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Testosterone Down-Regulates Expression of αvβ3-Integrin, E-Cadherin and Mucin-1 during Uterine Receptivity Period in Rats

(Testosteron Menindas Ekspresi αvβ3-Integrin, E-Cadherin dan Mucin-1 semasa Tempoh Reseptif Rahim dalam Tikus)

HELMY MOHD MOKHTAR, NELLI GIRIBABU & NAGUIB SALLEH*

ABSTRACT

Adequate development of uterine receptivity is crucial for establishment of pregnancy. Expression of uterine receptivity molecules i.e. αvβ3 integrin, E-cadherin and mucin-1 could be affected by testosterone. The objective of this study was to investigate effect of testosterone on expression of these molecules during early pregnancy. 30 ovariectomised female Sprague-Dawley rats were divided into 5 groups that consisted of vehicle control, rats received eight days sex-steroid replacement regime (intended to mimic the hormonal changes in early pregnancy) and three groups of rats given testosterone (1 mg/kg/day) subcutaneously with or without flutamide or finasteride between day 6 and 8 representing the period of uterine receptivity. At the end of the treatment, rats were sacrificed and uteri were removed. Expression and distribution of αvβ3 integrin, E-cadherin and mucin-1 were examined by immunoflourescence and levels of messenger RNA (mRNAs) were evaluated by real-time PCR. Expression of αvβ3 integrin, E-cadherin and mucin-1 in the uteri of rats receiving sex-steroid replacement regime increased significantly as compared to control ($p<0.05$*). In these rats, concomitant administration of testosterone between day 6 and 8 resulted in expression of αvβ3 integrin, E-cadherin and mucin-1 to decrease significantly (*$p<0.05$*) as compared to rats receiving sex-steroid replacement regime without testosterone treatment. Moreover, the testosterone effects were not antagonized by either flutamide or finasteride. As a result, reduced expression of uterine receptivity molecules by testosterone might interfere with early pregnancy establishment, therefore could adversely affect the female fertility.*

Keywords: αvβ3 integrin; E-cadherin; mucin-1; testosterone; uterine receptivity

ABSTRAK

Perkembangan yang mencukupi bagi reseptif rahim adalah penting untuk membolehkan kehamilan berlaku. Pengekspresan molekul reseptif rahim seperti αvβ3 integrin, E-cadherin dan mucin-1 boleh dipengaruhi oleh testosteron. Objektif kajian ini adalah untuk mengkaji kesan testosteron terhadap pengekspresan molekul ini semasa peringkat awal kehamilan. 30 ekor tikus Sprague-Dawley yang telah diovariektomi dibahagikan kepada 5 kumpulan terdiri daripada kumpulan kawalan, kumpulan tikus yang menerima regim penggantian steroid seks selama lapan hari (bertujuan untuk meniru perubahan hormon semasa peringkat awal kehamilan) dan tiga kumpulan tikus yang diberikan rawatan testosteron (1 mg/kg/hari) secara subkulitan dengan atau tanpa kehadiran flutamide atau finasteride antara hari ke-6 hingga ke-8 rawatan yang mewakili tempoh reseptif rahim. Di akhir rawatan, tikus telah dikorbankan dan uteri dikeluarkan. Pengekspresan dan taburan αvβ3 integrin, E-cadherin dan mucin-1 ditentukan dengan menggunakan immunoflourescence dan paras mRNA ditentukan dengan menggunakan Real-time PCR. Pengekspresan αvβ3 integrin, E-cadherin dan mucin-1 dalam uteri tikus yang menerima regim penggantian steroid seks didapati meningkat secara signifikan berbanding kawalan ($p<0.05$*). Pada tikus ini, pemberian bersama testosteron antara hari ke-6 hingga hari ke-8 telah menyebabkan pengekspresan αvβ3 integrin, E-cadherin dan mucin-1 menurun dengan signifikan (*$p<0.05$*) berbanding kumpulan yang menerima regim penggantian steroid seks tanpa rawatan testosteron. Selain itu, kesan testosteron ini tidak ditentang oleh flutamide atau finasteride. Kesimpulannya, penurunan pengekspresan molekul reseptif rahim oleh testosteron mungkin mengganggu perkembangan awal kehamilan dan seterusnya boleh menjejaskan kesuburan wanita.*

Kata kunci: αvβ3 integrin; E-cadherin; mucin-1; reseptif rahim; testosteron

INTRODUCTION

Uterine receptivity is a period where complex signals exist between the embryo and the maternal endometrium (Simon et al. 2000). The events that occur during uterine receptivity are precisely coordinated by the ovarian hormones, namely oestrogen and progesterone (Paulson 2011). During the uterine receptivity period, several molecules including integrins, E-cadherin and mucin-1 are expressed in the endometrium (Aplin 2000). Integrins are involved in the interactions between cell to cell and cell to matrix (Lessey

2003). It consists of two different, non-covalently linked subunits, i.e. α and β. Integrins have been identified as one of the markers of endometrial receptivity and can be found in the epithelium and stroma (Lessey et al. 1994). There are three integrins isoforms: α1β1, α4β1 and αvβ3 (Thomas et al. 2002). Amongst these, αvβ3 integrin can interact with the trophoblast during the uterine receptivity period (Apparao et al. 2001). αvβ3 integrin has been proposed to mediate attachment of the embryo to the receptive endometrium (Lessey 2003).

Cadherins are group of glycoproteins which play a role in calcium-dependent cell-to-cell adhesion (Kowalski et al. 2003; Nurismah et al. 2008). Cadherins can be divided into three types i.e. epithelial (E), placental (P) and neural (N) cadherins. E-cadherin is involved in embryo implantation (Achache & Revel 2006). It is a 120-kDa transmembrane protein that mediates epithelial cells adhesion (Yoshida-Noro et al. 1984). E-cadherin is expressed at the apical membrane of the endometrial luminal epithelium and participates in trophoblast attachment. Expression of E-cadherin between the adjacent epithelial cells is altered during trophoblast invasion (Li et al. 2002).

Mucin-1, a high molecular weight glycosylated macromolecule (> 200 kDa) is known to play a role as anti-adhesive agent. The presence of mucin-1 can interfere with cellular adhesion via a steric hindrance phenomenon and thus, this can prevent the adhesion of the implanting blastocyst to the endometrium. Additionally, mucin-1 can help to lubricate and protect the blastocyst from proteolysis and from microbial attacks (Brayman et al. 2004). In the uterus, mucin-1 is found to be expressed at the apical surface of the luminal and glandular epithelia. Down-regulation of mucin-1 expression will remove the barrier between blastocyst and endometrium, thus allowing the blastocyst to attach to the endometrium (Carson et al. 2000). High mucin-1 expression was reported during the peri-implantation period in humans (DeSouza et al. 1998).

Testosterone possesses strong androgenic effects which are important for both male and female (Mohamad et al. 2016). It has been reported to affect the reproductive processes in male and female. In female, the physiological testosterone level is much lower compared to male and the level rises immediately after ovulation (Davison et al. 2005; Nur Vaizura et al. 2018). In pathophysiological condition such as polycystic ovarian disease (PCO), plasma testosterone level is higher than normal (Apparao et al. 2002; Shafiee et al. 2013). The high level of testosterone can cause infertility among premenopausal women as it can suppress ovulation (Johansson & Stener-Victorin 2013; Mohamed-Hussein & Harun 2009) as well as decidualisation (Gonzalez et al. 2012). In addition, the high plasma testosterone level can also suppress the expression of genes that participate in uterine receptivity development (Yan et al. 2012). The effect of high plasma testosterone on expression of αvβ3 integrin, E-cadherin and mucin-1 which play a role during implantation is however unknown.

Our previous study showed the high plasma level of testosterone in female could suppress pinopode development and expression of MECA-79 under the influence of progesterone (Mohd Mokhtar et al. 2014). In this study, we hypothesized that high plasma testosterone level in female could affect expression of αvβ3 integrin, E-cadherin and mucin-1 during the uterine receptivity period. These could potentially interfere with blastocyst attachment and ultimately embryo implantation. Increased knowledge on the mechanism underlying these effects can lead to strategies for correcting implantation failure.

MATERIALS AND METHODS

ANIMALS & HORMONAL TREATMENT

Three-month-old adult female Sprague-Dawley (SD) rats (n=30), weighing 225 ± 25 g were caged under standard conditions (lights on 06:00 to 18:00 h; room temperature 24°C; 4 animals per cage). Animals were fed with rat chow diet (Harlan, Germany) and tap water *ad libitum*. All experimental procedures were approved by University of Malaya institutional ethics committee (2013-07-15/FIS/R/NS). Oestrogen, testosterone, flutamide, finasteride and peanut oil were obtained from Sigma–Aldrich, USA. Bilateral ovariectomy was performed twenty-one days prior to steroid treatment to eliminate the effect of endogenous sex-steroids (Salleh et al. 2005). Drugs were dissolved in peanut oil prior to subcutaneous administration behind the neck scruff.

In this study, ovariectomised rats were treated with sex-steroid regime to mimic the hormonal changes in early pregnancy. The regime include injection of 1.0 μg/kg/day estrogen at day 1 and day 2, 1.0 μg/kg/day estrogen and 4 mg/kg/day progesterone at day 3, no treatment at the 4th and 5th day, 16 mg/kg/day progesterone and 0.5 μg/kg/day estrogen between days 6 and 8 according to the established protocol by Kennedy (Kennedy 1986). Vehicle-treated animals which acted as control received 8 daily injections of peanut oil. 1 mg/kg/day testosterone, the dose regarded as supra-physiological in females (Dehghan et al. 2014) was given for three (3) days between days 6-8 that was considered as a 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.

IMMUNOFLUORESCENCE (IF) FOR DETECTION OF PROTEIN DISTRIBUTION

Uterine sections were blocked in 10% normal rabbit serum (sc-2338) (Santa Cruz, CA, USA) prior to incubation with rabbit polyclonal Integrin αv/β3 Antibody (Cat. No. 251672) (Abbiotec, 7985 Dunbrook Rd., Ste A San Diego, CA 92126, USA), goat polyclonal IgG E-cadherin primary antibody (sc-31020) and goat polyclonal IgG mucin-1 primary antibody (sc-6827) purchased from Santa Cruz, CA, USA. The antibodies were diluted at 1:100 in PBS with 1.5% normal blocking serum at room temperature for 1 h. After three times rinsing with PBS, sections were incubated

with rabbit anti-goat IgG–fluorochrome-conjugated secondary antibody (sc-2777) (Santa Cruz, CA, USA) 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, CA, USA). Counterstaining with DAPI was done to visualize the nuclei. All images were viewed under Nikon Eclipse 80i camera that was attached to a light microscope. Negative controls were performed by incubating the sections with non-immune IgG.

REAL-TIME PCR (QPCR) FOR 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 (Gene Quant 1300, Cambridge, UK). Gene expression was evaluated by using two steps real-time PCR and the 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 (-RT) acts as control. The amplified region of cDNA was probed with TaqMan fluorescence-labeled probe. TaqMan probe was very sensitive and is capable of detecting as few as 50 copies of RNA/mL and as low as 5-10 molecules (Tsai et al. 2012). The specificity of primer and probe ensures that expression of the target DNA was specifically evaluated. Validation was performed *in silico* by using whole rat genome and *in-vitro* by using whole rat cDNA (Applied Biosystems, Foster, CA, USA). These were to ensure that specific sequences were detected.

The assay used (TaqMan®-catalog number: Rn00596601_m1 for *Itgb3*, Rn00580109_m1 for *Cdh1* and Rn01462585_m1 for *Muc1* - Applied Biosystems, Foster City, CA, USA) amplifies 66 bp, 105 bp and 81 bp segments for *Integrin αV/β3*, *Cdh1* and *Muc1* respectively from the whole mRNA length of 787 bp (*Itgb3*), 4396 bp (*Cdh1*) and 2328 bp (*Muc1*). *Gapdh* (Rn99999916_s1) and *Hprt1* (Rn01527840-m1) (Applied Biosystems, Foster City, CA, USA) were used as reference.

PCR program included 2 min at 50°C Uracil N-glycosylase (UNG), 20 s, 95°C activation of ampliTaq gold DNA polymerase and 1 min denaturation at 95°C, 20 s and annealing/ extension at 60°C for 1 min. Denaturing and annealing were performed for 40 cycles. Negative controls were performed which include omission of reverse transcriptase (RT) or omission of cDNA. GenEx software (MultiD, Odingatan, Sweden) was used to normalized the measurements. This was followed by DataAssist v3 software (Applied Biosystems, Foster City, CA, USA) that were used to calculate the RNA fold changes. Data were analyzed according to comparative CT (2^^DDCT) method. The relative quantity of target in each sample was determined by comparing the normalized target quantity to average normalized target quantity of references.

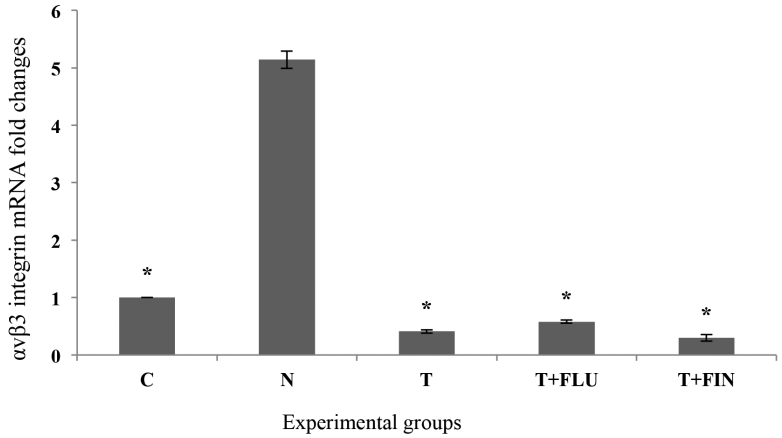
STATISTICAL ANALYSIS

Student's t-test and one-way analysis of variance (ANOVA) were used to determine the levels of significance. $p < 0.05$ was considered as significant. Post-hoc statistical power analysis was performed and all values were >0.5 which indicate adequate sample size.

RESULTS

EFFECTS OF TESTOSTERONE ON αvβ3 INTEGRIN MRNA EXPRESSION AND ITS PROTEIN DISTRIBUTION IN UTERUS

Effects on αvβ3 integrin mRNA expression Figure 1 shows the expression level of αvβ3 integrin mRNA in rats



A marked increase in αvβ3 integrin mRNA level was observed in rats received sex-steroid replacement without testosterone. Significant decrease in αvβ3 integrin mRNA level was observed in rats receiving testosterone treatment between days 6-8 with or without flutamide or finasteride. *p<0.05 compared to normal steroid replacement. C: vehicle control, N: sex-steroid replacement, T: testosterone only treatment, T + FLU: testosterone and flutamide injection, T + FIN: testosterone and finasteride injection. Data was obtained from six different rats and was expressed as mean ± S.E.M.

FIGURE 1. αvβ3 integrin mRNA expression level in uterus

receiving testosterone administered between days 6-8 of early pregnancy period. The highest mRNA levels were observed in non-testosterone treated rats received sex-steroid replacement where the levels were approximately 5.14 ± 0.15 fold significantly higher ($p<0.05$) as compared to ovariectomised control. Administration of testosterone between days 6 to 8 resulted in significant decreased ($p<0.05$) of αvβ3 integrin mRNA expression level in the uterus as compared to control. The effects of testosterone were neither antagonised by flutamide nor finasteride.

Effects on αvβ3 integrin protein distribution Figure 2 shows αvβ3 integrin protein was highly distributed in the endometrium and myometrium of non-testosterone treated ovariectomised rats receiving sex-steroid replacement regime mimicking hormonal changes in early pregnancy. In rats receiving testosterone during the uterine receptivity period, distribution of this protein in different uterine compartments was remarkably reduced. Concomitant administration of either flutamide or finasteride had not inhibited the effect of testosterone on αvβ3 integrin distribution.

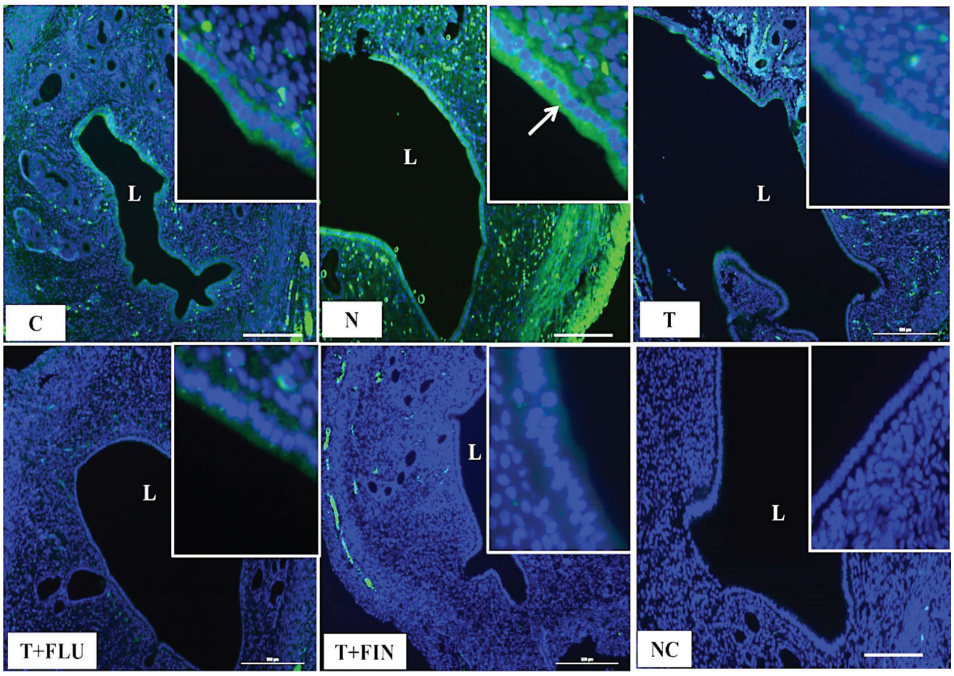
EFFECTS OF TESTOSTERONE ON E-CADHERIN mRNA EXPRESSION AND ITS PROTEIN DISTRIBUTION IN UTERUS

Effects on E-cadherin mRNA expression Figure 3 shows level of E-cadherin mRNA was highest in non-testosterone treated ovariectomised rats which received sex-steroid replacement regime mimicking hormonal changes in early pregnancy. This level was approximately 1.98 ± 0.15 fold significantly higher than ovariectomised control ($p<0.05$). Administration of testosterone between days 6 and 8 resulted in significantly reduced ($p<0.05$) E-cadherin mRNA expression. Concomitant administration of either flutamide or finasteride with testosterone did not cause significant changes in E-cadherin mRNA expression level in the uterus.

Effect on E-cadherin protein distribution Figure 4 shows the distribution of E-cadherin where this protein could be seen to be distributed in the luminal epithelium, stroma, stromal and myometrial blood vessels. The highest distribution could be seen in non-testosterone treated ovariectomised rats receiving hormonal changes mimicking early pregnancy period. Administration of testosterone between days 6 and 8 causes expression of E-cadherin to markedly reduced. The effect of testosterone was not antagonised by either flutamide or finasteride as no marked changes in E-cadherin distribution was observed.

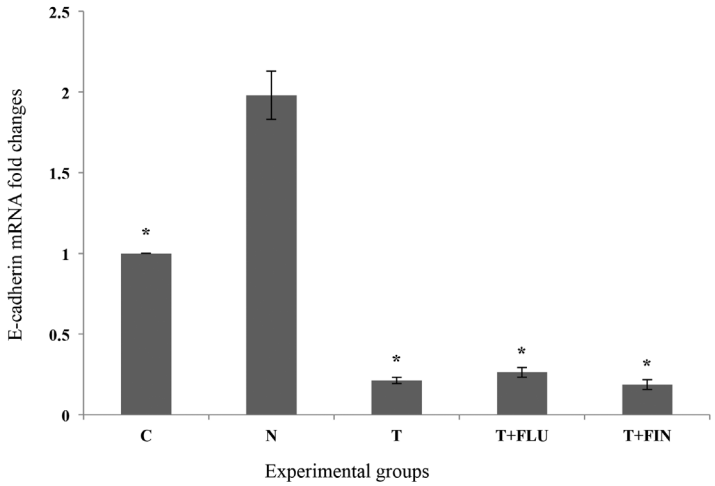
EFFECTS OF TESTOSTERONE ON MUCIN-1 mRNA EXPRESSION AND ITS PROTEIN DISTRIBUTION IN UTERUS

Effects on mucin-1 mRNA expression Figure 5 shows mucin-1 mRNA expression was highest in non-testosterone treated rats receiving sex-steroid replacement mimicking hormonal changes in early pregnancy. The expression level was approximately 1.53 ± 0.14 fold



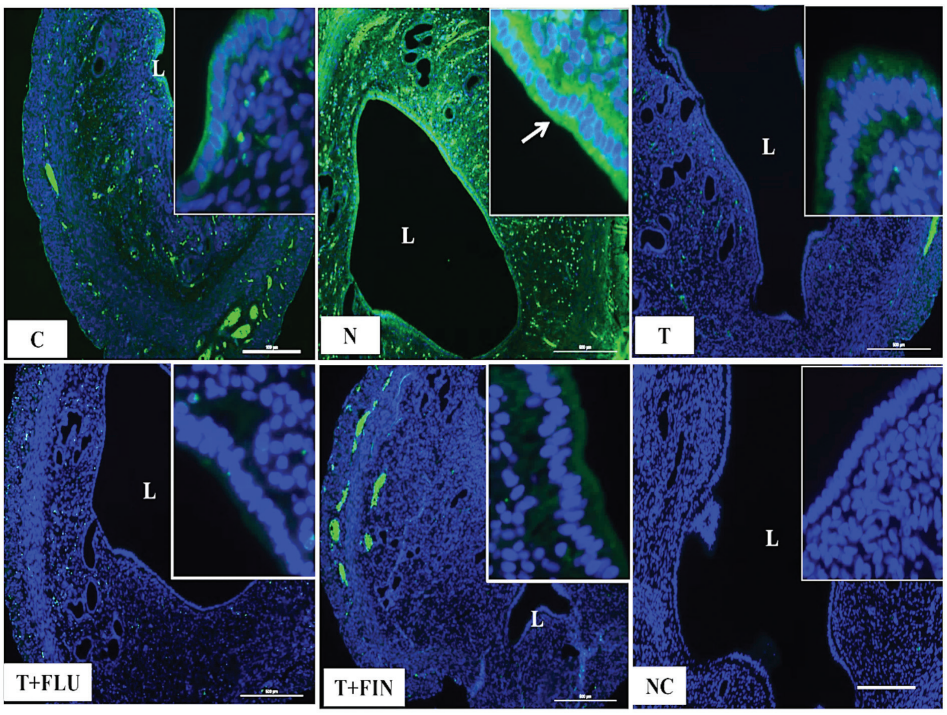
Immunofluorescence images showing high distribution of αvβ3 integrin in the luminal epithelium of rats which received sex-steroid replacement without testosterone. αvβ3 integrin was markedly reduced following testosterone treatment between days 6-8. C: vehicle control, N: sex- steroid replacement regime, T: testosterone only treatment, T + FLU: testosterone and flutamide and T + FIN: testosterone and finasteride. NC: negative control. Arrows pointing towards αvβ3 integrin distribution. L = lumen. Scale bar = 50 μm. *n*=6 per treatment group

FIGURE 2. Distribution of αvβ3 integrin protein in uterus



E-cadherin mRNA level was highest in rats receiving sex-steroid replacement without testosterone. E-cadherin mRNA levels were significantly decreased in rats receiving testosterone treatment with and without flutamide or finasteride. *p<0.05 compared to sex-steroid replacement. C: vehicle control, N: normal steroid replacement regime, T: testosterone only treatment, T + FLU: testosterone and flutamide injection and T + FIN: testosterone and finasteride injection. Data was obtained from six different rats and was expressed as mean ± S.E.M.

FIGURE 3. E-cadherin mRNA expression level in uterus

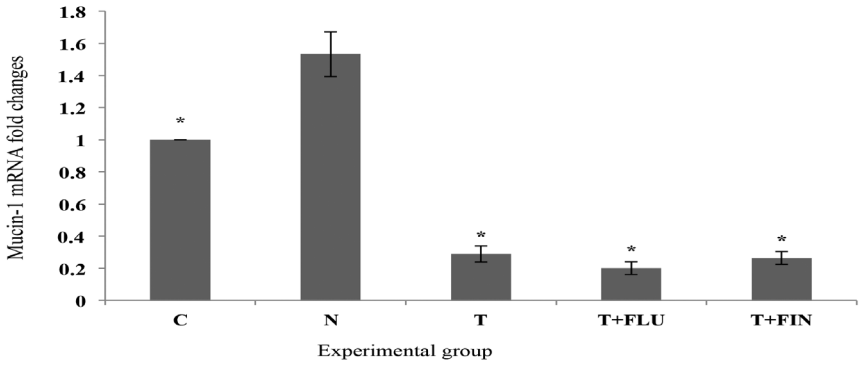


Immunofluorescence images showing high distribution of E-cadherin in luminal epithelium of rats which received sex-steroid replacement without testosterone. Distribution of E-cadherin was markedly reduced following testosterone treatment between days 6-8. C: vehicle control, N: sex-steroid replacement regime, T: testosterone only treatment, T + FLU: testosterone and flutamide and T + FIN: testosterone and finasteride. NC: negative control. Arrows pointing towards E-cadherin distribution. L = lumen. Scale bar = 50 µm. n=6 per treatment group

FIGURE 4. Distribution of E-cadherin protein in uterus

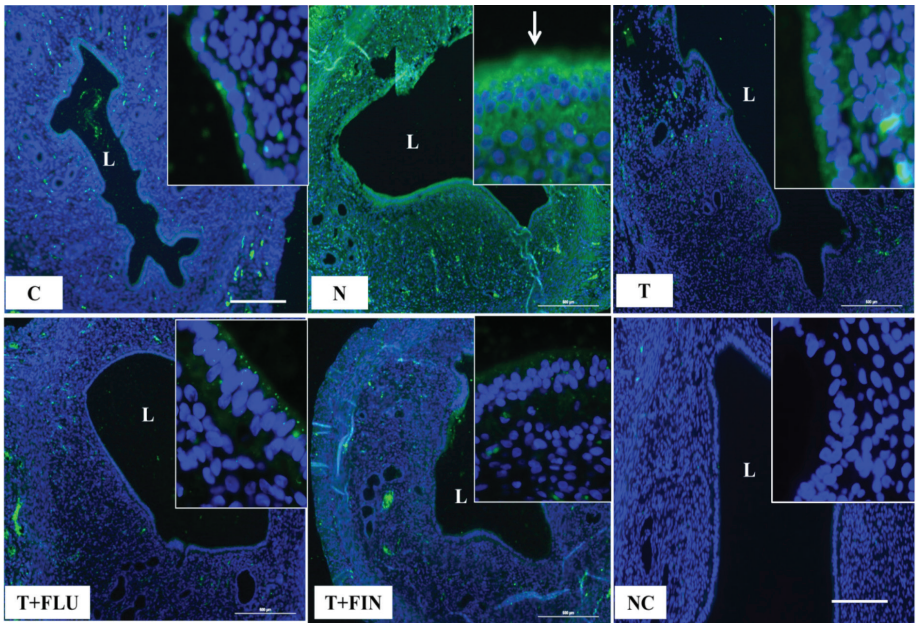
significantly higher than ovariectomised control (p<0.05). Administration of testosterone between days 6 and 8 resulted in significant decrease in mucin-1 mRNA level (p<0.05). Testosterone effect was neither antagonised by flutamide nor finasteride.

Effects on mucin-1 protein distribution Figure 6 shows mucin-1 was distributed in endometrial luminal epithelium, stroma and stromal blood vessels and myometrium. The high distribution of mucin-1 was observed in non-testosterone treated rats receiving sex-steroid replacement



The highest mucin-1 mRNA level was observed in rats receiving sex-steroid replacement without testosterone. Mucin-1 mRNA level was significantly decreased in rats receiving testosterone-only treatment with or without flutamide or finasteride. *p<0.05 compared to sex-steroid replacement. C: vehicle control, N: normal sex-steroid replacement, T: testosterone only treatment, T + FLU: testosterone and flutamide injection and T + FIN: testosterone and finasteride injection. Data were obtained from six different rats expressed as mean ± S.E.M.

FIGURE 5. Mucin-1 mRNA expression level in uterus



Immunofluorescence images showing distribution of mucin-1 which was highest in luminal epithelia of rats receiving sex-steroid replacement without testosterone. Low mucin-1 distribution was observed following testosterone treatment between days 6 - 8. C: vehicle control, N: sex-steroid replacement, T: testosterone only treatment, T + FLU: testosterone and flutamide injection and T + FIN: testosterone and finasteride injection. NC: negative control. Arrows pointing towards mucin-1 distribution. L = lumen. Scale bar = 50 μm. n=6 per treatment group

FIGURE 6. Distribution of mucin-1 protein in uterus

mimicking the hormonal changes in early pregnancy. Mucin-1 expression was markedly reduced following testosterone treatment between days 6 and 8 of the sex-steroid replacement. Concomitant administration of testosterone with either flutamide or finasteride did not result in any noticeable changes in mucin-1 distribution as compared to testosterone only treatment.

DISCUSSION

Blastocysts implantation occurs within a short window period known as uterine receptivity (Wang & Dey 2006). During this period, the uterus undergoes series of transformation and becoming receptive to the implanting blastocyst. Uterine receptivity period can be identified based on the presence of receptivity markers such as αvβ3 integrin, E-cadherin and mucin-1. In this study, expression of mRNAs and proteins of these markers was markedly increased between days 6 to 8 of sex-steroid replacement, intended to mimic the hormonal changes in early pregnancy. Our findings have shown that during this period, administration of testosterone has resulted in marked decrease in the level of expression of these markers in the uterus. Furthermore, testosterone effects were found neither involved genomic pathway nor mediated via the active testosterone metabolites, DHT as evidence from

the effect observed following administration of either flutamide (an androgen receptor blocker) or finasteride (a 5α-reductase inhibitor)

The possibilities that testosterone effect on uterine receptivity could involve the non-genomic pathway are supported by the reported non-genomic action of this hormone on the uterine smooth muscles (Perusquía et al. 1990). In addition, the non-genomic effect of testosterone on the uterus has also been shown by our previous findings in which this hormone down-regulates the expression of Cystic Fibrosis transmembrane regulator (CFTR) (Mohd Mokhtar et al. 2014), MECA-79 and pinopodes development during the uterine receptivity period in rats (Mokhtar et al. 2014). Absence or low level of 5α-reductase in the uterus might be the possible reason for the failure of testosterone to act on uterine cells via DHT.

αvβ3 integrin is one of the markers of uterine receptivity. This protein is involved in embryo recognition of the maternal endometrium (Reddy et al. 2001). Expression of αvβ3 integrin is markedly reduced in patient with infertility (Lessey 1998). Expression of αvβ3 integrin was found to be upregulated under the progesterone influence (Srinivasan et al. 2009). In this study, the up-regulated expression of αvβ3 integrin in the uterus which occur following progesterone replacement suggested that this hormone likely responsible for the observed increase in αvβ3 integrin level. Its down-regulated expression following administration of testosterone could be due to counteraction of the progesterone effects, leading to failure of maternal recognition of embryo which subsequently impairs the establishment of pregnancy.

Besides αvβ3 integrin, expression of E-cadherin in the uterus was also found to increase following progesterone replacement. This finding was consistent with a study which indicates that progesterone stimulates expression of E-cadherin in the uterus in mice (Jha et al. 2006). Expression of E-cadherin was also reported to increase at diestrus phase of the oestrous cycle, associated with high levels progesterone (Slater et al. 2002). The ability of testosterone to down-regulate expression of E-cadherin indicated that this hormone might impair embryo-uterus communication that is crucial for the successful establishment of pregnancy (Liu et al. 2006).

Expression of mucin-1 in the uterus during receptivity period is important as it forms scaffold that allows blastocyst to roll over the surface epithelium, thus preventing the embryo from sticking to the site other than that destined for implantation (Giudice 1999). The highest expression of mucin-1 was observed following progesterone replacement, consistent with the observation in humans in which mucin-1 was found to be highly expressed under the influence of progesterone (Aplin et al. 1998). Additionally, expression of mucin-1 which was highest at the apical membrane of uterine luminal epithelium supports its role in preventing blastocyst-endometrial adhesion (Horne et al. 2002). Down-regulation of mucin-1 by testosterone could result in premature adhesion of the embryo to the endometrium, which could halt the attachment and invasion.

CONCLUSION

In conclusions, decreased expression and distribution of αvβ3 integrin, E-cadherin and mucin-1 in uterus during receptivity period would impair successful embryo implantation and pregnancy establishment. This might contribute to infertility in women having high plasma testosterone in disease such as Polycystic Ovarian Disease or following excessive anabolic steroid intake.

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Helmy Mohd Mokhtar
Department of Physiology
Faculty of Medicine
Universiti Kebangsaan Malaysia Medical Center
Jalan Yaacob Latiff, Bandar Tun Razak
56000 Cheras, Kuala Lumpur, Federal Territory
Malaysia

Nelli Giribabu & Naguib Salleh*
Department of Physiology
Faculty of Medicine
University of Malaya
50603 Kuala Lumpur, Federal Territory
Malaysia

*Corresponding authors; email: naguib.salleh@gmail.com

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