A Preliminary Finding: Immunohistochemical Localisation and Distribution of Placental Angiotensin II Receptor Subtypes in Normal and Pre-Eclamptic Pregnancies

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Summary
Pre-eclampsia or pregnancy induced hypertension (PIH) affects 6-8% of all pregnancies. Although the underlying mechanism of PIH is still unknown, it is widely believed that the placenta plays an important role. It was thought that an ischemic placenta due to poor perfusion can precipitate the signs and symptoms of PIH. This study aims to investigate the possible role of Type 1 (AT1) and Type 2 (AT2) angiotensin II receptor subtypes in the mechanism of PIH. AT1 receptor stimulation causes vasoconstriction and AT2 receptor stimulation causes vasodilatation. Investigating the interactions of these two receptors in the placenta provides an insight as to the balance that may exist between AT1 and AT2 receptors in normal pregnancy. Any disruption to the balance might cause a disruption of the blood flow in the placenta, leading to PIH. Placentas were collected from 11 PIH patients and 11 normal patients. Immunohistochemistry techniques were performed on the placental tissue to determine the distribution of AT1 and AT2 receptors in the placental tissue qualitatively and quantitatively. It was observed that in normal patients, the balance between AT1 and AT2 receptors is that the level of AT2 receptors is higher than the level of AT1 receptors. However in the PIH patient, it was observed that the normal balance was disrupted. In PIH patients, the level of AT1 receptors was observed to be higher than the level of AT2 receptors. This study suggests that disruption of the balance between AT1 and AT2 receptors observed in PIH placentas might cause a decrease in blood flow to the placenta, causing it to be poorly perfused. This may cause placental ischemia which may lead to PIH.

Key Words: Placenta, Angiotensin receptors, Pre-eclampsia, Immunohistochemistry

Introduction
Pre-eclampsia is referred to as pregnancy induced hypertension (PIH) or acute hypertensive disease of pregnancy. The incidence rate for PIH is 6-8% of all pregnancies and 20% in pre-term birth. The mortality rate is 100,000 maternal deaths per year worldwide and it accounts for 10-20% of all maternal deaths yearly. It is seen more often in primigravida than in multiparous women and in extreme reproductive ages.

The placenta is a transient hormone secreting organ responsible for physiological exchange between the mother and the fetus. The placenta is widely considered to be the inciting organ of pre-eclampsia.
has been long believed that pre-eclampsia may be due to poorly perfused placenta, caused by defective trophoblast invasion of myometrial portion of the spiral arteries. In such conditions, the spiral arteries retain their musculo-elastic properties and responsiveness to vasoactive peptide substances, which may trigger placental ischaemia. Ischemic placenta may produce a circulating agent, which is currently unidentified but causes the widespread dysfunction of the maternal vascular endothelium that leads to the systemic manifestations of PIH.

Extensive studies have been done to determine the possible role of the renin angiotensin system of the placenta in PIH. There is increasing evidence of a placental renin angiotensin system that may be active during pregnancy, alterations of which could lead to PIH and other hypertensive disorders of pregnancy. Besides, researchers have localised the angiotensin II receptor subtypes (AT1), namely the AT1 receptor and more recently the AT2 receptor in the placenta. Research has conclusively shown an up-regulated expression of AT1 receptor subtype in the placenta of patients with pre-eclampsia, as compared to placenta of patients with uncomplicated pregnancy. The discovery of AT2 receptors in the placenta warrants the need to investigate the interactions and expressions of the two types of receptors as well its balance during normal pregnancy, and its possible disruption in pre-eclampsia.

The objective of this study is to examine the distribution of angiotensin receptors in the human placenta in normotensive and pre-eclamptic pregnancies in the context of the Malaysia population. This study also aims to determine whether differences in receptor immunoreactivity occur in pre-eclamptic as opposed to normotensive pregnancies. The hypothesis of this study is that there will be differences in the levels and distribution of the angiotensin II receptor subtypes, in placenta of pre-eclamptic women compared to normotensive women.

Materials and Methods

Collection and Processing of Human Placental Tissues

Placentas were collected from two groups of patients. The first group comprised of patients with uncomplicated pregnancy while the second group comprised of patients with pregnancy induced hypertension (PIH). Patients with other complication of pregnancy (e.g. gestational diabetes mellitus) were excluded in this study.

After delivery, the placenta was first checked for any abnormalities and the chorionic membrane on the maternal side was trimmed to reveal the cotyledons. A random spot was picked and a surgical blade was used to cut a 1cm x 1cm block of tissue through the whole thickness of the placenta. The sectioned placenta was washed in normal saline and immediately soaked in paraformaldehyde in a glass container. The placenta tissue was fixed using 4% paraformaldehyde in cacodylate buffer for at least 18 hours. The sample was sent to Gribbles Pathology Lab, Kuala Lumpur to be processed and blocked.

Immunohistochemistry

The sectioning of the specimen was done by utilising the Leica RM 2135 Rotary Microtome and Leica HI 1210 Flattening Bath. Paraffin blocks were placed in the fridge at 4°C to allow it to harden well. The Leica HI 1210 Flattening Bath was filled up with distilled water, and the water bath temperature was set at 45°C. The specimen was then sectioned at a thickness of 5μm and mounted on the APES coated slide.

AT1 and AT2 antibody used in this study was AT1 antibody (rabbit polyclonal IgG, obtained from Santa Cruz) and AT2 antibody (goat polyclonal IgG, obtained from Santa Cruz) were stored at 4°C. The titre of both AT1 and AT2 antibody was 200pg/mL. The AT1 antibody reagent was diluted 10x, while the AT2 antibody reagent, was diluted 100x. The substrate chromagen solution was prepared by mixing one drop (approximately 20μL) of liquid DAB chromagen with 1000μL of buffered substrate.

Immunohistochemistry staining was performed on the placental tissue. The slides were first soaked in xylene, followed by absolute alcohol, then sequentially transferred into 90% alcohol, 70% alcohol and distilled water.

Antigen retrieval was then performed on the placenta tissue. This was achieved using the heat method. The slides were soaked in 600mL of 0.1M citrate buffer and later heated in microwave oven (at 70% power for 15 minutes). Slides were then allowed to cool down for

Materials and Methods

Collection and Processing of Human Placental Tissues

Placentas used in this study were collected from patients in the Obstetrics and Gynaecology Department of Seremban Government Hospital, Malaysia with written and informed consent from the patients. Ethical approval was obtained from the Ethics Committee of the International Medical University in Kuala Lumpur, Malaysia.
15 minutes. Peroxidase block was then performed by applying a few drops of DAKO peroxidase block on the placental tissue, and leaving it on for 15 minutes. After that, the slides were jetwashed with TBS and soaked in TBS for five minutes.

Subsequently, a protein block was done using a few drops of DAKO protein block. After 15 minutes, the slides were jetwashed with TBS. 100μl of AT1 or AT2 antibody reagent was applied respectively to each slide, followed by incubation overnight at 4°C.

The next day, at room temperature, the slides were jetwashed with TBS and labelled polymer (horseradish peroxidase) was then applied on the placental tissue slides and incubated for another 30 minutes. The slides were then jetwashed with TBS and DAB chromagen was then added on the placental tissue for five minutes. The slides were then jetwashed with distilled water. Harris haematoxylin was used as counterstain.

When the staining process was completed, the slides were mounted with DPX and observed under the microscope. Images were captured by utilising a 5.1 mega pixel Evolution MP digital camera. Image Pro express software was used to capture the images which were then analysed.

The control tissue used in this project was the Macaca irus (long tail monkey) renal tissue, collected from the Anatomy Department of University Malaya. Both positive and negative controls gave appropriate findings when the optimal antibody dilution of 2.5x (for AT1) and 20x (for AT2) was applied.

Results

The distribution of AT1 and AT2 receptors in placenta tissues were determined qualitatively and quantitatively. The distribution was determined qualitatively by comparing microscopic images of slides stained for AT1 receptors and slides stained for AT2 receptors, which are taken from the same placenta samples. It was observed that in normal placenta, the level of AT2 receptors is higher than the level of AT1 receptors, as shown in figure 1(a) and 1(b). In the PIH placenta, it was observed that the level of AT1 receptors is higher than the level of AT2 receptors, as shown in Figure 2(a) and 2(b).

To determine the distribution of AT1 and A2 receptors quantitatively, three separate microscopic images were taken from the slide stained for AT1 receptors and slide stained for AT2 receptors. Both slides were from the same patient. Grids were then drawn on the images to divide it into 12 separate boxes. Four boxes were randomly picked and the number of brown spots in each box was counted and recorded (Table I and Table II).

As shown in Table I, the ratio of AT2 receptors to AT1 receptors in normal patients is 45:28, with a statistically significant p value of < 0.001. In Table II, the ratio of AT2 receptors to AT1 receptors in PIH patients is 31:44, with a statistically significant p value of < 0.001.

There are three main structures in a histological image of the placental tissue. They are the mesodermal core of the villus, blood vessels of the villus and the foetal maternal interface. Figure 3 shows AT1 receptors in a normal patient, was mostly localised in the blood vessels of the villus. The mesodermal core and the foetal maternal interface only showed traces of AT1 receptors. Figure 3 also shows AT1 receptors in a PIH patient was mostly localised in the blood vessels of the villus. The mesodermal core and the foetal maternal interface only showed traces of AT1 receptors.

Figure 4 shows AT2 receptor in a normal patient were mostly localised in the blood vessels and the mesodermal core of the villus. Foetal maternal interface of normal placenta only showed traces of AT1 receptors. Figure 4 also shows AT1 receptors in a PIH patient was mostly localised in the blood vessels of the villus. Foetal maternal interface and mesodermal core of PIH placenta only showed traces of AT2 receptors.
Table I: Distribution of AT1 and AT2 receptors in normal patients

<table>
<thead>
<tr>
<th>Normal patient</th>
<th>AT1</th>
<th>AT2</th>
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<tbody>
<tr>
<td>Patient 1</td>
<td>18</td>
<td>38</td>
</tr>
<tr>
<td>Patient 2</td>
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<td>Patient 3</td>
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<tr>
<td>Average</td>
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<td>45</td>
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<tr>
<td>Standard deviation</td>
<td>9.96</td>
<td>5.27</td>
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</table>

A two sampled equal variance t-test shows p value < 0.001, p value = 0.00063.

Table II: Distribution of AT1 and AT2 receptors in PIH patients

<table>
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<th>AT2</th>
</tr>
</thead>
<tbody>
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<td>Patient 1</td>
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<td>Patient 2</td>
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<td>Patient 3</td>
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<tr>
<td>Standard deviation</td>
<td>7.81</td>
<td>7.95</td>
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A two sampled equal variance t-test shows p value < 0.001, p value = 0.00074.

Fig 1(a) and 1(b): Immunohistochemical localisation of AT1 and AT2 receptors, in normal patient

Fig 2(a) and 2(b): Immunohistochemical localisation of AT1 and AT2 receptors, in PIH patient
Discussion and Conclusion

Although the aetiology of pregnancy induced hypertension (PIH) is not clearly understood, it is widely believed that the placenta is the inciting organ of this disease. This is based on observation that the features of PIH which include proteinuria, pathologic oedema and hypertension disappear after pregnancy, when the placenta is no longer present in the body. It was suggested that the PIH placenta is ischaemic, and ischaemic placenta may produce a circulating agent, although currently unidentified; causes the widespread dysfunction of the maternal vascular endothelium that leads to the systemic manifestations of PIH.

It is believed that the ischaemic placenta of PIH is caused by the absence of trophoblastic cells invasion of the spiral arteries. In normal placental development, there is a progressive loss of musculoelastic tissue in the spiral arteries. This causes a physiological dilatation of the spiral arteries, resulting in increase uterine blood flow. However, in PIH this physiological dilatation does not occur due to defective trophoblast invasion of spiral arteries. Instead, the spiral arteries retain their muscular structure, thus reducing normal blood flow to the placenta, causing it to be poorly perfused, resulting in PIH.
We have observed that there was a difference in the level of angiotensin II receptor subtypes, AT1 and AT2 receptors in both normal and PIH placenta. In the normal placenta, it was observed that the distribution of AT2 receptors is consistently higher than the distribution of AT1 receptors. This observation was seen using both qualitative and quantitative methods as described in the materials and methods section. There seems to be strong evidence suggesting the existence of a balance between the AT1 and AT2 receptors in normal placenta. The balance in normal placenta is that the number of AT2 receptors is higher than the number of AT1 receptors.

However, in a PIH placenta, it was observed that the distribution of AT1 receptors is consistently higher than the distribution of AT2 receptors. This further suggests that the balance between the AT1 and AT2 receptors is different in a PIH placenta as compared to a normal placenta, suggesting a disruption in the balance.

The findings of this study can be explained as follows. Stimulation of AT1 receptors causes vasoconstriction and stimulation of AT2 receptors causes vasodilatation. In normal placenta, there is a balance between the AT1 and AT2 receptors, thus a balance in vasoconstrictory and vasodilatory effect on the placental spiral arteries. This allows normal blood flow to the placenta, ensuring it to be well perfused. However, in PIH placenta, the balance is disrupted. This can affect the placental blood flow in two ways. Firstly, a higher level of AT1 receptors causes a greater vasoconstrictor effect. Secondly, a lower level of AT2 receptors causes a smaller vasodilatory effect. These two effects might give rise to an overall increase in vasoconstrictor effect on the placental spiral arteries. As a result, there is a decrease in blood flow to the placenta, giving rise to a poorly perfused placenta.

Recently, Thapa et al., (2004) observed that the expression of AT1 receptors is significantly increased in the Syncytiotrophoblast (STB) layer and the villous endothelium of human placenta with PIH. The authors noted that expression of AT1 receptors increases with the severity of the disease. In this study, it was also observed through immunohistochemical staining that both AT1 and AT2 receptors were predominantly located in the endothelial wall of the foetal vascular endothelium. This provides evidence that AT1 and AT2 receptors play an important role in the regulation of blood flow in the placenta. Localisation of AT1 receptors in this study is further supported by studies done by Cooper et al, 1999 and Cheong et al, 2000.
Both these studies show localised AT1 receptors in the foetal vascular endothelium. However the localisation of AT2 receptor in this study differs from the findings of Cheong et al, 2000. Cheong et al, 2000 reported no immunoreactivity in the placenta for the AT2 receptor. The lack of immunoreactivity maybe due to the AT2 antibody dilution used in their study, which is 100x dilution whereas in this study the AT2 antibody dilution was 20x.

It is acknowledged that one of the limitations of this study is the sample size. The sample size obtained for both normal and PIH placenta was 22 and 11 respectively. For the data to be reflective of the population, a larger sample size is needed. Another limitation of this study is the quantitative method used to determine the distribution of AT1 and AT2 receptors in placental tissue. This method may produce inaccuracies in the quantification of AT1 and AT2 receptors due to investigator bias. However, the method was chosen based on the resources available during this study. In future, these limitations can be overcome by increasing the sample size and the duration for sample collection and also by expanding the number of collaborating hospitals to collect placental samples. Limitations in the quantitative methods can be overcome by utilising computer-aided image analysis which can selectively quantify the brown spots on the capture image. This method will provide more accurate distributions of AT1 and AT2 receptors.

The findings in this study warrant further investigations at the molecular level to determine the mRNA expression of AT2 receptors and further analysing the balance between AT1 and AT2 receptors. Based on the identification of non-invasive measurements AT1 and AT2 receptors, the balance of these two receptors may be in future used as a risk stratification method in managing pre-eclampsia or PIH.

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References


