Estrogen receptor (ER)-α, β and progesterone receptor (PR) mediates changes in relaxin receptor (RXFP1 and RXFP2) expression and passive range of motion of rats’ knee

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Purpose: The high risk of knee injuries in female may be associated with sex-steroid hormone fluctuations during the menstrual cycle by its effect on ligaments and tendons stiffness. This study examined changes in knee range of motion in presence of estrogen and progesterone and investigated the interaction of their antagonists to relaxin receptors.

Method: Sixty WKY rats were divided into 10 different groups receiving 17β-estradiol (0.2, 2, 20 and 50 μg/kg), progesterone (4 mg/kg), estrogen receptor (ER) antagonist ICI 182/780, ERβ antagonist PHTPP, ERα antagonist MPP, and mifepristone in presence of estrogen and progesterone. Physiologic dose were injected subcutaneously 30 min before of hormone injection for 3 days consequently. Sham group received peanut oil (vehicle) also for 3 consecutive days. Following the treatment administrations, the knee range of motion and RXFP1/RXFP2 mRNA and protein expression were examined in the patellar tendon, lateral collateral ligament, and hamstring muscle.

Results: Our data showed that the knee range of motion was significantly increased in progesterone and high doses estrogen treatment but not significantly increased in low doses of estrogen treatment. The range of motion was decreased in the presence of estrogen receptor (ER) antagonist ICI 182/780, ERβ antagonist PHTPP, ERα antagonist MPP, and mifepristone, independently.

Conclusion: Progesterone and high doses of estrogen treatment resulted in the highest range of knee laxity correlated to expression of both relaxin receptor isoforms in knee tissues. Our findings thus suggested that female subjects are more vulnerable toward non-traumatic knee injury due to estrogen and progesterone fluctuation as compared to male subjects.

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1. Introduction

Sex-steroids have been reported to regulate changes in knee laxity (Dehghan et al., 2014a,b). E2 (estrogen) cause increased (Dragoo et al., 2009) while testosterone cause decreased (Dehghan et al., 2014a,b) knee laxity in rodents. In humans, knee laxity was reported to increase at around the time of ovulation, indicating of E2 influence (Zazulak et al., 2006). Knee laxity was also reported to be the highest level in the luteal phase of the menstrual cycle, suggesting of P4 (progesterone) influence (Heitz et al., 1999). Meanwhile, high level of E2 was reported to cause increased anterior tibia dislocation in rats during pregnancy (Charlton et al., 2001). The mechanisms underlying E2 and P4 effects on knee laxity are not well understood, however evidences suggested that these effects could be mediated via changes in collagen turnover (Song et al., 2014), modulation of enzymes activity involved in collagen metabolism (Shan et al., 2013) as well as response of tissues toward relaxin via changes in the amount of receptor expressed (Dehghan et al., 2014a,b).

The effect of sex-steroids is preceded by the binding to intracellular receptor, located either in the cytosol or nuclear compartments (Bayard et al., 1978). Binding of ligands to this receptor will initiate translocation of ligand–receptor complex to...
the nucleus where it acts as a transcription factor which binds to hormone responsive elements (HRE) on DNA, initiating gene transcription (Wierman, 2007). Two forms of ER (estrogen receptor) have been identified, i.e. ERα and ERβ, which varies in the tissue distribution. Meanwhile, PR also varies in tissues distribution (Edwards, 2005). ER and PR have been identified in human anterior cruciate ligament (ACL) (Liu et al., 1996) which could explain changes in knee laxity under different sex-steroid influence. Sex-steroids modulate joint laxity either directly or indirectly. E2 was reported to cause a significant loss of glycosaminoglycans (GAGs) and collagen content from the pubic symphysis, temporomandibular joint disk and articular cartilage of the knee in rabbits (Hashem et al., 2006). E2 also affects the expression of several isoforms of metalloproteinase (MMPs-1, 3 and 13) as well as tissue inhibitor of metalloproteinases (TIMP 1) (Lee et al., 2003) which were involved in collagen turnover. Meanwhile, E2 and P4 were also reported to induce protein and mRNA expression of relaxin receptors (RXFP1 and RXFP2) which result in increased tissue response toward relaxin (Dehghan et al., 2014a,b).

Relaxin, a 6-kDa polypeptide is structurally related to insulin and is produced by the corpus luteum and placenta. Relaxin exerts various effects on the musculoskeletal system via binding to several isoforms of relaxin receptor (RXFP1, RXFP2, RXFP3 and RXFP4) (Dehghan et al., 2013) and participates in extracellular matrix remodeling (Sherwood, 1994). Relaxin relaxin receptor expression has been identified in human female ACL (Drago et al., 2003). Relaxin regulates synthesis and/or degradation of matrix macromolecules via modulating the activity of several members of MMP enzymes (Nagvi et al., 2005). Serum relaxin levels have been reported to correlate with the levels of MMP-1 in anterior oblique ligament of thumb (Wolf et al., 2013) while relaxin effect on MMP-9 was found to be mediated via binding to RXFP1 (Ahmad et al., 2012).

We hypothesized that E2 and P4 effects on knee laxity and the expression of RXFP1 and RXFP2 receptors were mediated via ER isoforms (ERα and ERβ) and progesterone receptor (PR), respectively. The aim of this study is therefore to investigate the involvement of sex-steroid receptors in mediating E2 and P4 effects on knee passive ROM and relaxin receptor isoforms expression in the patellar tendon, collateral ligaments and hamstring muscles which are involved in controlling knee joint movement.

2. Material and methods

2.1. Animal preparation

The Faculty of Medicine Animal Care and Use Committee (ACUC) of University Malaya (UM) approved the procedures involving animals; with ethic number F/IS/22/11/2011/FD/R. Adult female Wistar Kyoto (WKY) rats 8–10 weeks of age, 180–200 g of weight, were obtained from the Animal House, university of Malaya, and caged in groups of six (n = 6 per group), in a clean and well ventilated standard environment of 12 h light:dark cycle. The animals had a free access to soy-free diet (Gold Coin Pellet) and tap water ad libitum. Based on treatment, the animals were distributed into different groups. Since serum 17 beta-estradiol concentrations vary markedly throughout the menstrual cycle, different doses of this hormone (0.2, 2, 20 and 50 μg/kg), progesterone (4 mg/kg) (Gholami et al., 2013) were injected subcutaneously at the neck cuff with a 24-hour interval for 3 days consecutively. Associated injections with the non-selective estrogen receptor (ER) antagonist ICI 182/780 25 mg/kg (Peng et al., 2010; Wakeling et al., 1991), a selective estrogen receptor beta antagonist (ERβ), 4-[2-phenylo-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP) 25 mg/kg, a selective estrogen receptor α antagonist (1,3-bis-(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1N-pyrazole dihydrochloride) MPP 25 mg/kg (Al-Nakkash, 2012), and mifepristone 7.5 mg/kg (Baan et al., 2005) were injected subcutaneously at the neck cuff 30 min before physiologic doses of estrogen and progesterone injection. The doses of hormones administered were based on physiological levels and antagonists doses were selected based on effective dose of previous studies. Control group, which received peanut oil (vehicle) also for 3 consecutive days. The drugs were dissolved in peanut oil and were subcutaneously administered. After measuring of knee range of motion for transcriptome and proteome studies, expression level of relaxin receptor isoforms mRNA was detected in the tissues of patellar tendon, collateral ligament and hamstring muscle.

2.2. Blood samples

Blood samples from rats groups were taken after 3 days treatment by heart puncture during sciarifying at the same hour of the treatment days. The blood samples were labeled for each hormone that injected and testing session. All blood samples were analyzed for estrogen and progesterone hormone using radioimmunoassay (RIA).

2.3. mRNA expression analysis by real time PCR (qPCR)

Patellar tendon, collateral ligament, and hamstring muscle were exposed after 3 days treatment from rat knee and kept in RNA Later. RNA later solution was used to stabilize and protect tissues cellular RNA (Ambion, L/N: 1206029B). Total RNA was isolated from 30 mg tissues (wet weight) using the RNeasy Fibrous tissue Mini kit (QIAGEN, Germany). The RNA purity and concentration was assessed by 260/280 UV absorption ratios (Gene Quant 1300, UK). Each RNA sample was assessed by integrity of Ethidium-Bromide agarose gel 1% and evaluated by electrophoresis then reverse transcribed into cDNA (Two-Step qRT-PCR Kit, High capacity RNA to DNA – Applied Biosystems, USA). Two-step real time PCR was performed to evaluate gene expression, with application of TaqMan® RT-qPCR. Hprt1 and GAPDH were used as reference genes. TaqMan primer and probe for RXFP1, RXFP2, GAPDH and Hprt1 were provided from predesigned assays from Applied Biosystems, USA. All experiments were done with 3 biological replicates. Real time-PCR program include 2 min at 50 °C reverse transcriptase, 20 s at 95 °C activation of polymerase, denaturation at 95 °C for 1 s and annealing at 60 °C for 20 s. Denaturing and annealing steps were performed for 40 cycles. Stepone plus real time PCR machine, master mix and assays were purchased from Applied Biosystems (USA). Data were analyzed according to Comparative Ct (2–ΔΔCt) method (Wong and Medrano, 2005), that amplification of the target and of the reference genes were measured in the samples and reference. Measurements were normalized using the GenEx software. The relative quantity of target in each sample was determined by comparing normalized target quantity in each sample to normalized target quantity average in the references. Data Assist v3 software from Applied Biosystems (USA) was used to calculate RNA folds changes.

2.4. Protein expression analysis by Western blotting

Patellar tendon, collateral ligament, and hamstring muscle tissues were separated from fat then rinsed with 0.1% phosphate buffer. These tissues were snapped frozen in liquid nitrogen and then stored at −80 °C prior to protein extraction. The total protein extracted from 50 mg (wet weight) of tissues. After extraction of total protein with PRO-PREP (Intron, UK), equal amount of protein from each tissue lysate were mixed with loading dye, boiled for 5 min and separated by SDS-PAGE 10%. The proteins were then transferred to the PVDF membrane (BIORAD, UK) and blocked with 5% BSA for 90 min at room temperature. The membrane was exposed for 90 min to rabbit polyclonal primary RXFP-1/LGR7 and P2X7 antibodies.
Table 1
Hormone level of blood serum in treated and non-treated groups.

<table>
<thead>
<tr>
<th>Treated samples</th>
<th>Hormone level (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrogen</td>
<td>39.4 ± 0.14 pg/mL</td>
</tr>
<tr>
<td>Control</td>
<td>69.7 ± 0.12 pg/mL</td>
</tr>
<tr>
<td>OIC 182/780 + 0.2 E</td>
<td>33.2 ± 0.09 pg/mL</td>
</tr>
<tr>
<td>MPP + 0.2 E</td>
<td>29.8 ± 0.06 pg/mL</td>
</tr>
<tr>
<td>PHTPP + 0.2 E</td>
<td>21.3 ± 0.11 pg/mL</td>
</tr>
<tr>
<td>Progesterone</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.18 ± 0.11 ng/mL</td>
</tr>
<tr>
<td>4 mg/kg P</td>
<td>23.67 ± 0.13 ng/mL</td>
</tr>
<tr>
<td>Mifepristone + 4 mg/kg P</td>
<td>3.5 ± 0.17 ng/mL</td>
</tr>
</tbody>
</table>

Table 1
Hormone level of blood serum in treated and non-treated groups.

3.2. Knee range of motion

In Fig. 1, the increase in knee passive ROM in E2 treated group (p < 0.05 as compared to control) was antagonized by MPP and PHTPP however not by ICI 182/780. Knee passive ROM was the highest following P4 treatment (mean = 92.4 ± 4.13) which was antagonized by mifepristone (p < 0.01).

3.3. RXFP1 and RXFP2 mRNA expressions in the patellar tendon

In Fig. 2, (A) the expression level of RXFP1 mRNA in the patellar tendon of ovariectomized rats received E2 was reduced by approximately 0.81, 1.4 and 1.62 fold in the presence of ICI 182/780, MPP, and PHTPP, respectively, as compared to 0.2 μg E2 treatment alone. The expression of RXFP1 mRNA was the highest in the P4 treated group, which was antagonized by approximately 2.31 fold in the presence of PR blocker, mifepristone.

(B) The expression level of RXFP2 mRNA in patellar tendon following E2 treatment was significantly reduced by approximately 0.35, 0.97 and 1.12 fold in the presence of ICI 182/780, MPP, and PHTPP, respectively, as compared to 0.2 μg E2 treatment alone. The expression of RXFP2 mRNA in the patellar tendon which was the highest following treatment with P4 was significantly reduced by approximately 1.45 fold following PR antagonist, mifepristone administration.

(C) Meanwhile, RXFP1 and RXFP2 protein expressions in the patellar tendon were the highest following P4 treatment. Mifepristone caused a significant decrease in both isoforms expression. The expressions of RXFP1 and RXFP2 under E2, which were significantly higher than control, were inhibited by ICI 182/780, MPP and PHTPP. PHTPP caused the highest inhibition on RXFP1 and RXFP2 protein expression.

3.4. RXFP1 and RXFP2 mRNA expressions in lateral collateral ligament

In Fig. 3, (A) the expression levels of RXFP1 mRNA in ovariecomized rats collateral ligaments of the knee were decreased in the presence of steroid receptor antagonists. The expression of RXFP1 mRNA was increased following estrogen treatment and was reduced in the presence of different antagonists, by approximately 0.96, 0.81 and 1.07 fold decrease following administration of ICI 182/780, MPP and PHTPP, respectively. The expression of RXFP1 mRNA in the collateral ligaments which was the highest following administration of P4 was reduced in the presence of PR antagonist, mifepristone by approximately 2.28 fold.

(B) The expression level of RXFP2 mRNA in ovariecomized rats received E2 treatment was decreased in the presence of ERα and ERβ antagonists. The expression of RXFP2 mRNA in the collateral ligament was approximately 0.93, 0.73 and 1.09 fold lower following administration of ICI 182/780, MPP and PHTPP, respectively, as compared to in the absence of these receptor blockers. The expression of RXFP2 mRNA in collateral ligament was the highest following P4 treatment which was reduced by approximately 1.09 fold following PR antagonist, mifepristone administration.

(C) Meanwhile, RXFP1 and RXFP2 protein expression in the collateral ligament was significantly increased under P4 influence and was antagonized by mifepristone. Under E2 influence, these isoforms expression were slight but significantly higher than the control group. Administration of ICI 182/780, MPP and PHTPP resulted in a marked inhibition of RXFP1 and RXFP2 proteins expression under E2 with PHTPP administration caused the greatest inhibition.
3.5. RXFP1 and RXFP2 mRNA expressions in lateral hamstring muscle

In Fig. 4, (A) the expression level of RXFP1 mRNA in ovariectomized rats’ hamstring muscle under the effect of E2 was reduced in the presence of ER antagonists, ICI 182/780, MPP, and PHTPP by approximately 0.92, 0.71 and 1.07 fold, respectively, as compared to 0.2 μg E2 treatment alone. Expression of RXFP1 mRNA in the hamstring muscle was the highest following treatment with P4 and was markedly reduced in the presence of PR antagonists, mifepristone by approximately 2.31 fold as compared to P4 treatment alone.

(B) The expression level of RXFP2 mRNA in ovariectomized rats hamstring muscle was reduced by approximately 0.91, 0.83 and 1.09 fold following treatment with ICI 182/780, MPP, and PHTPP...
respectively as compared to treatment with 0.2 μg E2 alone. The expression of RXFP2 mRNA was the highest following P4 treatment which was reduced by approximately 1.09 fold in the presence of PR antagonist, mifepristone.

(C) Meanwhile, in the hamstring muscle, RXFP1 and RXFP2 proteins were expressed the highest in P4 treated group, which were antagonized by mifepristone. E2 treatment resulted in slightly higher RXFP1 and RXFP2 protein expression that were markedly inhibited following administration of PHTPP, MPP and ICI 182/780.

4. Discussion

This study reveals the presence of ERα, ERβ and PR in patellar tendon, collateral ligaments and hamstring muscle of rats. In this study, the administrations of PHTPP, an ERβ antagonist markedly reduce the expression of RXFP1 and RXFP2 mRNA in the patellar tendon, collateral ligaments and hamstring muscles. PHTPP is a specific ERβ antagonist with a 36-fold selectivity over ERα, therefore its inhibition on estrogen effect would confirm the involvement of ERβ subtypes in mediating the biological effects of estrogen (Compton et al., 2004). Previous study has also reported the presence of ER (general) in the synovocytes, fibroblasts and ligament’s blood vessel of the knee joint (Liu et al., 1996) which could support our functional observations.

Meanwhile, the effect of ICI 182/780 (fulvestrant), a potent anti-estrogen which was known to disrupt receptor translocation into the nucleus as well as increase receptor degradation (Osborne et al., 2004; Wakeling, 2000) on E2-mediated expression of relaxin receptors was also documented in this study. ICI 182/780 binds to ER with affinity approximately 100 times greater than tamoxifen, a partial anti-estrogen however with no agonist activity on estrogen sensitive tissue such as uterus (Robertson, 2001). Our findings which indicate that ICI 182/780 administration did not result in a significant decrease in RXFP1 and RXFP2 mRNA expression in the patellar tendon of rats received physiologic dose of E2 treatment suggested that ligand-receptor complex translocation into the nucleus may not be required for mediating E2 effect in this tissue, most probably due to the presence of abundance nuclear ER. In mouse, ICI 182/780 has been reported to disrupt nuclear-cytoplasmic shuttling where the receptor could be retained in the cytoplasm, therefore blocking E2 effect (Dauvois et al., 1993). We speculated that in the tendon, cytoplasmic shuttling may not occur, therefore E2 effects could be minimally affected by ICI 182/780. There is also a possibility ICI 182/780 may not reached the target site in the tendon due its poor vascularization as compared to synovium (Rempel and Abrahamsson, 2001). In contrast, ICI 182/780 exerts a strong inhibition on RXFP1 and RXFP2 mRNA expressions in the collateral ligaments and hamstring muscles indicating that these tissues were responsive toward ICI 182/780 inhibition. Administration of a specific ERα antagonist, MPP also resulted in a significant inhibition of RXFP1 and RXFP2 mRNA in the patellar tendon, collateral ligament and hamstring muscles; however, the degrees of inhibition were significantly lesser than following PHTPP treatment. This finding indicates that despite of its presence, ERα is not the main isoform expressed in these tissues.

Our findings further revealed that RXFP1 and RXFP2 protein expressions follow an almost identical pattern to that of mRNA. Administration of ICI 182/780, MPP and PHTPP significantly inhibit...
these protein expression in the muscle, tendon and ligaments surrounding the knee joint. PHTPP exerts the strongest inhibition therefore confirmed that ERβ is the main ER isofrom expressed. Milder inhibition by MPP suggested that ERα was also involved in mediating RXFP1 and RXFP2 expression while variable degree of inhibition by ICI 182/780 indicates that E2 in general is required for the expression of relaxin receptor isoforms proteins. Meanwhile, the involvement of PR in mediating the up-regulation of RXFP1 and RXFP2 mRNA in the patellar tendon, collateral ligaments and hamstring muscles was confirmed from the inhibition by mifepristone. In addition to these knee-related structures, PR was also reported to be expressed in the synovium and ACL of human knees (Liu et al., 1996), which could explain P2g effect on knee laxity.

Our functional study revealed the lack of relaxin effect in rats received ERα, ERβ and PR antagonists which indicate that these receptors were required to mediate relaxin effects on the knee. P4 administration caused increased in knee passive ROM as well as in the groups treated with high doses of E2 however not in the control group, suggesting that these hormones were required to mediate relaxin effects in causing increase in knee passive ROM. Relaxin could act directly via inducing the activity of MMP, an enzyme responsible for collagen breakdown (Naqvi et al., 2005). E2 and P4 have been shown to up regulate the expression of relaxin receptors in ovariectomised rats’ knee (Dehghan et al., 2014a,b), therefore relaxin effects were augmented in the presence of these hormones. Our findings confirmed that both E2 and P4 up-regulate RXFP1 and RXFP2 in the patellar tendons, hamstring muscles and collateral ligaments via ERβ/ERα and PR which cause the increase in knee laxity under these hormones influences.

In conclusion, this study has provided evidences to support the involvement of ERα, ERβ and PR in mediating E2 and P4-induced increase in knee passive ROM and RXFP1 and RXFP2 expression in the structures controlling knee joint movements. We have shown that E2 effects were mediated mainly via binding to ERβ. Modulation of relaxin receptor expression by selective antagonists to ERβ and PR could be useful to prevent the increase in knee laxity under different sex-steroid influence thus could help to lower the incidence of non-contact knee injury which is related to different phases of the reproductive cycle.

Conflict of interest

There is no conflict of interest in this study.

Transparency document

The Transparency document associated with this article can be found in the online version.

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References
