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

MOHD HELMY MOKHTAR¹, NELLI GIRIBABU² and NAGUIB SALLEH²

¹Department of Physiology, Faculty of Medicine, University Kebangsaan Malaysia (UKM), Kuala Lumpur, Malaysia; ²Department of Physiology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

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).

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 co-related 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
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 flutamide (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% dodecenyl succinic anhydride, 19% nadic methyl anhydride, 3% benzylidynamilamine; Electron Microscope 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% H_2O_2 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 for *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).
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^ΔΔCT) 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 junctions 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 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 μm; n=6 per treatment group.
Administration of testosterone between days 6 and 8 resulted in a significant decrease in the uterine level of \textit{Cldn}4 mRNA. No significant changes in \textit{Cldn}4 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.

\textbf{Effect of testosterone on occludin mRNA expression and its protein distribution.} Figure 4 shows the levels of \textit{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 \textit{Ocln} mRNA level as compared to the rats receiving sex-steroid only treatment. No significant difference was noted in \textit{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.

\textbf{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
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
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.

**Acknowledgements**

This study was funded by PPP grant (PG007-2013B), University of Malaya, Kuala Lumpur, Malaysia.

**References**

1. Tranguch S, Daikoku T, Guo Y, Wang H and Dey SK: Molecular complexity in establishing uterine receptivity and implantation. Cell Mol Life Sci 62(17): 1964-1973, 2005. PMID: 16143898. DOI: 10.1007/s00018-005-5230-0
2. Ruan YC, Chen H and Chan HC: Ion channels in the endometrium: Regulation of endometrial receptivity and embryo implantation. Hum Reprod Update 20(4): 517-529, 2014. PMID: 24591147. DOI: 10.1093/humupd/dmu006
3 Fogle RH, Stanczyk FZ, Zhang X and Paulson RJ: Ovarian androgen production in postmenopausal women. J Clin Endocrinol Metab 92(8): 3040-3043, 2007. PMID: 17519304. DOI: 10.1210/jc.2007-0581

4 Kramen MA and Johnson DC: Uterine decidualization in rats given testosterone propionate neonatally. J Reprod Fertil 42(3): 559-562, 1975. PMID: 1123820. DOI: 10.1530/jrf.0.0420559

5 Concannon PW and Castracane VD: Serum androstenedione and testosterone concentrations during pregnancy and nonpregnant cycles in dogs. Biol Reprod 33(5): 1078-1083, 1985. PMID: 4074805. DOI: 10.1095/biolreprod.33.5.1078

6 Diao HL, Su RW, Tan HN, Li SJ, Lei W, Deng WB and Yang ZM: Effects of androgen on embryo implantation in the mouse delayed-implantation model. Fertil Steril 90(4 Suppl): 1376-1383, 2008. PMID: 18053999. DOI: 10.1016/j.fertnstert.2007.07.1341

7 Mohd Mokhtar H, Giribabu N, Kassim N, Muniandy S and Salleh N: Testosterone decreases fluid and chloride secretions in the uterus of adult female rats via down-regulating cystic fibrosis transmembrane regulator (Cfr) expression and functional activity. J Steroid Biochem Mol Biol 144 Pt B: 361-372, 2014. PMID: 25125390. DOI: 10.1016/j.jsbmb.2014.08.007

8 Tsukita S and Furuse M: Pores in the wall: Claudins constitute tight junction strands containing aqueous pores. J Cell Biol 149(1): 13-16, 2000. PMID: 2175101. DOI: 10.1083/jcb.149.1.13

9 Claude P and Goodenough DA: Fracture faces of zonulae occludentes from “tight” and “leaky” epithelia. J Cell Biol 58(2): 390-400, 1973. PMID: 2190950. DOI: 10.1083/jcb.58.2.390

10 Tsukita S and Furuse M: The structure and function of claudins, cell adhesion molecules at tight junctions. Ann N Y Acad Sci 915: 129-135, 2000. PMID: 1193568. DOI: 10.1111/j.1749-6632.2000.tb05235.x

11 Tsukita S, Furuse M and Itoh M: Multifunctional strands in tight junctions. Nat Rev Mol Cell Biol 2(4): 285-293, 2001. PMID: 11283726. DOI: 10.1038/35067088

12 Furuse M and Tsukita S: Claudins in occluding junctions of humans and flies. Trends Cell Biol 16(4): 181-188, 2006. PMID: 16537104. DOI: 10.1016/j.tcb.2006.02.006

13 Van Itallie CM and Anderson JM: Claudins and epithelial paracellular transport. Annu Rev Physiol 68: 403-429, 2006. PMID: 16460278. DOI: 10.1146/annurev.physiol.68.040310.131404

14 Anglew S, Ahlstrom R and Yu AS: Biology of Claudins. Am J Physiol Renal Physiol 295(4): F867-876, 2008. PMID: 18480174. DOI: 10.1152/ajprenal.90264.2008

15 Furuse M, Hirase T, Itoh M, Nagauchi A, Yonemura S and Tsukita S: Occludin: A novel integral membrane protein localizing at tight junctions. J Cell Biol 124(6 Pt 2): 1777-1788, 1993. PMID: 8276896. DOI: 10.1083/jcb.123.6.1777

16 Fujimoto K: Freeze-fracture replica electron microscopy combined with sds digestion for cytochemical labeling of integral membrane proteins. Application to the immunogold labeling of intercellular junctional complexes. J Cell Sci 108(11): 3443-3449, 1995. PMID: 8586656.

17 Murphy CR, Swift JG, Mukherjee TM and Rogers AW: The structure of tight junctions between uterine luminal epithelial cells at different stages of pregnancy in the rat. Cell Tissue Res 223(2): 281-286, 1982. PMID: 7066976. DOI: 10.1007/BF01258489

18 Salleh N, Baines DL, Naftalin RJ and Milligan SR: The hormonal control of uterine luminal fluid secretion and absorption. J Membr Biol 206(1): 17-28, 2005. PMID: 16440178. DOI: 10.1007/s00232-005-0770-7

19 Kenneth TG: Intrauterine infusion of prostaglandins and decidualization in rats with uteri differentially sensitized for the decidual cell reaction. Biol Reprod 34(2): 327-335, 1986. PMID: 3456802. DOI: 10.1095/biolreprod34.2.327

20 Dehghan F, Muniandy S, Yusof A and Salleh N: Sex-steroid regulation of relaxin receptor isoforms (rxfp1 & rxfp2) expression in the patellar tendon and lateral collateral ligament of female wky rats. Int J Med Sci 1(2): 180-191, 2014. PMID: 24465164. DOI: 0.7150/ijms.6283

21 Tsai YL, Wang HT, Chang HF, Tsai CF, Lin CK, Teng PH, Su C, Jeng CC and Lee PY: Development of taqman probe-based insulated isothermal PCR (IIPCR) for sensitive and specific on-site pathogen detection. PLoS One 7(9): e45278, 2012. PMID: 23049781. DOI: 10.1371/journal.pone.0045278

22 Leutenegger CM, Higgins J, Matthews TB, Tarantal AF, Luciv PA, Pedersen NC and North TW: Real-time Taqman PCR as a specific and more sensitive alternative to the branched-chain DNA assay for quantification of simian immunodeficiency virus RNA. AIDS Res Hum Retroviruses 17(3): 243-251, 2001. PMID: 11177407. DOI: 10.1089/0889222201750063160

23 Tandon R, Cattori V, Gomes-Keller MA, Meli ML, Golder MC, Lutz H and Hofmann-Lehmann R: Quantitation of feline leukemia virus viral and proviral loads by Taqman® real-time polymerase chain reaction. J Virol Methods 130(1-2): 124-132, 2005. PMID: 16054243. DOI: 10.1016/j.jviromet.2005.06.017

24 Lin P, Lan X, Chen F, Yang Y, Jin Y and Wang A: Reference gene selection for real-time quantitative pcr analysis of the mouse uterus in the peri-implantation period. PLoS One 8(4): e62462, 2013. PMID: 23638092. DOI: 10.1371/journal.pone.0062462

25 Murphy CR, Swift JG, Mukherjee TM and Rogers AW: Effects of ovarian hormones on cell membranes in the rat uterus. II. Freeze-fracture studies on tight junctions of the lateral plasma membrane of the luminal epithelium. Cell Biol 31(1): 55-62, 1975. PMID: 18075634. DOI: 10.1002/biolreprod.1117

26 Orchard MD and Murphy CR: Alterations in tight junction molecules of uterine epithelial cells during early pregnancy in the rat. Acta Histochem 104(2): 149-155, 2002. PMID: 12086335. DOI: 10.1078/0065-1281-00644

27 Gellersen B, Fernandes MS and Brosens JJ: Non-genomic progesterone actions in female reproduction. Hum Reprod 15(1): 119-138, 2009. PMID: 18936037. DOI: 10.1093/humupd/dm044

28 Salleh N, Mohd Mokhtar HM, Kassim NM and Giribabu N: Testosterone induces increase in aquaporin (AQP)-1, 5, and 7 expressions in the uteri of ovariectomized rats. J Membr Biol 248(6): 1097-1105, 2015. PMID: 26198330. DOI: 10.1007/s00232-015-9823-8

Received October 23, 2019
Revised November 7, 2019
Accepted November 12, 2019