Testosterone Reduces Tight Junction Complexity and Down-regulates Expression of Claudin-4 and Occludin in the Endometrium in Ovariectomized, Sex-steroid Replacement Rats

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Abstract. *Background/Aim: It was hypothesized that endometrial tight junction morphology and expression of tight junction proteins i.e., claudin-4 and occludin in the uterus, are affected by testosterone. Therefore, the effects of testosterone on these parameters in the uterus during receptivity period were investigated. Materials and Methods: Ovariectomized adult female rats were given testosterone (1 mg/kg/day) alone or in combination with flutamide or finasteride between days 6 to 8 of sex-steroid replacement treatment, which was considered the period of uterine receptivity. Ultramorphology of tight junctions was visualized by transmission electron microscopy while distribution and expression of claudin-4 and occludin were examined by immunofluorescence and real-time polymerase chain reaction respectively. Results: Administration of testosterone caused loss of tight junction complexity and down-regulated expression of claudin-4 and occludin in the uterus. Conclusion: Decreased endometrial tight junction complexity and expression of claudin-4 and occludin in the uterus during receptivity period by testosterone may interfere with embryo attachment and subsequent implantation.*

Implantation is a highly controlled process where successful interaction occurs between a blastocyst and the receptive endometrium. The blastocyst will only implant during a narrow period known as the uterine receptivity period (1). During this period, there is a reduction in the volume of uterine fluid,

which aids blastocyst attachment to the endometrium (2). Disturbances in the uterine fluid microenvironment during the uterine receptivity period might lead to implantation failure.

Testosterone is a male sex hormone that is also produced in ovaries in females (3) and decidual tissue. Testosterone is required for decidualization (4). In females, a rise in plasma testosterone level has been observed at around the time of implantation (5). However, an excessively high plasma testosterone level might interfere with pre-implantation embryo development as well as development of uterine receptivity (6). Our previous study showed that a high plasma testosterone level in rats suppressed pinopode development and expression of L-selectin ligand in the uterus in early pregnancy (7).

Tight junctions are the most apical part of cell-cell junctional complexes that are located next to adherence junctions, and represent a boundary between the plasma membranes of two adjacent cells (8). Tightness of the barrier formed by tight junction is determined by the number of strands and interconnections (9). Tight junctions have been reported to be involved in regulating diffusion of proteins and molecules between apical and basolateral membranes. In addition, they are also involved in controlling ion and H₂O transport across the paracellular space between epithelial cells (10). The composition and ratio of the transmembrane proteins, claudin-4 and occludin, in tight junctions determine their permeability and selectivity (11).

Claudin-4 is a key protein component of tight junctions. It polymerizes within plasma membrane as fibrils to generate tight junction strands (12-14). Occludin, which is a four transmembrane domain-containing protein is incorporated with and localized very close to claudin-based tight junction strands (15, 16). Expression of occludin is inversely correlated to the permeability of tight junctions. The permeability and selectivity of tight junctions can be controlled by hormones such as estrogen that causes tight junction strands to become parallel, with less branching and interconnections (17). Under progesterone influence, the

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network and depth of tight junction strands are increased with more branches and interconnections. Tight junctions are impermeable at the time of embryo implantation (17).

In this study, it was hypothesized that testosterone might affect tight junction morphology and expression of claudin-4 and occludin proteins in the uterus during the uterine receptivity. This study therefore investigated the mechanisms underlying adverse effects of testosterone on fertility by examining the effect of testosterone on tight junction morphology and expression of claudin-4 and occludin in the uterus during uterine receptivity.

Materials and Methods

Animals and hormone treatment. Three-month-old adult female Sprague-Dawley (SD) rats (n=6), weighing 225±25 g, were caged under standard conditions (lights on 06:00 to 18:00 h; room temperature 25±2°C; 5-6 animals per cage). Animals were fed with rat chow (Harlan, Germany) and tap water *ad libitum*. All experimental procedures were approved by the University of Malaya Institutional Ethics Committee (2013-07-15/FIS/R/NS). Estrogen, testosterone, flutamide, finasteride and peanut oil were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Rats were divided into five groups with six rats in each group. Bilateral ovariectomy was performed 21 days prior to steroid treatment to eliminate the effect of endogenous sex steroids hormones (18). Drugs were dissolved in peanut oil and injected subcutaneously behind the neck scruff in a volume of 0.1 ml.

In this study, ovariectomized rats were treated with a sex-steroid regime to mimic the hormonal changes in early pregnancy. The regime included the injection of 1.0 µg/kg/day estrogen on days 1 and 2, 1.0 µg/kg/day estrogen and 4 mg/kg/day progesterone on day 3, no treatment on days 4 and 5, and 16 mg/kg/day progesterone and 0.5 µg/kg/day estrogen between days 6-8 according to the established protocol by Kennedy *et al.* (19). Vehicle-treated animals received daily injections for 8 days of 0.1 ml peanut oil. Testosterone at 1 mg/kg/day, the dose regarded as supra-physiological in females (20), was given for 3 days (days 6-8) that was considered the period of uterine receptivity. Testosterone was given with flutamide (5 mg/kg/day) or finasteride (1 mg/kg/day). Both inhibitors were administered 30 min prior to testosterone injection. The rats from all groups were sacrificed and uterine horns were harvested 1 day after the last day of treatment.

Transmission electron microscopy (TEM). Uteri were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. 2 mm-cross sections of each uterine horn were cut and incubated overnight at 4°C in 2.5% glutaraldehyde buffer. Following removal of the buffer, the samples were rinsed three times, 15 min each, in 0.1 M phosphate buffer. Samples were then incubated in 1% osmium in 0.1 M phosphate buffer, rinsed and dehydrated in series of ethanol (70-100%). Samples were incubated twice for 5 min in propylene oxide and then transferred to a rotor for 1 h at room temperature in 1:1 mixture of propylene oxide and epon (47% Embed 812, 31% dodenyl succinic anhydride, 19% nadic methyl anhydride, 3% benzyldimethylamine; Electron Microscopy Sciences, Hatfield, PA, USA). This was followed by overnight incubation in 1:2 propylene oxide-epon, and finally 100% epon for 2-3 h. Individual uterine samples were embedded in 100% epon in silicon flat embedding molds, and capsules were polymerized at 60°C for 48 h. Ultrathin

transverse sections (70 nm) were prepared by using a diamond knife (Diatome, Hatfield, PA, USA) on a MT 6000-XL ultramicrotome, captured on 300-mesh copper grids, and stained with 2% uranyl acetate. The ultrathin sections were observed under TEM (Libra 120; Zeiss, Oberkochen, Germany) to assess the changes in tight junction morphology. The junctional regions of two randomly chosen villi were examined in each specimen.

Immunofluorescence detection of protein distribution. Uterine tissues were cut into 5-mm sections, deparaffinized in xylene, and rehydrated in decreasing concentrations of ethanol. Trisodium citrate (pH 6.0) was used for antigen retrieval, while 10% H₂O₂ in phosphate buffered saline (PBS) was used to neutralize endogenous peroxidase. Uterine sections were blocked in 10% normal rabbit serum (sc-2338; Santa Cruz Biotechnology, Santa Cruz, CA, USA) prior to incubation with goat IgG polyclonal primary antibody to claudin-4 (sc-17664) and goat IgG polyclonal primary antibody to occludin (sc-8145; Santa Cruz Biotechnology). The antibodies were diluted at 1:100 in PBS with 1.5% normal blocking serum at room temperature for 1 h. After rinsing three times with PBS, sections were incubated with rabbit anti-goat IgG-fluorochrome-conjugated secondary antibody (sc-2777; Santa Cruz Biotechnology) at a dilution of 1:250 in PBS with 1.5% normal blocking serum at room temperature for 45 min. The slides were rinsed three times with PBS and were mounted with Ultracruz mounting medium (Santa Cruz Biotechnology). Counterstaining with 4,6-diamidino-2-phenylindole was used to visualize the nuclei. All images were viewed under Nikon Eclipse 80i (Nikon, Tokyo, Japan) camera that was attached to a fluorescent microscope. Negative controls were performed by omitting the primary antibodies specific to claudin-4 and occludin or by using non-immune IgG; in these sections, no staining was observed.

Real-time PCR (qPCR) quantification of messenger RNA (mRNA). Whole uterine tissues were kept in RNALater solution (Ambion, Carlsbad, CA, USA) prior to RNA extraction. RNA was extracted by using RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) and its concentration was assessed by 260/280 UV absorption ratios (GeneQuant 1300; Biochrom, Cambridge, UK). Gene expression of claudin-4 (*Cldn4*) and occludin (*Ocln*) was evaluated using two-step real-time PCR. In this study, TaqMan1 RNA-to-CT 1-Step Kit (Ambion) was used. Firstly, cDNA was reversely transcribed to RNA by using high capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA). Amplifications on samples with no reverse transcriptase acted as control. The amplified region of cDNA was probed with TaqMan fluorescence-labeled probe. The TaqMan probe had a sensitivity of 100% and specificity of 96.67% (21) and was capable of detecting as few as 50 copies of RNA/ml (22) and as few as 5-10 molecules (23). The specificity of primer and probe ensured that expression of target DNA was specifically evaluated. Validation was performed *in silico* by using whole rat genome and *in-vitro* by using whole rat cDNA (Applied Biosystem) to ensure that specific sequences were detected. Thus, additional sequencing was not required.

The assay used TaqMan® Rn01196224_s1 for *Cldn4* and Rn00580064_m1 for *Ocln* (Applied Biosystems) which amplified 86 bp from the whole mRNA length of 1,824 bp of *Cldn4* and 4,148 for *Ocln*. In this study, glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) (Rn99999916_s1) and hypoxanthine phosphoribosyltransferase-1 (*Hprt1*) (Rn01527840-m1) (Applied Biosystems) were used as reference or house-keeping genes as their expression in uterine tissue was found to be most stable throughout the estrus cycle (24).

Figure 1. Ultramorphological appearance of uterine tight junctions in ovariectomized rats. Transmission electron microscopy images showing tight junction (TJ) morphology in the endometrium of different experimental groups. C: Vehicle control; N: normal sex-steroid replacement regime; T: testosterone; FLU: flutamide; FIN: finasteride. NP: normal early pregnant rat; M: microvilli; V: vacuole. Scale bar: 40 μ m; n=6 per treatment group.

PCR amplification program included 2 min at 50°C with uracil *N*-glycosylase, 20 s of 95°C activation with ampliTaQ gold DNA polymerase and 1 min denaturation at 95°C for 20 s and annealing/extension at 60°C for 1 min. Denaturing and annealing was performed for 40 cycles. Negative controls were performed which include omission of reverse transcriptase or omission of cDNA. All measurements were normalized by using GenEx software (MultiD, Odingatan, Sweden) followed by Data Assist v3 (Applied Biosystems) that was used to calculate the fold changes in RNA. Data were analyzed according to the comparative cycle threshold (CT) ($2^{\Delta\Delta CT}$) method. The relative quantity of target in each sample was determined by comparing the normalized target quantity in each sample to the average normalized target quantity of references.

Statistical analysis. Statistical differences were evaluated by Student's *t*-test and analysis of variance (ANOVA). A probability level of less than 0.05 ($p < 0.05$) was considered as significant. *Post-hoc* statistical power analysis was performed for all experiments and all values obtained were >0.8 , which were considered adequate.

Results

Effects of testosterone on tight junction ultramorphology. Figure 1 shows tight junction strands to be geometrically more complex and interconnected in the endometrium of rats receiving the sex-steroid replacement regime without testosterone. Testosterone administration between days 6 and 8 (regarded as the period of uterine receptivity) resulted in tight junction strands becoming more parallel. Concomitant administration of testosterone with either flutamide or finasteride also caused the tight junctions strand to become more parallel.

Effect of testosterone on claudin-4 mRNA expression and its protein distribution. Figure 2 shows the levels of *Cldn4* mRNA in the uterus was highest in rats receiving sex-steroid replacement regime without testosterone (approximately 11 times higher when compared to the ovariectomized controls).

Figure 2. Levels of claudin-4 mRNA in the uterus of ovariectomized rats. Claudin-4 mRNA levels were highest in rats receiving the normal sex-steroid replacement regime (N). In these rats, levels of claudin-4 mRNA were markedly reduced following testosterone (T) injection (days 6-8). C: Vehicle control; FLU: flutamide; FIN: finasteride. *Significantly different at $p < 0.05$ compared to normal sex-steroid replacement regime. Data are presented as the mean $\pm$ S.E.M of $n=6$ per treatment group.

Administration of testosterone between days 6 and 8 resulted in a significant decrease in the uterine level of *Cldn4* mRNA. No significant changes in *Cldn4* mRNA level was noted in rats receiving testosterone either with flutamide or finasteride as compared to those receiving testosterone only.

Figure 3 shows the highest claudin-4 protein distribution was observed in rats receiving the sex-steroid replacement regime without testosterone. The distribution was markedly reduced following testosterone treatment (days 6-8). In testosterone-treated rats, concomitant administration of either flutamide or finasteride on days 6-8 caused no relative change in the expression of claudin-4 protein in the endometrium.

Effect of testosterone on occludin mRNA expression and its protein distribution. Figure 4 shows the levels of *Ocln* mRNA in the uterus was highest in rats receiving the sex-steroid replacement regime without testosterone, and was approximately 5.5-fold higher than that of the ovariectomized controls. In these rats, administration of testosterone on days 6-8 reduced the *Ocln* mRNA level as compared to the rats receiving sex-steroid only treatment. No significant difference was noted in *Ocln* mRNA level in testosterone-treated rats following flutamide or finasteride injection.

Figure 5 shows the distribution of occludin protein in the uterus, which was highest in rats receiving sex-steroid replacement without testosterone. In these rats, the distribution of occludin was markedly reduced following injection of testosterone between days 6-8. There were no relative changes in the distribution of occludin between rats receiving sex-steroid replacement with testosterone with or without flutamide or finasteride injection.

Discussion

In this study, the findings were as follows: (i) Testosterone induced less complex tight junctions with more parallel strands during uterine receptivity; (ii) testosterone reduced expression and distribution of claudin-4 and occludin during uterine receptivity; (iii) the effect of testosterone was not antagonized by flutamide, suggesting that the genomic pathway was likely not involved in mediating the effect of testosterone; and (iv) the effect of testosterone was not antagonized by finasteride, suggesting that 5α -dihydrotestosterone (DHT) was likely not involved in mediating the testosterone effect.

We have shown that in rats which received normal sex-steroid replacement, tight junctions were complex, and interconnected with increased strand depth. These findings were consistent with previous reports which indicated that tight junction appears complex with increased in depth at the time of embryo implantation. These changes were found to occur under progesterone influence that helps to prevent diffusion of fluids and molecules through the paracellular pathway (17, 25). In addition, expression and distribution of claudin-4 and occludin were found to be markedly increased in rats receiving a normal sex-steroid replacement regime, in parallel with increased in complexity of the tight junctions. These findings were consistent with a previous study by Murphy *et al.* (25) which showed that in rats, higher expression of these proteins occurs under the influence of progesterone which contributed towards the formation of 'tight' tight junctions (25). Claudin-4 contributes towards adhesion and barrier properties of the tight junction (11). Occludin was reported to play a critical role in the development of uterine receptivity (26). Interaction between

Figure 3. Distribution of claudin-4 in the uterus of ovariectomized rats. Bright fluorescence signals indicate sites of expression of claudin-4 protein. C: Vehicle control; N: normal sex-steroid replacement regime; T: testosterone; FLU: flutamide; FIN: finasteride; NC: negative control; L: lumen. Arrows indicate claudin-4. Scale bar=50 μ m; n=6 per treatment group.

Figure 4. Levels of occludin mRNA in the uterus of ovariectomized rats. Occludin mRNA was highest in rats receiving normal sex-steroid replacement regime (N). Testosterone (T) administration on days 6-8 markedly reduced occludin mRNA levels. C: Vehicle control; FLU: flutamide; FIN: finasteride. *Significantly different at $p < 0.05$ compared to normal sex-steroid replacement regime. Data are presented as the mean $\pm$ SEM of n=6 per treatment group.

claudin-4 and occludin might regulate the permeability of paracellular pathways that control the volume and composition of the uterine fluid at the time of implantation. Administration of testosterone during the period of uterine receptivity, which

resulted in reduced complexity of tight junctions and expression and distribution of claudin-4 and occludin, might lead to a 'leaky' tight junction. The formation of leaky tight junctions would allow the movement of fluid through the paracellular

Figure 5. Distribution of occludin in uterus of ovariectomized rats. Bright fluorescence signals indicate sites of expression of occludin protein. C: Vehicle control; N: normal sex-steroid replacement regime; T: testosterone; FLU: flutamide; FIN: finasteride; NC: negative control; L: lumen. Arrows indicate occludin. Scale bar=50 μ m; n=6 per treatment group.

pathway. Therefore, testosterone could potentially disturb uterine fluid regulation during the uterine receptivity period *via* interference with the morphology of tight junctions.

We have shown that the effect of testosterone on tight junction morphology and expression of claudin-4 and occludin involved neither the genomic pathway, nor the active testosterone metabolite, DHT. These findings raise the possibility that testosterone might mediate its effect *via* a non-genomic pathway. Non-genomic effects of testosterone in the uterus have not been reported as far as we are aware, however, several effects of another sex steroid, progesterone, on the uterus have been found to involve non-genomic pathway (27). Other studies have reported that in the uterus, testosterone plays a role greater than DHT in affecting several uterine functions, including fluid and electrolyte secretion, as well as expression of proteins such as cystic fibrosis transmembrane regulator (7) and aquaporins (28). Testosterone, but not DHT was also reported to affect the expression of L-selectin ligand (MECA-79) which is of a marker of uterine receptivity (7).

Conclusion

The effect of testosterone on tight junction morphology and expression of claudin-4 and occludin in the uterus may disturb several implantation processes that are crucial for blastocyst attachment to the receptive endometrium. The changes induced by testosterone might ultimately lead to implantation failure,

which could contribute towards the high incidence of infertility associated with a high plasma testosterone level.

Conflicts of Interest

None of the Authors has any potential conflicts of interest associated with this research and performed final editing of the article.

Author's Contributions

N.S. designed the research. M.H.M collected tissue samples and performed experiments. M.H.M, N.G and N.S. analyzed data. M.H.M. and N.S. wrote the article. N.S supervised the research and performed final editing of the article.

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