9 expression in rat placental tissues was performed and found positive correlation between HPA and MMP-9 expression (r=0.388, P<0.05; r=0.312, P<0.05; r=0.391, P<0.05).

Discussion

PIH is one common complication of pregnancy. Most PIH cases occur at 20th gestation week and within 2 weeks after delivery, with incidence at about 5%. Without appropriate treatment, it can cause systemic spasm or even coma, thus occupying 15% of all maternal

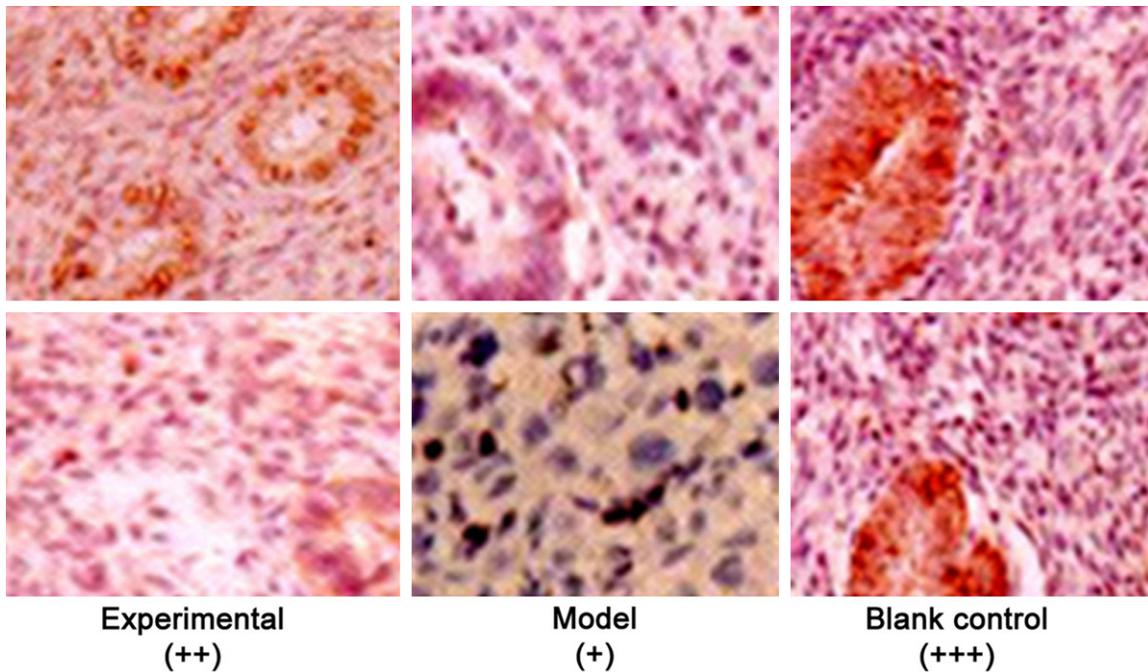


Figure 1. HPA and MMP-9 expression in rat placenta ($\times 400$). Upper panels: HPA. Lower panels: MMP-9.

death as the second common reason. It is one specific syndrome caused by organ hypo-perfusion after vascular spasm and coagulation activation [9, 10]. HPA is widely distributed in all cells and is one important transcriptional regulatory factor with close correlation with immune response, cell proliferation, growth/differentiation, cell cycle and apoptosis [11]. In human and primates, HPA exists in both placental and endometrial tissues [12]. During pregnancy, embryonic trophoblast invades into maternal decidua and spiral arteriole to replace vascular endothelial cells for establishing maternal/fetal circulation, thus providing nutrients for embryonic development. MMP-9 is one important member in matrix metalloproteinase system, and can hydrolyze multiple components of extracellular matrix, with close correlation with pathological process [13]. This study utilized low molecular heparin sodium to treat PIH rats, and measured HPA and MMP-9 expression to analyze mechanism of low molecular heparin sodium on PIH.

In this study, L-NAME was employed to generate PIH rat model. Experimental group utilized intervention by low molecular heparin sodium, whilst blank control group used normal pregnant rats. We further measured average artery pressure and urea protein in all rats. Results

showed decreased average artery pressure and protein urea levels in experimental group compared to model group, but still higher than blank group. These results suggested the successful generation of PIH rat model, plus the decrease of low molecular heparin sodium on average artery pressure and protein urea level in PIH rats, thus having certain treatment efficacy.

This study collected venous blood from rat fundus ocular venous plexus to separate serum and tested HPA and MMP-9 levels. Compared to blank control group, model rats had significantly lowered serum HPA and MMP-9 levels. After intervention by low molecular heparin sodium, experimental group rats had higher serum HPA and MMP-9 levels compared to model group, but still lower than blank group. Rats were further sacrificed to collect placental tissues, whose expression of HPA and MMP-9 was quantified. In model group, HPA and MMP-9 positive rates were significantly lower than those in blank group. Intervention by low molecular heparin sodium increased HPA and MMP-9 positive rates compared to model group, although still lower than blank control group. These results suggested that PIH rats had lower serum/tissue expressions of HPA and MMP-9. After low molecular heparin sodium

intervention, serum/tissue expression of HPA and MMP-9 were elevated. During pregnancy, HPA can regulate embryonic trophoblast cells, making them more easily to be attached and implanted into endometrium. Decreased expression of HPA leads to shallow infiltration of trophoblast, lower blood flow in placenta, thus causing PIH [14]. HS can bind trophic factors for tissues, cells and angiogenesis, and regulate effects on target cells to facilitate angiogenesis. HPA can degrade HS to affect embryonic implantation and placenta formation. Once HPA has down-regulation, shallow placenta implantation, hypoxia/ischemia of placenta occurs to aggravate PIH conditions [15, 16]. In a related study about MMP-9, PIH patients may have limited infiltration of trophoblast due to lower synthesis and secretion of MMP-9 by trophoblast, leading to under-development of cilia and vascular remodeling dysfunction of spiral arteriole, further causing hypo-perfusion of placenta, showing clinical symptoms of PIH [17]. Low molecular heparin sodium can enhance the anti-coagulation enzyme activity for impeding coagulation factor Xa and related enzyme, achieving rapid and persistent anti-clotting effects. Meanwhile it can also improve body hemodynamics without adverse effects on blood coagulation and platelet function [18-20], thus having certain treatment efficacy for PIH.

This study further analyzed the correlation between HPA and MMP-9 expression, and found positive correlation between HPA and MMP-9 levels. Previous studies showed that HPA could facilitate the release of urokinase type plasminogen and tissue type plasminogen binding on HS. These two factors can activate plasminogen to activate MMP-9 [21, 22]. This study suggested that lower HPA expression might down-regulate MMP-9 expression, further accelerating PIH occurrence. The intervention by low molecular heparin sodium elevated serum/tissue levels of HPA and MMP-9 in PIH rats, and influenced proliferation, invasion and migration of trophoblasts, thus impeding further progression of PIH.

Conclusion

Low molecular heparin sodium can elevate serum/tissue expression of HPA and MMP-9, and may inhibit occurrence and progression of PIH via affecting proliferation, infiltration and migration of trophoblast.

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Disclosure of conflict of interest

None.

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