Vascular dysfunction in the offspring of AT1 receptor antibody-positive pregnant rats during high-salt diet

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Abstract: Antibody against the angiotensin AT1 receptor (AT1-Ab) could disturb placental development. The placenta is the key organ between mother and fetus. Placental damage will seriously impair fetal growth and development in utero, leading to intrauterine growth restriction (IUGR). Based on the fetal origins of adult disease (FOAD) hypothesis, IUGR could increase a propensity to develop adult onset cardiovascular disease (CVD). The present study was designed to determine whether vascular function has changed in the adult offspring of AT1-Ab positive pregnant rats. Twenty four female rats (8-week-old, AT1-Ab negative) were randomly divided into two groups, immunized and vehicle groups. Immunized group received active immunization to establish AT1-Ab-positive model, while vehicle group was subjected to Freund’s adjuvant without antigen. After 8 weeks of immunization, the antibody titers in sera from the female rats were detected by enzyme-linked immunosorbent assay (ELISA). Then all the female rats were mated with normal Wistar male rats and became pregnant. Immunized/vehicle group offspring rats (I offspring/V offspring) were raised to 40-week-old under standard chow feeding. Then the two groups’ offspring rats were given a high-salt diet for 12 weeks (4% NaCl in chow feeding). Systolic blood pressure (SBP) was measured dynamically by noninvasive blood pressure system. The vascular ring experiment was performed to detect vascular function and reactivity. As detected by ELISA, the titers of antibody peaked at the 8th week (OD values: 2.75 ± 0.08 vs 0.33 ± 0.01, P < 0.01 vs vehicle group at the same time point). There was no significant difference of SBP between the two groups’ offspring rats during the high-salt diet (P > 0.05). Isolated thoracic aortic rings of I offspring had significantly decreased constriction under norepinephrine treatment (P < 0.01 vs V offspring) and significantly decreased dilation under acetylcholine treatment (P < 0.05 vs V offspring). These results suggest that the offspring of AT1-Ab-positive pregnant rats are more susceptible to vascular functional abnormality while being fed high-salt diet.

Key words: antibody; angiotensin; AT1 receptor; fetal origins of adult disease; high-salt diet; vascular ring
In the 1990s, Barker et al.\[1\] proposed the fetal origins of adult disease (FOAD) hypothesis, which suggests that intrauterine growth restriction (IUGR) would lead to permanent changes in the body’s structure and physiology, and increase the subsequent risk of adult onset of cardiovascular disease (CVD), such as hypertension\[2\], coronary heart disease\[3\] and stroke\[4\]. The placenta is the key organ between mother and fetus. Placental damage will seriously impair fetal growth and development in utero\[5\]. However, the factors contributing to placental distress have not been fully understood.

In 1999, Wallukat et al.\[6\] firstly reported that the antibody against the angiotensin AT1 receptor (AT1-Ab) was present in preeclamptic patients. This antibody could specifically recognize the functional epitope of the second extracellular loop of AT1 receptor (amino acid residues 165–191, AT1R-EC II) and possess AT1 receptor agonist-like effects. Up to now, a growing body of evidence suggested that AT1-Ab could impair placental development via various pathways, such as inducing excess soluble fms-like tyrosine kinase-1 (sFlt-1, a soluble form of the vascular endothelial growth factor-1 receptor) secretion\[7\] and anti-angiogenesis, stimulating plasminogen activator inhibitor-1 (PAI-1) secretion and shallow trophoblast invasion \[8\]. In addition, AT1-Ab increased placental apoptosis in human villous explants as well as trophoblast cells in vitro\[9\]. Moreover, the correlation of AT1-Ab levels in pregnant women and placental blood flow were negative\[10\], suggesting that AT1-Ab induced placental vessels constriction. All of these data indicate that AT1-Ab might have a detrimental effect on fetal growth and maturation, increasing the risk for future development of CVD consequences in adulthood.

Therefore, in this study, active immunization method was conducted to establish AT1-Ab-positive pregnant rat model. We sought to determine whether vascular function was abnormal in the offspring of AT1-Ab-positive pregnant rats in middle and late life.

1 MATERIALS AND METHODS

1.1 Materials

The peptide corresponding to the sequence of the second extracellular loop of the human AT1 receptor (AT1R-ECII, amino acid residues 165–191, IHRNVFFIINTNVTCAFHYESQNST) was synthesized using an automated peptide synthesizer by GL Biochem Ltd. (Shanghai). Norepinephrine (NE) was obtained from Harvest Pharmaceutical Ltd. (Shanghai), and acetylcholine (ACh, an endothelium-dependent vasodilator) was purchased from Sigma (St Louis, Missouri, USA). All chemicals utilized in this study were of analytical grade.

1.2 Animals

The research procedures complied with the “Guiding Principles in the Use and Care of Animals” published by the National Institutes of Health (NIH Publication No. 85–23, Revised in 1996), and agreed by the Institutional Animal Care and Use Committee of Capital Medical University. AT1-Ab-negative female Wistar rats (8-week-old, 180–200 g) and healthy male Wistar rats (220–250 g) were selected for this study [Grade II, Certificate number of the breeder: SCXK (Jing) 2009-0001]. The female rats were used for establishing an active immunization model, and the male rats were chosen for mating.

1.3 Active immunization model

Twenty-four female Wistar rats were randomly divided into two groups. In immunized group, rats were actively immunized with synthetic AT1R-ECII peptide fortnightly in accordance with our previous method\[11\]. After 8 weeks, the immunized female rats were mated with normal male rats (unimmunized). Blood was
drawn from the caudal vein each time before immunization. The serum was collected to monitor the titers of antibody. Vehicle group rats were treated with a mixture of Freund’s complete/incomplete adjuvant (without antigen) and saline in the same manner.

Immunized/vehicle group offspring rats (I offspring/V offspring, both male) were raised to 40-week-old under the same conditions. All of them had free access to water and were housed individually under controlled environmental conditions (19–22 °C, 30%–40% humidity) and 12 h/12 h light/dark cycle. After reaching 40 weeks of age, the two groups’ offspring rats were given a high-salt diet containing 4% salt (by weight) in chow feeding for 12 weeks.

1.4 Enzyme-linked immunosorbent assay (ELISA)
Serum AT1-Ab levels were detected by ELISA as we used previously[12], and the results were expressed as optical density (OD) values. Briefly, synthetic peptide (5 mg/mL) in a 100 mmol/L Na2CO3 solution (pH 11.0) was coated on microtiter plates overnight at 4 °C. The wells were then saturated with 0.1% PMT buffer [0.1% (w/v) albumin bovine V, 0.1% (v/v) Tween 20 in phosphate-buffered saline (PBS), pH 7.4] for 1 h at 37 °C and washed three times with PBST. The sera dilutions were added to the saturated microtiter plates for 1 h at 37 °C. After PBST washing, the wells were incubated with biotinylated goat anti-human IgG antibodies (Sigma) (1:1 000 dilutions in PMT) for 1 h at 37 °C. Following three times of washing, streptavidin-peroxidase conjugate (Sigma) at 1: 2 000 dilution in the same buffer was added to the wells and incubated under the same conditions. Finally, 2,2-azino-di-(3-ethylbenzothiazoline) sulphonate (ABTS)-H2O2 (Roche, Basel, Switzerland) substrate buffer was added and incubated for 30 min in the dark at room temperature. The absorbance was measured at 405 nm using an ELISA reader (Spectra Max Plus, Molecular Devices, Sunnyvale, California, USA). We also calculated positive/negative (P/N) ratio [(specimen OD value – blank control OD value) / (negative control OD value – blank control OD value)], and those samples with a P/N value of at least 2.1 were considered as AT1-Ab positive.

1.5 Blood pressure measurement in vivo
Systolic blood pressure (SBP) was estimated by a tail-cuff method (II TC noninvasive blood pressure system; Life Science Instruments, Gene&I, China) in offspring rats. The reported blood pressure value is the mean of five systolic measurements.

1.6 Thoracic aorta preparations and measurement of vascular function in vitro
The rats were anesthetized and killed by cervical dislocation. Each thoracic aorta was quickly removed and placed into ice-cold 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) solution (in mmol/L: NaCl 144, KCl 5.8, CaCl2 2.5, MgCl2 1.2, HEPES 5, and D-glucose 11.0, pH 7.4). The aortas were cut into rings of 3–4 mm length. The rings were then suspended on two wire hooks in water-jacketed tissue baths containing 10.0 mL HEPES solution bubbled with 95% O2 and 5% CO2 and maintained at 37 °C. The upper hook was connected to a force transducer, and changes in isometric force were recorded by a PowerLab system (AD Instruments Co., Ltd., Sydney, Australia). Passive tension was adjusted to 2.0 g, and all subsequent measurements represented force generated above this baseline. A 1.5 h equilibration period was allowed before any experimental intervention, and the bath was flushed every 20 min with the fresh HEPES solution during equilibration. After equilibration, the rings were maximally constricted with KCl (60 mmol/L) followed by extensive washing, and the procedure was repeated three to five times until a stable vasoconstriction was observed[13]. The contraction was generated by NE (1 × 10–9–1 × 10–6 mol/L). The relaxation was induced by ACh (1 × 10–9–1 × 10–5 mol/L) on the contraction generated by NE (1 × 10–6 mol/L).

1.7 Statistical analysis
All data were described as means ± SEM. Results were analyzed by two-way ANOVA using the statistics software of SPSS 13.0. \( P < 0.05 \) was considered statistically significant, and \( P < 0.01 \) was considered highly significant.

2 RESULTS

2.1 High levels of AT1-Ab were generated in immunized mother rats
As illustrated in Fig. 1, all of the immunized female rats received AT1R-ECII peptide generated high serum levels of AT1-Ab at the 2nd week after initial immunization, which was detected by ELISA method. The titers of antibody peaked at the 8th week (OD value: 2.75 ± 0.08 vs 0.33 ± 0.01, \( P < 0.01 \) vs vehicle group at the same time point). However, the titers of AT1-Ab were close to background level in the concurrent control, suggesting that the active immunization model was
successfuly established.

2.2 Arterial blood pressure showed no change in I offspring

Both immunized and vehicle groups’ offspring rats were raised to 40-week-old under standard chow feeding. Then the two groups’ offspring rats were given a high-salt diet (4% NaCl in chow diet) for 12 weeks. The SBP was measured by noninvasive blood pressure system at 40, 44, 48, and 52 weeks of age. Interestingly, the SBP showed no significant difference between two groups \((P > 0.05)\). Although I offspring were given a high-salt diet, the BP was not elevated (Table 1).

2.3 NE-induced vasoconstriction decreased in I offspring

The vascular ring experiment was performed to detect vascular function and reactivity. Isolated thoracic aortic rings were treated with cumulative concentration \((1 \times 10^{-9} - 1 \times 10^{-6} \text{ mol/L})\) of NE. The contraction of aortic rings is defined by the ratio of NE- to KCl-induced vasoconstrictions. Figure 2 showed that the response to

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<th>Table 1. Measurement of systolic blood pressure (SBP)</th>
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The offspring rats’ SBP was monitored dynamically by noninvasive blood pressure system. Data are expressed as means ± SEM, \(n = 8\). V offspring, vehicle group offspring rats; I offspring, immunized group offspring rats.
NE-induced vasoconstriction of thoracic aortic rings in I offspring decreased markedly, compared with that in V offspring.

2.4 ACh-induced vasodilatation decreased in I offspring

To clarify vasodilatation function of I offspring, the relaxing effect of ACh was studied. First, isolated thoracic aortic rings were precontracted by NE (1 × 10⁻⁶ mol/L). Then aortic rings were treated with cumulative concentration (1 × 10⁻⁹–1 × 10⁻⁵ mol/L) of ACh. As shown in Fig. 3, ACh-induced vasodilatation also decreased significantly in I offspring, compared with that in V offspring.

3 DISCUSSION

AT1-Ab was first detected in the sera of preeclamptic patients[6], then in malignant hypertension[14], refractory hypertension[15] and renal-allograft rejection[16]. Especially, accumulative evidence suggests that AT1-Ab has a detrimental impact on placental development. The placenta is a key organ for fetus nutrient supply, and placental damage could contribute to IUGR. According to FOAD hypothesis, IUGR increases the susceptibility to cardiovascular events in adult life. In the current study, we found that the SBP of AT1-Ab-positive pregnant rats’ offspring was not elevated despite provision of a high-salt diet, but the vascular function reduced.

Active immunization is a commonly used means to study the pathophysiological significance of antibodies. The synthetic peptides corresponding to the sequences of receptors were used to immunize rats and induced large numbers of antibodies, which displayed similar biological behaviors with antibodies purified from patients[17,18]. The epitope peptide of extracellular second loop of AT1 receptor (AT1R-ECII) was used as antigen to establish AT1-Ab-positive pregnant rat model in our study.

In the present study, the two groups’ offspring rats were given mild high-salt diet (4% NaCl). So the salt intake is just an interference factor, far below hypertensive model dose (8% NaCl)[19,20]. However, the SBP still did not rise in I offspring. It is widely accepted that the decreased elasticity of the arterial wall is responsible for the elevation of BP, but the regulation of BP is a complicated process in which a host of neural and hormonal factors are involved. Thus, there may be vascular lesion in spite of normal BP.

Furthermore, AT1 receptor is mainly located on vascular smooth muscle and endothelium. So we need to investigate vascular function and reactivity with the vascular ring experiment. NE is a kind of catecholamine neurotransmitter, and also a hormone[21]. Circulating NE is primarily from the adrenal medulla, producing vasoconstriction and causing the elevation of blood pressure. NE is recognized to be a kind of conventional vasoconstrictive drug in basic level. We observed that NE-induced vasoconstriction decreased in I offspring. The reduced thoracic aorta contraction could explain why the BP was not high to a certain extent. The impairment of endothelium or smooth muscle may cause the reduced aortic contraction, such as the release of vascular active molecules out of balance in endothelium, and the changes of receptors or channels in smooth muscle. The underlying mechanisms require further study.

As the results showed, ACh-induced vasodilatation also decreased in I offspring. ACh is a neurotransmitter which binds specially with various cholinergic receptors[21], which promotes the release of NO through nitric oxide synthase (NOS) and thus induces vasodilatation. In healthy individuals, endothelial cells regulate a host of functions including vasomotor tone, thrombosis/fibrinolysis and cell-cell interactions[22]. The development of endothelial dysfunction may be a common pathway by which cardiovascular risk factors impact plaque formation, growth and rupture. Endothelial dysplasia causes the disorder of regulation as above and leads to the occurrence of diseases, such as atherosclerosis[23].

In conclusion, the present study found that thoracic aorta vasoconstriction and vasodilatation decreased significantly in the offspring of AT1-Ab-positive pregnant rats, although the BP was not elevated during a high-salt diet. It can be inferred that AT1-Ab may be a risk factor for fetal intrauterine growth, and thus programming vascular lesion in adulthood.

Vascular lesion could increase the risk for further development of CVD. Because AT1-Ab also partially exists in healthy pregnancies, we suggest that AT1-Ab could be applied as a common parameter for pregnant women in clinic, protecting fetus growth in utero and reducing the morbidity to some CVD in adult life.

There are also some limitations in our study. We just observed the basic level of vasoconstriction induced by NE. In the next step we will observe the vascular response to AT1-receptor agonist, angiotensin II. Consid-
ering the changes of BP originally, we found that the BP was still normal whereas the vascular dysfunction under a high-salt diet. However, we ignored the observation of vascular function under standard chow feeding. Actually, the vessels of I offspring under chow feeding should be acted as controls to high-salt intake group. The present study was lack of this kind of control. We will improve this point in further study. Furthermore, the underlying mechanisms of AT1-Ab-induced vascular changes need to be discovered.

REFERENCES