THE EFFECTS OF SEX-STEROIDS AND MENSTRUAL CYCLE/OESTROUS PHASES ON KNEE LIGAMENT LAXITY IN HUMANS AND RODENTS

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THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

FACULTY OF MEDICINE

UNIVERSITY OF MALAYA

KUALA LUMPUR

2014

ORIGINAL LITERARY WORK DECLARATION

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Name of Degree: PhD

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"): THE EFFECTS OF SEX-STEROIDS AND MENSTRUAL CYCLE/OESTROUS PHASES ON KNEE LIGAMENT LAXITY IN HUMANS AND RODENTS

Field of Study: EXERCISE PHYSIOLOGY

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ACKNOWLEDGEMENTS

It is my pleasure to thank the people who have made this thesis possible. First of all, I would like to express my genuine gratitude to my supervisor, Dr. Naguib Salleh, my project leader from Department of Physiology, Faculty of Medicine, University of Malaya for his excellent supervision, advice, and guidance from the beginning stages of this study as well as providing me with valuable experience throughout the work.

A special thanks to my supervisor, Dr. Ashril Yusof from Department of Exercise Science, Sport Centre, University of Malaya for the privilege of working under exemplary guidance and his advice, patience and encouragement throughout this study.

I would like to take this opportunity to appreciate Prof. Dr. Sekaran Muniandy from Department of Molecular Medicine, Faculty of Medicine, University of Malaya for his support and providing me such a friendly environment in his lab to work.

I highly appreciated to AP Dr. Abdul Halim Mokhtar from Department of Sport Medicine, University of Malaya for his kindly support in this project.

My deepest and most heart-felt thanks go to my most beloved family, for their endless love and support.

To those directly and indirectly involved in this study, your contribution shall not be forgotten, and please accept my utmost appreciation.

ABSTRAK

Insiden kecederaan lutut "*non-contact*" dilaporkan lebih tinggi pada wanita berbanding lelaki. Bagi wanita, insiden ini adalah berkaitan dengan fasa kitaran haid. Memandangkan ini, kami menyiasat perkara berikut: (i) perubahan dalam kelalaian lutut dalam model tikus di bawah pengaruh seks-steroids yang berbeza; (ii) Mekanisme perubahan dalam kelalaian lutut yang melibatkan ungkapan pembezaan reseptor relaxin dan (iii) antara lutut asas kelalaian dan serum tahap hormon seks - steroids dan relaxin di fasa kitaran haid. Dalam bahagian pertama kajian, tikus ovariectomized WKY telah dibahagikan kepada kumpulan rawatan yang berbeza (n = 6 bagi setiap kumpulan): dos berbeza 17 β-oestradiol, progesteron dan testosteron (125 & 250 μg/kg). Hormon telah disuntik subcutaneously selama 3 hari berturut-turut. Pada masa yang sama, steroid hormon penghalang reseptor dan penghalang enzim (ICI 182780, PHTPP, MPP, mifepristone, Flutamide, dan Finasteride) juga telah disuntik dengan rawatan agonist steroid masing-masing. Pada masa yang sama, tikus-tikus utuh telah dikumpulkan berdasarkan peringkat kitaran oestrous mereka: proestrus , estrus, metestrous dan diestrus. Dalam ovariectomized steroid tikus dirawat dan utuh, pelbagai lutut gerakan (ROM) telah diukur dengan menggunakan goniometer kecil digital. Tikus-tikus telah dikorbankan dan ungkapan isoforms relaxin reseptor, RXFP1 dan RXFP2 mRNAs dan protein dalam ligamen lutut dan tendon ditentukan.Sementara itu, pada manusia, perubahan dalam kelalaian lutut di fasa kitaran haid diperhatikan dalam atlet wanita.Varus lutut dan valgus sudut telah ditentukan dalam atlet dan bukan atlet fasa kitaran haid mereka dengan menggunakan goniometer pemerintah ortopedik.Tahap serum oestrogen, progesteron dan relaxin telah ditentukan dalam subjek manusia di pelbagai fasa kitaran haid mereka.Hasil: data kami menunjukkan bahawa ROM lutut pada tikus telah meningkat dengan ketara berikutan rawatan oestrogen

dan progesteron bagaimanapun susut berikutan rawatan testosteron.Perubahan ROM lutut telah dihalang oleh antagonis terhadap reseptor oestrogen, progesteron dan androgen. ROM lutut adalah tinggi di peringkat proestrus dan diestrus kitaran oestrus. Progesteron dan oestrogen merangsang manakala testosteron menghalang perubahan RXFP1 dan RXFP2 mRNA dan ungkapan protein yang dihalang oleh penghalang reseptor masing-masing.Sementara itu, rawatan dengan relaksin meningkatkan ROM lutut oestrogen dan progesteron dirawat tetapi tidak testosteron dirawat ditunjukkan daripada perubahan pengawalaturan reseptor relaksin.Pada manusia, perbezaan yang signifikan dalam varus dan valgus sudut diperhatikan dalam wanita di pelbagai fasa kitaran haid dengan yang tertinggi pada fasa ovulasi dan haid kitaran.Kajian ini memberikan gambaran tentang kesan seks steroid pada ROM lutut dan RXFP1 dan ungkapan RXFP2. Dalam tikus, ungkapan peningkatan RXFP1 dan RXFP2 boleh menjelaskan peningkatan dalam lutut kelalaian bersama. Pada manusia, meningkat kelalaian di ovulasi dan fasa luteal pertengahan kitaran tersebut selaras dengan tahap yang tinggi oestrogen dan progesteron. Dalam kedua-dua tikus utuh dan manusia, korelasi yang kuat antara kelalaian lutut dan tahap serum relaksin telah diperhatikan. Penemuan kami boleh menjelaskan kelemahan yang mendasari dasar wanita terhadap kecederaan lutut bukan traumatik di fasa kitaran reproduktif mereka.

ABSTRACT

The incidence of non-contact knee injury was reported higher in female than male. In female, the occurrence of this injury was related to different phases of the menstrual cycle. In view of this, we investigated the followings: (i) changes in knee laxity in rodent model under different sex-steroid influence; (ii) mechanisms underlying changes in knee laxity, which involves differential expression of relaxin receptors and (iii) correlation between knee laxity and serum levels of sex-steroids and relaxin at different phases of the menstrual cycle. In first part of the study, ovariectomized WKY rats were divided into different treatment groups (n=6 per group): different doses of 17 β-oestradiol, progesterone and testosterone were administered. The hormones were injected subcutaneously for 3 consecutive days. In parallel, steroid hormone receptor blockers and enzyme inhibitor (ICI 182780, PHTPP, MPP, Mifepristone, Flutamide, and Finasteride) were also injected with the respective agonist. In parallel, intact rats were grouped based on their oestrous cycle stages: proestrus, estrus, metestrus and diestrus. In ovariectomized steroid treated and intact rats, knee range of motion (ROM) was measured by using a digital miniature goniometer. The rats were sacrificed and expression of relaxin receptor isoforms, RXFP1 and RXFP2 mRNAs and proteins in knee ligaments and tendons were determined. Meanwhile, in humans, changes in knee laxity were observed at different phases of the menstrual cycle in the female athletes. The knee varus and valgus angles were determined at different phases of the menstrual cycle by using an orthopedic goniometer ruler. Blood was withdrawn and serum levels of oestrogen, progesterone and relaxin were determined in human at different phases of their menstrual cycle. Our findings showed a significant increase in knee ROM in rats following oestrogen and progesterone treatment however was decreased following testosterone treatment. Changes in knee ROM was antagonized by the concomitant

administration of oestrogen, progesterone and androgen receptor antagonists. Knee ROM was high at proestrus and diestrus stages of the oestrous cycle. Progesterone and oestrogen up-regulated while testosterone down-regulated RXFP1 and RXFP2 mRNA and protein expressions which were antagonized by the respective steroid hormone antagonist. Meanwhile, relaxin administration increases knee ROM in oestrogen and progesterone treated but not testosterone treated groups indicating relaxin receptor up-regulation. In humans, significant difference in knee varus and valgus angles were observed in female athletes and non-athletes at different phases of the menstrual cycle which was the highest in the ovulatory and menstrual phases. Non-athletes have higher medial and lateral knee laxity as compared to athletes. This study has provided an insight into the mechanism underlying sex-steroid control of knee ROM via modulating the expression of RXFP1 and RXFP2 receptors. In rodents, increased RXFP1 and RXFP2 expressions could contribute to increase in knee laxity. In humans, the increase in laxity at ovulatory and mid-luteal phases of the cycle was consistent with high level of oestrogen and progesterone. In both intact rats and humans, strong correlations was noted between knee laxity and serum relaxin levels. Our study has provided the basis underlying female susceptibility towards non-traumatic knee injury at different phases of their reproductive cycle.

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LIST OF SYMBOLS ANDABBREVIATIONS

AC	Adenylate cyclase
ACL	Anterior cruciate ligament
APS	Ammonium per sulphate
APS	Ammonium Persulphate
BBT	Basal body temperature
BF	Beef Heifer
BSA	Bovine Serum Albumin
D	Dog
Ds	Diestrous
E2	Oestrogen/oestradiol
ELISA	Enzyme-linked immunosorbent assay
Es	Estrus
F	Fish
Fin	Finasteride
Flu	Flutamide
G	Guinea pig
GPCRs	G-protein-coupled receptors
H	Human
ICI 182/780	7α,17β-[9-[(4,4,5,5,5-Pentafluoropentyl) sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol
INSL	Insulin-like peptide
IP	Intraperitoneal
LCL	Lateral collateral ligament
M	Mouse
MCL	Medial collateral ligament
Mife	Mifepristone
MMPs	Matrix metalloproteinases
MPP	1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1N-pyrozole dihydrochloride
Ms	Metestrous
NO	Nitric oxide

OPG	Osteoprotegerin
P	Progesterone
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase Chain Reaction
PHTPP	4-[2-phenyo-5,7-bis(trifluoromrthyl) pyrazolo(1,5-a) pyrimidin-3-yl] phenol
Ps	Proestrous
PT	Patellar tendon
PVDF	Poly Vinylidene fluoride membrane
R	Rat
RA	Rheumatoid arthritis
RANKL	Receptor activator of nuclear factor κB ligand
Rb	Rabbit
RIA	Radioimmunoassay
RLN	Relaxin
ROM	Range of motion
RXFP	Relaxin family peptide receptors
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel
SST	Sample separator tube
T	Testosterone
TNF	Tumour necrosis factor
WB	Western blotting
α	Alpha
β	Beta

CHAPTER 1 INTRODUCTION

1.1 Introductions

Musculoskeletal injuries are common problem among athletes and are not gender specific (Arendt *et al.*, 1999), however, female have been reported to have higher risk of ligament injury as compared to male (Ireland, 2002; Myklebust *et al.*, 1998). Sprains of the knee joint are common in athletes who participate in direct contact sports such as football or soccer, who are more likely to injure their collateral and anterior cruciate ligaments. It has been proposed that the incidence of knee injury in female is related to phases of the menstrual cycle. The softening of ligaments in the luteal phase of the cycle has long been attributed to an increase in collagenase enzyme activity (Qin *et al.*, 1997). An increasing trend of non-contact knee injury has also been reported in the ovulatory phase and this decrease during the follicular phase however due to the conflicting reports, no conclusion could be drawn from the previous studies on the effect of menstrual cycle phases on this injury in female (Wojtys *et al.*, 1998).

The amplitude of joint laxity is related to the joint movement which was limited by ligaments and tendons. The relationship between different phases of menstrual cycle and knee joint laxity is still uncertain. Some researchers demonstrated that no association between knee laxity and phases of the menstrual cycle (Belanger *et al.*, 2004; Karageanes *et al.*, 2000). Meanwhile, knee laxity has been reported to be high in the ovulatory phase with a peak in oestrogen concentration (Heitz *et al.*, 1999; Romani *et al.*, 2001). Studies have revealed that these differences could be related to the differential expression of sex-steroid receptors in the skeletal muscle (Dragoo *et al.*, 2003; Lemoine *et al.*, 2003) in both humans

and animals (Sciore *et al.*, 1998), which could explain differences in the incidence of non-contact knee injury at different phases of the cycle.

Fluctuation in relaxin receptor expression in the knee was postulated based on the observed fluctuation in its expression in the uterus, being the lowest during the proliferative phase and rises sharply at the time of ovulation (Bond *et al.*, 2004). In neonatal porcine uterus, oestrogen increases RXFP1mRNA expressions, while in cervix, oestrogen increases and relaxin decreases these receptors expression (Yan *et al.*, 2008). During late pregnancy in mice, relaxin has no influence on RXFP1 expression (Siebel *et al.*, 2003). Progesterone has been reported to increase the expression of RXFP1 in the pregnant rat uteri (Vodstrcil *et al.*, 2010a) while oestrogen was found to regulate RXFP1 mRNA expression in human cervix at term (Maseelall *et al.*, 2009). Relaxin receptors expression may vary in the musculoskeletal tissues and these changes may affect joint laxity. Nonetheless, the effect of sex steroids on joint laxity in female remains inconclusive.

The laxity of the knee joint could potentially be affected by relaxin. Currently, little is known with regards to RXFP1 and RXFP2 expressions in the knee. Relaxin levels rise in the second half of the menstrual cycle in parallel with an increase in progesterone level. Wang *et al*. (2009b) reported relaxin receptors were expressed in knee fibrocartilage in mouse. In humans relaxin receptor expression have been identified in the ACL in male and female knees (Faryniarz *et al.*, 2006), with higher expression in female (Dragoo *et al.*, 2003). This gender specific difference suggests the influence of circulating female sex hormones on the expression of relaxin receptor in the knee. In view of that relaxin has been implicated in controlling joint laxity and relaxin receptor expression is under sex-steroid influence, therefore correlation between plasma levels of relaxin and sex-steroids in female

need to be established in order to gain insight into the possible association between these hormones in the pathogenesis underlying non-traumatic knee injury in female.

Knee joint relies on ligaments and surrounding muscles for stability. Sex-steroids have been reported to influence strength and laxity of knee ligaments and possibly affecting the muscles. The incidence of knee injury as a result of anterior cruciate ligament (ACL) tear has been widely reported and this was high during the luteal phase of the menstrual cycle (Pehrsson *et al.*, 2007). Relaxin receptors have been reported to be expressed in guinea pig ACL (Dragoo et al, 2003). Concomitant expression of relaxin and oestrogen receptors have also been reported in the ACL (Fairyniarz *et al*, 2006). Apart from ACL, the expression of relaxin receptors in other knee structures including patellar tendon and collateral ligaments is currently unknown. Together with ACL, collateral ligaments participate in the control of knee stability during movement. Patellar tendon is an important part of the extensor mechanism of the lower extremity and is also involved in preventing excessive knee hyperextension.

We hypothesized that the expression of relaxin receptors, RXFP1 and RXFP2 in the knee collateral ligaments, patellar tendon and hamstring muscles is under the influence of sex-steroids (oestrogen, progesterone and testosterone), and changes in these receptors expression may explain changes in knee laxity under different sex-steroid effect and at different phases of the menstrual/ oestrous cycle. We further hypothesized that relaxin levels correlate with the levels of sex-steroids in plasma and this would complement the influence of sex-steroids on the changes in RXFP1 and RXFP2 expressions. We also hypothesized that there is a correlation between rotational laxity of the knee and the levels of circulating sex-steroids and relaxins in females which could explain changes in knee

laxity at different phases of the menstrual cycle which were observed in both athletes and non-athletes females. In this study, ACL was not investigated since the relationship between sex-steroids and changes in ACL laxities have been well studied previously.

The specific objectives of this study are:

1- to identify the effects of sex steroids (oestrogen, progesterone, and testosterone) on the expression of RXFP1 and RXFP2 in the patellar tendon, collateral ligament, and hamstring muscles in rodents.

2- to confirm the effect of individual sex steroids on RXFP1 and RXFP2 expressions in the patellar tendon, collateral ligaments and hamstring muscles via concomitant administration of their respective antagonists. Additionally, the effect of sex-steroids with and without their respective antagonist on knee passive range of motion (ROM) will also be investigated.

3- to observe the relationship between plasma sex-steroid and relaxin levels with the expression of relaxin receptor isoforms and changes in knee passive ROM at different phases of oestrous cycle of rats.

4- to investigate changes in medial and lateral knee laxity during phases of the menstrual cycle in athletes and non-athletes and to observed the correlation between serum oestrogen, progesterone, testosterone and relaxin levels with knee laxity at different phases of menstrual cycle in both groups.

1.2 Significance of the study

Many common non-traumatic joint injuries in females especially during sports are attributed to the fluctuation in the reproductive hormones profiles. Women at a specific period of their menstrual cycle may be more prone to some form of non-traumatic knee injury. This study identifies changes in knee laxity and explore mechanisms underlying these changes which was related to differential expression of relaxin receptor isoforms, RXFP1 and RXFP2 and the fluctuation of sex-steroids and realxin levels. In view of this, detailing the mechanism underlying sex steroid influence on knee joint laxity is important in order to explain the high incidence of knee-related injury of the reproductive age female especially during sports. This information is valuable to the athletes and trainers in dedicating a specific time during the menstrual cycle period to performed exercise in order to reduce the risk of the non-traumatic joint injuries.

CHAPTER 2 LITRATURE REVIEW

2.1 Introductions

This chapter presents information background, which is tailored to the specific objectives of this study.

2.2 Overview of non-traumatic knee injury in female

The most common injuries related to joints have been reported to involve the ankles and the knees. Knee injuries which occur during sports is mostly associated to sub-luxation or dislocation. In view of this, a clear understanding on the injury pattern, the mechanisms underlying this injury and the risk factors is crucial in exercise physiology and sports medicine (Junge *et al.*, 2008). Knee injuries undoubtedly affect the athlete performances. The most common type of injuries are the non-contact, which occurs during activities such as decelerating, landing, cutting, and pivoting (Traina & Bromberg, 1997). The risk factors for this injury could be related to the equipments, shoe-surface and internal factors including the anatomical and hormonal defects (Griffin *et al.*, 2000).

A remarkable number of non-traumatic injuries among women during sports over the years has led to multiple studies being performed in order to better understand the underlying mechanism involved. Females are known to be 2 to 9 times more vulnerable than males towards knee injury (Arendt *et al.*, 1999; Dragoo *et al.*, 2009). In female, the occurrence was related to different phases of the menstrual cycle (Dragoo et al, 2011a). Several reports indicated that high incidence of non-contact knee injury happened during the follicular phase of the cycle, while others reported that the incidence is the highest at ovulation and in the luteal phase of the cycle (Shultz *et al.*, 2004; Shultz *et al.*, 2005). These raised the possibility that female sex hormones could be involved in this injury. The role of sex steroids on knee injury remains poorly understood and represent an area for

investigation. Table 2-1 below summarizes the study related to sex-steroid influence on knee injury in female.

Table 2-1 Previous reported data of the non-traumatic knee injury and menstrual cycle phases

Author	**Year**	**Target**	**Sampling**	**Model**	**Hormone**	**Conclusion**
(Fouladi *et al.*)	2012	Knee joint position sense (JPS)	menstrual cycle phases	healthy female athletes	oestrogen/ progesterone	different levels of knee JPS across a menstrual cycle
(Ruedl *et al.*)	2011	ACL injury	questionnaire	skiers female non-contact ACL injury	NI	ACL injuries in skiers are intrinsic / extrinsic risk factors
(Dragoo *et al.*)	2011	ACL injury	mid-luteal phase/ questionnaire	female athletes	Relaxin/progesterone	correlation progesterone /relaxin with ACL injury
(Montgomery & Shultz)	2010	knee flexors and extensors	menstrual cycle phases	active women	oestradiol/ progesterone /testosterone	no change from time of menses Isometric knee-extension /flexion torque
(Cesar *et al.*)	2011	knee valgus angles	menstrual cycle phases	non-athletic females	progesterone	valgus angles were less in the luteal phase compared to both follicular phases
(Bell *et al.*)	2009	hamstring muscle properties change across menstrual cycle	menstrual cycle phases	Normal women	oestrogen	hamstring muscle stiffness not changed in menstrual cycle/ extensibility increased at ovulation with oestrogen increases
(Park *et al.*)	2009	knee laxity and stiffness	menstrual cycle phases	healthy female	Oestradiol/ progesterone	increased knee joint laxity at ovulation
(Kerksick *et al.*)	2008	muscle injury, in eccentric exercise	NI	healthy men and eight women	Oestradiol/ lactate dehydrogenase	muscle strength changes were similar among genders
(Beynnon *et al.*)	2006	ACL injury	menstrual cycle phases	Female recreational alpine skiers	progesterone	preovulatory phase were more likely to ACL tear than postovulatory phase
(Agel *et al.*)	2006	ACL injury and ankle sprains	menstrual cycle phases	Female basketball and soccer players	NI	ACL injury /ankle sprains rate not linked to hormonal therapy

2.2.1 Anatomy of the knee joints

Knee being one of the largest joint in the body is vital for movement. The anatomy of this joint is reflective of its function in ambulation. The knee joint is a relatively complex anatomical structure. Knee joint connects thigh bone (femur) to the shin bone (tibia) with smaller bone that runs alongside the tibia (fibula) and the knee cap (patella) that make the knee joint. The patella bone is located into the thin anterior wall of the knee joint capsule. The ligaments, tendons, and capsule are components of joints guard the joint stiffness and laxity (Schmitz *et al.*, 2013). In addition, knee joint is controlled by a variety of surrounding connective tissues including ligaments, muscles, tendons, menisci, cartilage, and bursae to maintain stability.

The ligaments surrounding the knee connect bones and provide stability by limiting movements and together with menisci and bursae protect the articular capsule. These ligaments consist of: cruciate ligaments that avoid femur from sliding forward / backward on the tibia and collateral ligaments that avoid femur from sliding side to side. Collateral ligaments provide additional stabilization of the knee and direct movement in a correct direction. They are divided into medial and lateral collateral ligaments that resists knee rotational movement. The suprapatellar bursae prevents the knee from being pinched during extension (Gill *et al.*, 2009). Tendons connect the bones to the leg muscles that move the knee joint. A tendon that extends down from the quadriceps muscle incorporate the patella bone and attach to the tibia, providing extension at the knee joint. The patellar tendon is also called patellar ligament because it connects patella to the tibia (Dye *et al.*, 1998).

The elastic cartilage ensures knee movement and protects the bone to slide easily of the joint surfaces. The menisci protects the ends of bones from rubbing on each other and to

effectively deepen the tibial sockets into which the femur attaches. Numerous bursae, or fluid-filled sacs, help the knee move smoothly (Lewin, 1952). Knee joint consist of connective tissue with extensive collagen fibres containing cartilage-like cells. Strong fibres run along the menisci from one attachment to the other, while weaker radial fibres are interlaced with the former. The joint is bathed in synovial fluid, which is contained inside the joint capsule. The muscles surrounds the knee consist of the hamstrings, quadriceps, and calf muscles. These muscles support knee to flex, extend, stabilize, and work in groups to allow the body to perform important movements such as walking, running, kicking, and jumping (Amis *et al.*, 2003).

Knee is one of the most distressed joints in the physical activity due to large extreme of activities and its principal movements (flexion/extension/abduction/adduction). This joint was mainly designed to support locomotion movement and weight stability of the body posture. However, it is especially unstable medially and sideways. The knee traumatic and non-traumatic injuries caused by ligamentous instability can be evaluated to determine any differences in their stability. Ligamentous instability can be classified as either straight or rotatory instability (Granchi *et al.*, 2008). Special tests for ligaments assessment exists to disclose of knee joint function instability. Lachman drawer and pivot-shift test are performed to elicit cruciate ligaments laxity, while varus/valgus stress test for assessment of collateral ligaments integrity. These tests are usually performed with the knee in extension at 90 and 30 degrees (Devan *et al.*, 2004).

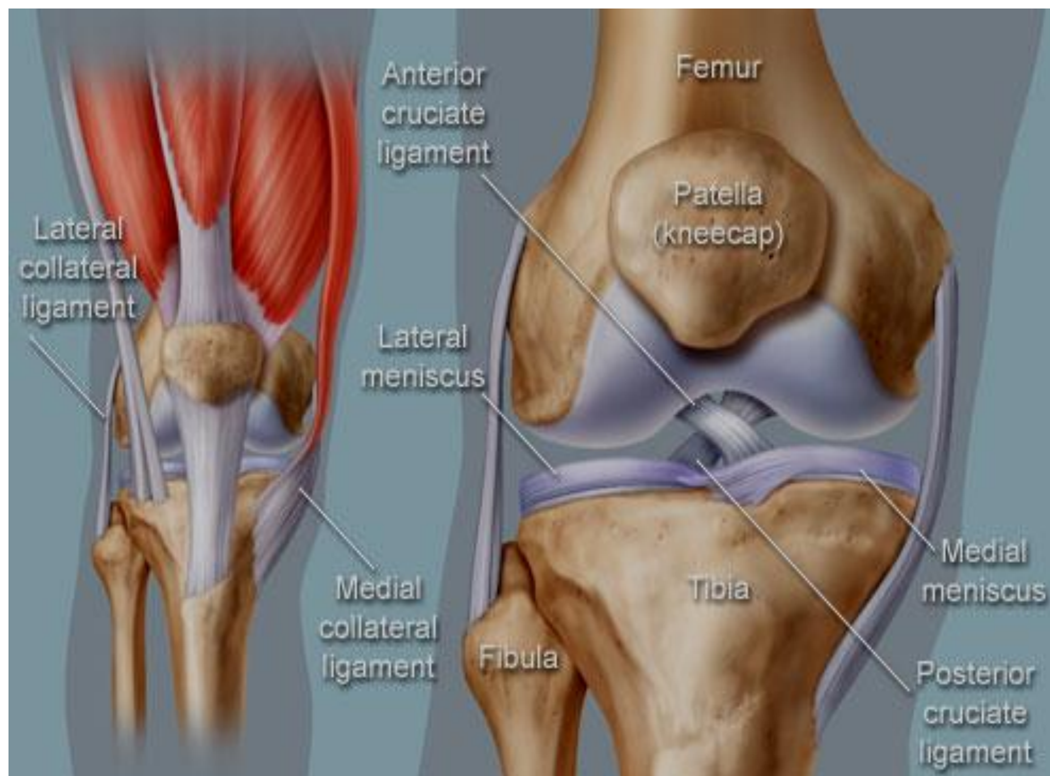


Figure 2-1 Knee anatomical perspective (www.webmd.com)

There are evidences to suggest that knee laxities are gender specific and the effect on female knee are greater than male (Hsu *et al.*, 2006; Wojtys *et al.*, 2003). Additionally, greater knee and ankle laxities have been reported in women as compared to men (Beynnon *et al.*, 2005). Range of motion (ROM) is defined as a movement potential of a joint from full flexion to full extension. Many reports have suggested that hormonal fluctuation could affect knee laxity and subsequently knee range of motion (ROM). Overuse, age and traumatic injuries can cause structural damage to the knee that may limit its function. Therefore, a thorough understanding of the anatomy of the knee is essential to properly diagnose and treat disorders related to knee joint movements (Herrmann *et al.*, 2013).

2.2.2 Physiological control of knee joint movement

The knee joint is an organ, which simultaneously guarantees stability and movement. The tissues surrounding this joint are highly vascularised and the joint cavity contains fluid that have important roles in many physiological function and metabolism of the joint. The fluid or exudates produced by the synovial layer of the tissues distribute mineral and nutrients to the different parts of the joint. The decrease in synovial blood flow may cause reduced joint irrigation and may contribute to tissue injury during trauma, where this may also be related to vasoconstriction (Ar'Rajab *et al.*, 1996).Adequate blood supply is also important to deliver hormones to their specific receptors in different parts of the joints which regulates laxity, thus its movement(Junqueira *et al.*, 1986). Knee stability is also controlled by the nerve innervating the knee joint. Proprioceptors are found in the knee which sense changes in joint position and the information is relayed via the spinal nerve to the higher centre in the cerebelum (Marieb, 2009). Input from the higher centre is sent to the muscles that control knee joint stability which is achieved via varying degree of contraction of different groups of muscles (Marieb, 2009). Female is more susceptible to various knee disorders such as arthritis (Kumar *et al.*, 2012). Pathophysiological changes including degenerative disorders could affect joint metabolism and can cause joint inflammation. In addition, collagen content of the knee could also be affected therefore may interfere with knee laxity and joint movements.

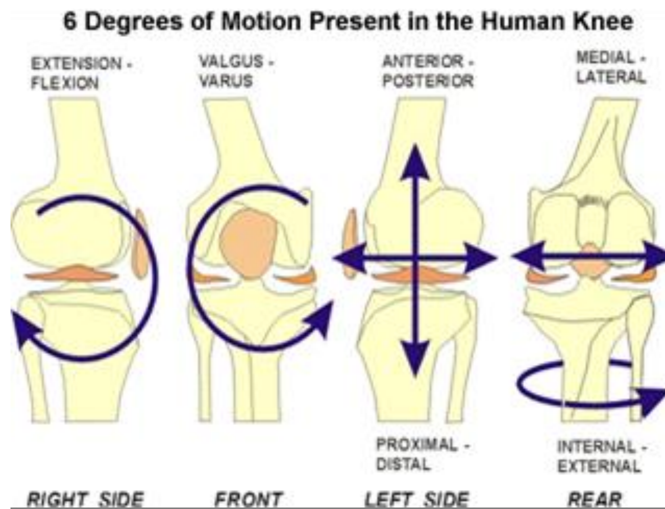


Figure 2-2 Motion degrees of human kneehttp://www.jointinjury.com/knee

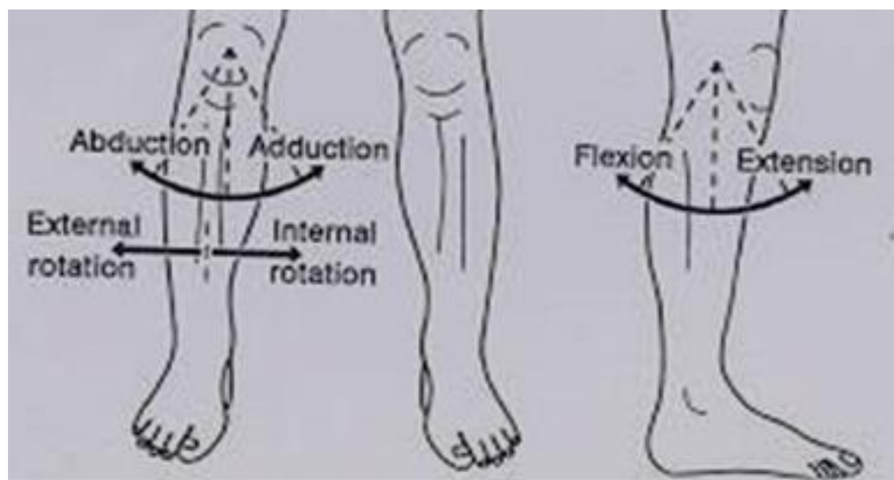


Figure 2-3 Knee rotational movement www.faculty.utpa.edu/rafree/res/biomechanics

2.2.3 Knee movement and its connective tissue mechanism

Tendons and ligaments are elastic connective tissue surrounding the knee joint that give support and are vital for knee movements. Knee ligaments connect the thigh to lower leg bones(Marieb & Hoehn, 2013). Sprains or tears of these ligaments are common in sports injury. Athletes who participate in direct contact sports such as football or soccer are more likely to injure their collateral ligaments and anterior cruciate ligament (Dragoo *et al.*, 2011b). Knee joint relies on these ligaments and the surrounding muscles for stability. Cruciate ligaments are found inside and collateral ligaments (medial and lateral) are found on the sides of the knee (Snell, 2011). The cruciate ligaments control back and forth motion while collateral ligaments control sideway motion of the knee. Tendons are stiff cords of tissue that connect muscles to bones. The patellar tendon is an important part of the extensor mechanism of the lower extremity (Snell, 2011). Meanwhile, the role of the hamstring muscles as a dynamic stabilizer of the knee joint rests in its importance as a joint compressor and restraining mechanism for anterior motion of the tibia on the femur. These complexes of connective tissues participate in the control of knee stability during movement and support it against unusual movement (Marieb, 2009). Cruciate and collateral ligaments prevent knee anterior-posterior and lateral/medial dislocation respectively. Basic movement of the knee joint is shown in Figure 2-2 and 2-3 while Figure 2-4 shows the location of the collateral ligament and patella tendon.

Collateral ligament **Patellar tendon**

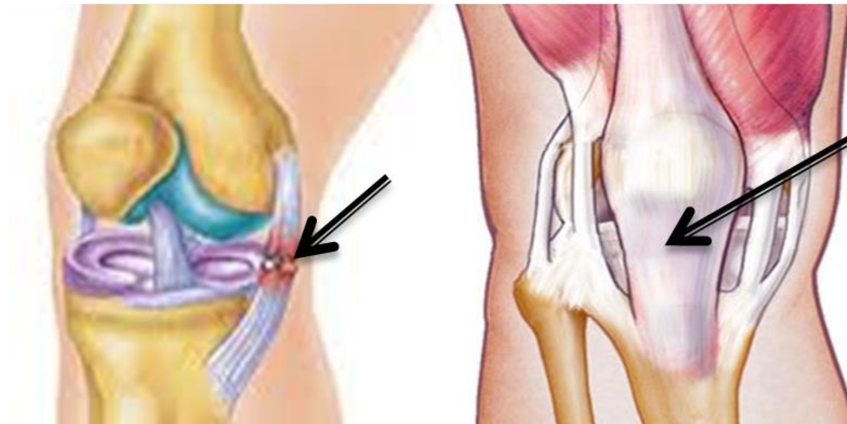


Figure 2-4 Knee patellar tendon and collateral ligament
(Dehghan *et al.*, 2013b)

2.3 Menstrual cycle

Menstrual cycle is the changing phase that happens in the uterus and ovary for sexual reproduction purposes and occurs only in parturient female and other female primates. This necessary period need to produce oocytes and prepare the uterus for pregnancy (Johnson & Martin, 2012). The length of the cycle varies greatly among women and fluctuates 25 to 35 days, with 28 days nominated as an average length. Based on the events in the ovary, each cycle can be divided into three phases; the follicular phase, ovulation, and luteal phase, which are controlled by normal hypothalamic-pituitary-ovarian axis (Klump *et al.*, 2013). The menstrual cycle begins from the first day of bleeding and is associated with increasing amounts of oestrogen in the follicular phase. Approximately mid-cycle, 24–36 hours after the luteinizing hormone (LH) surges, the dominant follicle releases an egg in an event called ovulation(Johnson & Martin, 2012). The corpus luteum which is formed post-ovulation produced large amount of progesterone where under progesterone influence, the endometrium undergoes changes to the receptive state in preparation for embryo implantation and the establishment of pregnancy (Marieb, 2009). In an event of no

implantation, the corpus luteum degenerates which causes a sharp drop in both oestrogen and progesterone levels that precede the onset of the next cycle (Sherwood, 2011). Figure 2-5 shows changes in the hormonal profiles (LH, FSH, oestrogen and progesterone), ovary and endometrium throughout the menstrual cycle.

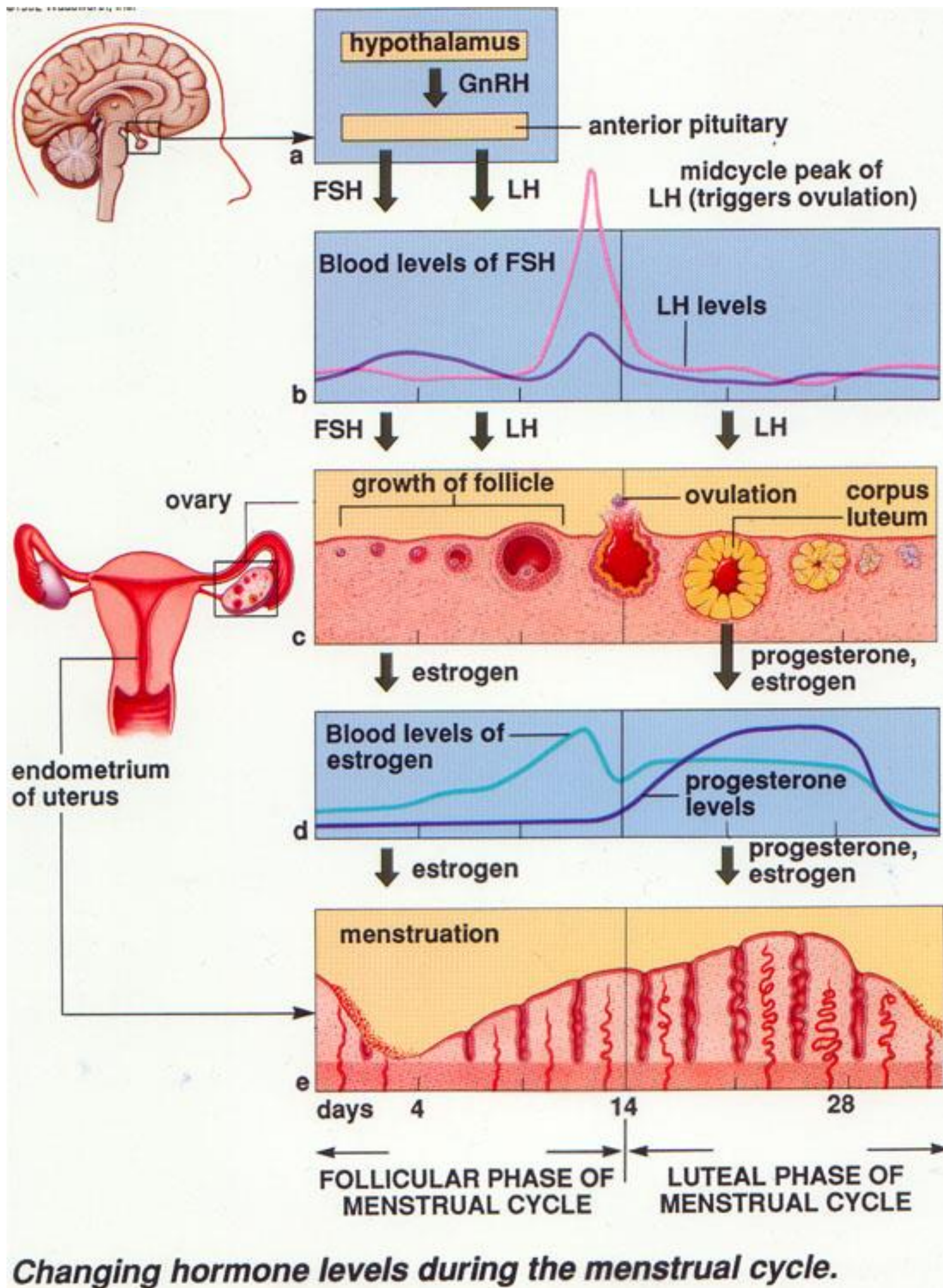
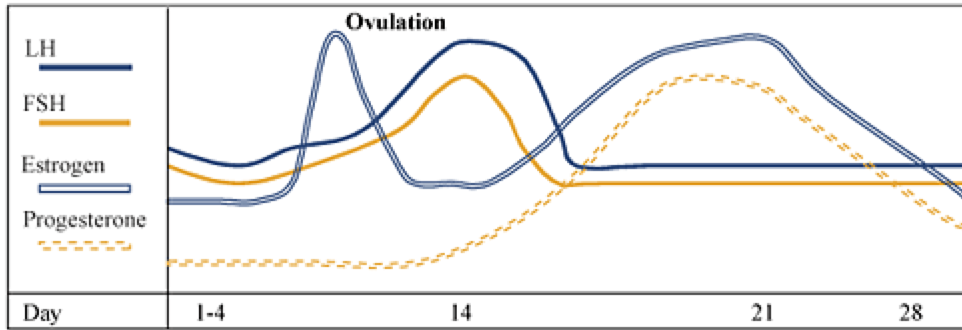


Figure 2-5 Changing hormone levels, ovarian and uterine events during the menstrual cycle
dentistryandmedicine.blogspot.com

2.4 Oestrous cycle

Non-primate mammals such as rodents do not display menstrual bleed or menstrual cycle. In these animals, the reproductive cycle is known as oestrous cycle. In rodent, oestrous cycle occurs 4 to 5 days, which can be divided into proestrous, oestrus, metestrous and diestrous (Staley & Scharfman, 2005). Proestrous occurs in the first 12 hours of the cycle where oestrogen levels peak and was confirmed from the presence of a predominantly nucleated epithelial cells. Oestrus phase occurs 12 hours following proestrous and is indicated by the presence of cornified cells in the vaginal smear. Ovulation occurs at the beginning of oestrus and the end of proestrus phases. Meanwhile, a combined of leucocyte, cornified and nucleated epithelial cells in the vaginal smear indicate metestrous phase. This phase occurs for 21-hour period following oestrus. The diestrous phase has the longest interval time of 57 hours and during this phase, vaginal cells displays predominantly leucocytes. Corpus luteum activity occur in metestrous and diestrous phases associated with high progesterone secretion. Due to its short cycle length, rat is a perfect animal model for investigating changes that occur during the reproductive cycle (Marcondes *et al.*, 2002).



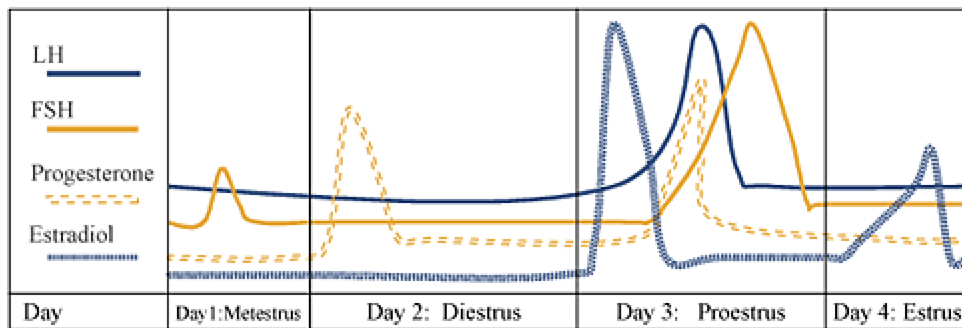


Figure 2-6 Human and rat reproductive menstrual cycle
pubs.niaaa.nih.gov

2.5 Overview of Sex-Steroid Biochemistry and Physiology

In females, sex-steroids are produced from the adrenal glands, ovaries and regulate wide range of physiological functions. The major female sex-steroids are oestrogen and progesterone, testosterone are also produced in a small amount by the ovary and adrenal glands. Steroid hormones bind to steroid hormone binding globulin (SHBG) where it is transported to the target organs through the circulatory system to the specific site of actions. The binding of sex hormones to SHBG are of different affinity. The free hormones can leave the circulation and enter into the target cells where they can bind to specific

intracellular receptors to initiate the biochemical expression of specific genes (Devlin, 2011).

Free sex hormones, being lipophilic can readily cross the cell membranes and bind to the intracellular receptors. Steroid receptors are markedly different from gonadotropin receptors, where the latter are localized in the cell membrane and have several second-messenger systems as mediators of receptor binding (Marieb, 2009). Following receptor binding, steroid-receptor complex crosses the nuclear membrane to bind to the nuclear target which results in the expression of specific genes that can be translated to specific steroid actions (Devlin, 2011). Steroids regulate largely gene expression at a transcriptional level. mRNAs are then exported to the cytoplasm, where protein synthesis takes place, resulting in alterations in cell growth or physiology that are characteristic of the steroid hormone for that target issue. Figure 2-7 shows the mechanism by which sex-steroid exerts its intracellular effect.

Upon release into the circulation, the free steroid hormones exert a negative feedback effect on the pituitary and hypothalamus. At a very high dose however, a positive feedback effect occur on LH secretion from the pituitary gland, which in turn induces ovulation (Devlin, 2011). In the second half of the menstrual cycle, oestrogenis also produced by the corpus luteum (Marieb, 2009). Progesterone which is produced by steroidogenic activity in the ovary is also a major female sex hormones and is important in the maintenance of pregnancy (Devlin, 2011). This hormone acts on the reproductive organs, brain, kidney, lungs and joints especially during pregnancy (Santiago *et al.*, 2001). Elevation of the basal body temperature following ovulation is due to the thermogenic effect of progesterone in the hypothalamus. Testosterone is an anabolic hormone which participates in multiple organs functions in male and is also secreted in a small amount in female mainly by the

ovaries and adrenal glands (Cox & John-Alder, 2005). In female, testosterone is involved in decidualisation while adrenal androgens participate in pubic hair growth and in the post-menopausal period, is a major precursor for oestrogen (Devlin, 2011).

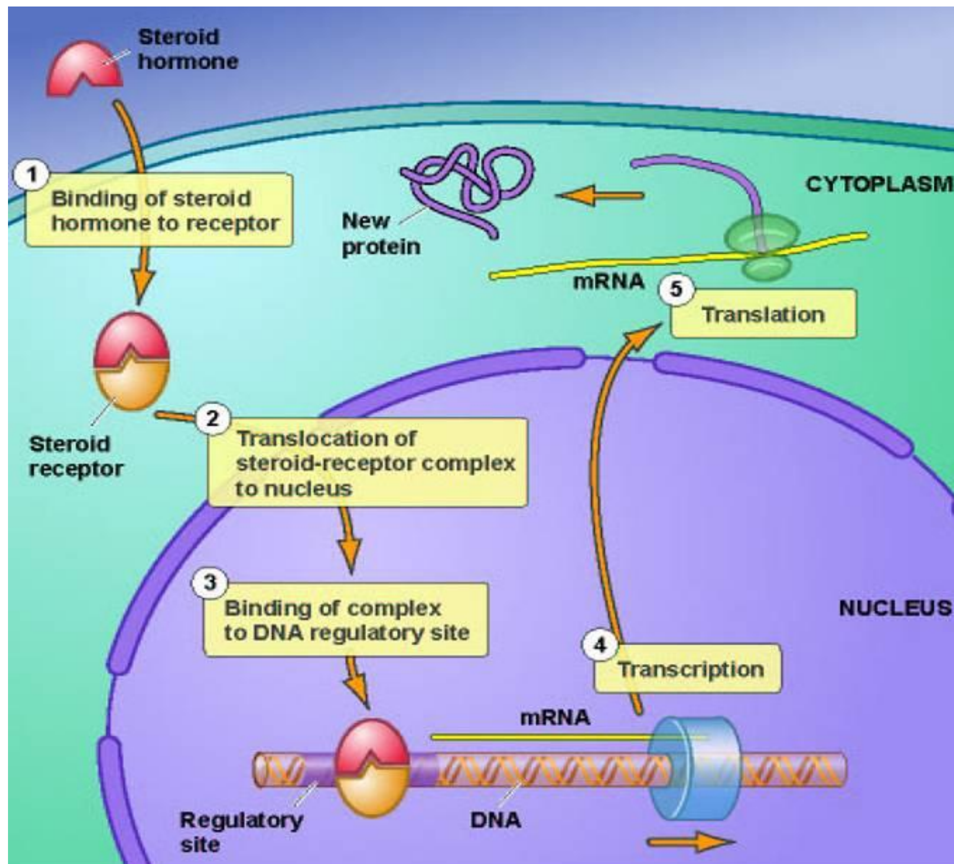


Figure 2-7 Sex steroid hormone response (www.thepepproject.net)

2.5.1 Sex-steroids and Knee Laxity

Females are exposed to rhythmic rise and fall in the levels of oestrogen and progesterone throughout the course of the menstrual cycle. These hormones can influence metabolism of the anterior cruciate ligament (Romani *et al.*, 2003; Yu *et al.*, 1999). Sex steroids fluctuation during the menstrual cycle has been known to be a risk factor for ACL injury in females (Slauterbeck *et al.*, 2002). Beside progesterone, oestrogen has also been reported to have influence on knee laxity. This was based on the findings by Dragoo et al, (2003) who reported that simultaneous administration of oestrogen and relaxin in guinea pigs resulted in increased ligament laxity. Meanwhile, in female, testosterone which levels fluctuates during the menstrual cycle (Dawood & Saxena, 1976) promotes increase muscle and bone mass and prevent osteoporosis (Cox & John-Alder, 2005; Mooradian *et al.*, 1987). Testosterone has also been used for postmenopausal hormone therapy in female (Bolour & Braunstein, 2005).

2.6 Relaxin

Relaxin, a polypeptide hormone which level rises in the second half of the menstrual cycle increases with a rising level of progesterone. This hormone (with a classical structure of insulin) interacts with G-protein-coupled receptors (GPCRs) which exist in various tissues, including the musculoskeletal and non-musculoskeletal systems. Meanwhile, the presence of relaxin receptor as evidence from relaxin binding assay and immunostaining has been reported higher in female ACL as compared to male (Faryniaz *et al*, 2006), which suggest that female sex-steroids could affect relaxin receptor expression in the ACL. Relaxin has been widely implicated in the control of knee joint laxity.

Relaxin, the mammalian 6-kDa heterodimeric polypeptide hormone, is a member of the insulin-like superfamily (HISAW, 1926) and consists of seven peptides of high structural but low sequence similarity. Relaxin is belongs to a family of peptide family hormones, which is believed to have evolved from insulin early in the evolution of vertebrates (Bathgate *et al.*, 2006). Relaxin family peptides interact with their receptors, which exist in various tissues, including musculoskeletal and non-musculoskeletal systems.

The actions of relaxin receptor are mediated by different signalling pathways (Kong *et al.*, 2010). Relaxin plays an essential role in the biological processes such as metabolism, growth, pregnancy, and parturition in different species including humans and rodents. Relaxin circulates in these species during pregnancy emanating from the corpus luteum (Conrad & Baker, 2013) and placenta (Goh *et al.*, 2013), however temporal pattern of change and serum concentrations of this hormone are different. In rodents, circulating relaxin peak concentrations at the end of pregnancy reaches 100 ng/ml, two times greater than in human (Sherwood OD, 1994). While relaxin plays important role in collagen catabolism of the pubic symphysis during gestation in lower mammals such as mice and rats (Samuel *et al.*, 1998), the role of this hormone on pubic symphysis of human is however unknown (Hashem *et al.*, 2006).

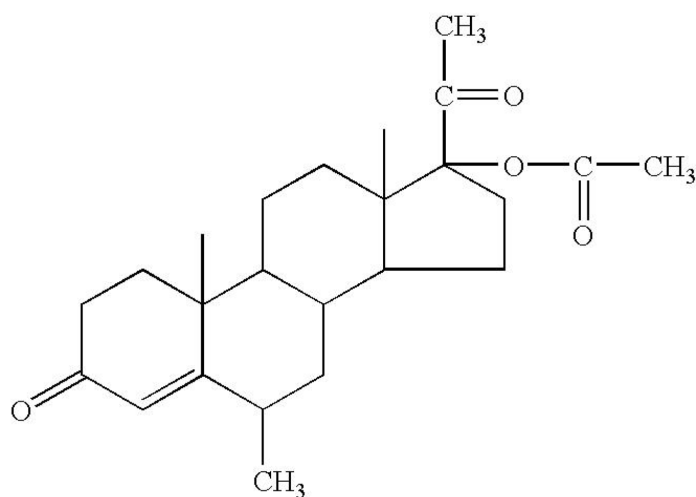


Figure 2-8 Relaxin structure

2.6.1 Relaxin roles in different tissues

Several studies have highlighted the therapeutic potential of relaxin for ectopic pregnancy, male infertility, and heart failure, cardiovascular and musculoskeletal diseases. Currently, there are seven known relaxin family peptides which are structurally related to insulin which include relaxin (RLN)1, RLN2, RLN3, insulin-like peptide (INSL)3, INSL4, INSL5 and INSL6 (Bathgate *et al.*, 2013). RLN1 and RLN2 are strong regulators of collagen expression and metabolism in fibroblasts, andare differentially expressed in the corpus luteum, decidua, and endometrium, as well as prostate tissue while RLN3 is specific to the brain (Sherwood, 2005). RLN1 and RLN2 reconcile the hemodynamic changes occurring during pregnancy such as cardiac output, renal blood flow, and arterial compliance (Kirk, 2011) as well as weakening the pelvic ligaments for parturition in species such as guinea pigs and mice (Sherwood OD, 1993). RLN3 is a highly conserved neuropeptide in vertebrates, and is involved in a wide range of neuroactivities such as response to stress and cognition, as well as in neurological disease (Smith *et al.*, 2011).

Relaxin binds to relaxin family peptide receptors (RXFP) and exerts its action through a ligand-receptor system in multiple pathways. The relaxin receptor is involved in signal transduction between extracellular/intracellular domains. Relaxin1 to 4 hormones are ligands for the RXFP1, RXFP2, RXFP3, and RXFP4, respectively (Figure 2.8). This family peptides act on four GPCRs (LGR7, LGR8, GPCR135, and GPCR142) (Kong *et al.*, 2010).RXFP1 and RXFP2 are composed of large extracellular domains which encompass of leucine-rich repeats. On the other hand, RXFP3 and RXFP4 proteins are more similar to small peptide ligands (Summers *et al.*, 2009). Recently, it has been shown that there is a difference in the ligand binding mode between RXFP1 and RXFP2 (Scott *et al.*, 2012). RXFP1 and RXFP2 exist in uterus, cervix, vagina, brain, and heart of a number of animal species. However, production of these proteins differs among tissues of various species. For example RXFP1 is expressed in rats and mice myometrium (Vodstrcil *et al.*, 2010b), whereas in human, this receptor is mainly localized to the endometrium (Campitiello *et al.*, 2011). Moreover, RXFP1 is expressed in the rats and mice heart localized to the atria where it mediates positive inotropic and chronotropic responses (Piedras-Renteria *et al.*, 1997), while there is currently no report of this receptor binding or function in the human heart.

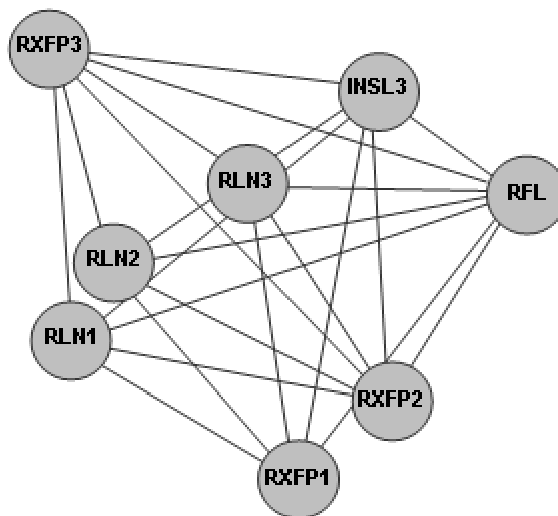


Figure 2-9 Interaction of RLN1, LN2, and RLN3 proteins with their receptors RXFP1, RXFP2, and RXFP3, respectively, as well as with insulin growth factor (INSL3)

Evidence also suggests that the functional domains of RXFP1, the cell type in which it is expressed, and the ligand used to activate the receptor, all have important roles in the musculoskeletal system (Figure 2.9). Relaxin alters cartilage and tendon stiffness by activating collagenase. Relaxin is also involved in bone remodelling process and in healing of injured ligaments and skeletal muscles (Dragoo *et al.*, 2009; Li *et al.*, 2005). The soft tissue-healing cascade is composing of three phases: inflammation, regeneration, and fibrosis. Relaxin is a regulator of both inflammation and fibrosis (Mu *et al.*, 2010). Relaxin also acts as antifibrotic agent, and favours muscle regeneration and against muscle fibrosis to promote regrowth of myofibers in skeletal muscle healing.

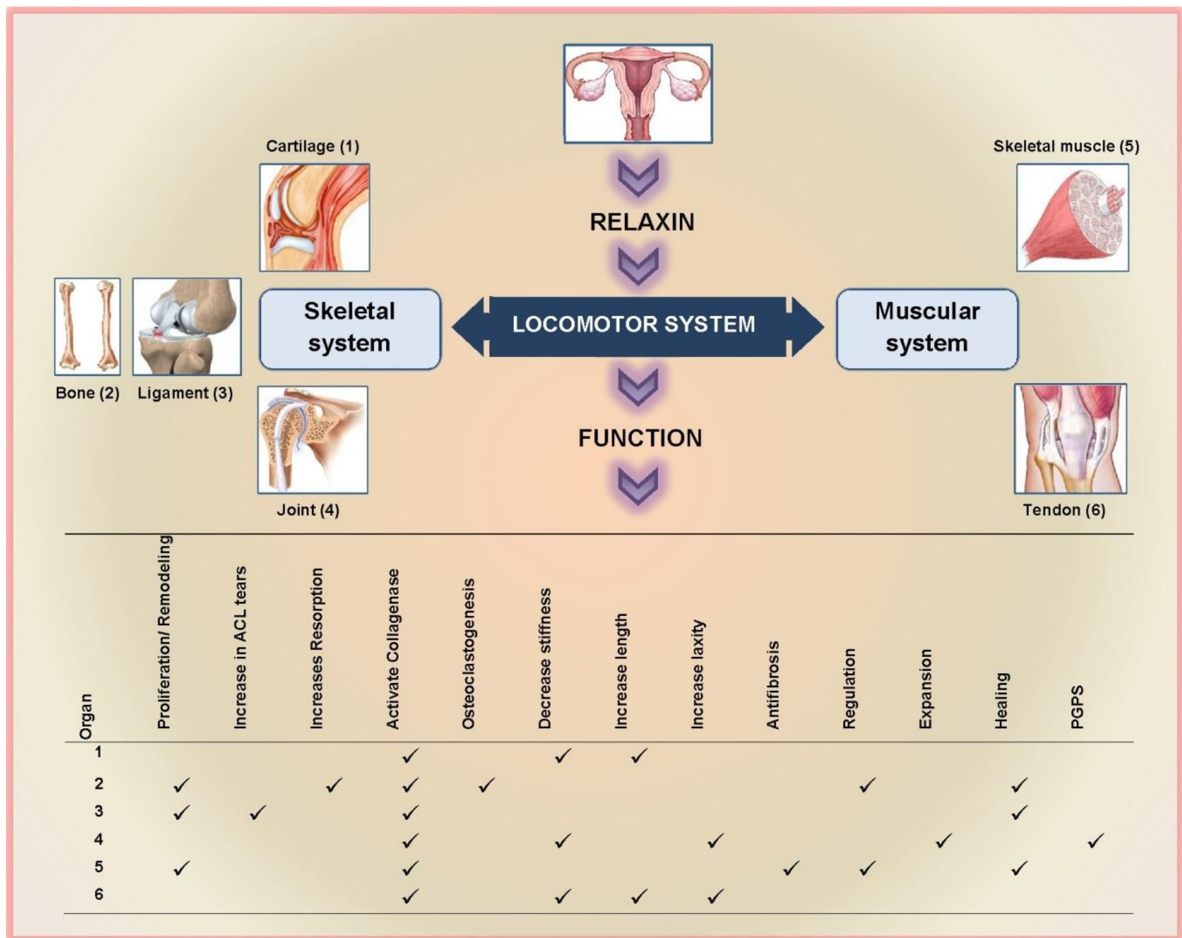


Figure 2-10 A summary of relaxin role in the locomotor system (Dehghan *et al.*, 2013b)

2.6.2 Relaxin roles in musculoskeletal tissues

2.6.2.1 Relaxin effects on joints

RXFP1 and RXFP2 receptors orchestrate several activities in joints laxity through combination with sex-steroids hormones. Relaxin in combination with oestrogens may also have therapeutic value in the treatment of rheumatoid arthritis (RA) (Ho *et al.*, 2011; Santora *et al.*, 2005). RA is a chronic and systemic inflammatory disorder that may affect many tissues and organs, but also causes bone destruction through synovial hypertrophy.

However, the incidence and severity of this disease during pregnancy is lower than normal. During pregnancy, relaxin and oestrogen levels in the serum are elevated leading to decrease in inflammation in RA patients (D'Elia *et al.*, 2003; Ho *et al.*, 2011). Relaxin exerts its anti-inflammatory effect through down-regulation of neutrophil function (Bani *et al.*, 1998) and stimulates leukocyte adhesion and migration in human mononuclear cells (Figueiredo *et al.*, 2006). A combined treatment using relaxin and oestrogen appears to reduce circulating tumor necrosis factor (TNF)-α level in rat adjuvant-induced arthritis model of RA and increased the anti-inflammatory cytokine IL-10 in human cells.(Figueiredo *et al.*, 2006; Santora *et al.*, 2005). In view of this, relaxin has a potential beneficial effect in the treatment of synovial diseases.

2.6.2.2 Relaxin effects on bones

Relaxin along with hormones such as oestrogen and growth factors such as TGF-β help orchestrate the bone remodelling process. These factors regulate a cytokine system containing three fundamental molecules, the receptor activator of nuclear factor κB ligand (RANKL), RANK, and osteoprotegerin (OPG). In the RANKL/RANK/OPG system, RANKL on the preosteoblastic/stromal cells binds to its receptor (RANK) on the osteoclastic precursor cells and induces expression of a variety of genes to provide the crucial signal to drive osteoclast recruitment and development (Facciolli *et al.*, 2009). OPG regulates the system through blocking the effects of RANKL and interfering with RANK signalling.

Relaxin facilitates differentiation of peripheral blood mononuclear cells (PBMCs) into mature osteoclasts during osteoclastogenesis by stimulating osteoblastic/stromal cell production, while oestrogen inhibits this process through increasing OPG production

(Facciolli *et al.*, 2009). Therefore, relaxin is one of the osteoclast-activating factors that increases bone resorption. It is also overexpressed in tumours that promote growth, differentiation, and invasiveness, which lead to osteolytic metastases (Clezardin & Teti, 2007). Together, these data indicate a possible role of relaxin in osteoclastogenesis (Facciolli *et al.*, 2009; Ferlin *et al.*, 2010). Relaxin 2 (RLN2) regulates bone metabolism and proliferation in human osteoblasts. Stimulation of osteoblasts with RLN2 activates adenylate cyclase and increases cAMP production by G- proteins and thereby increases cell proliferation (Ferlin *et al.*, 2009). Previous studies have identified an inactivating mutation in the RXFP2 gene (T222P), which caused idiopathic osteoporosis in young men through functional osteoblast impairment and reduced bone density (Ferlin *et al.*, 2009). A similar result was also observed in knockout mouse model (Ferlin *et al.*, 2008). There is also some evidence to suggest that higher levels of oestrogen and relaxin in pregnant women correlated with an increased prevalence of congenital dysplasia of the hip (CDH) in neonates (Saugstad, 1991; Steinetz *et al.*, 2008). In view that relaxin affects both osteoclast and osteoblast, therefore this hormone is involved in bone remodelling process, and stimulation of osteoblast by relaxin-2 suggest that this hormone is potentially useful in the treatment of bone condition such as osteoporosis.

2.6.2.3 Relaxin effects on ligaments

Relaxin hormone alters ligament mechanics due to its collagenolytic effect mediated by discharge of matrix metalloproteinases (MMPs) (Qin *et al.*, 1997), collagenase (Wiqvist *et al.*, 1984), and plasminogen activator (Koay *et al.*, 1983). Relaxin treatment in pregnant cattle increased pelvic width and height (Musah *et al.*, 1986; Perezgrovas & Anderson, 1982), but not in other joints such as wrist and knee (Marnach *et al.*, 2003; Weinberg, 1956). Increase in serum relaxin concentration may also correlate with joint

laxity (Dragoo *et al.*, 2011b; Lubahn *et al.*, 2006), but this effect during pregnancy is controversial (Forst *et al.*, 1997). Some studies have reported higher relaxin levels in pregnant women with pelvic joint instability or hip joint laxity as compared to controls (Saugstad, 1991; Steinetz *et al.*, 2008), while other studies did not (Ohtera *et al.*, 2002). Two studies on the relationship between serum relaxin levels and joint laxity reported no significant association between this hormone level and knee and generalized joint laxity (Wolf *et al.*, 2013). Studies have also suggested a relationship between higher relaxin and progesterone serum levels in pregnant females with pelvic girdle pain (PPGP) syndrome (Kristiansson *et al.*, 1999; MacLennan *et al.*, 1986; Wreje *et al.*, 1995) and pelvic floor dysfunction (Harvey *et al.*, 2008), whereas other studies have not found such a relationship (Crelin & Brightman, 1957; Petersen *et al.*, 1994; Vollestad *et al.*, 2012). Study design and methodological differences may account for some of the conflicting data.

Relaxin appears to play a role in anterior cruciate ligament (ACL) injury (Dragoo *et al.*, 2009). Oestrogen and relaxin receptors have been found in the human female ACL (Faryniarz *et al.*, 2006). Studies on the mechanical properties of human ACLs illustrate that those treated with relaxin have reduced ligament integrity and may be at higher risk of injury (Dragoo *et al.*, 2011b; Toth & Cordasco, 2001). This finding was also replicated in an animal model, where rabbits treated with relaxin had significantly weaker ACL's compared with controls (Dragoo *et al.*, 2009). Additionally, there was increased anterior tibia displacement on radiographic assessment, indicating ACL laxity, in animals treated with relaxin (Dragoo *et al.*, 2009).

There also may be an association between ACL injuries and stages of menstrual cycle. Occurrence of ACL injuries during the ovulatory phase (midcycle) are more frequent than the luteal phase (Wojtys *et al.*, 2002). During this period, oestrogen and relaxin levels

are high, therefore, activation of the oestrogen and relaxin receptors may be increased (Min & Sherwood, 1996). Relaxin activates collagenolytic system, which increases collagenase synthesis and finally degrades the extracellular matrix composition (Garibay *et al.*, 2004; Guttridge, 2004).

A prospective study of elite female athletes illustrated that players with increased serum relaxin levels had an increased risk of an ACL tear compared with females with lower relaxin levels (Dragoo *et al.*, 2011a). Players having a relaxin concentration of greater than 6.0 pg/ml had more than a 4 times greater risk of ACL injury. Other studies have collaborated these findings (Beynnon *et al.*, 2006a). Relaxin appears to affect other ligaments such as volar oblique in perimenopausal women via a receptor-mediated process. In this ligament relaxin particularly binds and probably reveals in presence of cellular or extracellular matrix receptors (Lubahn *et al.*, 2006).Taken together, these findings indicate that while relaxin effects are beneficial to the lower animals especially during pregnancy, its proposed effect on the peripheral ligament laxity in humans and animals may predispose the joint to a non-traumatic injury.

2.6.2.4 Relaxin effects on skeletal muscles

Relaxin helps to regulate normal skeletal muscle through two principle-signalling pathways: adenylate cyclase (AC) and nitric oxide (NO). Relaxin activates the AC signalling pathway in skeletal muscles through the following signal chain: (Pertseva *et al.*, 2006; Plesneva *et al.*, 2008; Shpakov *et al.*, 2006; Shpakov *et al.*, 2007; Shpakov *et al.*, 2004). Relaxin also activates the NO pathway in skeletal muscle via relaxin mediated activation of receptor tyrosine kinase (Plesneva *et al.*, 2008). NO regulates various biological processes, and is produced by NO synthase (Stamler & Meissner, 2001). There

are data which indicate relaxin stimulates NO synthase signalling in the skeletal muscles of type 2 diabetic rats, leading to NO dysfunction (Kuznetsova *et al.*, 2010).

Relaxin may be implicated in the skeletal muscle healing process by regulating inflammation, tissue remodelling and, fibrosis (Formigli *et al.*, 2005; Sherwood, 2005). The degree of fibrotic response varies with the level of inflammation and injury. Relaxin may improve spontaneous regeneration of injured skeletal muscle as illustrated in an injured muscle mouse model (Kazumasa *et al.*, 2001; Sato K *et al.*, 2003).During this process, skeletal muscle cells regenerate and repair to reduce the size of a damaged or necrotic area and replace it with new living tissue. Degeneration/inflammation is retrogressive changes in cells and tissues characterized by abnormal structural changes and decreased functions (Li *et al.*, 2005; Merchav *et al.*, 2005; Mu *et al.*, 2010; Negishi *et al.*, 2005).

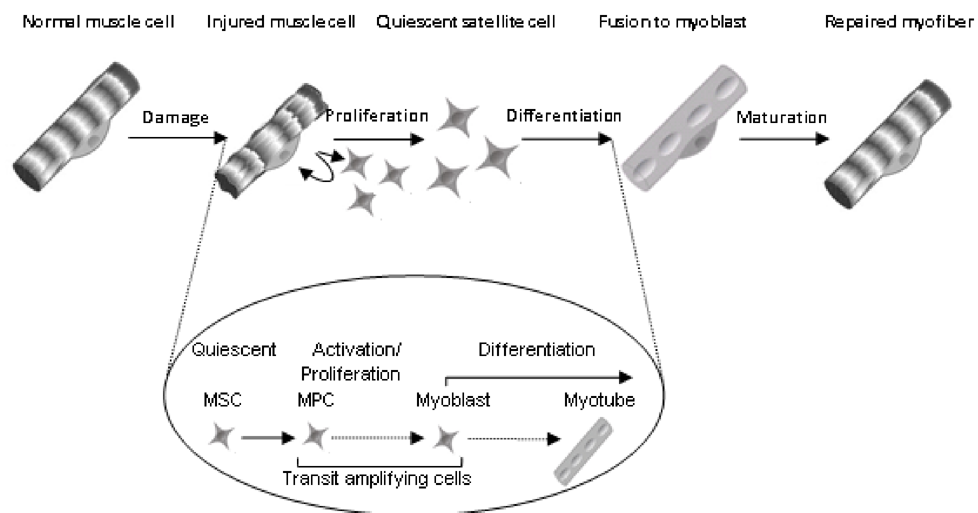


Figure 2-11 Mechanism of regeneration in damaged muscle. MSC (muscle satellite cell), MPC (myogenic progenitor cell)

In regeneration phase, immature granulation tissue containing active fibroblasts produce abundant type III collagen, which fills the defect left by an open wound (Volk *et al.*, 2011). Granulation tissue moves, as a wave, from the border of the injury towards the centre. As granulation tissue matures, fibroblasts produce less collagen and become more spindly in appearance, which then begin to produce a much stronger type I collagen (Syed *et al.*, 2011). Some of the fibroblasts mature into myofibroblasts containing similar action to the smooth muscle, which enables them to contract and reduce the size of the wound (Sarrazy *et al.*, 2011).Fibrosis is the last phase of healing where a non-functional scar tissue is formed caused by excessive accumulation of connective tissue following damage. Fibrosis often delays and impairs the recovery of damaged tissue (Diegelmann & Evans, 2004). Relaxin has been shown to inhibit fibrosis formation through several mechanisms which include neutralization of the effect of TGFβ1 (transforming growth factor, beta1) and activation of the collagenolytic system, which increases collagenase synthesis (Garibay *et al.*, 2004; Guttridge, 2004; Mendias *et al.*, 2012; Mendias *et al.*, 2004; Mu *et al.*, 2010; Vinall *et al.*, 2011). Through these mechanisms, relaxin reduces the formation of fibrous scar tissue. In view of that relaxin plays important role in the healing process, it can potentially be used as a therapeutic agent to treat damaged skeletal muscle (Negishi *et al.*, 2005).

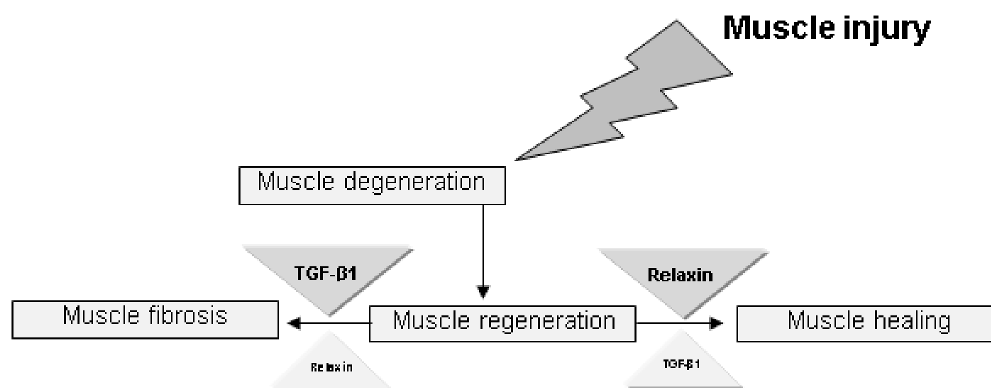


Figure 2-12 Mechanism of regeneration in damaged muscle

2.6.2.5 Relaxin effects on tendons

Relaxin has been reported to effect tendon metabolism by controlling the length of tendon growth (Wood *et al.*, 2003) and reduce tendon stiffness by increasing tendon laxity through activation of collagenase. An *in-vivo* study investigating the growth of rat tails and human patellar tendons showed that relaxin levels correlate with tendon length (Wood *et al.*, 2003). Rat tail tendons treated with relaxin exhibited alterations in collagen through interfering with fibril association and collagen sliding (Wood *et al.*, 2003). Another study in women with normal menstrual cycle, who did not take any contraception pills, demonstrated a significant link between serum relaxin levels and patellar tendon stiffness. Besides, of the reported effects of relaxin on the tendon, potential benefits of relaxin on tendon repair and remodelling is largely unknown.

2.6.2.6 Relaxin effects on Cartilage

Relaxin appears to decrease knee articular cartilage stiffness (Bonaventure *et al.*, 1988; Hellio Le Graverand *et al.*, 1998) through induction of collagenase-1, matrix

metalloproteinase (MMP-1) and MMP-3, which reduces collagen content and expression in fibrocartilaginous cells. Modulation of MMPs to loss of collagen by hormones may contribute selectively to degeneration of specific joints fibrocartilaginous explants facilitated by proteinases (Hashem *et al.*, 2006; Naqvi *et al.*, 2005). The degradation of extracellular matrix in fibrocartilage is synergized by β-oestradiol. Relaxin exerts its effect through binding to RXFP1 and RXFP2 receptors. The ratio of RXFP2 in knee meniscus of pregnant rabbits was shown to be more than RXFP1, which may indicate differential role of these receptors in the remodelling of fibrocartilage (Wang *et al.*, 2009b). Comparison of collagen content in articular cartilage of non-pregnant and pregnant rabbits showed that the total RNA levels and chondrocyte metabolism decreased during pregnancy. Depending on the level of skeletal maturity, pregnancy can exert both general and specific effects on the RNA levels in articular cartilage of the rabbit knee. Thus, relaxin may play a role in women' susceptibility to musculoskeletal disease. Taken together, these findings suggested that in female, increased relaxin level may result in undesirable effects on the articular cartilage.

Table 2-2 Review of previously reported data of relaxin in locomotor system

Organ	Author	Year	Sample	Model	Treatment	Relaxin	Role of relaxin
Skeleton							
Cartilage	Bonaventure *et al.*	1988	Chondrocytes cell	Rb / vitro	Porcine relaxin	NI	Modulation of type I, II, III collagen expression
	Naqvi*et al.*	2004	Joint fibrocartilaginous cells	Rb / vitro	Human relaxin, β-oestradiol	NI	No increase collagenase1 & MMP3 expression
	Hashem *et al.*	2006	Knee meniscus fibrocartilage & articular cartilage	Rb/ vitro	Human relaxin, β-oestradiol, progesterone	NI	No significant change of GAGs (glycosaminoglycans) & collagen
	Wang *et al.*	2009	Joint fibrocartilaginous cells	M / vitro	NI	1,2	Expression of RXFP2 > RXFP1
Bone	Santora *et al*	2005	Arthritis paw	R / vivo	Porcine relaxin & 17-β–oestradiol	NI	Combination hormone therapy reduced arthritis inflammation
	Kristiansson *et al.*	2005	Normal osteoblasts cell	H / vitro.	Relaxin	1,2	Bone resorption by mediators
	Ferlin *et al.*	2008	Bone densitometry, cryptorchidism, osteoblast cell	H,M/vivo, vitro	Agonists INSL3, relaxin, forskolin	2	Links RXFP2 gene mutations with human osteoporosis
	Facciolli *et al.*	2009	Osteoclasts cell	H/ vitro	Relaxin	1	Facilitation the differentiation of osteoclasts
	Ferlin *et al.*	2009	Osteoclasts cell	H/ vitro	Relaxin	1	Relaxin is a potent stimulator of osteoclastogenesis
	Ferlin *et al.*	2011	Femoral heads osteoblast cell	H/ vitro	Relaxin 2	2	RXFP2 system is involved in bone metabolism
	Ho *et al.*	2011	Joint tissues, Murine osteoblast cells	R/ vivo & vitro	17-β- oestradiol, porcine relaxin	NI	Modulation of RANKL-OPG system
Joint	Weinberg *et al.*	1956	4 nonpregnant &11 pregnant	H/vivo	Relaxinas releasin	NI	No chang in pelvic measurement
	Cerlin *et al.*	1957	Pelvic joints	R/vivo	Relaxin, oestrogen	NI	No difference in pelvic joint flexibility
	Perezgrovas *et al.*	1982	Pelvic joint	BF/ vivo	Porcine relaxin	NI	Expansion of the pelvic area(p)
	MacLennan *et al.*	1986	Patients with late pregnancy	H/vivo	NI	NI	High relaxin link between pelvic pain and joint laxity during late pregnancy
	Musah*et al.*	1986	Pelvic joint	BF/vivo	Relaxin	NI	Induction pelvic expansion, highly significant interaction(p)
	Udén *et al.*	1988	CDH patients	H/vivo	NI	NI	Increased sensitivity of the receptors of the fibroblasts
	Saugstad*et al.*	1991	153 Pregnant women	H/vivo	NI	NI	Congenital hip dysplasia rate, consistent withoestrogen and relaxin levels
	Petersen *et al.*	1994	472 pregnant women	H/vivo	Ni	NI	Not associated with pregnancy pelvic pain
	Wreje *et al.*	1995	19 women	H/vivo	Oral contraceptive	NI	Higher relaxin with posterior pelvic and lumbar pain
	Schauberger *et al.*	1996	21 women	H/vivo	NI	2	No correlation with serum relaxin & joint laxity
	Forst *et al.*	1997	90 newborn children	H/vivo	NI	NI	rElaxin in newborns c presentation hip
	Vogel*et al.*	1998	12 girls, three boys newborn	H/vivo	NI	2	Reduction of relaxin concentration with increasing sonographic hip
	Kristiansson *et al.*	1999	200 pregnant women	H/vivo	NI	NI	Relaxin correlated with pelvic pain in early pregnancy

	Ohtera *et al*.	2001	Knee joint of nonpregnant and pregnant	R/vivo	NI	NI	Relaxin preventing the development of joint contracture
	Arnold *et al*.	2002	Athlete eumenorrheic women and men	H/vivo	NI	NI	No effect on knee laxity
	Marnach *et al*.	2003	Pregnant women	H/vivo	NI	NI	No correlation wrist joint laxity and relaxin level
	Harvey *et al*.	2008	Pregnant women	H/vivo	NI	NI	Higher relaxin and fall significantly faster in women with PFD
	Vøllestad *et al*.	2012	212 women pelvic joints	H/vivo	NI	NI	Contribution with pelvic joints laxity but no responses to pain & disability
Ligament	Albert *et al*.	1997	455 pregnant women	H/vivo	NI	NI	No difference in serum relaxin concentration with pelvic pain
	Dragoo *et al*.	2003	ACL specimens	H/vitro	Human recombinant relaxin		Binding of relaxin female ACL tissues but not in men ACL
	Galey*et al*.	2003	ACL specimens	H/vitro	Human relaxin	NI	Competence of the ACL to sequester relaxin
	Faryniarz*et al*.	2006	ACL injuries cells	H/vitro	NI	NI	Increased binding of relaxin-labeled in ACL fibroblasts cellsmore in female
	Lubahn *et al*.	2006	Volar oblique ligament(VOL)	H/vitro	NI	NI	Relaxin binds to (VOL) perimenopausal women.
	Dragoo *et al*.	2009	ACL specimens	G/vivo	Porcine relaxin/oestrogen	NI	Alternation mechanical properties of the ACL
	Dragoo *et al*.	2011	Female athletes	H/vivo	NI	2	Higher relaxin in ACL tears than without tears
Muscular							
Muscle	Kuznetsova *et al*.	1999	Type 1,2diabetes skeletal muscles	R/vitro	Insulin/ relaxin/IGF-1	NI	No regulation activity of glycogen synthase by relaxin
	Shpakov *et al*.	2004	Skeletal muscles	R/ vitro	Inhibitor	NI	Inhibitor blocked stimulatory effect of relaxin on adenylate cyclase
	Li *et al*.	2005	Tibialis anterior muscles	M/ vivo	Relaxin	NI	Regeneration and prevention of fibrosis
	Merchav *et al*.	2005	Internal obturator muscles withPH	D/vivo	NI	1	Pathogenesis role of relaxin on PH
	Negishi *et al*.	2005	Skeletal muscles, Myoblasts (C2C12 cells)	R/vivo & vitro	Relaxin	NI	Antifibrosis agent and improve the healing
	Shpakov *et al*.	2006	Skeletal muscles	R/vivo	Pig relaxin2	1,2	Relaxin2stimulated the AC activity
	Shpakov *et al*.	2007	Myoblastic cell line	R/ vitro	Relaxin	1	Stimulation of adenylyl cyclase activity
	Shpakov *et al*.	2007	Skeletal muscles	R/ vivo	Porcine relaxin2	2	Regulation of adenylyl cyclasesystem under hyperglycemia
	Chistyakova *et al*.	2009	Skeletal muscles	F/ vitro	Relaxin	2	Relaxin in increasing G-6-PDH activity
	Kuznetsova *et al*.	2010	Type 2 diabetes skeletal muscles	R/vitro	Streptozotocin	NI	Stimulation NO synthase in rat skeletal muscles
	Mu *et al*.	2010	C2C12 mouse/ myoblasts cell , muscle	H,M/vivo,vitro	Relaxin	1	Regeneration/increased angiogenesis/revascularization
Tendon	Wood *et al*.	2003	Tail tendon	R/vivo	Pentapeptide NKISK, relaxin	NI	Significantly more creep & changes in tendon length
	Pearson*et al*.	2011	Patellar& medial gastrocnemius tendon,	H/ vivo	NI	NI	Correlations between relaxin & patellar tendons stiffness

2.7 Summary of literature findings

Gender related hormonal influence on the knee joint may have important role in higher risk for injury in female athletes population. Knee joint is believed to be a critical joint which injuries may cause permanent disability to the female athletes. Alterations in hormonal patterns during the menstrual cycle might contribute to non-contact injury in female at different phases of the menstrual cycle. The mechanisms that contribute to the higher incidence of injury rates among the female athletes at different phases of the menstrual cycle remains poorly understood. Besides sex-steroids, relaxin is also involved in modulating knee laxity. Correlation and interaction between relaxin and sex-steroids in females therefore need to be establish in order to gain an insight into the mechanism underlying the pathogenesis of non-traumatic knee injury in female. The current study hypotheses the relationship between progesterone, oestrogen, testosterone, relaxin, and relaxin receptor expression with knee laxity with a particular attention being given to lateral and medial knee laxities.

CHAPTER 3 MATERIALS AND METHODS

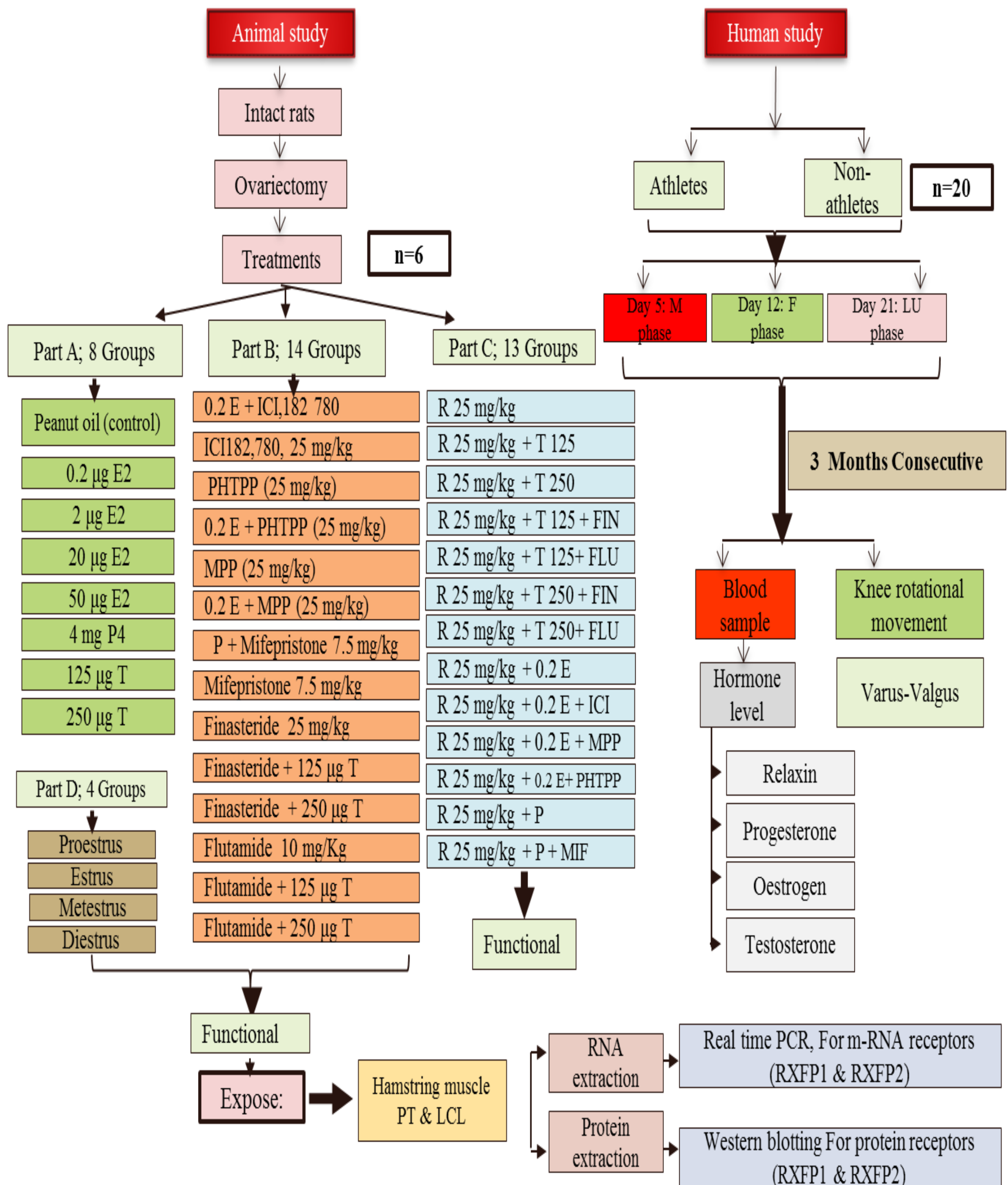


Figure 3-1 Schematic overview and research methods of the study

3.1 Materials

3.1.1 Animal

All procedures involving experimental animals were approved by the Faculty of Medicine Animal Care and Use Committee (ACUC), University of Malaya (UM) with ethics number: FIS/22/11/2011/FD(R). Adult female WKY rats weighing 180-220 grams, obtained from the Animal House, Faculty of Medicine, UM were caged in a group of six, in a clean and well ventilated standard environment of 12 hours light: dark cycle. The animals had free access to soy-free diet (Gold Coin Pellet) and tap water ad libitum.

3.1.2 Chemicals and consumable

The chemicals and consumables used in this project are shown in Table 3.1.

Table 3-1 Chemicals and consumable used in this project

No	Chemicals	Company
1	Progesterone (100 mg, purity minimum 99 %)	Sigma, St. Louis, MO
2	Testosterone (5 g,purity > 99 %)	Sigma, St. Louis, MO
3	17 Beta-oestradiol	Sigma, St. Louis, MO
4	Relaxin	Sigma, St. Louis, MO
5	Peanut oil (P2144, 1L)	Sigma, St. Louis, MO
6	Absolute Ethanol (purity > 99 %)	Sigma, St. Louis, MO
7	Pro-Prep protein extraction solution	Intron Biotechnology, Korea
8	Micro BCA protein assay Kit	Thermo Scientific, Rockford, USA
9	7α,17β-[9-[(4,4,5,5,5-Pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol (ICI 182/780) purity ≥ 98 %	Santa-Cruz Delaware CA, USA
10	4-[2-phenyo-5,7-bis(trifluoromrthyl)pyrazolo(1,5-a) pyrimidin-3-yl] phenol (PHTPP) purity 99.8 %	Tocris, Bioscience USA
11	1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1N-pyrozole dihydrochloride (MPP) purity 99.8 %	Tocris, Bioscience USA
12	Mifepristone purity 99.8 %	Tocris, Bioscience USA
13	Flutamide purity≥ 98 %	Sigma, St. Louis, MO
14	Finasteride purity ≥ 98 %	Sigma, St. Louis, MO
15	Beta Actin antibody	Abcam, UK
16	RXFP1 antibody	Abcam, UK
17	RXFP2 antibody	Abcam, UK
18	4CN optic substrate kit	Bio Rad, Alfred Nobel Drive Hercules, CA, USA
19	Poly Vinylidene fluoride membrane (PVDF)	Bio Rad, Alfred Nobel Drive Hercules, CA, USA
20	Bovine Serum Albumin (BSA)	Innovative, Peary Court Novi, Michigan, USA
21	Spectra multicolor broad range	Fermentas, USA
22	Tetramethylenediamine (TEMED)	Sigma, St. Louis, MO
23	RNA later	Ambion,Lincoln Center drive, USA
24	RNase Free water	Ambion,Lincoln Center drive, USA
25	Micro 96 well plate and cover	Applied Biosystems, USA
26	High capacity RNA to cDNA	Applied Biosystems, USA
27	Fast advanced Master Mix (Taq man)	Applied Biosystems, USA
28	RNA primers assays	Applied Biosystems, USA
29	Rat Enzyme-linked immunosorbent assay (ELISA) kit	CUSABIO, USA
30	Human Enzyme-linked immunosorbent assay (ELISA) kit	CUSABIO, USA

3.1.3 Sterilization

All experiments matherials (plastic tips, collection tubs, glass disposables) were sterilized by autoclaving at 1.05 kg/cm^2 (15 psi) on liquid cycle for 20 min. Distilled water (dH2O) and ultrapure water (Mili-Q or ddH2O) were used for critical parts of the study.

3.2 Methods

3.2.1 Ovariectomy surgical procedures

Ovariectomy is used to omit animal’s internal steroid hormones. To remove animal’s ovary, rats were anaesthetized with ketamine (80 mg/kg) and xylazine (8 mg/kg). Ketamine is a hydrochloric salt and used for general anesthesia in combination with a sedative such as xylazine. Xylazine is a drug that used for relaxation, painkilling, and general anesthesia in animals. The combination of these two drugs were injected intraperitoneally (IP). After injection of ketamine and xylazine rat was placed in left lateral position. Before starting, the rat’s operation area was shaved and swabbed with 70 % ethanol to remove filth. Small incisions were made in skin and muscle; ovary can be seen between fats in the peritoneal cavity. The ovaries were removed and remaining part was returned into the peritoneal cavity. Then incision parts of muscle and skin was sutured. Ovariectomy operation was completed by dorso-lateral incisions, with same method for opposite side (Parhizkar *et al.*, 2008). Two weeks after surgery, rats were prepared to continue into further experiment.

3.2.2 Animal preparation and administration of sex steroid hormone

In first part of the study, rats were ovariectomizedtwo weeks prior to steroid treatments. Based on the treatment, animals were distributed into different groups (n = 6per group). Hormones were injected subcutaneously at the neck cuff with a 24 hours interval for 3 days consecutively. Antagonists were also administrated subcutaneously at the neck cuff, 30 minutes before the hormone injection. For transcriptome and proteomic studies expression level of relaxin receptor isoforms (RXFP1 and RXFP2) mRNA and protein were detected in the hamstring muscle, patellar tendon, and collateral ligament tissues. Control group, which received peanut oil (vehicle), was also treated for three consecutive days. The drugs were dissolved in peanut oil and were subcutaneously administered.

3.2.3 Identification of oestrous cycle stages

Vaginal secretions were collected by using a plastic pipette filled with 10 µL of normal saline (NaCl 0.9%). The tip of the pipette was inserted into rat vagina, but not deeply to avoid cervical stimulation. Unstained material was placed onto a slide and was observed under a light microscope. The proportion of different cells was used to determine oestrus cycle phases, in which round and nucleated cells are epithelial cells which define proestrus; irregular shapes without nuclei are the cornified cells that were observed during estrus; little round cells are the leukocytes that characterize diestrus. At metestrus, however, three different kinds of cells could be identified

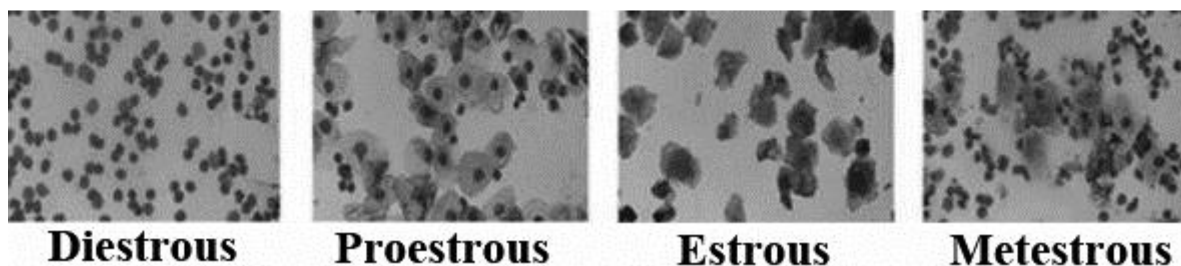


Figure 3-2 Identification of the oestrous cycle in rat

3.2.4 Animal study groups

3.2.4.1 Oestrogen treated group

- Control group
- 4 doses of oestrogen (0.2, 2, 20, 50 μg/kg)
- 7α,17 β-[9 - [(4,4,5,5,5-Pentafluoropentyl)sulfinyl]nonyl] estra - 1, 3, 5(10) - triene-3,17 - diol (ICI 182/780) (25 mg/kg)
- Physiological dosage of oestrogen (0.2 μg/kg) + ICI 182/780
- 4 - [2 – phenyo - 5, 7 – bis (trifluoromrthyl) pyrazolo (1, 5 - a) pyrimidin – 3 - yl]phenol (PHTPP), an ER - β blocker (25 mg/kg), as internal control
- Physiological dosage of oestrogen + PHTPP (25 mg/kg)
- 1, 3 – bis (4 - hydroxyphenyl) – 4 – methyl – 5 - [4 - (2 -piperidinylethoxy) phenol] - 1N - pyrozole dihydrochloride (MPP), an ER - α blocker (25 mg/kg) as internal control
- Physiological dosage of oestrogen + MPP (25 mg/kg)

3.2.4.2 Progesterone treated group

- Control group
- Physiological dosage of progesterone (4 mg/kg)
- Mifepristone (7.5 mg/kg)as internal control
- Progesterone (4 mg/kg) + Mifepristone (7.5 mg/kg)

3.2.4.3 Testosterone treated group

- Control group

- 2 doses of testosterone (125 and 250 μg/kg)
- 2 doses of testosterone (125 and 250 μg/kg)
- 2 doses of testosterone + Flutamide (10mg/kg)
- Flutamide (10 mg/kg) as internal control
- 2 doses of testosterone Finasteride (20 mg/kg)
- Finasteride (20 mg/kg) as internal control

3.2.4.4 Oestrous cycle

- Prostrous
- Estrus
- Metestrous
- Diestrous

3.2.5 Investigation of blood serum hormone level

Blood samples were collected into a separator tube (SST) and allowed to clot for 30 minutes at room temperature. The samples were centrifuged at 3000×g, for 15 minutes. Serum samples were aliquoted and stored at -20° C. Serum samples were analysed in duplicate for relaxin by using enzyme-linked immunosorbent assay (ELISA) kit (CUSABIO - USA). ELISA was performed according to manufacturer's guidelines. Absorbance for relaxin was determined by using a microplate reader (iMark; Bio - Rad, Hercules, CA, USA) at a wavelength of 450 nm. A set of standard serial dilutions of known concentrations were provided by the manufacturer and were used to construct a standard curve to determine the hormone levels. The overall intra-assay percent coefficient of variation for rats were 150, 75, 37.5, 18.75, 9.4, 4.7, 2.4, 0 ng/ml.Standard curves were

created by serial dilution and hormones concentration in the samples were determined from the standard curve.

3.2.6 Knee range of motion (ROM)

Rat's knee ROM was measured using digital miniature goniometer. A day after the last day of drug treatment, the rats were anesthetized using ketamine and xylazine (80 + 8 mg/kg). The depth of unconsciousness was confirmed from the lack of response to painful stimuli, which was usually applied at the plantar surface of the foot (Flecknell, 2009). It is important to maintain the animal in deep anaesthesia in order to prevent active muscle contraction in response to pain which will increased resistance towards passive traction (Bazin *et al.*, 2004). The hip and knee joints were fixed and rest on the sensor. Meanwhile, the lower leg (knee up to the ankle) was tied *in-situ* to the device arm. Rat knee ROM was measured by pulling the device arm in a clockwise direction at a minimum constant force (12 ± 1 N) using a mini digital Newton meter (American Weight, USA; model: AMW – SR - 1KG). Once the force exceed 13 N, the traction applied was immediately terminated and the angle obtained was recorded which represents passive extension. Assessment of the angle was made in different groups of treatment. This device and its function are shown in Figure 3.3.

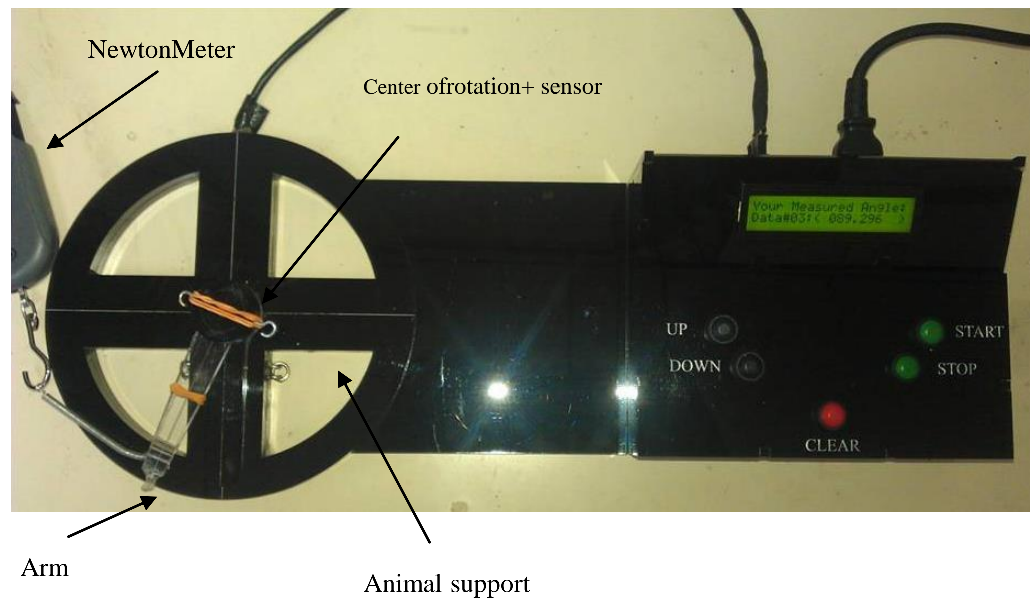


Figure 3-3 Measurement of rat's ROM by digital miniature goniometer
(Patent IC/No: PI 2013701411). The image of digital goniometer is shown. The device consisted of an arm that is attached to a sensor, which can detect changes in the angle once the arm is rotated. The sensor is connected to software which analysed the angle using Torque principle. "Torque, is the tendency of a force to rotate an object around a fixed axis and is defined by "$\tau = r\ F \sin\ \theta$" formula. r: rat leg length (r), F: force (A), θ: angle between the applied force and rat's ROM (A and B distance).

Figure 3-4 Digital Newton meter
(American Weight, USA; model: AMW-SR-1KG)

3.2.7 Real Time PCR (qPCR)

3.2.7.1 Real Time PCR, principle

The Polymerase Chain Reaction (PCR) is a process for the amplification of specific fragments of DNA / RNA. Real time PCR is a specialized technique that allows a PCR reaction to be visualized "in real time" as the reaction progresses and known as a sensitive technique to calculate gene expression in the data that were collected during the PCR amplification process. Quantitative PCR (qPCR) is used in variation of molecular biology studies such as Gene expression analysis: Cancer and Drug research, Disease diagnosis (Viral quantification), Food testing, animal and plant breeding (Gene copy number), and for determining of DNA / RNA interfering (Palmer *et al.*, 2003). Although in normal PCR, the product can only measure at the end of process, in qPCR it can be measured while it occurs. The development of fluorogenic labelled probes also made it possible to eliminate post-PCR processing for the analysis of probe degradation. This method able to detect small difference between samples compare to other methods, however it needs high cost reagents and equipment (Wong & Medrano, 2005).

Reverse transcription-quantitative of mRNA or Real-time can be achieved as a one-step or two-step procedure. The most common method for gene expression investigation is two-step RT-qPCR. With one-step method, the reverse transcription and PCR amplification steps are performed in a single buffer system while two-step procedure is performed in two separate reactions that allow storage of cDNA for long time (Wong & Medrano, 2005). Two-step RT-qPCR was performed in this study. There are two different types of methods for detection of PCR: TaqMan and SYBR Green. Each method has its limitations and benefits. TaqMan chemistry (known as fluorogenic 5´ nuclease) enables you to perform

multiplex PCR, SYBR Green method (highly specific, double standard DNA binding) has only Quantification probe. TaqMan assay was used in this study. Real time PCR amplification is shown in Figure 3.5.

3.2.7.2 Real-time PCR demonstration

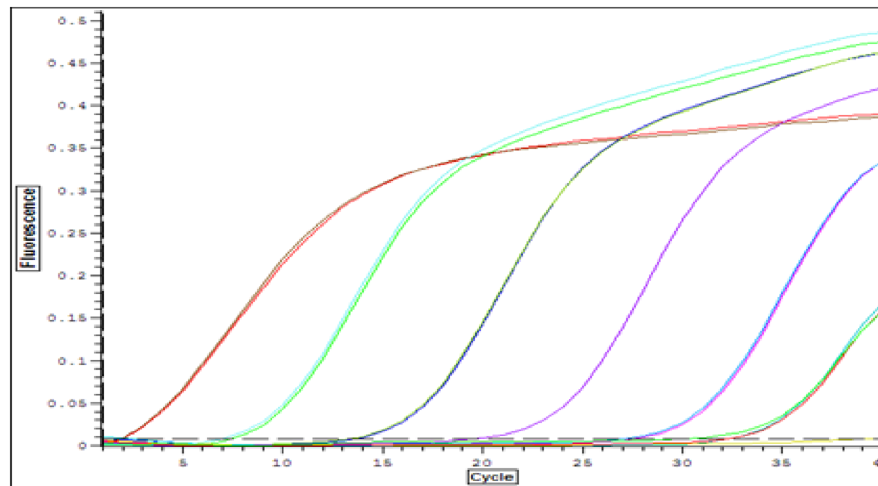


Figure 3-5 Real time PCR amplification view

3.2.7.3 Sample collection and Tissue processing for RNA isolation

Patellar tendon and collateral ligament tissues were exposed after 3 days treatment and from the rat knee washed with PBS buffer and soaked in five volume of RNA Later®. RNA later® solution was used to stabilize and protect tissues cellular RNA (Ambion, L/N: 1206029). Samples can be preserved in RNA Later® solution at – 20 ° C or below for up to 2 years. Total RNA was extracted from 30 mg tissues (wet weight) that were floating into the RNA later® solution using the RNeasy Fibrous tissue Mini kit (QIAGEN, Germany).

3.2.7.4 RNA extraction

RNeasy® Fibrous Tissue kit (QIAGEN, Germany) specific kit for fibre-rich tissues was used to isolate RNA from hamstring muscle, patellar tendon, and lateral collateral ligament. The kit contains are: Buffer RLT, Buffer RW1, Buffer RPE, RNase-Free Water, Proteinase K, RNeasy Mini Spin Columns, Collection Tubes (1.5 ml), RNase-Free DNase I (lyophilized), Buffer RDD. The tissues were retrieved from RNA later® solution with sterile forceps and drip-dried immediately with an absorbent paper towel, then soak into the lysis RLT Buffer added with β-mercaptoethanol. A 30 mg (wet tissue) was homogenized using a rotor–stator TissueRuptor (230V, 50-60 Hz_ QIAGEN, Germany). Homogenate was incubated at 55°C for 20 minutes with added 590 µl RNase-free water and 10 µl proteinase K solution to the lysate. The supernatant transferred after Centrifuging (20 – 25°C for 3 minutes) to the new tube and then 0.5 volumes (usually 450 µl) of pure ethanol added to cleared lysate. It was mixed well by pipetting and Transferred 700 µl of the sample, to the RNeasy Mini spin column placed in a 2 ml collection tube (2 times), by discarding the flow through. After washing with RW1 buffer, mixed of 10 µl DNase I stock solution and 70 µl buffer RDD was added directly to the RNeasy spin column membrane to remove DNA. DNase I is sensitive to physical denaturation. Mixing should carry out by gently inverting the tube. This mixture was incubated for 20 minutes at room temperature (20 – 30°C). The membrane then was washed twice with 500 µl of RPE washing buffer. RNA was collected by Placing of RNeasy spin column in a new 1.5 ml collection tube and adding 30 – 50 µl RNase - free water.

3.2.7.5 RNA quality control

The pure isolated RNA may be contaminated by DNA, protein or phenol that could inhibited the RNA downstream solicitation. Therefore, evaluating of RNA integrity and quality is necessary. The purity and concentration of RNA was evaluated by A260/280, A260/230 UV absorption ratios (Gene Quant 1300, UK). Agarose gel is the most common way to evaluate RNA integrity. Each RNA sample was assessed by integrity of Ethidium-Bromide agarose gel 1 % and evaluated by electrophoresis in TBE buffer. After run the agarose gel for 45 minutes in 90 V, gel was observed in the specific gel documentation system (Vilber Lourmat, Fisher Scientific). RNA sharp and clear bands can be seen in 18S and 28S rRNA. To catch the RNA bands location 1Kb DNA ladder was used.

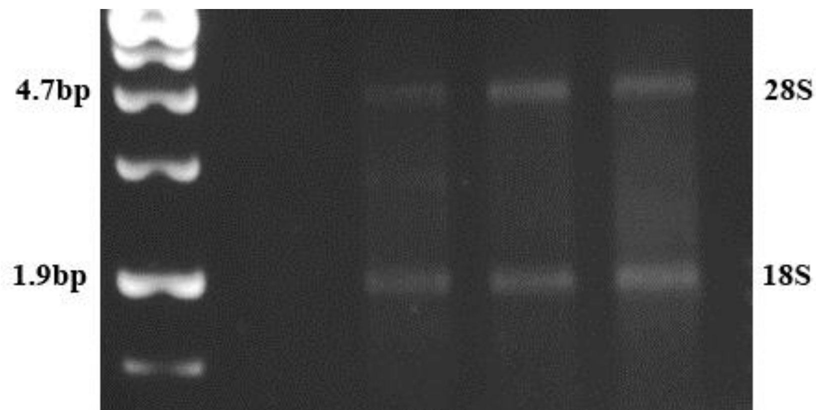


Figure 3-6 RNA gel electrophoresis

3.2.8 RNA to cDNA converssion

To perform the two-step Real time PCR method, RNA samples need to be converted to cDNA. Reverse transcription into cDNA was performed by using Two-Step qRT-PCR kit, High capacity RNA to cDNAby Applied Biosystems_US. This kit contains reagents for reverse transcription (RT) buffer and enzyme to convert total RNA to single-stranded

cDNA. According to the manufacturer's guidelines total RNA converted to cDNA by adding of up to 9 µl RNA (1000 ng), 10 µl of RT buffer, and 1 µl of RT enzyme. To perform reverse transcription of converting, optimized program the thermal cycler conditions was used. The program was included 37°C for 60 minutes, 95°C for 5 minutes and hold in 4°C (Kwok & Higuchi, 1989). The total tube of cDNA can be stored in –80 ° C for long term storage.

3.2.9 Real time PCR running

After converted total RNA to cDNA and their quantification, two-step Real time PCR was run to evaluate the mRNA gene expression by following the manufacturer procedures. The amplified region of cDNA was tagged with fluorescence-labeled probe. The specificity of the primer and the probe ensures that the expression of the target DNA was specifically evaluated. Real time PCR does not require a time consuming post amplification gel electrophoresis due to its high sensitivity. TaqMan probe has a sensitivity of 100 % and a specificity of 96.67 % (Tsai *et al.*, 2012) and is capable of detecting as few as 50 copies of RNA/ml and as low as 5 - 10 molecules (Hofmann-Lehmann *et al.*, 2002).

All reagents including probes and primers were obtained from Applied Biosystems, USA. Primers were designed by the same company for specific targets: RXFP1: Rn01495351; Lot no: 926762, and RXFP2: Rn01412901; Lot no: 651878, amplifies 116 bp segment of RXFP1 from the whole mRNA length of 2277 bp, and 138 bp segment of RXFP2 from the whole mRNA length of 2214 bp. Hprt1 and GAPDH were used as reference genes. Expression levels of Gapdh andHprt1 in each sample were determined for normalisation as housekeeping genes. The catalogue number for the housekeeping genes are: GAPDH, Rn99999916_S1, Lot no: 10377343, which amplifies 87 bp segment of

GAPDH from the mRNA length of 1307 bp and Hprt1, Rn01527840, Lot No: 1118680, which amplifies 67 bp, segment from the mRNA length of 1260 bp. The target assay was validated in-silico using whole rat genome sequences and *in - vitro* using whole rat cDNA sequences to ensure target sequences were detected (Applied Biosystems, USA). Nuclease-free water was used throughout Real-time PCR and qPCR experiments.

To carry out the reaction combination 50 ng of cDNA in 1 µl, 5 µl of master mix buffer, 1 µl of each target primer, and 3.5 RNase free water was loaded in 96 well plates. The total volume of PCR mixture was 10 µl in each well. All experiments samples were loaded in three biological replicates. After prepared the reaction plate, plate was loaded into the Applied Biosystem Step One Plus thermal cycler. Real time-PCR program was set according to manufacture protocol included 2 minutes at 50ºC reverse transcriptase, 20 seconds at 95ºC activation of polymerase, denaturation at 95ºC for 1 second and annealing at 60ºC for 20 seconds. Denaturing and annealing step was performed for 40 cycles.

3.2.10 Data Analysis for Quantification of Gene Expression

Data was analysed according to Comparative fold changes in expression were method (Wong & Medrano, 2005):

Δ Ct = Ct of target gene – Ct of housekeeping genes average

$\Delta\Delta$ Ct = ΔCt of treated samples – Δ Ct of non-treated samples

Fold changes = Ct ($2^{-\Delta\Delta Ct}$)

Amplifications of the target and reference genes were measured in the samples and reference. Measurements were standardized 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 was used to calculate RNA folds changes.

3.2.11 Protein expression by Western blotting

3.2.11.1 Western blotting- principle

Western blotting or protein immunoblot is an analytical technique that is commonly accepted to detect specific proteins in the total extracted protein from tissue or cell lines. This method separates the protein by running the gel electrophoresis and identifies with specific antibodies according to their size. The bands blot is on a membrane (nitrocellulose or PVDF). The first step in western blotting is running the gel electrophoresis to separate the total protein, then antigen was transferred to the blotting membrane electrophoretically. In this study PVDF (polyvinylidene difluoride) membrane was used. In the next step, protein was transferred to PVDF membrane; the remaining protein site was blocked to reduce nonspecific bands. After blocking, membrane was soaked into the primary and secondary antibodies to bind and specify the target protein. Finally, the target protein was visualized with incubation of membrane into the substrate detection reagent (Walker, 1996).

3.2.11.2 Sample collection and Tissue processing for protein extraction

Hamstring muscle, patellar tendon, and collateral ligament tissues were exposed and immediately frozen in liquid nitrogen (- 196°C) and then stored at – 80°C prior to protein extraction.

3.2.11.3 Protein extraction

Total protein was extracted with using PRO-PREP (Intron, UK) solution kit. This kit is able to isolate protein from whole cell of tissue. This solution included is of highly denature ionic detergent such as sodium dodecyl sulphate (SDS), lithium dodecyl sulphate and sodium deoxycholate that can isolate protein as a monomeric for any protein molecular weight analysis and western blot. It also contain zwitterioninc detergent CHAPS (3 - [(3 - Choamidopropyl) dimethylammonio] - 1 - propanesulfonate) which is more effective in disrupting protein interaction. Total protein was isolated by following the protocol:

1 - 30 mg of hamstring muscle (wet tissue) was separated and soaked into the 400 µl of extraction solution. Whole part of patellar tendon and collateral ligament were cut and placed into the 250 µl into the solution. The maximum weight of patellar tendon and collateral ligament were 15 mg.

2- The tissues were homogenised with using an ultrasonic cell disruptor (Branson) by putting on ice to disrupt tissues.

3- After 10 minutes homogenising; samples were incubated at – 20°C for 30 minutes to persuade cell lysis. This step was performed two times to increase protein concentration.

4- The samples were centrifuged 15 minutes, at 4°C, 13000 rpm to separate supernatant. The supernatant then transferred into the new 1.5 tube and stored in – 20°C.

3.2.11.4 Protein quantification

Protein quantification was performed to determine samples concentration by using Micro BCA Protein Assay kit (Thermo Scientific™ - US). This kit contains Micro BCA Reagent A (MA), Micro BCA Reagent B (MB), Micro BCA Reagent C, andBovine Serum Albumin (BSA) as Standard Ampules at 2.0 mg/mL in a solution of 0.9 % saline and 0.05

% sodium azide. The Kit is a detergent-compatible bicinchoninic acid formulation for the colorimetric detection and quantitation of total protein.A purple-colored reaction product is formed by the chelation of two molecules of BCA with one cuprous ion (Cu^{+1}). This water-soluble complex exhibits a strong absorbance at 570 nm wavelength that is linear with increasing protein concentrations. The samples concentration were calculated from a linear plot of BCA standard concentration (Axis X) of the intensity by changing of colour in absorbance. This absorbance obtained from the reaction between BSA and BCA reagents. Standard concentrations of BSA were prepared at 10, 8, 6, 4, 2, 1, 0.5, and 0 µg/ml. The samples then were diluted 1 in 50 with PBS buffer. 50µl of total protein samples or standard as a serial dilution were added to 50 µl of BCA reagents in a Greiner UV Transparent 96 Well Plate (Thermo Scientific™ - US) in triplicate. The plate was incubated for 2 hours at 37 ° C, then the intensity of changed colours were qualified by using a plate reader at wavelength of 570 nm. Standard curve was generated by serial dilution and samples protein concentration calculated from the standard curve.

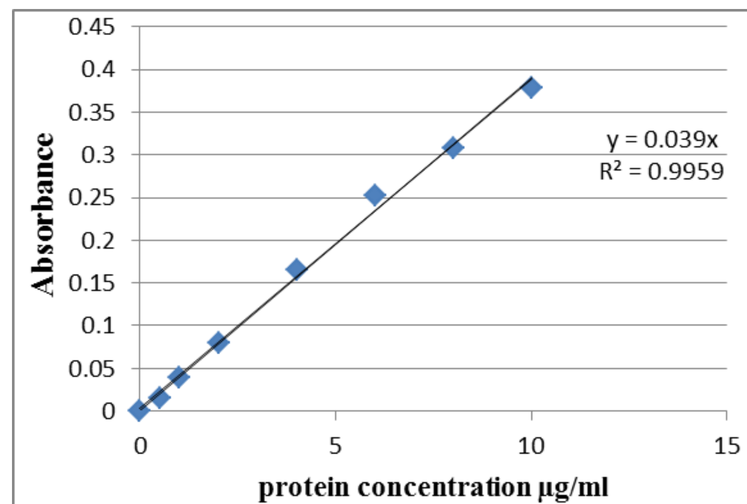


Figure 3-7 Standard curve for protein determination

3.2.12 Western blot running

SDS-PAGE running: Electrophoresis gel or SDS-PAGE gel (sodium dodecyl sulfate polyacrylamide gel electrophoresis) can be one-dimensional or two-dimensional technique to detect and characterize of specific protein. One-dimensional electrophoresis is used for most protein and nucleic acid separations. Relocation of protein in electrophoresis is based on charges, mass, size and strength of electrical field (Bolt & Mahoney, 1997).

The matrix is polyacrylamide gel that is polymer of acrylamide and bis- acrylamide (Sigma-US), the presence of N, N, N′, N′ - tetramethyleethylenediamine (TEMED), and ammonium per sulphate (APS), catalyses the gel polymerization (Walker, 2007). According to the protein size, samples separate within the gel and determine by their molecular weight. High percentage of gel for low molecular weight and low percentage for high molecular weight is recommended. Ionic detergent SDS is used in polyacrylamide gel, which denatures the protein and makes them negatively charged. The charge is equally distributed to protein molecule based on their length and lead to migration according to the size.

Polyacrylamide gel contain of two different parts (resolving and stacking) with separate formulation. Resolving part used at the bottom of gel caster with a pH of 8.8 and stacking part is placed above the resolving with a pH of 6.8. In this study, resolving gel with 12 % for (RXFP1, RXFP2, and Beta Actin) was used. Stacking gel is used to pack protein together after loading and mostly prepare in 4 %. The gel was prepared in gel cast and kept overnight at 4°C. The compositions of each gel are shown in Table 3.2.

Table 3-2 Composition of 12 % resolving and 4 % stacking gel

Stock solution	Resolving gel 12 %	Stacking gel 4 %
Acrylamide	8.3 ml	0.665 ml
4X resolving	4 ml	_
4X Stacking	_	1.25 ml
10 % SDS	0.2 ml	50 µl
10 % APS	0.1 ml	25 µl
TEMED	7 µl	5 µl
Double distilled H2O	6.4 ml	3 ml

The following day equal volume of samples (20 µg) were mixed with loading dye, then boiled for 5 minutes and loaded in the gel. Gel cast then placed inside the electrophoresis tank and bathed in migration buffer with the recommended time as instructed by the manufacturer. Pre-stained protein marker was loaded into the one of the empty wells to assess protein size bands. Rabbit polyclonal Beta Actin (Abcam, UK) was used as loading control, to check whether all samples have been loaded equally, and compare the expression of different samples. Electrophoresis was disconnected when the front dye reached at the bottom of the gel.

Transfer to membrane: The separated samples by electrophoresis, the protein transferred to PVDF (BIORAD, UK) membrane for immunoblotting. A hydrophobic membrane needs to prewet into pure methanol (2 minutes) and then into the twobin buffer (5 minutes) which contain 20 % methanol. The presence of methanol in twobin buffer (transfer buffer) is to promote dissociation of SDS from the protein and improve adsorption of protein onto the membrane (Pettegrew *et al.*, 2009). The PVDF membrane was placed next to the gel. The two were sandwiched between absorbent materials, and clamped between solid supports to

maintain tight contact between the gel and membrane without air bubbles. Samples transformation can perform in wet or semi-dry conditions. In this study, wet condition tank is used. The gel membrane sandwich was placed in wet transfer tank for electro blotting at constant voltage of 100 V for 90 minutes. After transferred membrane was exposed and washed with PBST (phosphate buffer saline-tween 20) for 3 times, 5 minutes.

Blocking step: To block the membrane, two blocking solutions were traditionally used: non-fat milk or BSA (5 %). Milk is cheaper but is not recommended for studies of phospho-proteins, it cause high background because the phospho-specific antibody detects the casein present in the milk. BSA was used to block the membrane 90 minutes at 4°C under agitation. Washing with PBST 3 times, 5 minutes is necessary on this step before incubation with primary antibody.

Incubation with antibodies: primary antibody was diluted 1:1000 in PBST buffer and incubated with membrane 60 minutes at 4°C under agitation. After washing with PBST buffer, secondary antibody (attached to horseradish peroxidase-HRP) was diluted 1:2000 and incubated for 60 minutes under agitation.

Visualizing:tovisualize the target protein band Opti - 4CN ™Substrate kit was used. This kit is colorimetric (HRP) substrate and detected band can be seen by necked eyes. Primary and secondary antibodies that used in this study are shown in Table 3.3.

Table 3-3 primary and secondary antibodies that have been used in western blot.

Target protein	Primary antibody	Secondary anti body
RXFP - 1/LGR7	Rabbit polyclonal	Mouse anti-rabbit HRP
RXFP - 2/ LGR8	Mouse polyclonal	Rabbit anti-mouse HRP
Beta Actin	Rabbit polyclonal	Goat anti-rabbit HRP

3.2.13 Western blot data Analysis

The visualized blots of target protein, membrane were captured by a gel documentation system (Vilber Lourmat, from Fisher Scientific, USA). The density of each band was determined using Image J software (National Institutes of Health, USA). The ratio of each target band over Beta actin was calculated and considered as the expression level of the target proteins.

3.3 Human study

Fortyhealthy females (20 athletes; 20.3 ± 1.28 years, 163 ± 2.75 cm height and 20 non-athletes; 21.7 ± 2.27 years, 158.5 ± 5.28 cm heights) who were not on hormonal contraceptives and were confirmed to have regular menstrual cycles after 3 cycles were monitored for 3 consecutive months volunteered to participate in this study. The inclusion criteria include no history of knee surgery or injury or history of injury or chronic pain in both lower extremities for the past 1 year. Subjects were not on specific medications. Eumenorrhic, Amenorrheic, Polymenorrhoeic subjects were excluded. The participants were informed the detail of the study from the information sheet, and written informed consent was provided. This study was approved by the Institutional of Medical Centre Board, University Malaya with Medical Ethics No: 1010.90.

3.3.1 Blood sample collection and serum hormones' analyses

Blood samples were collected 3 times per month: on day first of menstruation, day 12 which was in the follicular phase, and day 20 which was in the mid luteal phase (Widmaier *et al.*, 2006). The phases were determined using fertility chart and blood samples were withdrawn via venepuncture. After collection into a separator tube (SST), blood was allowed to clot for 30 minutes at room temperature. The blood was then centrifuge at 3000 g, for 15 minutes. Serum samples were then aliquoted and were stored at – 20°C. Enzyme-linked immunosorbent assay (ELISA) was performed using Elisa kit (CUSABIO - USA) to measure the levels of relaxin. Meanwhile, Radioimmunoassay was used to measure the level of oestrogen, progesterone and testosterone.

3.3.2 Basal Body Temperature

The basal body temperature (BBT) is used to determine whether the cycle is an ovulatory cycle. An ovulatory cycle was featured by a slight increase ~0.5^{o}C of BBT which usually occur in the mid of the cycle. BBT was measured when the body is at rest. In determining BBT, rectal temperature was obtained. The temperature was taken at 7 o'clock in the morning for 1 month duration. The sample of BBT recorded is shown in Figure 3.8.

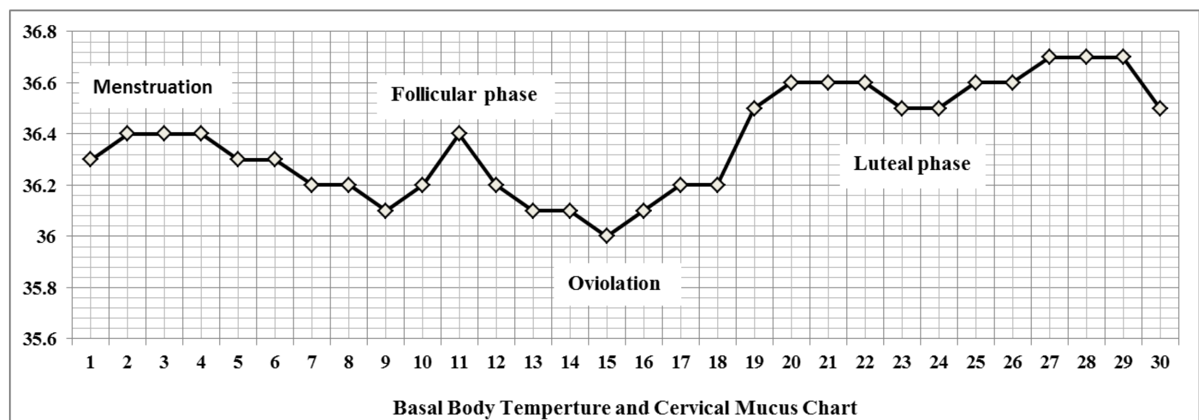


Figure 3-8 Basal body temperatures chart

3.3.3 Knee rotational movement

In this part of study we were examined the knee rotational movement by performing a varus-valgus examination using a manual orthopaedic goniometer ruler (model: MR0104PVC material 180°). These tests were used to measure the varus/valgus angles and the internal/external rotational laxity of the knee joint.Data were collected by measuring subjects both knees. In order to establish the reliability of the data, varus-valgus test were accomplished by fixing the knee at 30° and 0°. All procedures were identical to those described in the clinical examination textbook (Bendjaballah *et al.*, 1997). Varus-Valgus test were performed by placing unusual stresses forces on the articular surfaces, joint capsule and ligaments.

Varus test is a stress force applied from the lateral side of the femur by adducting the ankle. Testing LCL (lateral collateral ligament) is performed by flexing the knee at 30 ° and 0 °, with subjects lying flat. One hand of the examiner was placed over the lateral joint line while another hand holds the lower leg firmly at the ankle. Meanwhile, valgus force is a stress force applied on the lateral side by abducting the ankle. Stretching of MCL (medial collateral ligament) was performed in medial side of the knee by flexing the knee at 30 and 0 °, with patient lying flat with one examiner hand was placed over the medial joint line and another hand holds the lower leg firmly. In order to ior to check for the relaibility of the data, these tests were performed by 3 different examiners and the data was gathered prior to analyses.

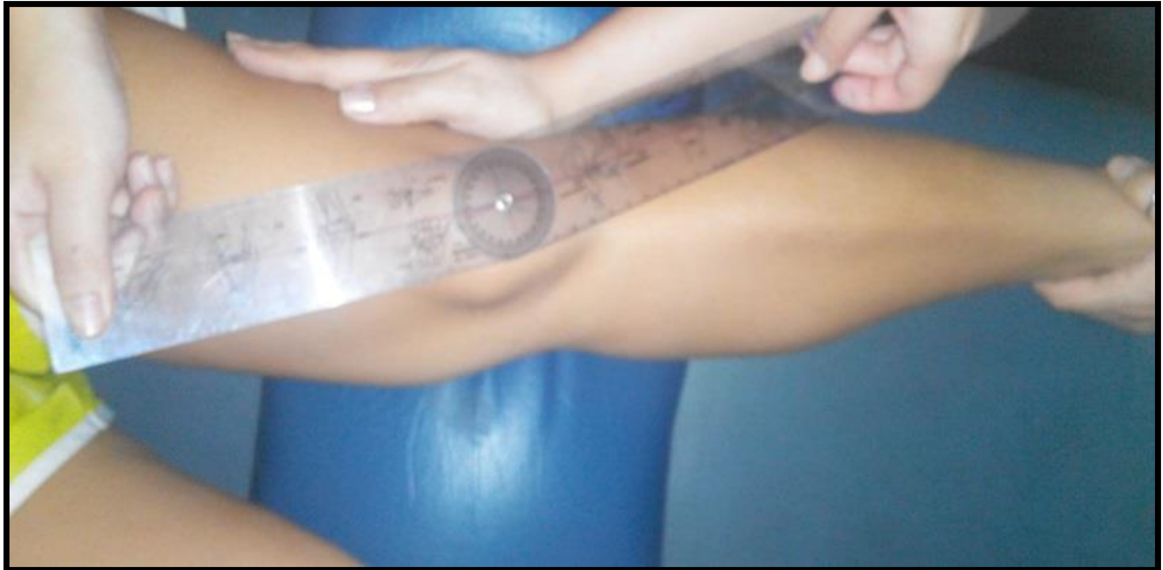


Figure 3-9 Knee rotational movement investigation by Varus-Valgus test at 30°

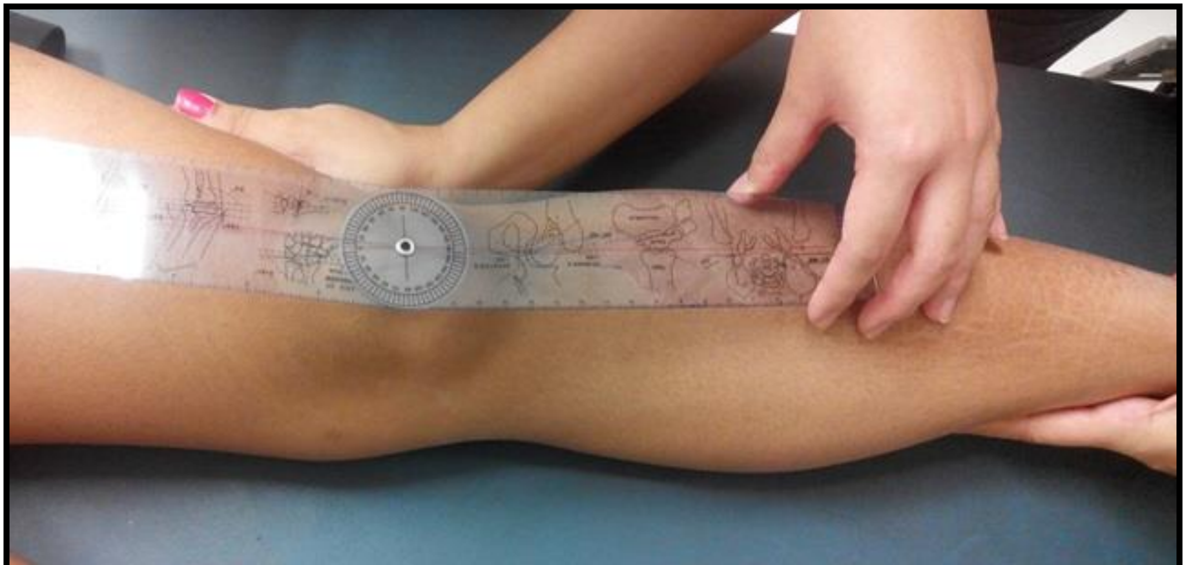


Figure 3-10 Knee rotational movement investigation by Varus-Valgus test at 0°

3.3.4 Data analysis of knee laxity in human

All the data were presented as mean ± standard deviation, analysed by descriptive analysis to determine hormone levels and rotational angles of each subject throughout the menstrual cycle.One-way ANOVA was usedto ascertain the correlation by using SPSS 18.0. $p < 0.05$ was considered as statistically significant.As for each variables, 3 measurements was used to evaluate the differences between 3 observations, one way repeated measure Anova was applied and the results revealed that there were no significant differences among these three observations.Therefore, the mean of these observations was computed and used for data analysis. Two-way ANOVA was usedto compare athletes and non-athletes groups. For variables with a normal distribution, Pearson correlation coefficient and for non-normal distributed variable, Spearman correlation coefficient was applied.

CHAPTER 4

Sex-Steroid Regulation of Relaxin Receptor Isoforms (RXFP1 & RXFP2) Expression in the Patella Tendon and Lateral Collateral Ligament of Female WKY Rats

4.1 Introduction

Relaxin which consist of relaxin-1, -2 and -3 and insulin-like peptides (INSL3, 4, 5 and 6) (Park *et al.*, 2005) is a polypeptide hormone that possesses structural similarity to insulin and is primarily synthesized by the corpus luteum and placenta (Conrad & Baker, 2013). Relaxin binds to and activates G-protein-coupled receptors (GPCRs): RXFP1(LGR7) and RXFP2(LGR8) (Scott *et al.*, 2012). Human relaxins H1 and H2 activate both RXFP1 and RXFP2 (Nair, 2012), while rat relaxin1 binds weakly to RXFP2 (Scott *et al.*, 2005). Relaxin H3 binds selectively to RXFP1 as well as RXFP3 (GPCR135) and RXFP4 (GPCR142) (Rosengren *et al.*, 2006). RXFP1 and RXFP2 are the two main relaxin receptor isoforms (Schwabe & Büllesbach, 2007).

Relaxin activity has been reported in both pregnant and non-pregnant female primates and non-primate vertebrates (Cernaro *et al.*, 2013; Hayes, 2004). In primates, ovarian relaxin is not required for successful term pregnancy (Hayes, 2004) however is required to maintain pregnancy in rats and pigs (Sherwood OD, 1993). During pregnancy in mice, relaxin is involved in transformation of pubic joint cartilage into a more flexible and elastic interpubic ligament in order to accommodate the enlarging uterus (Lippert *et al.*, 1987). In non-pregnant rats, relaxin administration alone or in combination with oestrogen reduces pubic symphysis collagen content, with progesterone antagonizing this effect (Samuel *et al.*, 1996). Meanwhile, during pregnancy in rats, relaxin was reported to regulate collagen catabolism in the pubic symphysis (Samuel *et al.*, 1998). In lower species such as rodents, relaxin action may be more reflective than in humans where serum relaxin level was found to have no link to the generalized joint laxity (Wolf *et al.*, 2013).

Relaxin affects tissues' extracellular matrix components including collagen, H_2O and hyaluronan. Relaxin increases cervical hyaluronan content by up-regulating the expression of hyaluronic acid synthase (Soh, 2012). This effect will result in loosening of tissues' dense collagen fiber network. Relaxin has been shown to possess anti-fibrotic action by down-regulating fibroblast activity, increasing collagenases synthesis and inhibit transforming growth factor (TGF-β)-stimulated collagen-l lattice contraction in rat kidney (Masterson *et al.*, 2004). Relaxin up-regulates matrix metalloproteinases (MMP), a family of extracellular proteases that interfere with matrix remodeling by their ability to degrade extracellular matrix components including collagen (Chow *et al.*, 2012). Relaxin has been reported to stimulate the expression of collagenase-1, MMP-1, MMP-3 (Hashem *et al.*, 2006), MMP-9, and MMP-13 in fibrocartilaginous cells (Ahmad, 2012) and MMP-2 in periodontal ligament cells (Ho, 2009; Miao, 2011).

Sex-steroids have been reported to affect relaxin receptor expression. In the human endometrium, relaxin receptor isoforms expression vary across the menstrual cycle with low level expressed during the proliferative phase and rises sharply at the time of ovulation (Bond *et al.*, 2004). In neonatal porcine uterus, oestrogen increases *RXFP1*mRNA expression, while in cervix, oestrogen increases and relaxin decreases the expression of this receptor (Yan *et al.*, 2008).During late pregnancy in mice, relaxin has no influence on RXFP1 expression (Siebel *et al*., 2003). Progesterone has been reported to increase the expression of RXFP1 in the pregnant rat uteri (Vodstrcil*et al*., 2010a) while oestrogen was found to regulate RXFP1 mRNA expression in human cervix at term (Maseelall, 2009).

Knee is another potential target for relaxin action. Currently, little is known with regards to the expression of RXFP1 and RXFP2 in the knee. Wang et al. (Wang *et al.*, 2009a) reported that relaxin receptors were expressed in mouse knee fibrocartilage. In humans, relaxin receptors have been identified in the anterior cruciate ligament (ACL) in male and female knee (Faryniarz *et al.*, 2006), with higher expression in female (Dragoo *et al.*, 2003). This gender specific difference suggests the influence by female sex hormones. Apart from ACL, the expression of relaxin receptors in other knee structures including patellar tendon and lateral collateral ligament is currently unknown. Together with ACL, they participate in the control of knee stability during movement.

We hypothesized that female sex hormones affect RXFP1 and RXFP2 expression in the patella tendon and lateral collateral ligament, thus affecting knee laxity. This could explain differences in the incidence of non- contact knee injury between male and female (Sutton & Bullock, 2013), and in female at different phases of the menstrual cycle (Hansen *et al.*, 2013). In view of this, we investigated these isoforms expression in the patella tendon and lateral collateral ligament in rat model in order to provide the mechanism underlying changes in knee laxity under different sex-steroid influence.

4.2 Results

4.2.1 Plasma Sex-Steroid Levels following Subcutaneous Hormone Injection

In Table 4-1, the average hormones level in the blood in different groups of treatment is shown. Our findings indicate that serum levels of oestrogen and testosterone correlate with the doses administered. Subcutaneous injection of 0.2μg/ml

oestrogenresulted in plasma level of approximately two times higher than the control while injection of 4 mg progesterone resulted in five times higher level than the control.

Table 4-1 Plasma sex steroid level following subcutaneous hormone injection.
Values were given as mean ± standard error of mean. E – Oestrogen, P – progesterone and T – testosterone. The level of hormones in the blood correlates with the amount injected via the subcutaneous route.

Treated samples	Hormone level (Mean ± SD)
Estrogen	
Control	39.4 ± 0.14 pg/ml
0.2 μg/kg E	69.7 ± 0.12 pg/ml
2 μg/kg E	195.2 ± 0.31 pg/ml
20 μg/kg E	267.7 ± 0.76 pg/ml
50 μg/kg E	329.4 ± 2.33 pg/ml
Progesterone	
Control	4.18 ± 0.11 ng/ml
4 mg/kg P	23.67± 0.13 ng/ml
Testosterone	
Control	0.25 ± 0.004 ng/ml
125 μg/kg T	0.89 ± 0.005 ng/ml
250 μg/kg T	1.07 ± 0.002 ng/ml

4.2.2 RXFP1 and RXFP2 Proteins Expression in the Patellar Tendon and Lateral Collateral Ligament

In Figure4-1, the expression of RXFP1 protein is the highest in the lateral collateral ligament of progesterone treated rats with 1.75 fold increase as compared to control. Treatment with oestrogen at 0.2 μg/ml resulted in a slight but significant increase in the expression of RXFP1 protein as compared to control ($p<0.05$). Treatment with higher

oestrogen doses (>2µg/ml) resulted in a dose-dependent increase in RXFP1 protein expression with 43 and 100 percent increase following treatment with 20 and 50µg/ml oestrogen respectively. Testosterone treatment resulted in a significant decrease in the expression of RXFP1 (0.54 and 0.45 fold increase following subcutaneous administration of 125 and 250µg/ml testosterone respectively).

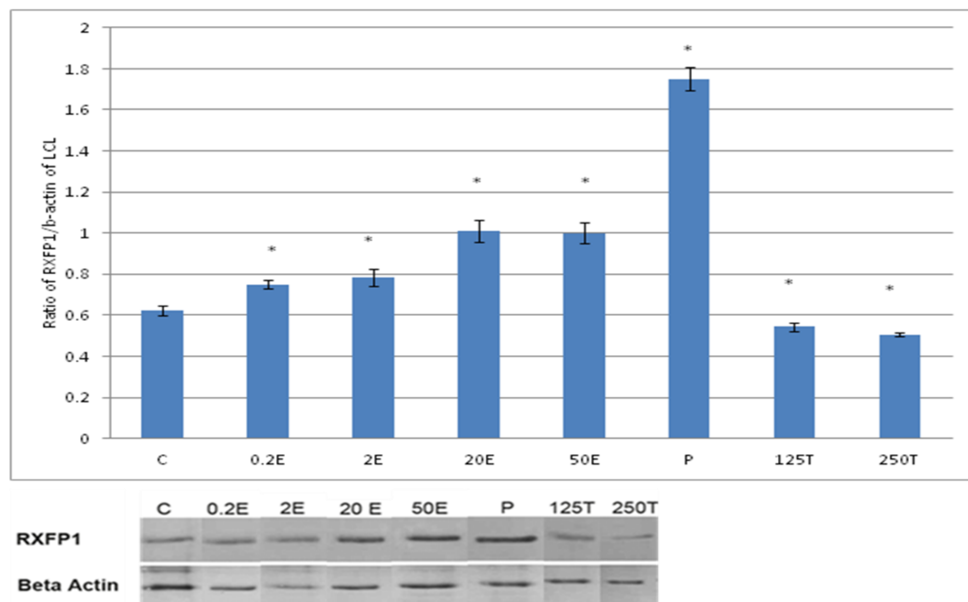


Figure 4-1 The expression of RXFP1 protein in the total homogenate of collateral ligament from ovariectomised rats treated with different sex-steroids: C- control; 0.2E – 0.2µg/ml oestrogen; 2E - 2µg/ml oestrogen, 20E - 20µg/ml oestrogen; 50E –50µg/ml oestrogen; P – 4mg progesterone; 125T – 125µg testosterone; 250T – 250µg testosterone, LCL-lateral collateral ligament. Data were expressed as mean ± SEM, n=6 per treatment group. * $p<0.05$ as compared to control, Shapiro-Wilk test, p= 0.319732. A dose-dependent increase in RXFP1 protein expression was observed following oestrogen treatment. Progesterone caused the highest increase while testosterone caused a decrease in this isoform expression. The image was cut in order to arrange the bands in sequence with the label on the x-axis of the bar chart

In Figure 4-2, the expression of RXFP1 protein in the patellar tendon following treatment with 4mg progesterone is 2.71 folds higher than the control. Oestrogen treatment resulted in a dose-dependent increase in this protein expression (1.25 to 2.67 fold increase following treatment with oestrogen at doses between 0.2 to 50µg/ml). Testosterone

treatment resulted in a dose-dependent decrease in RXFP1 protein expression (0.65 and 0.51 fold decrease) following treatment with 125 and 250µg testosterone respectively. The expression of RXFP1 protein in the patella tendon was higher than the lateral collateral ligament.

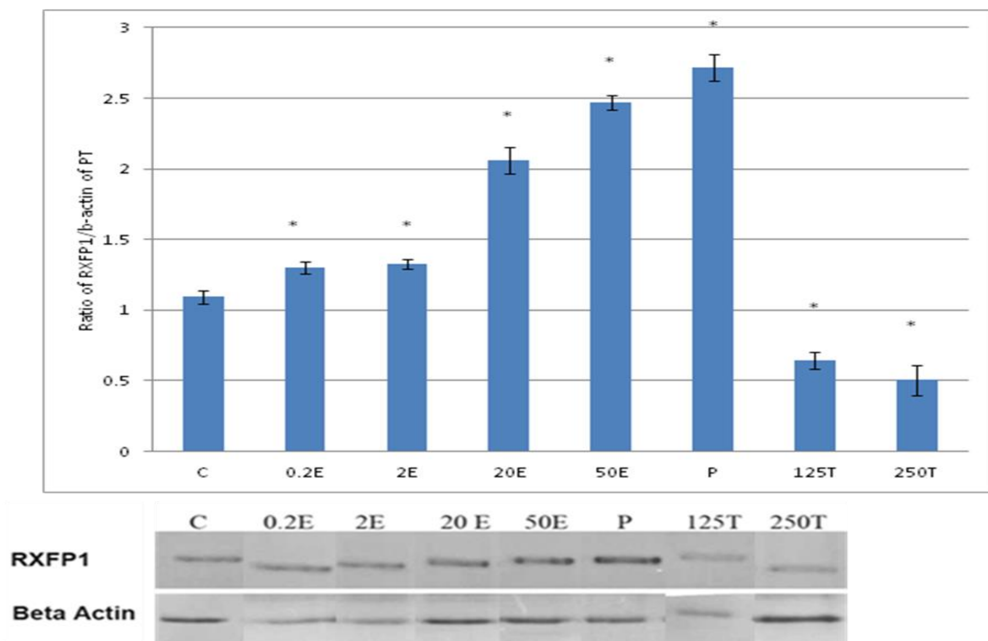


Figure 4-2 The expression of RXFP1 protein in the total homogenate of the patellar tendon from ovariectomised rats treated with different sex-steroids: C- control; 0.2E – 0.2µg/ml oestrogen; 2E - 2µg/ml oestrogen, 20E - 20µg/ml oestrogen; 50E –50µg/ml oestrogen; P – 4mg progesterone; 125T – 125µg testosterone; 250T – 250µg testosterone PT- patella tendon. Data were expressed as mean ± SEM, n=6 per treatment group. * $p<0.05$ as compared to control, Shapiro-Wilk test, p= 0.282109. A marked increase in the expression of RXFP1 protein occurs following treatment with high doses of oestrogen (20 & 50µg/ml). Meanwhile, progesterone treatment caused the highest increase while testosterone caused a decrease in RXFP1 protein expression in the tendon. The image was cut in order to arrange the bands in sequence with the label on the x-axis of the bar chart.

In Figure 4-3, the expression of RXFP2 protein in the lateral collateral ligament was the highest following treatment with progesterone (0.93 fold increase as compared to control) ($p<0.05$). A dose-dependent increase in the expression of RXFP2 protein was observed following treatment with increasing doses of oestrogen (0.93 to 1.09 fold increase with oestrogen doses between 0.2 to 50µg/ml). Treatment with 0.2µg/ml oestrogen did not result in a significant increase in RXFP2 protein expression as compared to control. Treatment with 125 and 250µg/ml testosterone resulted in a significant decrease in the expression of RXFP2 protein, with 13 and 14 percent reduction as compared to control.

In Figure 4-3, the expression of RXFP2 protein in the patellar tendon was the highest following progesterone treatment (1.28 fold increase as compared to control). Treatment with increasing doses of oestrogen resulted in a dose-dependent increase in RXFP2 protein expression; although the increase observed following 0.2µg/ml oestrogen treatment was not significantly differ from the control. A significant reduction in RXFP2 protein expression was noted following treatment with 250µg testosterone, which was 21.9 percent lower than the control. In the patella tendon, the expression of RXFP1 exceeds RXFP2 by more than two times in particular following treatment with high oestrogen doses (20 and 50µg/ml) and progesterone.

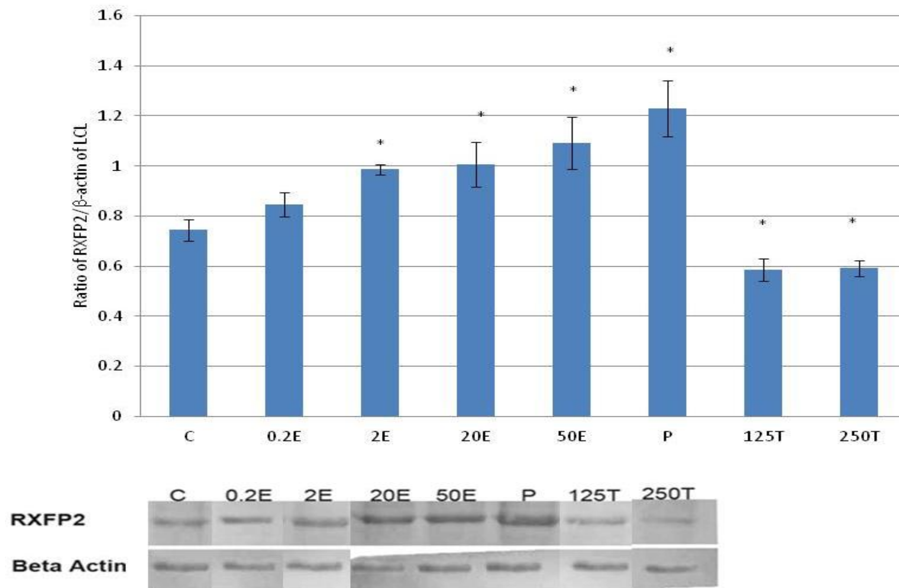


Figure 4-3 The expression of RXFP2 protein in the total homogenate of collateral ligament from ovariectomised rats treated with different sex-steroids: C- control; 0.2E – 0.2μg/ml oestrogen; 2E - 2μg/ml oestrogen, 20E - 20μg/ml oestrogen; 50E –50μg/ml oestrogen; P – 4mg progesterone; 125T – 125μg testosterone; 250T – 250μg testosterone, LCL-lateral collateral ligament. Data were expressed as mean ± SEM, n=6 per treatment group. * $p<0.05$ as compared to control, Shapiro-Wilk test, p= 0.776415. The expression of RXFP2 protein was increased following treatment with oestrogen at doses exceeding 2μg/ml. Meanwhile, progesterone caused the highest increase while testosterone caused a decrease in RXFP2 protein expression. The image was cut in order to arrange the bands in sequence with the label on the x-axis of the bar chart.

4.2.3 RXFP1and RXFP2 mRNAs Expression in the Patellar Tendon and Lateral Collateral Ligament

In Figure 4-4, following progesterone treatment, the expression of RXFP1 mRNA in the lateral collateral ligament was 2.71 folds higher as compared to control. The lowest expression was noted following treatment with 250μg testosterone, which was 27 percent lesser than the control. Treatment with oestrogen resulted in a dose-dependent increase in RXFP1 mRNA expression (1.21 to 2.13 fold increase following treatment with 0.2 to 50μg/ml oestrogen).

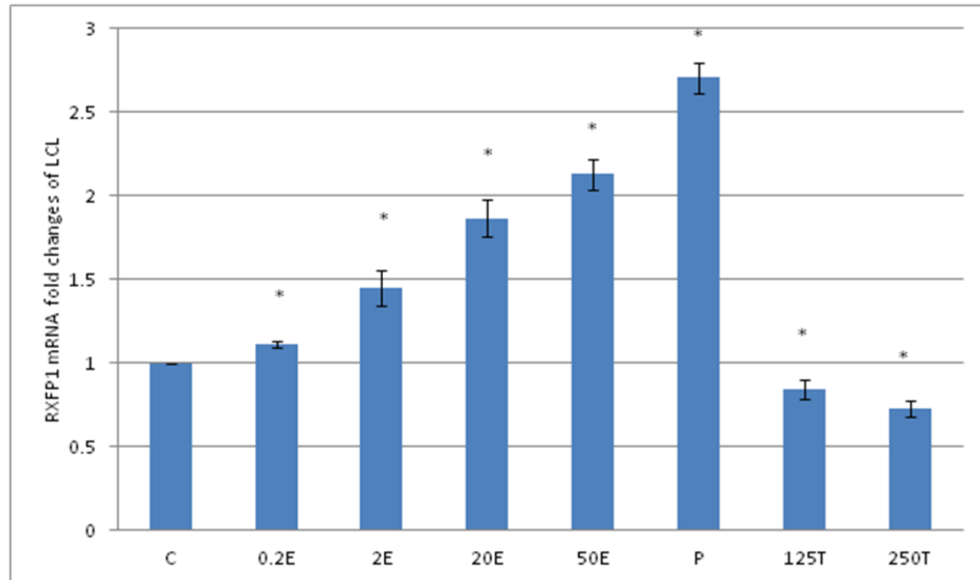


Figure 4-4 The expression of RXFP1 mRNA in the lateral collateral ligament fromovariectomised rats treated with different sex-steroids: C- control; 0.2E – 0.2µg/ml oestrogen; 2E - 2µg/ml oestrogen, 20E - 20µg/ml oestrogen; 50E –50µg/ml oestrogen; P – 4mg progesterone; 125T – 125µg testosterone; 250T – 250µg testosterone, LCL-lateral collateral ligament. Data were expressed as mean ± SEM, n=6 per treatment group. * $p<0.05$ as compared to control, Shapiro-Wilk test, p= 0.313319. A dose-dependent increase in RXFP1 mRNA expression was noted with increasing doses of oestrogen. Progesterone treatment caused the highest increase while testosterone caused a dose-dependent decrease in RXFP1 mRNA expression

In figure 4-5, the expression of RXFP1 mRNA in the patellar tendon was the highest following treatment with progesterone (2.86 fold increase as compared to control). Treatment with testosterone at 125 and 250µg/ml resulted in a significant reduction in RXFP1 mRNA expression as compared to control ($p<0.05$), with 16 and 26 percent decrease respectively. Oestrogen administration resulted in a dose-dependent increase in the expression of RXFP1 mRNA (1.63 to 2.23 fold increase following treatment with 0.2 to 50µg/ml oestrogen), which were significantly higher than the control ($p<0.05$). The level of expression of RXFP1 in the patellar tendon was higher than its expression in the lateral collateral ligament.

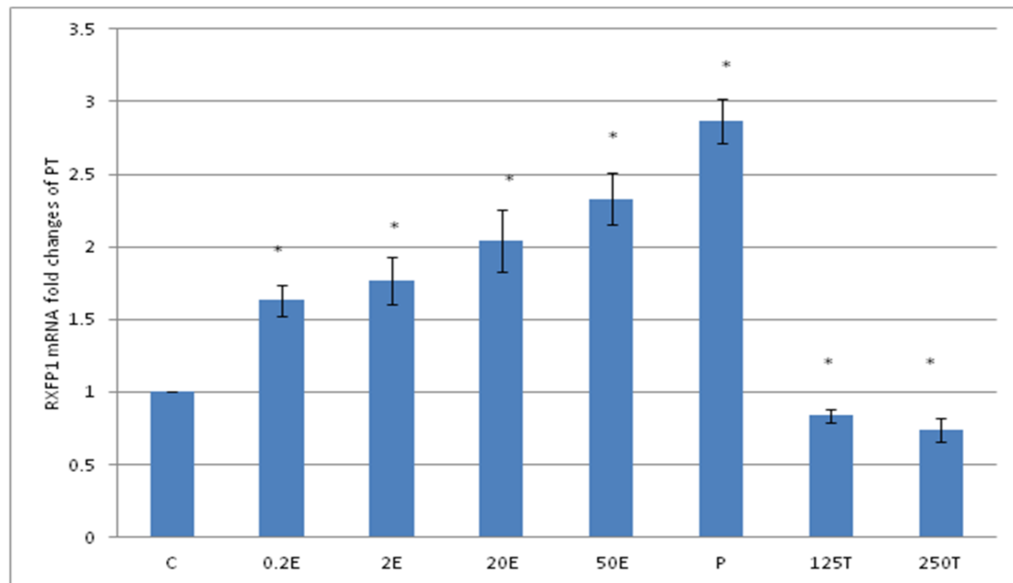


Figure 4-5 The expression of RXFP1 mRNA in the patellar tendon from ovariectomised rats treated with different sex-steroids: C- control; 0.2E – 0.2µg/ml oestrogen; 2E - 2µg/ml oestrogen, 20E - 20µg/ml oestrogen; 50E –50µg/ml oestrogen; P – 4mg progesterone; 125T – 125µg testosterone; 250T – 250µg testosterone, PT-patella tendon. Data were expressed as mean ± SEM, n=6 per treatment group. * $p<0.05$ as compared to control, Shapiro-Wilk test, p= 0.207574. A dose-dependent increase in RXFP1 mRNA was noted following increasing dose of oestrogen. Meanwhile progesterone treatment resulted in the highest increase while testosterone reduced the mRNA expression.

In figure 4-6, the expression of RXFP2 mRNA in the lateral collateral ligament was increased following progesterone treatment which was 1.58 fold higher than the control. Oestrogen treatment resulted in a dose-dependent increase in RXFP2 mRNA expression (1.13 to1.43 fold increase following treatment with 0.2 to 50µg/ml oestrogen). Treatment with low dose oestrogen (0.2µg/ml) however did not result in any significant change in the mRNA expression, while treatment with higher oestrogen doses (>2µg/ml) resulted in a significant increase in the mRNA level as compared to control. Testosterone treatment at 125 and 250µg/ml resulted in a significant decrease in RXFP2 mRNA expression as compared to 0.2µg/ml oestrogen and the control ($p<0.05$).

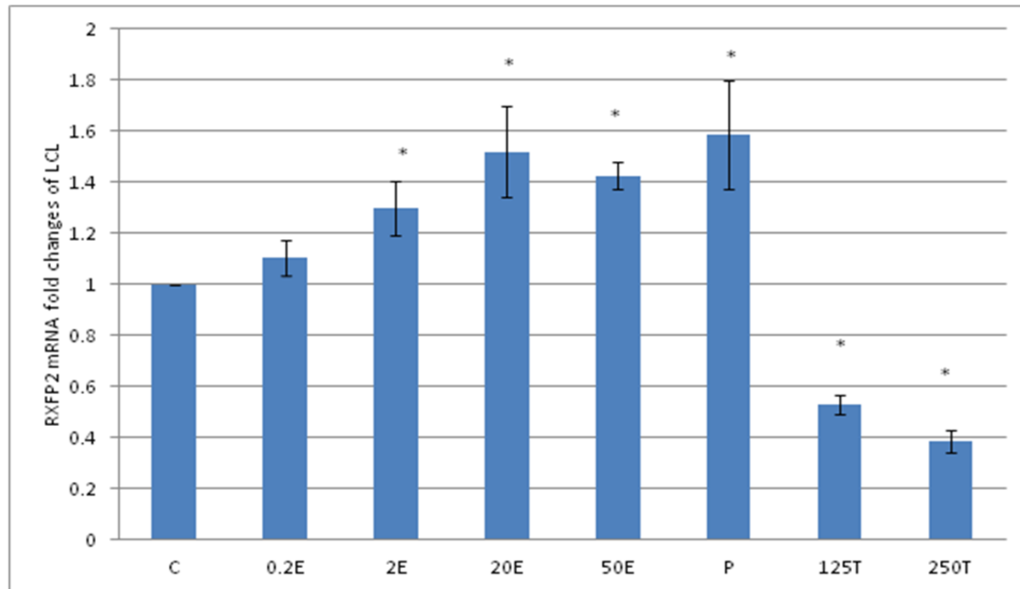


Figure 4-6 The expression of RXFP2 mRNA in the lateral collateral ligament from ovariectomised rats treated with different sex-steroids: C- control; 0.2E – 0.2µg/ml oestrogen; 2E - 2µg/ml oestrogen, 20E - 20µg/ml oestrogen; 50E –50µg/ml oestrogen; P – 4mg progesterone; 125T – 125µg testosterone; 250T – 250µg testosterone, LCL-lateral collateral ligament.Data were expressed as mean ± SEM, n=6per treatment group. * $p<0.05$ as compared to control, Shapiro-Wilk test, p= 0.668854. Treatment with oestrogen at doses exceeding 2µg/ml resulted in a dose-dependent increase in RXFP2 mRNA expression. Meanwhile, progesterone caused the highest increase while testosterone treatment resulted in a decrease in this isoform mRNA expression.

In figure 4-7, the expression of RXFP2 mRNA in the patella tendon was the highest following treatment with progesterone (2.28 fold increase as compared to control). Oestrogen treatment resulted in a dose-dependent increase in RXFP2 expression (1.13 to 2.01 fold increase following treatment with 0.2 to 50µg/ml oestrogen). Treatment with 125 and 250µg/ml testosterone resulted in a significant reduction in RXFP2 mRNA expression (0.79 and 0.5 fold- respectively) as compared to control. RXFP2 mRNA expression in the patella tendon exceeds its expression in the lateral collateral ligament only following treatment with 50µg/ml oestrogen and progesterone.

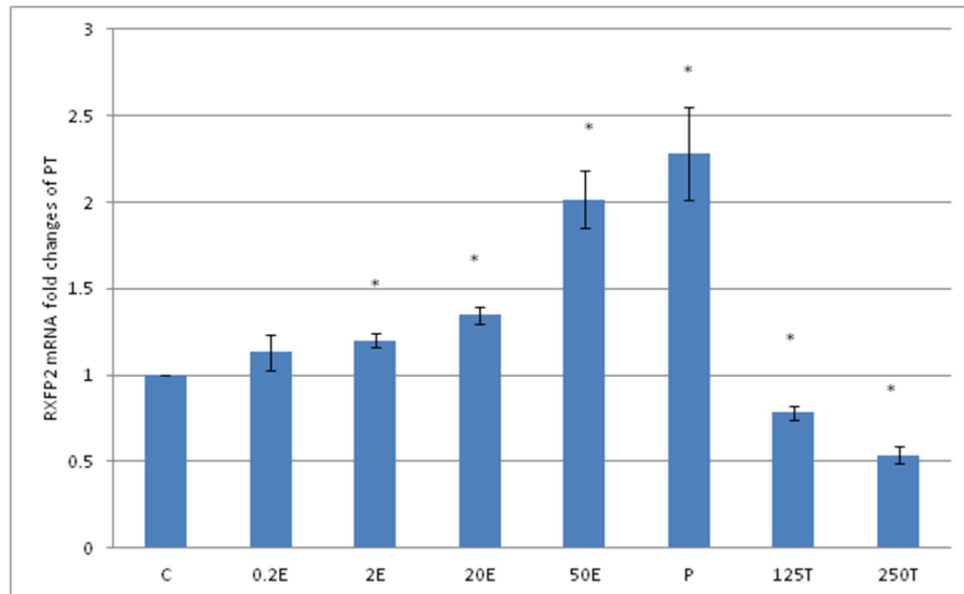


Figure 4-7 The expression of RXFP2 mRNA in the patellar tendon from ovariectomised rats treated with different sex-steroids: C- control; 0.2E – 0.2µg/ml oestrogen; 2E - 2µg/ml oestrogen, 20E - 20µg/ml oestrogen; 50E –50µg/ml oestrogen; P – 4mg progesterone; 125T – 125µg testosterone; 250T – 250µg testosterone, PT-patellar tendon. Data were expressed as mean ± SEM, n=6 per treatment group. * $p<0.05$ as compared to control, Shapiro-Wilk test,p= 0.842243. An increase in the RXFP1 mRNA was noted following treatment with oestrogen at doses exceeding 2µg/ml. Progesterone treatment resulted in the highest increase while testosterone caused a dose-dependent decrease in RXFP2 mRNA expression.

4.3 Discussion

Our findings have revealed the followings: (i) The expression of RXFP1 protein and mRNA in knee lateral collateral ligament and patellar tendon were up-regulated by oestrogen and progesterone, and were down-regulated by testosterone (ii) RXFP2 protein and mRNA expression were up-regulated by oestrogen at doses exceeding 2µg/ml and progesterone. Treatment with 0.2µg/ml oestrogen however had no significant effect on the expression of this receptor isoform, (iii) The expression of RXFP1 protein and mRNA in the patellar tendon under the influence of oestrogen and progesterone was higher than its expression in the lateral collateral ligament, (iv) No significant changes in RXFP2

expression was noted between patella tendon and lateral collateral ligament except following treatment with 50μg/ml oestrogen and progesterone, where higher expression was noted in the former than the latter and (iv) RXFP1 is the main isoform expressed in the patella tendon and lateral collateral ligament of the rat knee joint.

The effect of relaxin on ligament laxity has long been reported(Becker GJ, 2001). The first evidence was documented by Hisaw in 1926 where relaxin infusion in mice was found to cause relaxation of the pelvic ligament (MacLennan, 1981). Research related to relaxin effect on knee joint laxity has become increasingly important in the recent years due to the reported increase in the incidence of non-contact knee injury among the female athletes. Recent evidence suggested that plasma relaxin level correlates with the increase in incidence of ACL tear in elite female athletes (Dragoo *et al.*, 2011b). In addition to knee, the laxity of trapezial-metacarpal joint in humans (Wolf *et al.*, 2013) and pubic ligaments in mice (Lippert *et al.*, 1987)has also been reported to change with changes in plasma relaxin level.

The influence of female sex-hormones on relaxin action in the knee ligaments has been proposed. *In-vitro* binding assay has revealed that relaxin binding to the female ACL was higher than male ACL. This finding was supported by the increase in the number of relaxin receptors expressed in the female ACL as compared to male (Dragoo *et al.*, 2003). Quantitation of relaxin receptors by radioligand binding assay in the human ACL fibroblast cells has revealed that relaxin binding was present in four out of five female ACL fibroblast as compared to one out of five male ACL fibroblast (Faryniarz *et al.*, 2006). The direct influence of oestrogen on ACL laxity has been documented by Dragoo et al (Dragoo *et al.*, 2009) where three weeks combined infusion of oestrogen and relaxin to the guinea pigs has

resulted in increased anterior tibial dislocation, indicating an increase in ACL laxity. Meanwhile, in rabbit temporomandibular joint and pubic symphysis, concomitant administration of relaxin with oestrogen was found to cause loss of glycosaminoglycans and collagen from these tissues, which will lead to an increase in the ligament laxity. Progesterone administration meanwhile prevented this effect (Hashem *et al.*, 2006).

Although this study has not provided direct evidence of an increase in the expression of relaxin receptors in the ACL under sex-steroid effect, we have for the first time shown that oestrogen and progesterone upregulate the expression of relaxin receptor isoforms (RXFP1 and RXFP2) in the patella tendon and lateral collateral ligament. In other tissues including uterus (Vodstrcil *et al.*, 2010a) and cervix (Maseelall *et al.*, 2009), oestrogen was also reported to upregulate the expression of the relaxin receptors. In this study, modulation of RXFP1 and RXFP2 expression by sex-steroids could provide the basis underlying the effect of these hormones on knee joint laxity. Recently, relaxin was also found to have significant effect on the patellar tendon stiffness in healthy, young and normally menstruating women (Pearson *et al.*, 2011). Although the actual mechanism is unknown, this effect could possibly be mediated through upregulation of RXFP1 and RXFP2 by sex-steroids, which levels fluctuate throughout the menstrual cycle. The mechanisms that lead to the changes in knee ligament laxity following the binding of relaxin to its receptors have been earlier discussed (*introduction section*).

Our findings have important implication as it could help to explain differences in the incidence of non-contact knee injury between male and female. Female was reported to have two to ten times higher risk of injury than male and is related to phases of the menstrual cycle (Hansen *et al.*, 2013) which pointed towards the involvement of sex-

steroids. The effect of oestrogen on RXFP1 and RXFP2 expression in the patellar tendon and lateral collateral ligament of rats' knee confirmed the involvement of this hormone in modulating knee laxity. At low dose (0.2µg/ml), which correlate with the plasma oestrogen level of 69.7 ± 0.12pg/ml (table 1), the expression of RXFP1 was higher than the control although no significant changes in RXFP2 expression were noted. The mean reported plasma oestrogen level in the early follicular phase of the menstrual cycle was 31 pg/ml (0.31 X 10^{-4}µg/ml) (Fenichel *et al.*, 1989). In rat at proestrous, oestrogen level was found to be higher at 40.5 pg/ml (0.45 X 10^{-4}µg/ml) (Walf *et al.*, 2009). In view that 0.2µg/ml oestrogen administration resulted in the plasma oestrogen level of 69.7 pg/ml, which was slightly higher than the level observed in humans and rats at the follicular phase and proestrus stage respectively, there is a possibility that this level could affect knee ligament laxity via upregulating the expression of relaxin receptor isoforms rendering the tissue to be more sensitive towards relaxin action.

Meanwhile, administration of 2, 20 and 50 µg/ml oestrogen resulted in the circulating plasma level of 195.2 ± 0.31, 267.7 ± 0.76 and 329.4 ± 2.33 pg/ml respectively (table 1). Treatment with oestrogen at doses exceeding 2µg/ml resulted in higher expression of RXFP1 and RXFP2 as compared to the control and 0.2µg/ml oestrogen.High oestrogen level in the blood could be achieved at the time of ovulation, with the reported level of 400pg/ml (0.4 X 10^{-3}µg/ml) (Fenichel *et al.*, 1989). Shultz et al (Shultz *et al.*, 2010) reported that high incidence of non-contact knee injury occur in the pre-ovulatory phase, which suggested that increased oestrogen level may have significant impact on the knee ligament laxity. Several other findings have also reported that increased incidence of non-contact knee injury during the ovulatory phases of the cycle (Park *et al.*, 2009b; Romani *et*

al., 2003; Wojtys *et al.*, 2002) was related to the effect of high oestrogen doses on knee ligament laxity. Oestrogen- induced upregulation of relaxin receptors will augment relaxin effect on the knee.

High plasma oestrogen can be achieved following consumption of oral contraceptive pills (OCP) that contains 15 to 50 µg oestrogen (Dhont, 2010). In the female athletes, OCP is consumed for birth control purpose and to prevent menstruation in order to improve their physical performance (Frankovich & Lebrun, 2000). Few studies have reported that OCP intake could influence knee laxity; however their findings seem to be conflicting. Hicks-Little et al, (Hicks-Little *et al.*, 2007) and Cammarata et al (Cammarata & Dhaher, 2008) reported that female on OCP tend to have higher knee laxity, while others reported the opposite (Martineau *et al.*, 2004). Meanwhile, few other studies found no relationship between OCP consumption and knee ligament laxity (Agel *et al.*, 2006; Dhont, 2010). Differences in sex-steroid content between different OCP formulations could be the reason for this variability.

While oestrogen has been shown to affect ACL laxity in the animal model (Dragoo *et al.*, 2009), little is known with regards to the effect of progesterone and testosterone on knee ligament laxity. Dragoo et al, measured serum relaxin concentration (SRC) in elite collegiate female athletes and had observed a positive correlation between SRC and serum progesterone concentration in athletes who are oligomenorrheic, eumenorrheic, not on oral contraceptive and have ovulatory cycle (Dragoo *et al.*, 2011a). In this study, serum progesterone level was measured to confirmed luteal phase measurement of relaxin as relaxin levels are known to be higher in this phase. Other studies involving female athletes has also indicated that knee laxity was increased during ovulation and in the luteal phase of

the menstrual cycle (Hicks-Little *et al.*, 2007). Meanwhile, a positive correlation between changes in knee laxity and knee joint load has been reported from follicular phase to ovulation, and from ovulation to the luteal phase (Park *et al.*, 2009b), where the latter phase is associated with high serum progesterone level. Our findings which revealed an up-regulation of RXFP1 and RXFP2 isoforms in the patella tendon and lateral collateral ligament by progesterone further support these observations. The increase in these isoforms expression in the collateral ligaments predisposes the knee towards non-contact injury especially during excessive varus or valgus movement.

Progesterone upregulation of RXFP1 and RXFP2 expression in the knee was supported by findings in other tissues including the pregnant rat uteri, where this hormone was found to cause an increase in RXFP1 expression in the myometrium (Vodstrcil *et al.*, 2010a). In the mammary glands, progesterone was reported to stimulate relaxin receptor expression which resulted in reduced breast laxity. This effect is due to a decrease in the collagen content of the mammary gland's extracellular matrix in the presence of relaxin (Winn *et al.*, 1994). In this study, the plasma level achieved following subcutaneous progesterone injection was 23.67 ± 0.13 ng/ml, which was slightly higher than the maximum plasma level reported during the luteal phase of the cycle in premenopausal female, at 17.02 ng/ml (Shultz *et al.*, 2006). This indicate that the level of progesterone in the luteal phase can cause an increase in knee laxity, rendering the knee to be more susceptible towards a non-contact injury.

Finally, we have also observed that RXFP1 and RXFP2 expression in both patellar tendon and lateral collateral ligament was down-regulated by testosterone and this effect was dose-dependent. In the female, the presence of androgen receptor was reported in the

ACL (Lovering & Romani, 2005), suggesting that testosterone could influence knee laxity. Testosterone level was found to fluctuate throughout the menstrual cycle, with the highest level noted in the luteal phase (Shultz *et al.*, 2004).Down-regulation of RXFP1 and RXFP2 expression by testosterone in the patella tendon and lateral collateral ligament indicated that knee laxity could be reduced under the influence of this hormone. A recent finding by O'Leary et al, (O'Leary *et al.*, 2013) indicated that prolonged aerobic exercise in women with normal menstrual cycle can induce a short-term elevation in plasma testosterone level. We hypothesized that a continuous short-burst in plasma testosterone may result in down-regulation of relaxin receptors in these knee structures, thus could help to reduce knee laxity in exercising women. In addition to this, testosterone has also been reported to increase the strength of the muscles that control knee joint movement (Bhasin *et al.*, 1996). Taken together, these findings may explain increased knee joint stiffness in female following testosterone administration.

In conclusion, our study has provided novel evidences that sex-steroid affect RXFP1 and RXFP2 expression in the patellar tendon and lateral colateral ligament of the rat knee joint.These findings are important as it may help to provide the basis underlying increased in incidence of non-contact knee injury in female as compared to male and in female at different phases of the menstrual cycle. Precaution however should be taken when projecting these findings to humans as recent evidence suggested that there was apparently no relationship between serum relaxin and joint laxity (Wolf *et al.*, 2013).

CHAPTER 5

Testosterone Reduces Knee Passive Range of Motion and Expression of Relaxin Receptor Isoforms via 5α-Dihydrotestosterone and Androgen Receptor Binding

5.1 Introduction

Range of motion (ROM), defined as the movement potential of a joint from full flexion to full extension is controlled by the connective tissue, muscles, tendons, and ligaments(Schmitz *et al.*, 2013). Knee joints connect femur to tibia and fibula through the collateral and anterior cruciate ligaments (Marieb & Hoehn, 2013). Medial and lateral collateral ligaments (MCL and LCL) prevent excessive varus-valgus-forces while anterior cruciate ligament (ACL) prevents knee hyperextension (Marieb & Hoehn, 2013).The patellar tendon, which connects patella to tibia tuberosity stabilizes the patella and also prevents knee hyperextension(Janousek, 1999). Evidence suggests that gender could influence knee laxity. Females have been reported to have greater knee laxity than males(Hsu *et al.*, 2006; Wojtys *et al.*, 2003). Additionally, females have also been reported to have greater ankle laxity (Beynnon *et al.*, 2005). These gender specific differences suggest that male and female sex hormones participate in controlling the generalized joint laxity. So far, studies investigating the relationship between sex hormones and joint laxity mainly focused on the knee and the findings remain inconclusive.

Relaxin, a member of the insulin-like superfamily, has been implicated in the modulation of joint laxity (Dragoo *et al.*, 2009). In primates, relaxin-1 (Rln1), 2 (Rln2), and 3 (Rln3) are the main isoforms (Chen *et al.*, 2012) while in rats, mice and pigs, Rln1 and Rln3 are the main subtypes(Sherwood, 2004). Relaxin increases the joint laxity via up regulating expression and stimulating activity of matrix metalloproteinases (MMPs), collagenases which stimulates collagen degradation and inhibit collagen synthesis by fibroblasts (Lee *et al.*, 2012). Relaxin effect is mediated via binding to the relaxin receptor, which consists of the two main isoforms, RXFP1 and RXFP2 (Scott *et al.*, 2012). Relaxins

binding to RXFP1 and RXFP2 are species specific. In humans, Rln1 and Rln2 have similar binding affinity to RXFP1 and RXFP2, while in rats Rln1 binds weakly to RXFP2 (Scott *et al.*, 2005). Meanwhile, Rln3 has been reported to bind to RXFP1, RXFP3, and RXFP4 (Bathgate *et al.*, 2005) while insulin-like factor 3 (Insl3), whichis structurally related to relaxin, selectively binds to RXFP2 (Kumagai *et al.*, 2002). Serum relaxin level fluctuates throughout the reproductive cycle and is markedly increased during pregnancy (Forst *et al.*, 1997). Studies have shown a higher plasma relaxin level in pregnant women with pelvic joint instability and increased hip joint laxity(Saugstad, 1991; Steinetz *et al.*, 2008). While reports have indicated a positive correlation between the serum relaxin concentration and joint laxity (Dragoo *et al.*, 2011b), relaxin effect on the joint is more reflective in rodents than in humans (Sherwood, 2004).

Testosterone, an anabolic steroid produced by the testes, ovaries and adrenal glands has serum concentration seven to eight times greater in male than in female (He *et al.*, 2007).Testosterone promotes the development of secondary male sexual characteristics such as increased in muscle and bone mass and induced male sexual behavior while its active metabolite, 5α-DHT promotes male pattern hair distribution. Additionally, testosterone also stimulates erythropoiesis and increase muscle strength (Barrett *et al.*, 2009). Despite being produced in low amounts, testosterone is essential for several key reproductive processes in female such as decidualization (Ishikawa *et al.*, 2007). The classical effect of testosterone is mediated via a genomic pathway involving the androgen receptor (AR)while a non-genomic pathway mediates its rapid effect(Kouloumenta *et al.*, 2006). An understanding of the testosterone effect on joint laxity is currently not fully understood. Few studies have suggested that testosterone could influence knee laxity however, its exact role remain elusive. Shultz *et al.*(Shultz *et al.*, 2004) reported that testosterone has a positive rather than

a negative relationship with changes in knee laxity in the presence of oestrogen and progesterone. On the other hand,Rozzi *et al*.(Rozzi *et al.*, 1999) reported that male athletes have greater knee joint laxity that female athletes, suggesting a negative influence of testosterone on laxity.

The notion that testosterone decreases joint laxity is further supported by the reported increase in the collagen content of the prostate, breast and capsular tissue and an increase in knee ligament repair strength by testosterone (Lovering & Romani, 2005). In view of the fact that relaxin increases while testosterone may decrease joint laxity(Shultz *et al.*, 2004), we hypothesized that testosterone down regulates the expression of the relaxin receptor in the joint, rendering this tissue to be insensitive towards relaxin action. This study therefore aimed to investigatethe effect of testosterone on knee joint laxity and changes in relaxin receptor expression under testosterone influence. Additionally, the involvement of the androgen receptor and testosterone active metabolite, 5α-DHT, in mediating testosterone effect was also investigated.

5.2 Results

5.2.1 Passive Knee ROM in Testosterone-Treated Ovariectomised Rats

The degree of knee joint angle (differences between passive flexion and extension) is shown in Figure 5-1. There was a significant difference in the angle between testosterone-treated and control groups where testosterone caused a decrease in the passive ROM. Testosterone effect was antagonized by flutamide (FLU) and finasteride (FIN). Following treatment with 125 and 250 μg/kg testosterone, the ROM was 6.22° and 6.45°

lower respectively than the non-treated group (p <0.05). The degree of knee joint angle in the groups receiving 125 and 250μg/kg testosterone was greater in the presence of FLU, which were 13.49° and 15.87°higher respectively (p <0.05) as compared to without FLU treatment. The angles in the groups receiving 125 and 250μg/kg testosterone were also noted to be greater in the presence of FIN, which was 12.84° and 14.82°higher respectively (p <0.05)as compared to without FIN treatment. Knee joint angle was significantly higher in the presence of relaxinin all groups except in the group receiving both doses of testosterone where no difference in the angles was noted. The angle was the highest in the control group, reduced in the presence of testosterone and significantly increased in the presence of FLU and FIN.

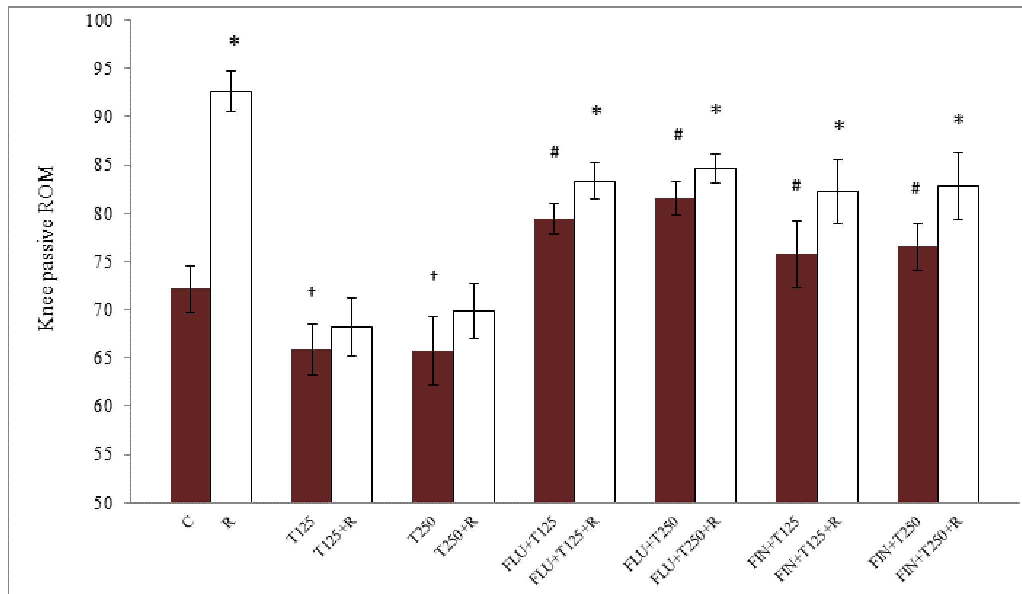


Figure 5-1 Passive ROM of the rat knee
Treatment with peanut oil (control), testosterone, testosterone plus FLU or FIN with and without relaxin. Our findings indicated that treatment with 125 and 250 µg/kg testosterone significantly reduced the knee ROM as compared to control. In the presence of relaxin, knee ROM in the control group was significantly higher than in the testosterone-treated group. Relaxin administration to the group treated with testosterone did not result in a significant increase in the passive knee ROM as compared to without relaxin. The presence of FLU and FIN significantly increase the knee ROM which was further increased following relaxin administration (p <0.05). * p <0.05 as compared to in the absence of relaxin;†p <0.05 as compared to control; $^{\#}$p <0.05 as compared to in the absence of FLU or FIN.C: control, R: relaxin, T125: 125 µg/kg/day testosterone, T250: 250 µ/kg/day testosteroneFLU: flutamide, FIN: finasteride.

5.2.2 RXFP1 and RXFP2 mRNA Expression in Patellar Tendon

In Figure 5-2, *RXFP1* mRNA expression in the patellar tendon following 125 and 250 µg/kg testosterone treatment was lower than the control (*p* <0.05). Administration of FLU to 125 and 250µg/kg testosterone treated groups resulted in an increase in *RXFP1*mRNA expression by approximately 0.69 and 0.86 fold respectively (*p* <0.05). Finastride administration to these groups also resulted in 0.20 and 0.22 fold increase in *RXFP1* mRNA expression respectively. The expression of *RXFP2* mRNA in the patellar tendon in 125 and 250µg/kg testosterone-treated groups following FLU administration was 0.30 and 0.71 fold higher respectively (*p* <0.05). The expression of *RXFP2* mRNA in the group

receiving 125 and 250µg/ kg testosterone treatment plus FIN administration was also increased by 0.38 and 0.39-fold respectively (p <0.05).

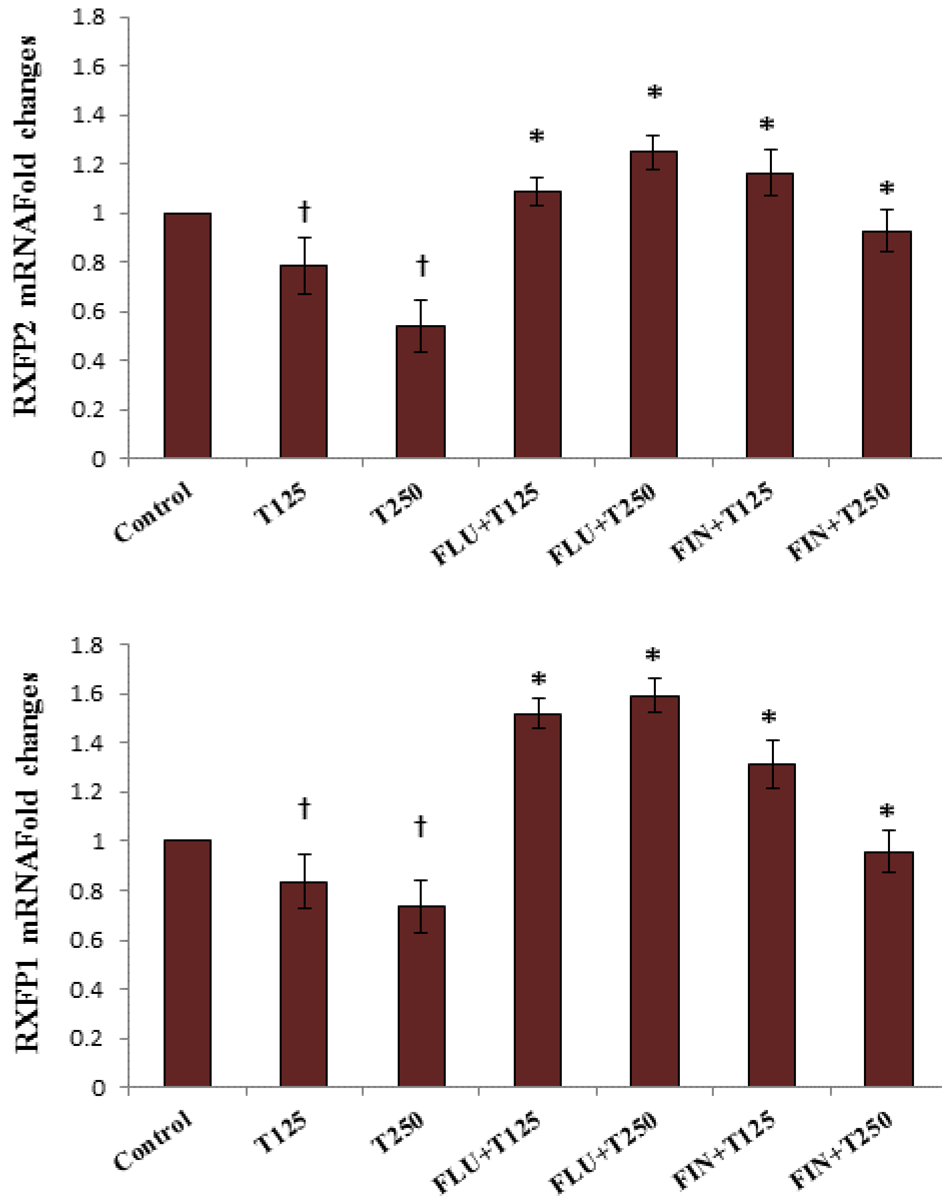


Figure 5-2 Changes in RXFP1 and RXFP2 mRNA expression in the presence of FLU and FIN in the patellar tendon of steroid-replaced ovariectomised rats. Treatment with 125 and 250µg/kg/day testosterone caused a significant decrease in RXFP1 and RXFP2 mRNA levels as compared to control. Administration of flutamide and finasteride significantly antagonized the inhibitory effect of testosterone on *RXFP1* and *RXFP2* expression with FLU effect relatively higher than FIN for *RXFP1*, however no significant differences were noted for *RXFP2* mRNA.$^{\dagger}p$ <0.05 as compared to control *p <0.05 as compared to testosterone only treatment for the respective group. T125: 125 µg/kg/day testosterone, T250: 250 µg/kg/day testosterone, FLU: flutamide, FIN: finasteride.

5.2.3 RXFP1 and RXFP2 Protein Expression in Patellar Tendon

In Figure 5-3, the expression of RXFP1 protein in the patellar tendon was higher in the group receiving FLU with testosterone (0.79 and 0.83 fold increase respectively as compared to 125 and 250μg/kg testosterone only treatment (p <0.05). The expression of RXFP1 protein in the groups receiving FIN with testosterone was also significantly increased as compared to without FIN(0.59 and 0.52-fold increase respectively as compared to 125 and 250μg/kg testosterone only treatment)(p <0.05). The expression of RXFP2 protein in the patellar tendon was increased following treatment with FLU plus the two doses of testosterone, (0.29 and 0.36-fold increase respectively as compared to 125 and 250μg/kg testosterone treatment alone) (p <0.05). The expression of RXFP2 protein was significantly increased following treatment with FIN plus testosterone as compared to testosterone only treatment (0.2 and 0.24-fold increase respectively as compared to 125 and 250μg/kg testosterone) (p <0.05).

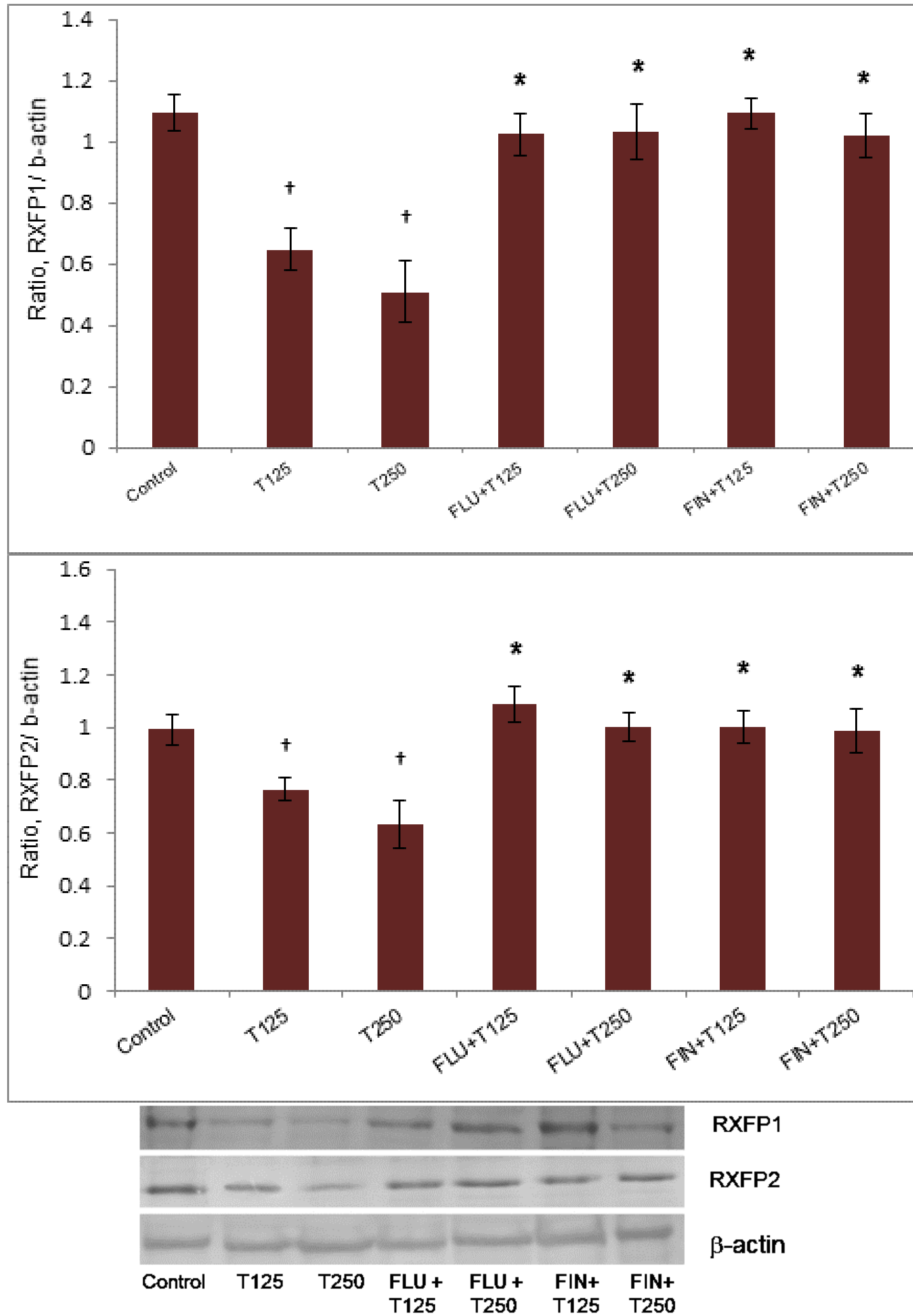


Figure 5-3 The expression of RXFP1 and RXFP2protein in the patellar tissue homogenate from ovariectomised rats treated with testosterone with and without FLU and FIN. A decreasein RXFP1 protein expression was noted following testosterone treatment which was antagonized by FLU and FIN. The expression of RXFP2 protein was reduced following testosterone treatment which was also antagonized by FLU and FIN. The antagonizing effect of FIN indicated that DHT and not testosterone mediates the inhibition of RXFP1 and RXFP2 protein expression(Data were expressed as mean ± SEM, n = 6 per treatment group; 125T: 125μg testosterone; 250T: 250μg testosterone; FLU: 10 mg/kg flutamide; FIN: 20 mg/kg finasteride). †p <0.05 as compared to control *p <0.05 as compared to testosterone only treatment for the respective group.

5.2.4 RXFP1 and RXFP2 mRNA Expression in Lateral Collateral Ligament

In Figure 5-4, the expression of *RXFP1* mRNA in the lateral collateral ligament was reduced following treatment with testosterone at 125 and 250µg/kg/day. Administration of FLU and FIN resulted in a significant increase in mRNA expression where effect of the latter suggested DHT-mediated testosterone effect. Meanwhile, decreased *RXFP2* mRNA expression was also noted following testosterone treatment alone (0.31 and 0.33 fold decrease respectively following exposure to 125 and 250µg/kg testosterone) which was also antagonized by FLU and FIN ($p < 0.05$).

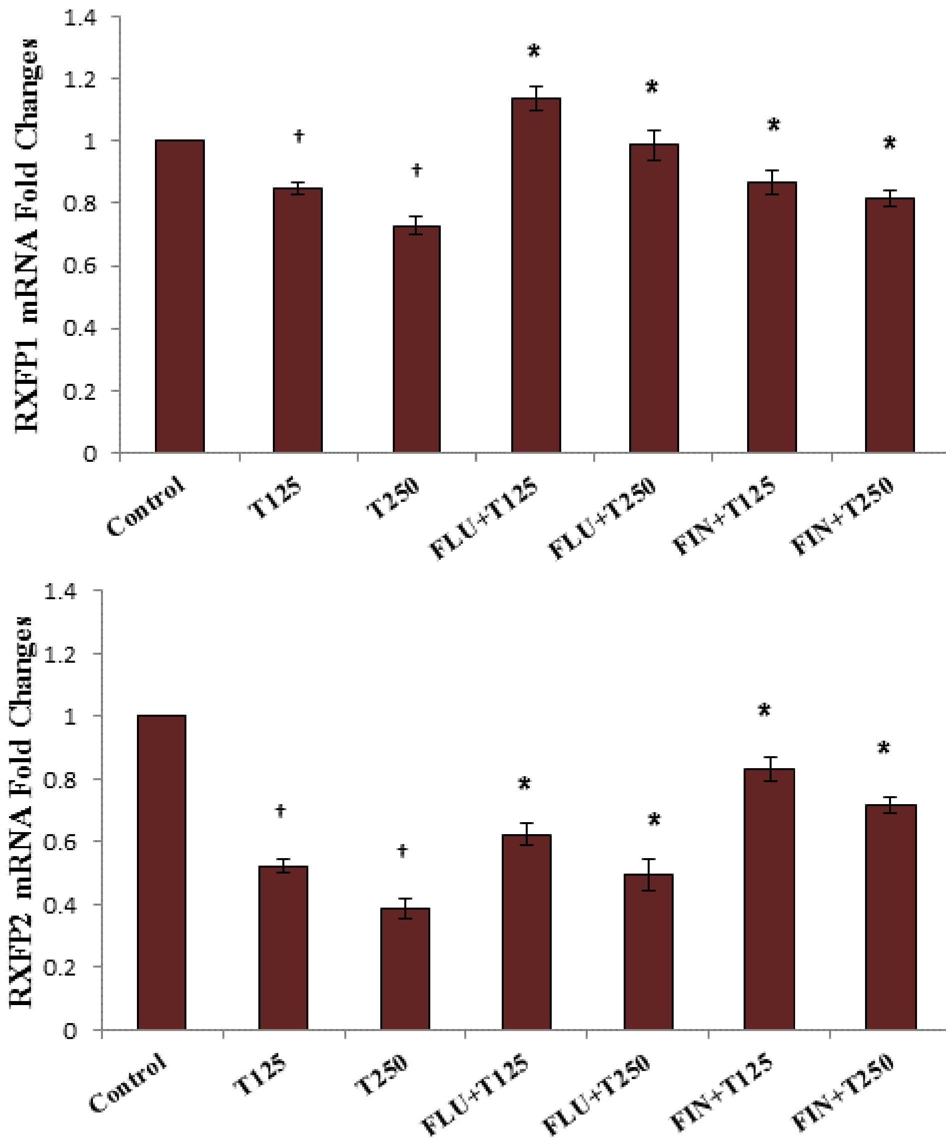


Figure 5-4 Changes in RXFP1 and RXFP2 mRNA expressionin the lateral collateral ligament of steroid-replaced ovariectomised rats in the presence of FLU and FIN. Treatment with 125 and 250µg/kg/day testosterone caused a decrease in*RXFP1* and *RXFP2* mRNA expression as compared to control (testosterone only treatment) (p <0.05). Administration of flutamide and finasteride caused a significant increase in the expression of *RXFP1* and *RXFP2* with FLU inhibition was relatively greater than FIN for *RXFP1*, however FIN inhibition was relatively higher than FLU for *RXFP2*mRNA expression.$^{\dagger}p$ <0.05 as compared to control.*p <0.05 as compared to testosterone only treatment for the respective group.

5.2.5 RXFP1 and RXFP2 Protein Expression in Lateral Collateral Ligament

In Figure 5-5, the expression of RXFP1 protein in the lateral collateral ligament was reduced following testosterone only treatment, which was antagonized by FLU and FIN. FLU administration resulted in 0.28 and 0.48-fold increase in RXFP1 expression as compared to 125 and 250μg/kg testosterone only treatment respectively($p < 0.05$). The reduced RXFP1 expression following 125 and 250 μg/kg testosterone administration was antagonized by FIN ($p > 0.05$). A decrease in the expression of RXFP2 protein in the lateral collateral ligament following administration of both doses of testosterone was also antagonized by FLU and FIN.

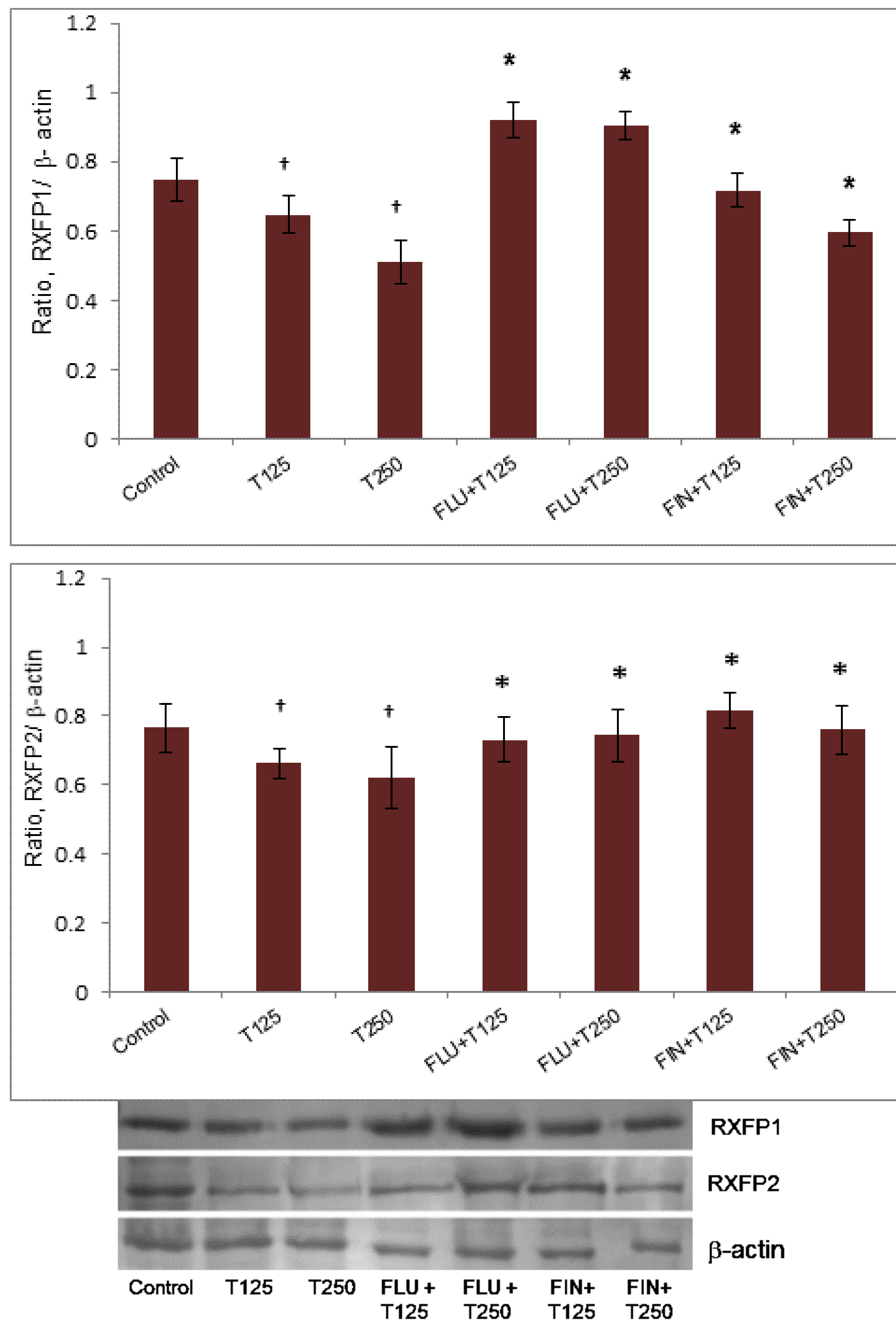


Figure 5-5 The expression of RXFP1 and RXFP2 protein
RXFP1 and RXFP2 protein from the homogenate of lateral collateral ligaments of ovariectomised rats treated with 125 and 250 µg/kg testosterone with and without FLU and FIN administration. A dose-dependent decrease in RXFP1 protein expression was noted following treatment with both doses of testosterone which was antagonized by FLU and FIN. Similarly, a decrease in RXFP2 protein expression was observed following treatment with both doses of testosterone which was antagonized by FLU and FIN. Data were expressed as mean ± SEM, n = 6 per treatment group; 125T: 125µg testosterone;250T: 250µgtestosterone; FLU: 10 mg/Kg FLU; FIN: 20 mg/kg FIN.$^{\dagger}p$ <0.05 as compared to control;*p <0.05 as compared to testosterone only treatment for the respective group.

5.3 Discussion

To the best of our knowledge, this study is the first to demonstrate the effect of testosterone on knee laxity in an ovariectomised rat model. Ovariectomy was performed to investigate sex-steroid effect on the ligament laxity, as removal of endogenous hormone and replacement with exogenous steroid would ensure that the effect observed was due to the action of individual steroid rather than the combined effect of multiple steroids produced by the ovaries. In the female of reproductive age, 25% of the circulating testosterone originates from the ovaries while the remaining is derived from conversion of androsteinedione into testosterone in the adrenal gland(Speroff *et al.*, 1989). In humans, the level of endogenous testosterone has been reported to fluctuate throughout the menstrual cycle which was the highest at around the time of ovulation (Rao & Kotagi, 1982). A recent finding by O'Leary *et al*.(O'Leary *et al.*, 2013) reported that a prolong edaerobic training in the eumenorrheic women induces a short-term elevation in the plasma testosterone levels, which appears to be unrelated to the level of oestrogen and the phases of menstrual cycle.There is currently no report on changes in the testosterone level throughout the oestrous cycle.

Testosterone has been proposed to influence knee laxity in female. A positive correlation between plasma testosterone, free androgen index (FAI) and anterior cruciate ligament stiffness has been reported in young females near ovulation (Lovering & Romani, 2005). The mechanisms underlying testosterone effect on ligament laxity is unknown, however a limited finding in the prostate gland indicated that testosterone as opposed to oestrogen affects collagen metabolism (Srinivasan *et al.*, 1986) via down regulating the expression of oestrogen receptor (ER)-α(Asano *et al.*, 2003). So far, no study has reported

testosterone effect on relaxin receptor expression in the joints, although relaxin has been shown to affect ligament laxity and its receptor was expressed in female knee joint of both humans(Dragoo *et al.*, 2003) and rodents (Dragoo *et al.*, 2009). Our findings which revealed down regulation of relaxin receptor expression in the knee ligaments and tendons by testosterone has provided explanation for the observed decrease in knee passive ROM under testosterone influence. A decrease in knee passive ROM could be due to decreases in the relaxin effect on ligament, rendering the tissue to have decreased laxity.

Our study has provided the first direct evidence on testosterone effect on the passive ROM in rats' knee. We have shown that testosterone reduces the ROM, which was inhibited by FLU and FIN. In the group receiving 125 and 250μg/kg/day testosterone treatment, the presence of relaxin did not significantly increase knee passive ROM. This finding suggested that knee was not responsive to relaxin in the presence of testosterone due to relaxin receptor down regulation. The knee ROM was however increased following FLU or FIN administration, suggesting that inhibition of androgen binding to its receptor and conversion of testosterone to DHTcaused the increase in knee laxity. FLU inhibition confirmed the release of DHT-mediated inhibition on relaxin receptor expression through androgen receptor binding. Relaxin administration resulted in further increase in knee laxity in the groups receiving both testosterone doses with FLU or FIN, which again suggested the release of DHT-mediated inhibition on relaxin receptor expression. Meanwhile, increased knee laxity in the groups receiving testosterone with FLU or FIN without relaxin suggested that endogenously produced relaxin from sources such as breast(Speroff *et al.*, 1989)might influence knee laxity.

The presence of AR has been reported in human female knee joint (Fytili *et al.*, 2005; Lovering & Romani, 2005). In this study, inhibition of testosterone-mediated down-regulation of RXFP1 and RXFP2 protein and mRNA expression in the patellar tendon and lateral collateral ligament by FLU confirmed AR involvement in mediating testosterone effect. Our findings have also confirmed DHT involvement in mediating testosterone action as evidenced from the antagonizing effect of FIN on testosterone-mediated decrease in knee passive ROM as well as testosterone-mediated down-regulation ofRXFP1 and RXFP2 protein and mRNA expression in these knee structures. FIN is a competitive, selective, and reversible inhibitor of 5α-reductase, an enzyme that converts testosterone to DHT (Frye *et al.*, 1998). In females, DHT exerts multiple physiological effects mainly in the uterus and ovaries (Cárdenas & Pope, 2005). DHT has also been reported to cause an increase in the twitch and titanic contraction of fast-twitch skeletal muscle in mice (Hamdi & Mutungi, 2010). Although we have shown evidence that DHT is most likely involved in causing a decrease in knee laxity, further studies are needed to support DHT participation such as identifying the expression and measuring the activity of 5α- reductase enzyme in the knee. In our study, a direct effect of DHT on knee laxity and relaxin receptor isoforms expression was unable to be investigated since this prohibited compound was not commercially available.

Our findings have important implications in the field of exercise physiology. Testosterone could affect female knee laxity as its level was reported to be the highest in the ovulatory phase of the menstrual cycle (Lovering & Romani, 2005). Although we have found that testosterone decreases knee laxity, the findings by Shultz *et al.*(Shultz *et al.*, 2005) who reported a positive correlation between plasma oestrogen and testosterone level with knee laxity throughout the menstrual cycle suggested dominant effects of oestrogen over

testosterone. In female athletes, anabolic steroids are widely used to boost their physical performances. Despite reported adverse effects, this drug could help to strengthen the knee via its effect on ligament and tendon laxity, which could improve athletes' short-term performance as well as reduce the risk of non-contact knee injury. Additionally, the strength of the muscles controlling knee joint movement was also increased(Bhasin *et al.*, 1996). This study has shown for the first time the direct effect of testosterone on knee passive ROM and RXFP1 and RXFP2 protein and mRNA expression in a rat model, which was mediated via DHT and involved androgen receptor binding. These therefore could explain the protective role of testosterone against non-contact knee injury in female. Precaution however is needed when extrapolating these data to humans since relaxin action on human joints might not be as pronounced as those observed in rodents (Wolf *et al.*, 2013).

CHAPTER 6

Oestrogen 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

6.1 Introduction

Sex-steroids have been reported to regulate changes in knee laxity (Dehghan *et al.*, 2014). E_2 cause increased (Dragoo *et al.*, 2009) while testosterone cause decreased (Dehghan *et al.*, 2014) knee laxity in rodents. In humans, knee laxity was reported to increase at around the time of ovulation, indicating of E_2 influence (Zazulak *et al.*, 2006). Knee laxity was also reported to be the highest in the luteal phase of the menstrual cycle, suggesting of P_4 influence (Heitz *et al.*, 1999). Meanwhile, high E_2 level was reported to cause increased anterior tibial dislocation in rats during pregnancy (Charlton *et al.*, 2001). The mechanisms underlying E_2 and P_4 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 towards relaxin via changes in the amount of receptor expressed (Dehghan *et al.*, 2014).

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 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. E_2 was reported to cause a significant loss of glycosaminoglycans

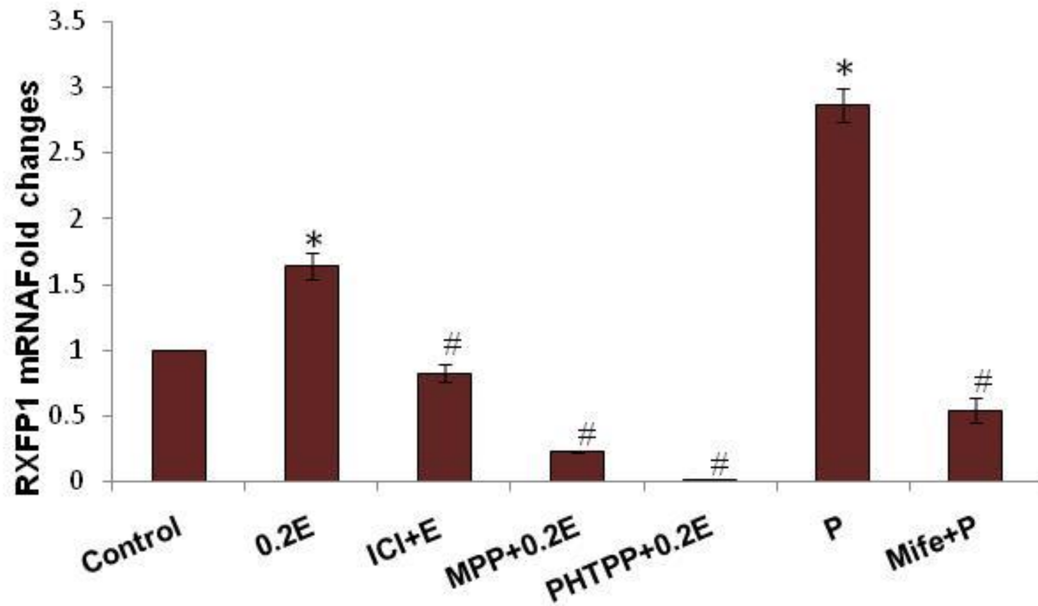
(GAGs) and collagen content from the pubic symphysis, temporomandibular joint disc and articular cartilage of the knee in rabbits (Hashem *et al.*, 2006).E_2 also affects the expression of several isoforms of metalloproteinase (MMPs-1, 3 and 13) as well as tissue inhibitor of metalloproteinases (TIMP 1) (Lee *et al.*, 2003) which were involved in collagen turnover. Meanwhile, E_2 and P_4were also reported to induce protein and mRNA expression of relaxin receptors (RXFP1 and RXFP2) which result in increase tissue response towards relaxin (Dehghan *et al.*, 2014).

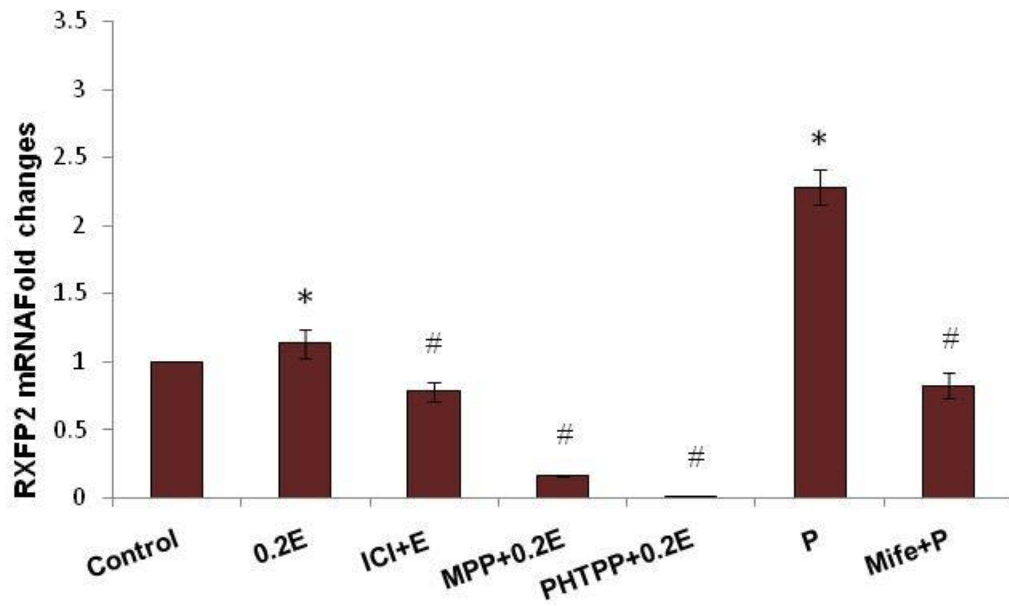
Relaxin, a 6-kDa polypeptide is structurally related to insulin and is produced by the corpus luteum and placenta . Relaxin exerts various effects on the musculoskeletal system via binding to several isoforms of relaxin receptor (RXFP1, RXFP2, RXFP3 and RXFP4) (Dehghan *et al.*, 2013a) and participates in extracellular matrix remodeling (Sherwood, 1994). Relaxin receptor expression has been identified in human female ACL (Dragoo *et al.*, 2003). Relaxin regulates synthesis and/or degradation of matrix macromolecules via modulating the activity of several members of MMP enzymes (Naqvi *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 E_2 and P_4 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 E_2 and P_4 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.

6.2 Results

6.2.1 Effect of steroid receptor antagonists on RXFP1 and RXFP2 mRNA and proteins expression in the patellar tendon





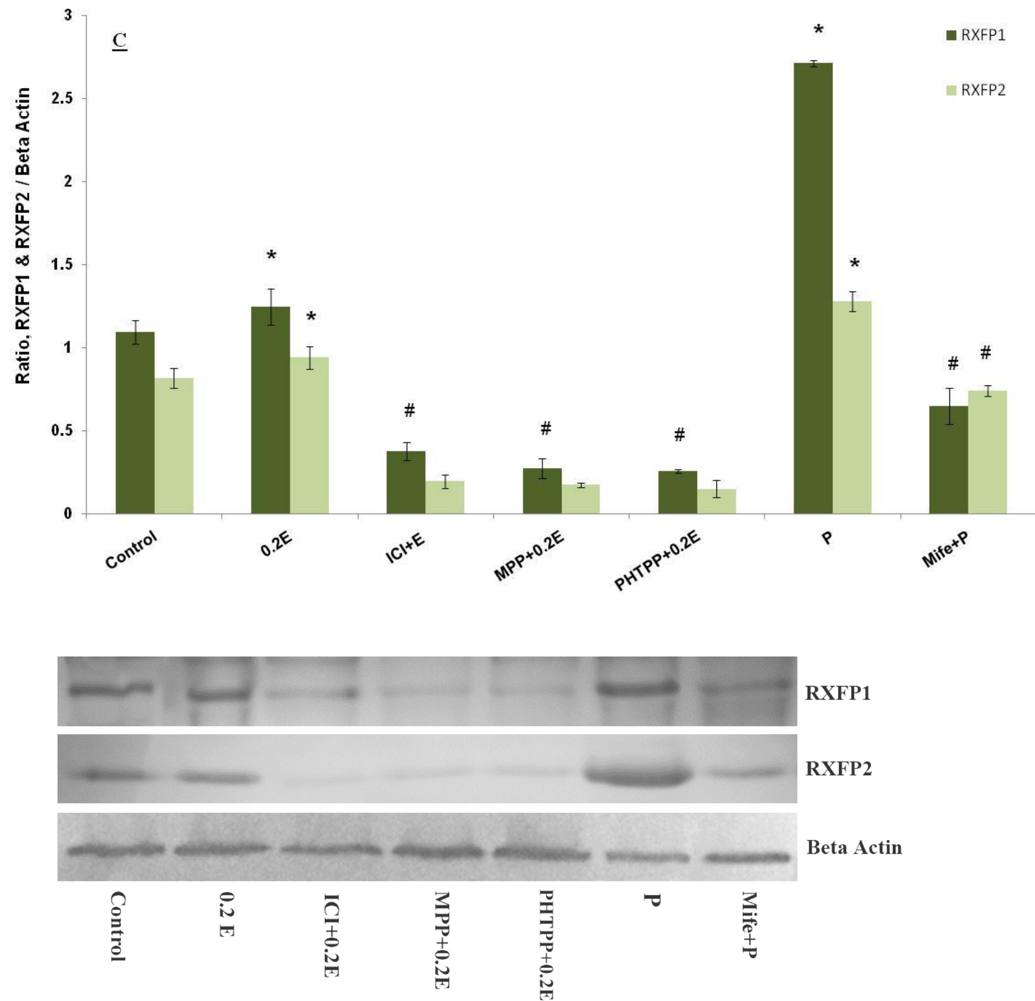


Figure 6-1 RXFP1& RXFP2 mRNA and protein expression

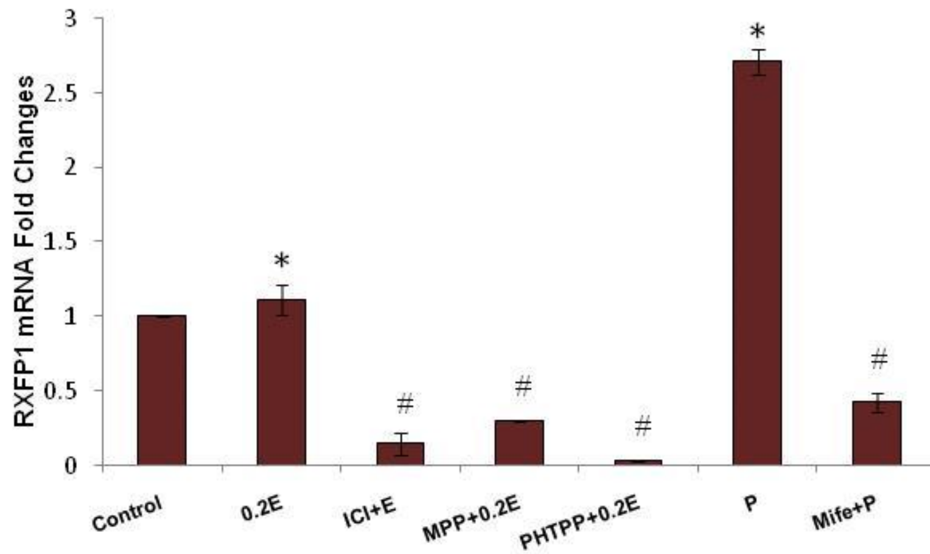
(A) The expression of RXFP1 mRNA in the patellar tendon of ovariectomized rats treated with E_2, P_4 and their antagonists. Control; 0.2E – 0.2µg/kg E_2; ICI+0.2E- ICI 182/780 (25 mg/kg) + 0.2 µg/kg E_2; MPP+0.2E – MPP (25mg/kg) + 0.2µg/kg E_2; PHTPP+0.2E – PHTPP (25 mg/kg) + 0.2 µg/kg E_2; P- 4mg/kg P_4; Mife+P- 7.5 mg/kg mifepristone +4 mg/kg P4. Data were expressed as mean ± SEM, n = 6 per treatment group and * $p < 0.05$ as compared to control, #$p<0.05$ as compared to the respective agonist only treatment. **(B)** The expression of RXFP2 mRNA in the patellar tendon of ovariectomized rats treated with E2, P4 and their antagonists: Control; 0.2E – 0.2 µg/kg E2; ICI+0.2E- ICI 182/780 (25 mg/kg) + 0.2 µg/kg E2; MPP + 0.2E – MPP (25 mg/kg) + 0.2 µg/kg E2; PHTPP+0.2E – PHTPP (25 mg/kg) + 0.2 µg/kg E2; P- 4 mg/kg P4; Mife + P- 7.5 mg/kg mifepristone +4 mg/kg P4. Data were expressed as mean ± SEM, n = 6 per treatment group and * $p<0.05$ as compared to control, #$p<0.05$ as compared to the respective agonist only treatment. **(C)** Ratio ofRXFP1& RXFP2/ β-actin protein expression levels in patellar tendon of ovariectomized rats treated with E2, P4 and their antagonists. Control; 0.2 E – 0.2 µg/kg E2; ICI+0.2 E- ICI 182/780 25 mg/kg + 0.2 µg/kg E2; MPP+0.2 E – MPP 25 mg/kg + 0.2 µg/kg E2; PHTPP+0.2 E – PHTPP 25 mg/kg + 0.2 µg/kg E2; P- 4 mg/kg progesterone; Mife + P- 7.5 mg/kg mifepristone + 4 mg/kg P4. Data were expressed as mean ± SEM, n=6 per treatment group and * $p < 0.05$ as compared to control, #$p<0.05$ as compared to the respective agonist only treatment.

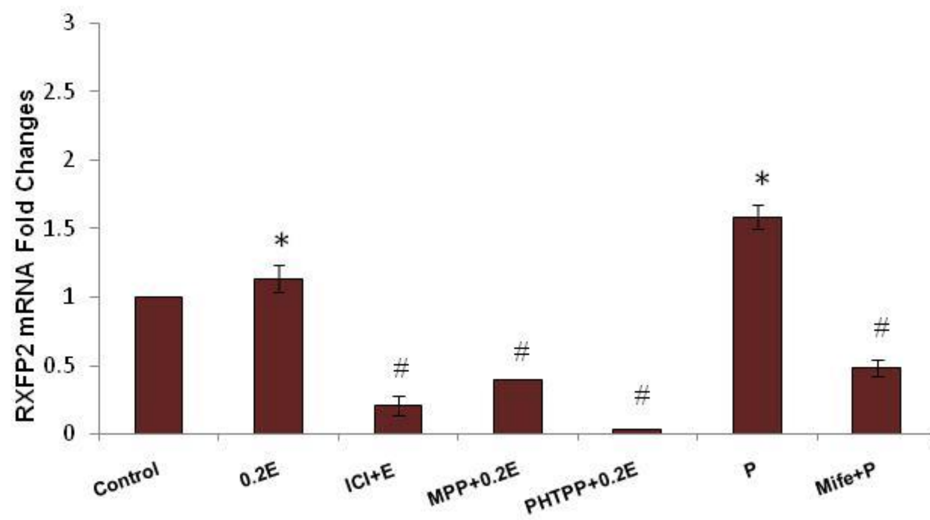
In figure 6-1, the expression level of *RXFP1* mRNA in the patellar tendon of ovariectomized rats received E2 was reduced by approximately 0.81, 1.4 and 1.62 fold in the presence of ICI 182/780, MPP, and PHTPP respectively as compared to 0.2 μg E_2 treatment alone. The expression of *RXFP1* mRNA was the highest in the P_4 treated group which was antagonized by approximately 2.31 fold in the presence of PR blocker, mifepristone.

The expression level of *RXFP2* mRNA in patellar tendon following E_2 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 E_2 treatment alone. The expression of *RXFP2* mRNA in the patellar tendon which was the highest following treatment with P_4 was significantly reduced by approximately 1.45 fold following PR antagonist, mifepristone administration.

Meanwhile, RXFP1 and RXFP2 protein expressions in the patellar tendon were the highest following P_4 treatment. Mifepristone caused a significant decrease in both isoforms expression. The expressions of RXFP1 and RXFP2 under E_2, which were significantly higher than control, were inhibited by ICI 182780, MPP and PHTPP. PHTPP caused the highest inhibition on RXFP1 and RXFP2 protein expression.

6.3 Effect of steroid receptor antagonists on RXFP1 and RXFP2 mRNA and proteins expression in the collateral ligaments





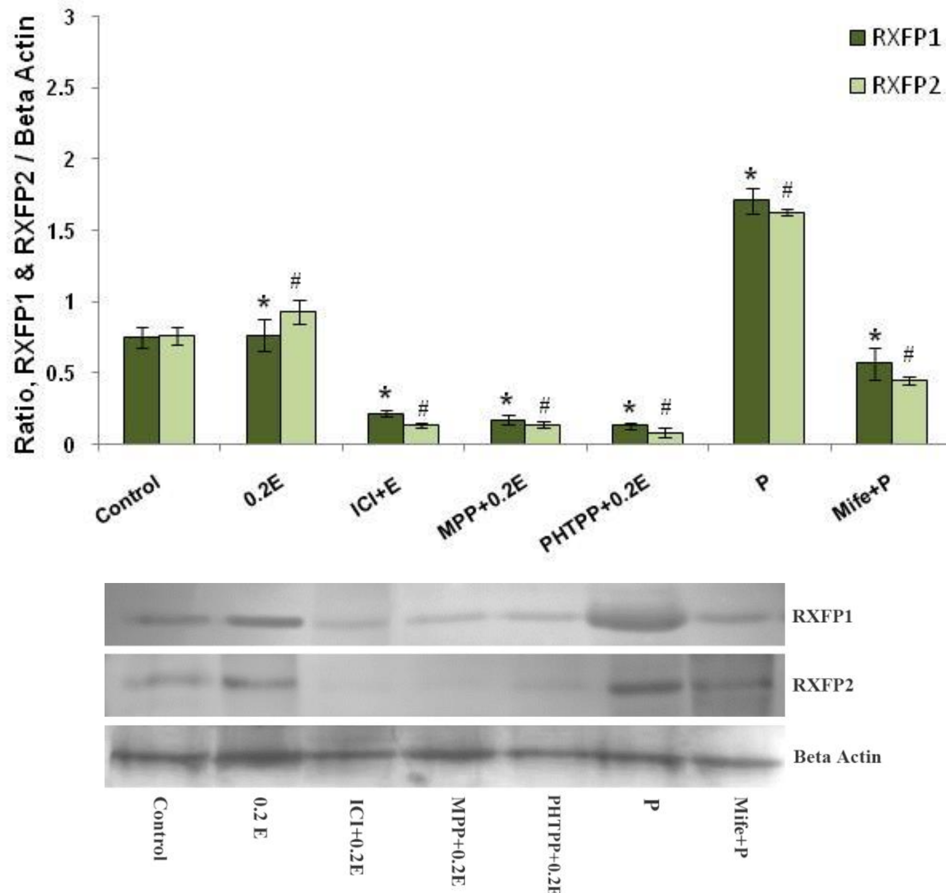


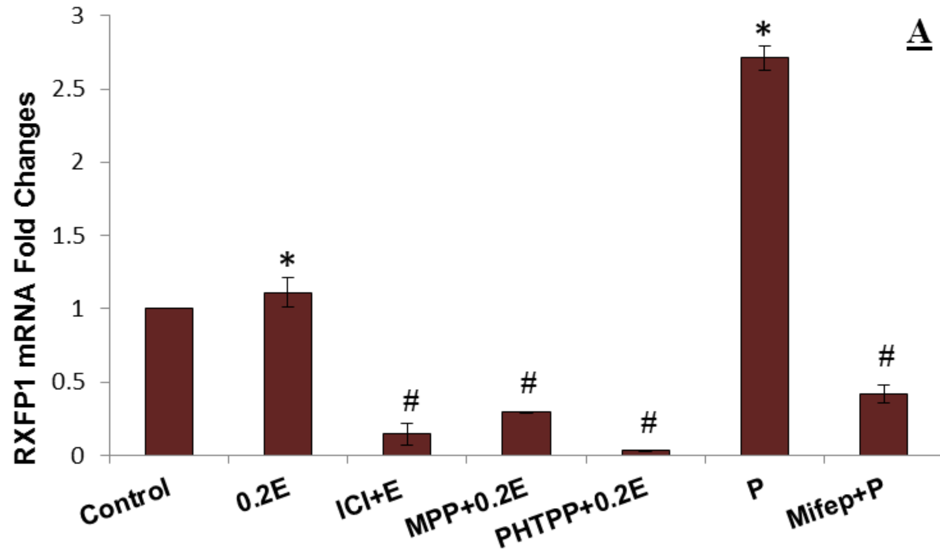
Figure 6-2 RXFP1& RXFP2 mRNA and protein levels in collateral ligament
(A) The expression of RXFP1 mRNA in collateral ligament of ovariectomized rats treated with E2, P4 and their respective antagonists. Control; 0.2E – 0.2 µg/kg oestrogen; ICI + 0.2E - ICI 182/780 (25 mg/kg) + 0.2 µg/kg E2; MPP + 0.2 E – MPP (25 mg/kg) + 0.2 µg/kg E2; PHTPP + 0.2 E – PHTPP (25 mg/kg) + 0.2 µg/kg E2; P - 4mg/kg P4; Mife + P - 7.5 mg/kg mifepristone +4 mg/kg P4. Data were expressed as mean ± SEM, n=6 per treatment group and * $p < 0.05$ as compared to control, #$p<0.05$ as compared to the respective agonist only treatment.**(B)** The expression of RXFP2 mRNA in collateral ligament of ovariectomized rats treated with E2, P4 and their antagonists: Control; 0.2 E – 0.2 µg/kg E2; ICI+0.2 E- ICI 182/780 (25 mg/kg) + 0.2 µg/kg E2; MPP+0.2 E – MPP (25 mg/kg) + 0.2 µg/kg E2; PHTPP+0.2 E –PHTPP (25 mg/kg) + 0.2 µg/kg E2; P-4 mg/kg P4; Mife + P-7.5 mg/kg mifepristone + 4 mg/kg P4. Data were expressed as mean ± SEM, n=6 per treatment group and * $p < 0.05$ as compared to control, #$p<0.05$ as compared to the respective agonist only treatment.. **(C)** Ratio ofRXFP1& RXFP2/ β-actin protein levels in collateral ligament in presence of oestrogen& progesterone antagonists. Control; 0.2 E – 0.2 µg/kg E2; ICI + 0.2 E- ICI 182/780 (25 mg/kg) + 0.2 µg/kg E2; MPP+0.2 E – MPP (25 mg/kg) + 0.2 µg/kg E2; PHTPP+0.2 E – PHTPP 25 mg/kg + 0.2 µg/kg E2; P-4 mg/kg P4; Mife + P-7.5 mg/kg mifepristone + 4 mg/kg P4. Data were expressed as mean ± SEM, n=6 per treatment group and * $p < 0.05$ as compared to control, #$p<0.05$ as compared to the respective agonist only treatment.

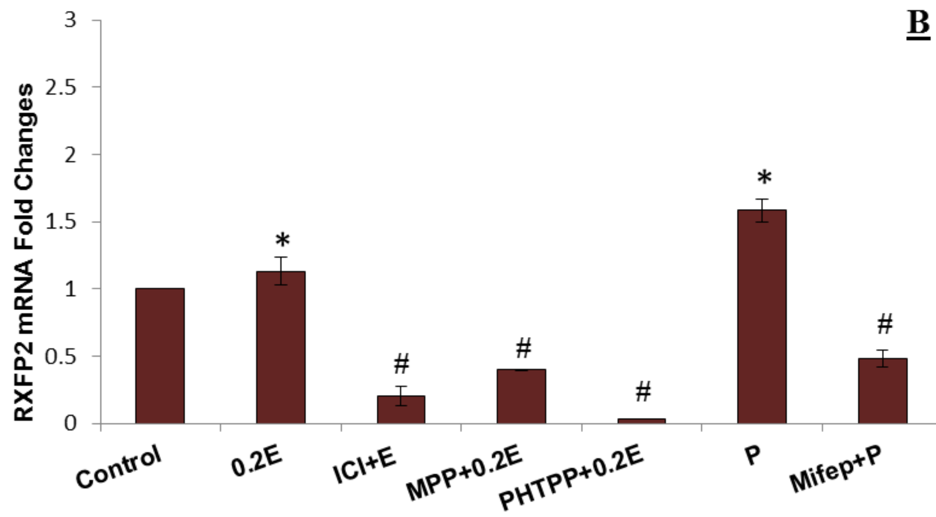
In Figure 6-2, the expression levels of *RXFP1* mRNA in ovariectomised rats collateral ligaments of the knee were decreased in the presence of steroid receptor antagonists. The expression of *RXFP1* mRNA was increased following oestrogen treatment and was reduced in the presence of different antagonists, 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 P_4 was reduced in the presence of PR antagonist, mifepristone by approximately 2.28 fold.

The expression level of *RXFP2* mRNA in ovariectomized rats received E_2 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 P_4 treatment which was reduced by approximately 1.09 fold following PR antagonist, mifepristone administration.

Meanwhile, RXFP1 and RXFP2 protein expression in the collateral ligament was significantly increased under P_4 influence and was antagonized by mifepristone. Under E_2 influence, these isoforms expression were slight but significantly higher than the control group. Administration of ICI 182780, MPP and PHTPP resulted in a marked inhibition of RXFP1 and RXFP2 proteins expression under E_2 with PHTPP administration caused the greatest inhibition.

6.4 Effect of steroid receptor antagonists on RXFP1 and RXFP2 mRNA and proteins expression in the hamstring muscles





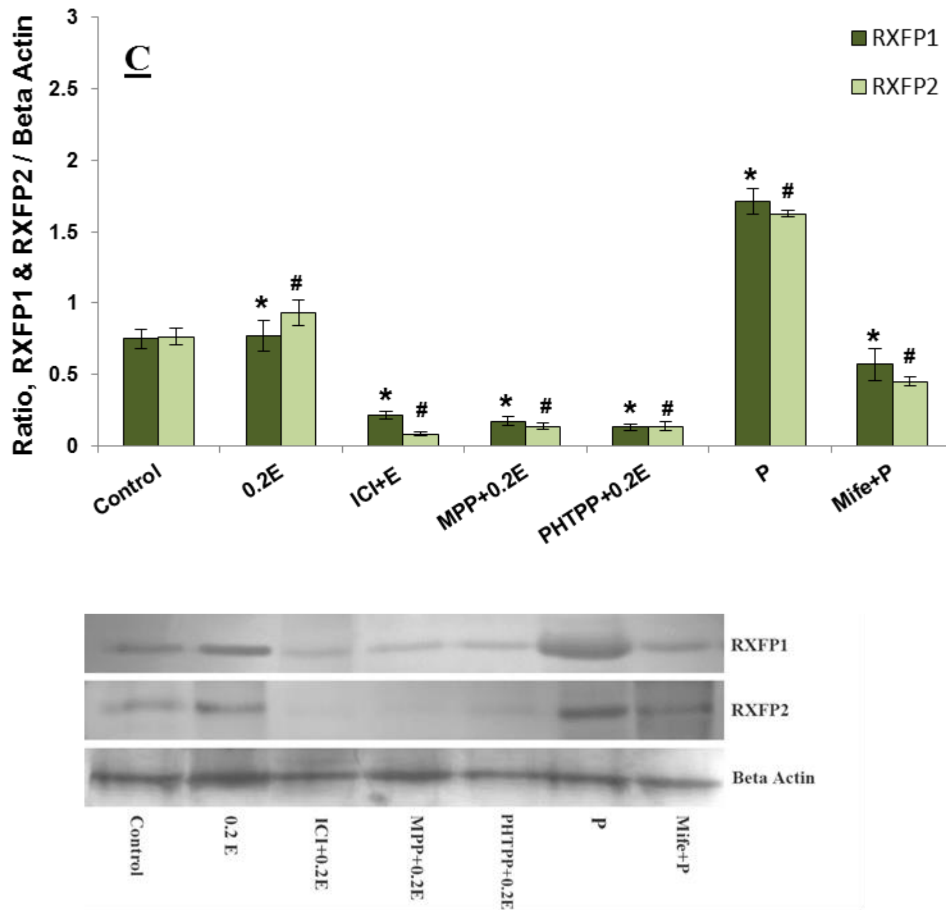


Figure 6-3 RXFP1& RXFP2 mRNA and proteins levels in the hamstringmuscle

(A) The expression of RXFP1 mRNA in the hamstring muscle of ovariectomized rats treated with E2, P4 and their antagonists. Control; 0.2 E – 0.2 μg/kg E2; ICI+0.2 E- ICI 182/780 25 mg/kg + 0.2 μg/kg E2; MPP+0.2 E – MPP (25 mg/kg) + 0.2 μg/kg E2; PHTPP+0.2 E –PHTPP (25 mg/kg) + 0.2 μg/kg E2; P-4 mg/kg P4; Mife + P- 7.5 mg/kg mifepristone +4 mg/kg P4. Data were expressed as mean ± SEM, n=6 per treatment group and * $p < 0.05$ as compared to control. **(B)** The expression of RXFP2 mRNA in the hamstring muscle of ovariectomized rats treated with E2, P4 and their antagonists: Control; 0.2 E – 0.2 μg/kg E2; ICI+0.2 E- ICI 182/780 (25 mg/kg) + 0.2 μg/kg E2; MPP+0.2 E – MPP (25 mg/kg) + 0.2 μg/kg E2; PHTPP+0.2 E –PHTPP (25 mg/kg) + 0.2 μg/kg E2; P-4 mg/kg P4; Mife + P- 7.5 mg/kg mifepristone + 4 mg/kg P4. Data were expressed as mean ± SEM, n=6 per treatment group and * $p < 0.05$ as compared to control. **(C)** Ratio ofRXFP1& RXFP2/ β-actin protein expression levels in hamstring muscle of ovariectomized rats treated with E2, P4 and their antagonists. Control; 0.2 E – 0.2 μg/kg E2; ICI+0.2 E- ICI 182/780 (25 mg/kg) + 0.2 μg/kg E2; MPP + 0.2E – MPP (25 mg/kg) + 0.2 μg/kg E2; PHTPP+0.2E –PHTPP (25 mg/kg) + 0.2 μg/kg E2; P- 4mg/kg P4; Mife + P- 7.5 mg/kg mifepristone + 4 mg/kg P4. Data were expressed as mean ± SEM, n = 6 per treatment group and * $p < 0.05$ as compared to control, #$p<0.05$ as compared to the respective agonist only treatment.

In figure 6-3, the expression level of *RXFP1* mRNA in ovariectomized rats' hamstring muscle under the effect of $E_2$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 E_2 treatment alone. Expression of *RXFP1* mRNA in the hamstring muscle was the highest following treatment with P_4 and was markedly reduced in the presence of PR antagonists, mifepristone by approximately 2.31 fold as compared to P_4 treatment alone.

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 E_2 alone. The expression of *RXFP2* mRNA was the highest following P_4 treatment which was reduced by approximately 1.09 fold in the presence of PR antagonist, mifepristone.

Meanwhile, in the hamstring muscle, RXFP1and RXFP2 proteins were expressed the highest in P_4 treated group which were antagonized by mifepristone. E_2 treatment resulted in slightly higher RXFP1 and RXFP2 protein expression which were markedly inhibited following administration of PHTPP, MPP and ICI 182 780.

6.5 Knee passive ROM in the presence of steroid receptor antagonist

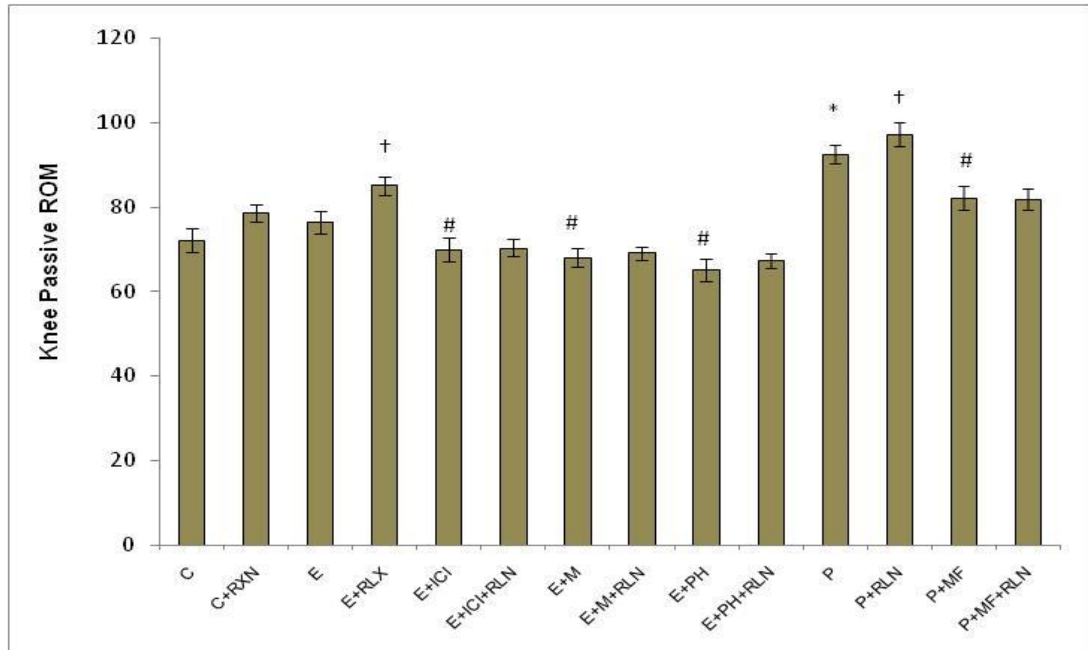


Figure 6-4 Knee passive ROM of ovariectomised rats

Rats received different doses of E2, P4 and the receptor antagonists with and without relaxin. Control; E – 0.2 μg/kg E2; E+RLN- E2 plus Relaxin, E+ICI- E2 plus 25 mg/kg ICI 182/780; E+ICI+RLN- E2 plus 25 mg/kg ICI 182/780 plus Relaxin, E+M- E2 plus 25 mg/kg MPP, E+M+RLN- E2 plus 25 mg/kg MPP+Relaxin, E+PH- E2 plus 25 mg/kg PHTPP, E+PH+RLN- E2 plus 25 mg/kg PHTPP plus Relaxin, P-4 mg/kg P4; P+RLN-ICI-P4 plus Relaxin, P+MF- P4 plus Mifepristone (7.5 mg/kg) and P+MF+RLN- P4 plus Mifepristone (7.5 mg/kg) plus Relaxin. Data were expressed as mean ± SD, n=6 per treatment group and * $p < 0.05$ as compared to control, #$p<0.05$ as compared to the without receptor blocker, †$p<0.05$ as compared to withour relaxin. Knee passive ROM was following E2 and P4 treatments.

In Figure 6-4, the increase in knee passive ROM in E_2 treated group ($p<0.05$ as compared to control) was antagonized by MPP and PHTPP however not by ICI 182780. Knee passive ROM was the highest following P_4 treatment (mean = 92.4 ^{o} ±4.13) which was antagonized by mifepristone ($p<0.01$). Relaxin administration caused an even higher increase in knee passive ROM in the control, E_2 and P_4-treated groups, however no significant changes in knee ROM were observed in the groups received E_2 plus MPP, E_2

plus PHTPP treatments as compared to similar groups without relaxin. Meanwhile, relaxin administration in the group received P_4plus mifepristone treatment also did not cause any significant changes in knee passive ROM.

6.6 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 administration 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 oestrogen effect would confirm the involvement of ERβ subtypes in mediating the biological effects of oestrogen (Compton *et al*., 2004). Previous study has also reported the presence of ER (general) in the synoviocytes, 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 182780 (fulvestrant), a potent anti-oestrogen which was known to disrupt receptor translocation into the nucleus as well as increase receptor degradation (Osborne *et al.*, 2004; Wakeling, 2000) on E_2-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-oestrogen however with no agonist activity on oestrogen sensitive tissue such as uterus (Robertson, 2001). Our findings which indicate that ICI 182780 administration did not result in a significant decrease in *RXFP1* and *RXFP2* mRNA expression in the patellar tendon of rats received E_2 treatment, suggested that ligand-receptor complex translocation into the nucleus may not be required

for mediating E_2 effect in this tissue, most probably due to the presence of abundance nuclear ER. In mouse, ICI 182780 has been reported to disrupt nuclear-cytoplasmic shuttling where the receptor could be retained in the cytoplasm, therefore blocking E_2 effect (Dauvois *et al.*, 1993). We speculated that in the tendon, cytoplasmic shuttling may not occur, therefore E_2 effects could be minimally affected by ICI 182780. There is also a possibility ICI 182780 may not reached the target site in the tendon due its poor vascularization as compared to synovium (Rempel & Abrahamsson, 2001). In contrast, ICI 182780 exerts a strong inhibition on *RXFP1* and *RXFP2* mRNA expressions in the collateral ligaments and hamstring muscles indicating that these tissues were responsive towards ICI 182780 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 degree 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 182780, MPP and PHTPP significantly inhibit these proteins expression in the muscle, tendon and ligaments surrounding the knee joint. PHTPP exerts the strongest inhibition therefore confirmed that ERβ is the main ER isoform expressed. Milder inhibition by MPP suggested that ERα was also involved in mediating RXFP1 and RXFP2 expression while variable degree of inhibition by ICI 182780 indicates that E_2 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 P_4 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. Relaxin administration caused increased in knee passive ROM in the groups treated with E_2 and P_4 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). E_2 and P_4have been shown to up-regulate the expression of relaxin receptors in ovariectomised rats' knee (Dehghan *et al.*, 2014), therefore relaxin effects were augmented in the presence of these hormones. Our findings confirmed that both E_2 and P_4 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 E_2 and P_4-induced increase in knee passive ROM and RXFP1 and RXFP2 expression in the structures controlling knee joint movements. We have shown that E_2 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.

CHAPTER 7

Changes in Laxity and Expression of Relaxin Receptor Isoforms (*RXFP1* and *RXFP2*) of Rats' Knee at Different Phases of Oestrus Cycle

7.1 Introduction

Joint laxity has been reported to be modulated by hormones. In lower species such as rodents, relaxin, a polypeptide produced by the corpus luteum in non-pregnant females (Dehghan *et al.*, 2014)was found to reduce laxity of the pelvic joint ligament during pregnancy in species such as guinea pigs, mice and bats (Sherwood *et al.*, 1992). In rats, relaxin administration has been reported to reduce the organization and mechanical strength of periodontal ligaments (Madan *et al.*, 2007). In human females, serum relaxin levels was found to positively correlate with the incidence of anterior cruciate ligament tear, suggesting of relaxin influence on knee laxity (Dragoo *et al.*, 2011a). Additionally, relaxin receptor has also been reported to be expressed in the anterior cruciate ligament (Dragoo *et al.*, 2003). Meanwhile, in rats, both relaxin receptor isoforms, RXFP1 and RXFP2 were found to be expressed in knee collateral ligaments and patellar tendon (Dehghan *et al.*, 2014).

There were evidences, which suggest the involvement of female hormones in modulating joint laxity. Following pregnancy in rats, knee joint contracture which was created by prolonged immobilization was reduced as compared to the non-pregnant rats, suggesting the influence of progesterone in the decrease in joint laxity (Ohtera *et al.*, 2002). A pregnancy-associated increase in laxity of medial collateral ligament was also observed in rabbits (Hart *et al.*, 2000). Increased in the incidence of back pain in humans during pregnancy is also related to increase in pelvic ligament laxity, influence by progesterone with little influence by relaxin (Aldabe *et al.*, 2012). In ovariectomised sheep, administration of physiologic dose of oestrogen and oestrogen receptor antagonist was found to have no significant effect on anterior cruciate and medial colateral ligaments of the knee (Strickland *et al.*, 2003). Several studies in humans have indicated the influence of

female sex hormones i.e. oestrogen and progesterone on knee laxity. Zazulak et al, (Zazulak *et al.*, 2006) and Park et al, (Park *et al.*, 2009a) reported that laxity was the highest at ovulation indicating of oestrogen influence. Knee laxity was also high in the luteal phase of the cycle, indicating of progesterone influence (Shultz *et al.*, 2005).

Despite of its use as experimental model, changes in knee laxity in rodent throughout phases of the oestrus cycle is widely unknown. Additionally, mechanisms underlying these changes have not been fully elucidated. Based on the reported modulation of relaxin receptor expression by sex-steroids (Dehghan *et al.*, 2014), we hypothesized that expression of relaxin receptor isoforms in the knee and muscles controlling knee joint movement changes at different phases of the oestrus cycle. The aims of this study were therefore to investigate changes in knee passive range of motion (ROM) and expression of relaxin receptor isoforms, RXFP1 and RXFP2 in the patellar tendon, collateral ligaments and hamstring muscles which could contribute to the observed changes in knee laxity at different phases of the cycle.

7.2 Results

7.2.1 Knee Passive ROM at different phases of the oestrus cycle

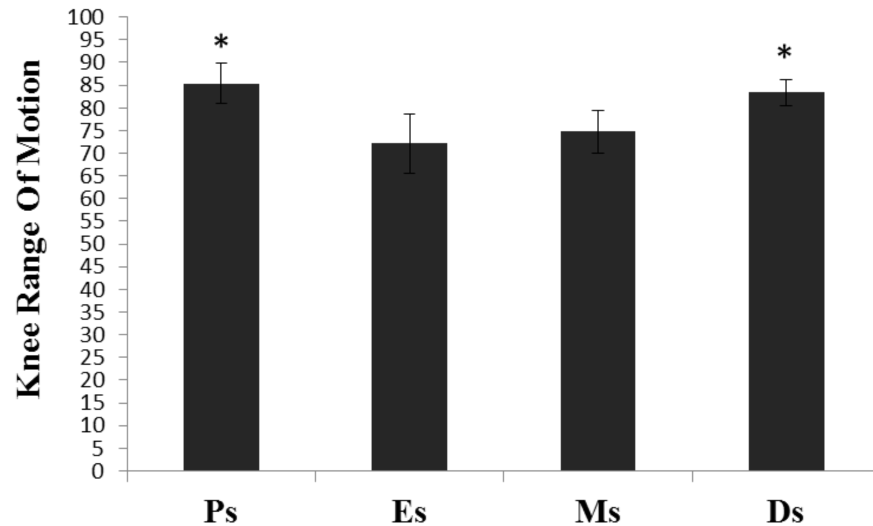
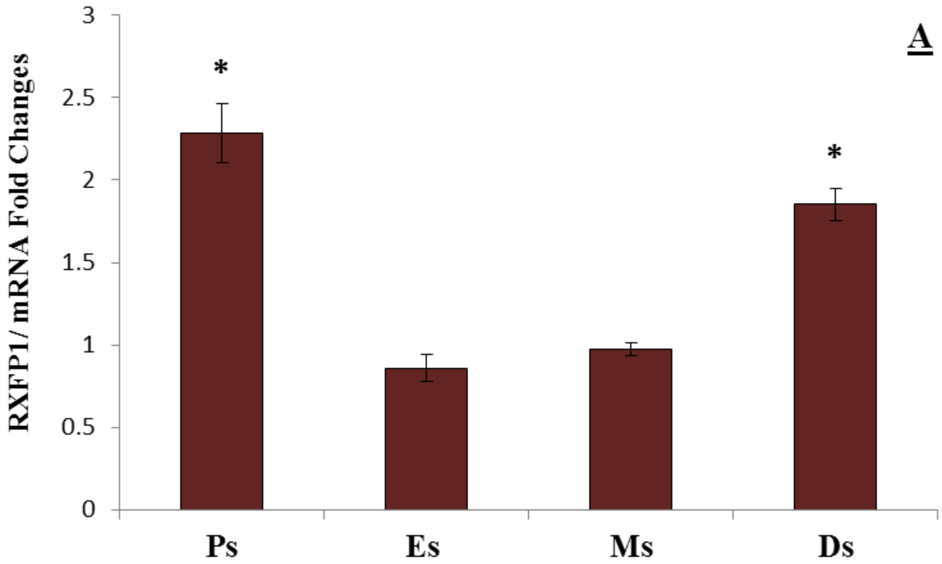
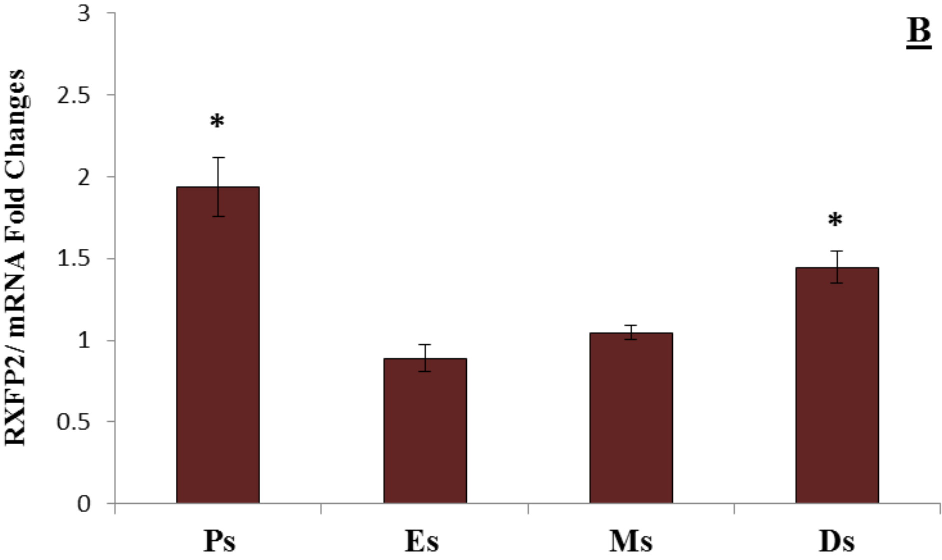


Figure 7-1 Knee passive ROM at different phases of the oestrus cycle
Ps-proestrus; Es-estrus; Ms; metestrus; Ds; diestrus. Data were expressed as mean ± SEM and n = 6 per treatment group. Knee ROM was the highest at proestrus and diestrus phases and the lowest at estrus and metestrus phases.

Figure 7-1 shows knee passive ROM in intact female rats at different phases of the oestrus cycle. The ROM was the highest at proestrus and diestrus. Meanwhile, knee ROM was the lowest at estrus and metestus which were significantly lower than proestrus and diestrus.

7.2.2 RXFP1 & RXFP2 expressions in hamstring muscle at different phases of the oestrus cycle





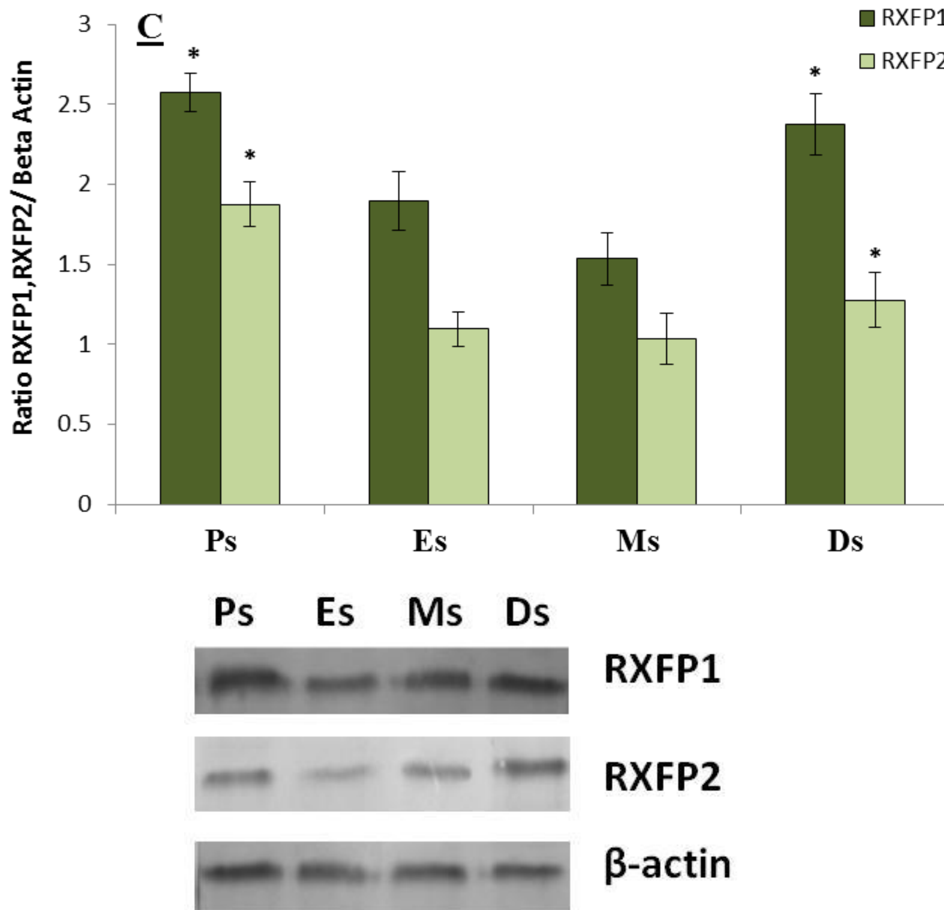
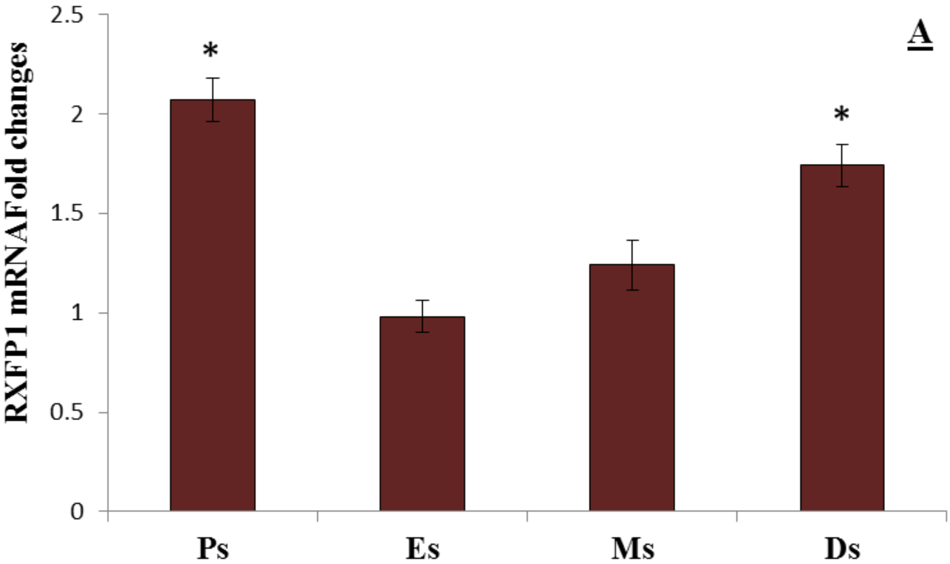
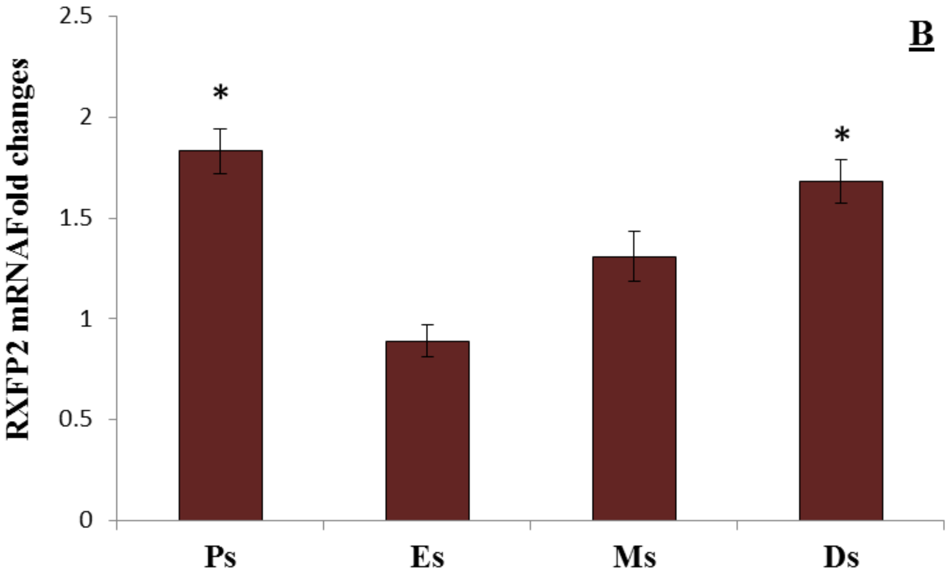


Figure 7-2 RXFP1& RXFP2 mRNA and protein expression in the hamstring muscle at different phases of the oestrus cycle. The expression of **(A)** RXFP1 mRNA **(B)** RXFP2 mRNA and **(C)** RXFP1& RXFP2 proteins in the hamstring muscle. The highest mRNA and protein expression levels were noted at proestrus phase. Ps-proestrus; Es-estrus; Ms; metestrus; Ds; diestrus. Data were expressed as mean ± SEM and n = 6 per treatment group.

Figure 7- 2 shows (A) RXFP1 mRNA, (B) RXFP2 mRNA and (C) RXFP1 and RXFP2 over β-actin protein expressions in the hamstring muscles of intact rats at different phases of the oestrus cycle. Our findings indicate that RXFP1 and RXFP2 mRNA levels were the highest at proestrus and diestrus (proestrus >> diestrus) followed by metestrus and estrus. The expression was the lowest at oestrus. In the hamstring muscle, RXFP1 mRNA level was higher than RXFP2. Meanwhile, RXFP1 and RXFP2 proteins were expressed the highest during proestrus followed by diestrus. The expression was the lowest at metestrus. The levels of RXFP1 protein expressed in the muscles were higher than RXFP2.

7.2.3 RXFP1 & RXFP2 expression in patellar tendon at different phases of the oestrus cycle





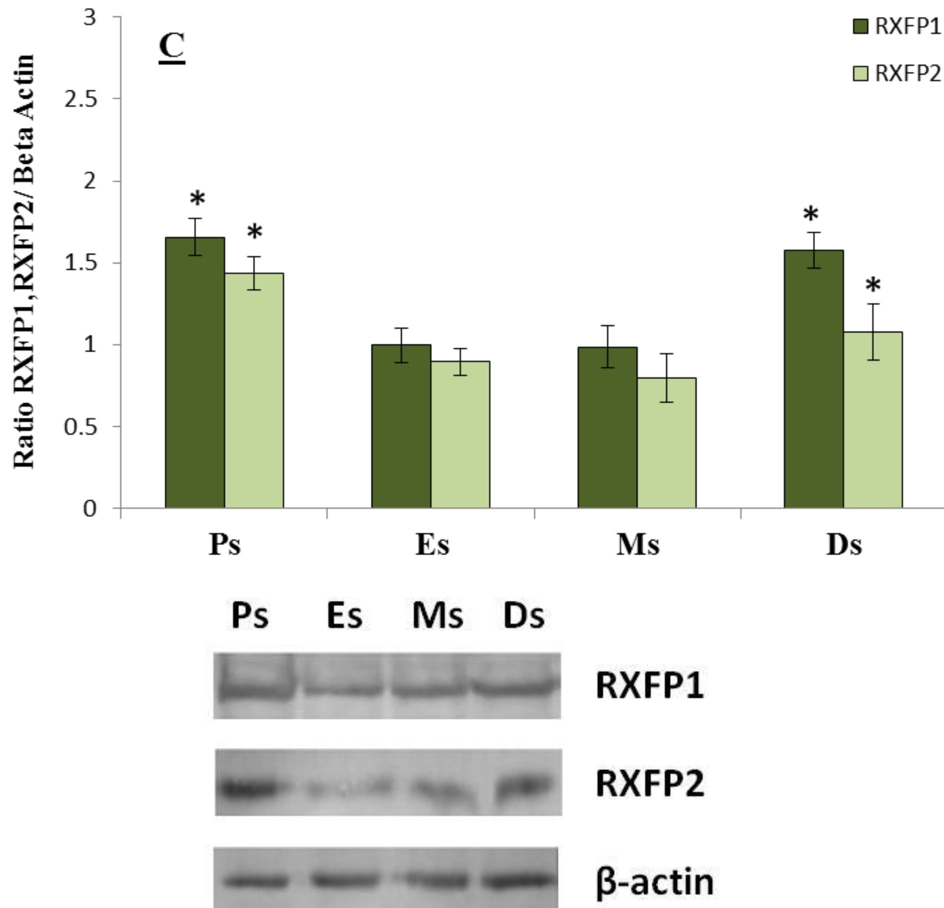
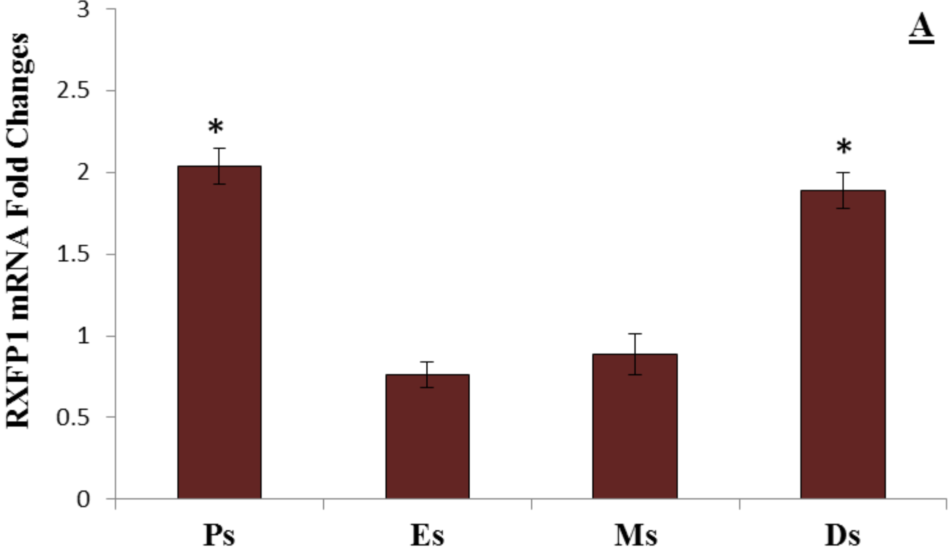
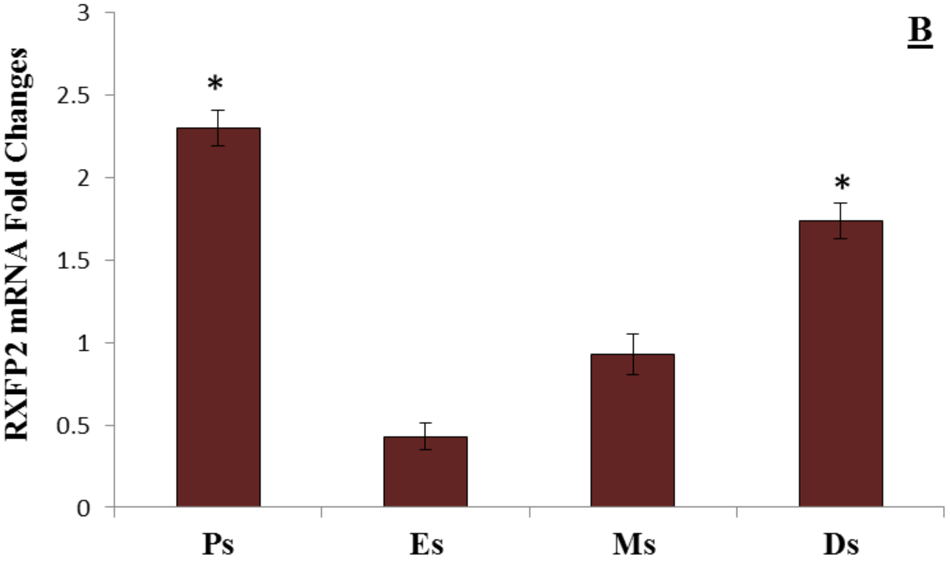


Figure 7-3 RXFP1& RXFP2 mRNA and protein expression levels in patellar tendon at different phases of the oestrus cycle. The expression of **(A)** RXFP1 mRNA **(B)** RXFP2 mRNA and **(C)** RXFP1& RXFP2 protein in patellar tendon. mRNA and protein expression levels were highest at proestrus and diestrus phases. Ps-prosterous; Es-estrus; Ms; metestrus; Ds; diestrus. Data were expressed as mean ± SEM and n =6 per treatment group.

Figure 7-3 shows (A) RXFP1 mRNA, (B) RXFP2 mRNA and (C) RXFP1 and RXFP2 over β-actin protein expressions in the patellar tendon of intact rats at different phases of the oestrus cycle. Our findings indicate that expression of RXFP1 mRNA was the highest during proestrus followed by diestrus phases of the cycle. The expression was the lowest at estrus phase. RXFP2 mRNA expression was also the highest at proestrus followed by diestrus and the lowest at estrus. Meanwhile, RXFP1 and RXFP2 proteins were expressed the highest at proestrus followed by diestrus and the lowest at estrus and metestrus phases. No differences in expression levels of RXFP1 were noted between proestrus and diestrus phases of the cycle.

7.2.4 RXFP1 & RXFP2 expression in collateral ligament at different phases of the oestrus cycle





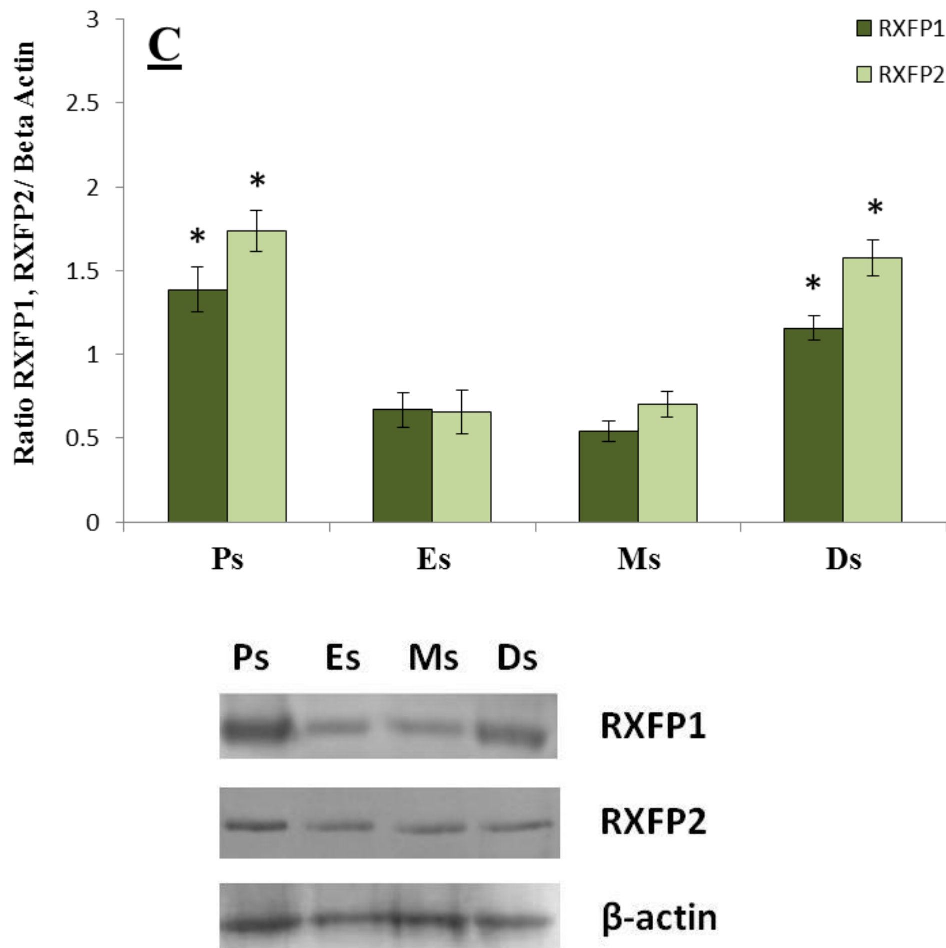


Figure 7-4 RXFP1& RXFP2 mRNA and protein expression levels in collateral ligament at different phases of the oestrus cycle. The expression of (A) RXFP1 mRNA (B) RXFP2 mRNA and (C) RXFP1& RXFP2 protein expression in collateral ligament. mRNA and protein expressions were the highest at proestrus phase followed by diestrus phase. Ps- proestrus; Es- estrus; Ms; metestrus; Ds; diestrus. Data were expressed as mean ± SEM and n = 6 per treatment group.

Figure 7-4 shows (A) RXFP1 mRNA, (B) RXFP2 mRNA and (C) RXFP1 and RXFP2 over β-actin protein expressions in the collateral ligaments of intact rats at different phases of the oestrus cycle. Our findings indicate that RXFP1 mRNA expression was the highest at proestrus and slightly lower at diestrus. However, no significant different were noted in RXFP1 expression between these cycle phases. RXFP1 mRNA expression was the lowest at estrus and metestrus. Meanwhile, RXFP2 expression was the highest at proestrus

which was approximately 1.5 fold higher than diestrus. The expression was the lowest at estrus. RXFP1 and RXFP2 proteins expressions were also the highest at proestrus followed by diestrus and the lowest at estrus and metestrus. It was noted that the levels of expression of RXFP2 mRNA and protein in the ligament exceed RXFP1 which was in contrast to the patellar tendon and hamstring muscles where RXFP1 isoform was the most dominant.

Table 7-1 Serum hormone level in non ovariectomized rat at different phases of the oestrus cycle

Oestrus cycle	**Hormone level (Mean ± SD)**
Oestrogen:	
- Proestrus	39.376± 4.56 pg/ml
- Estrus	12.64± 2.98 pg/ml
- Metestrus	8.035± 1.09 pg/ml
- Diestrus	16.487± 2.32 pg/ml
Progesterone	
- Proestrus	22.87± 2.65 ng/ml
- Estrus	14.92± 2.054 ng/ml
- Metestrus	16.96± 2.73 ng/ml
- Diestrus	24.03± 3.085 ng/ml
Relaxin	
- Proestrus	12.34 ± 3.96 pg/ml
- Estrus	8.04 ± 1.17 pg/ml
- Metestrus	9.48 ± 1.75 pg/ml
- Diestrus	18.03 ± 2.85 pg/ml

Table 7 shows the levels of oestrogen, progesterone and relaxin at different phases of the cycle in intact rats. The level of oestrogen was the highest at proestrus which was 2.3 fold higher than at diestrus. The level at estrus was lower than diestrus. Oestrogen level was the lowest at metestrus. Progesterone level was the highest at diestrus which was 1.7 times higher than at estrus. The level at proestrus was slightly lower than at diestrus. Progesterone level was the lowest at estrus. Meanwhile relaxin level was the highest at diestrus followed by proestrus. Relaxin level was the lowest at estrus which was nearly 2.5 times lower than at diestrus.

7.2.5 Correlation between serum relaxin and progesterone

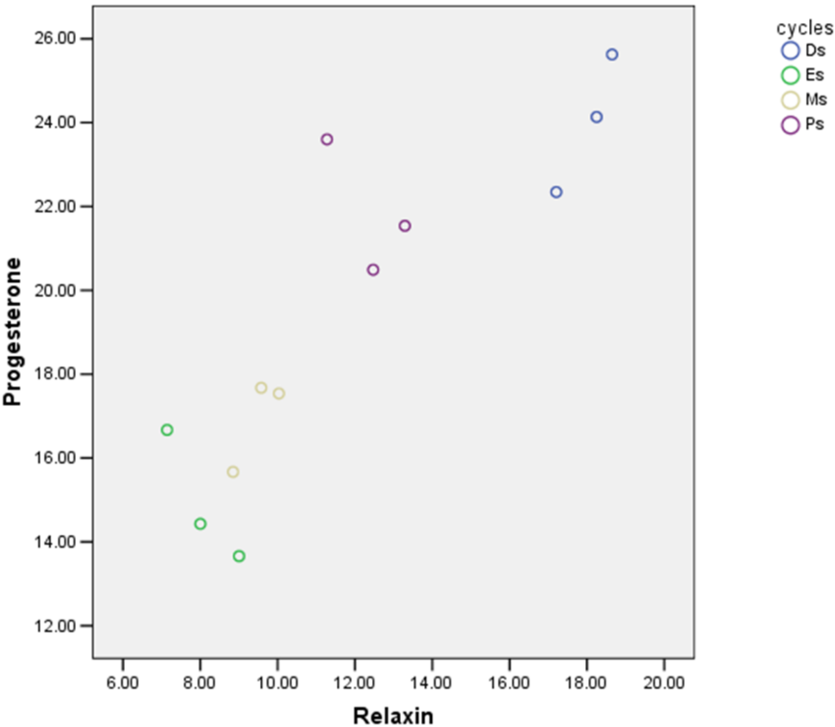


Figure 7-5 Correlation between serum relaxin and progesterone levels.

A strong positive correlation (r = 0. 901) was observed between serum progesterone and relatin levels throughout the oestrus cycle.

7.3 Discussion

This study reported for the first time changes in knee passive ROM in rats at different phases of the oestrus cycle. Our findings indicate that the ROM was the highest at proestrus which correlates with the highest serum oestrogen and progesterone levels and at diestrus which correlate with the highest serum progesterone levels. The serum hormone levels observed in this study were consistent with the reports in rats where progesterone and oestrogen levels peak at proestrus with another progesterone peak was observed at diestrus (Staley & Scharfman, 2005). The influence of female sex-hormone on knee laxity has been demonstrated by Woodhouse et al, (Woodhouse *et al.*, 2007) where intact female rats received daily injection of synthetic oestrogen (ethinylestradiol) and progesterone (levonogestrel) have a significantly reduced ACL stiffness as compared to the non-treated rats during femur-ACL-tibia loaded-to-failure test. Dragoo et al, (Dragoo *et al.*, 2009) demonstrated that combined administration of relaxin and oestrogen to guinea-pigs resulted in increased anterior tibial translation and a significantly weaker ACL on load-to-failure testing. Oestrogen at increasing doses were also reported to cause decreased human and rabbit ACLsfibroblast proliferation and collagen synthesis in culture (Thornton *et al.*, 2007). In humans, reports have indicated that high oestrogen levels in the ovulatory phases of the menstrual cycle were associated with high incidence of ACL injury (Beynnon *et al.*, 2006a) most probably due to decreased ACL laxity under the influence of this hormone (Shultz *et al.*, 2005).

Progesterone has also been reported to affect ligament laxity. In humans, correlation between serum progesterone levels and ACL stiffness has been suggested from the observed decreased in ACL laxity in the post-ovulatory (luteal) phase of the menstrual cycle (Park *et al.*, 2009b; Romani *et al.*, 2003). Human female athletes on oral

contraceptive pills containing high doses oestrogen and progesterone have greater ACL laxity than athletes not on OCPs (Lee *et al.*, 2014). The mechanisms underlying oestrogen and progesterone effect on knee laxity is not fully understood, however there were evidences which indicate that oestrogen stimulates collagenase, an enzyme responsible for collagen breakdown (Rajabi, 1991). Oestrogen and progesterone have recently been shown to up-regulate the expression of the receptors for relaxin, RXFP1 and RXFP2 in knee structures of rats (Dehghan *et al.*, 2014). Oestrogen and progesterone have also been shown to inhibit collagen synthesis in the tendon and skeletal muscles (Hansen *et al.*, 2009), which could contribute to the decreased in knee laxity.

Our findings also indicate that serum relaxin level was the highest at diestrus phase of the cycle. This was consistent with the reported increase in relaxin synthesis by the corpus luteum which was formed following ovulation and was maintained throughout diestrus phase of the cycle (Fields & Fields, 1985). In lower species such as rodents, relaxin affects laxity of the joints (Sherwood *et al.*, 1992) although no correlation between peripheral joint laxity and serum relaxin levels were noted in human females during pregnancy (Schauberger *et al.*, 1996). Despite of the lack of correlation, relaxin receptor expression has been identified in humans' ACL ligaments (Dragoo *et al.*, 2003)and ligament fibroblast (Faryniarz *et al.*, 2006) apart from the reported expression in the rats' patellar tendon and collateral ligaments (Dehghan *et al.*, 2014). Relaxin has been reported to induce activity of matrix metalloproteinases (MMPs) which are involved in the collagen breakdown (Hashem *et al.*, 2006; Naqvi *et al.*, 2005). The high levels of relaxin at diestrus and proestrus as observed in this study could contribute to the increase in knee passive ROM. A strong correlation between serum relaxin and progesterone levels suggested that

both hormones might be involved in the increase in knee laxity especially during diestrus phases of the cycle.

Our findings further indicated the changes in expression of two main receptors for relaxin, RXFP1 and RXFP2 in the hamstring muscles, patellar tendon and collateral ligaments, which participate in maintaining knee stability. We have shown that RXFP1 and RXFP2 expressions were the highest during proestrus and diestrus phases of the cycle, with the expression levels during the former were slightly greater than the latter. In muscles and tendon, RXFP1 was the main isoform while in the ligament, RXFP2 levels was the highest. Increased expression of these receptor isoforms could explain the augmented response of the hamstring muscle, patellar tendon and collateral ligament to relaxin in causing a reduction of knee laxity as reflected from the increase in passive ROM.

In conclusion, our study has shown that knee passive ROM in rats changes at different phases of the oestrus cycle, which most likely correlates with the changing levels of sex-steroid hormones. Additionally, fluctuation in relaxin level might also contribute to these observed effects. Differential regulation of RXFP1 and RXFP2 in the knee and its related structures by the changing levels of sex-steroids could complement changes in the relaxin levels to produce maximum effect on knee laxity.

CHAPTER 8

Changes in Serum Sex-Steroid and Relaxin Levels throughout the Menstrual Cycle and its Relationship with Lateral and Medial Knee Laxities in Athlete and Non-Athlete Females

8.1 Introduction

Knee joint, being the largest in the body, has its stability maintained by the shape of condyles and menisci and the surrounding structures. The four knee ligaments i.e. anterior cruciate ligament (ACL), posterior cruciate ligament (PCL), medial collateral ligament (MCL) and lateral collateral ligament (LCL), capsules (posteromedial and posterolateral) and iliotibial tract significantly contribute to maintaining knee stability (Fuss, 1989). In full extension, posterior oblique ligament and posteromedial capsule resist valgus stress while at 30 degrees flexion, MCL is the primary restraint (Messner & Gao, 1998). MCL together with posterior oblique ligament also resists abnormal internal tibial rotation (Kakarlapudi & Bickerstaff, 2000). Lateral collateral together with popliteofibular and cruciate ligaments act as a primary stabilizer to the lateral opening of the joint (Ullrich *et al.*, 2002). The laxity and integrity of MCL and LCL could be assessed by varus and vulgus stress tests (Romero *et al.*, 2002).

Menstrual cycle is associated with fluctuations in the levels of sex-steroids i.e. oestrogen and progesterone (Bäckström *et al.*, 1982). In addition, testosterone (Liening *et al.*, 2010) and relaxin (Johnson *et al.*, 1993) levels were also reported to fluctuate. For a 28 days menstrual cycle, days 1–9 marked the follicular phase with oestrogen predominates, days 10–14 marked the ovulatory phase, where oestrogen continues to prevail and reaches its peak while days 15–28 is the luteal phase where progesterone levels surpass that of oestrogen (Heitz *et al.*, 1999). Relaxin, which is secreted by the ovaries and reaches its peak during the luteal phase, exerts multiple systemic effects especially during pregnancy (Parry & Vodstrcil, 2007). Meanwhile, a very low level of testosterone fluctuates throughout the cycle and is involved in oestrogen formation and preparation of the endometrium for decidualization (Belanger *et al.*, 2004).

Female has two to ten times greater risk of non-traumatic knee injury than male (Hansen *et al.*, 2013). The reason for this is unclear, however evidences suggested that female sex-hormones could influence knee laxity (Park *et al.*, 2009a). There were conflicting reports with regards to the effect of sex-hormones on knee laxity. While some reported that female anterior knee laxity was the highest in the follicular (pre-ovulatory) phase (Beynnon *et al.*, 2006b; Hewett *et al.*, 2007), others reported that laxity was the greatest in the ovulatory (Adachi *et al.*, 2008; Park *et al.*, 2009a; Wojtys *et al.*, 1998) and luteal phases (Dragoo *et al.*, 2011a; Hicks-Little CA, 2007) of the cycle. There were also reports which indicate that no differences in laxity was observed across phases of the menstrual cycle (Karageanes *et al.*, 2000). In addition to sex-steroids, relaxin was also reported to influence knee ligament laxity where high relaxin levels correlate with the increase in laxity (Dragoo *et al.*, 2011a).

While most studies reported the influence of sex-steroids on ACL laxity, changes in LCL and MCL laxities remains largely unknown. Additionally, relationship between sex-steroids and relaxin with medial and lateral knee laxities have never been reported. We hypothesized that medial and lateral knee laxities changes at different phases of the menstrual cycle, therefore contributes towards difference in the incidence of non-contact knee injury with cycle phases. This study therefore aimed to investigate changes in medial and lateral knee laxities in female athletes and non-athletes at different menstrual cycle phases and their relationship with the levels of sex-steroids and relaxin in the circulation.

8.2 Results

8.2.1 Body Composition Analyses

Table 8-1 Characteristics and demographics at different phases of the menstrual cycle. *p<0.05 as compared to the athletes/non-athletes group

Variables	Athletes Mean ± SD	Non-Athletes Mean ± SD
Age (years)	20.3 ± 1.28	21.7 ± 2.27
Height (cm)	163 ± 2.75	158.5 ± 5.28
Weight (kg)		
- **Menstruation**	58 ± 7.93	56.5 ± 9.96
- **Follicular phase**	57.5 ± 7.75	55.7 ± 9.99
- **Luteal phase**	58 ± 7.93	56.1 ± 10
Total Body Water (kg)		
- **Menstruation**	30.8 ± 2.42*	27 ± 3.19
- **Follicular phase**	30.4 ± 2.33*	27 ± 3.1
- **Luteal phase**	30.6 ± 2.45*	27.1 ± 3.2
Total Protein (kg)		
- **Menstruation**	7.18 ± 0.75	8.24 ± 0.66*
- **Follicular phase**	7.21 ± 0.81	8.24 ± 0.66*
- **Luteal phase**	7.21 ± 0.85	8.22 ± 0.65*
Total Mineral (kg)		
- **Menstruation**	3.08 ± 0.25*	2.73 ± 0.32
- **Follicular phase**	3.03 ± 0.26*	2.69 ± 0.3
- **Luteal phase**	3.05 ± 0.27*	2.71 ± 0.33
Skeletal Muscle Mass (kg)		
- **Menstruation**	23 ± 2.05*	19.7 ± 2.55
- **Follicular phase**	22.7 ± 2.02*	19.7 ± 2.47
- **Luteal phase**	22.8 ± 1.91*	19.8 ± 2.58
Body Fat Mass (kg)		
- **Menstruation**	15.7 ± 5.34 (26.7%)	19.1 ± 7.02(33.4%)*
- **Follicular phase**	15.9 ± 5.44 (26.7%)	19 ± 7.34 (33 %)*
- **Luteal phase**	16.1 ± 5.5 (27.3%)	19 ± 7.06 (33 %)*
Body Mass Index (kg/m^2)		
- **Menstruation**	21.9 ± 2.55	22.2 ± 3.37
- **Follicular phase**	21.8 ± 2.59	22.2 ± 3.51
- **Luteal phase**	22 ± 2.67	22.2 ± 3.39
Waist Hip Ratio	0.82 ± 0.04	0.84 ± 0.06*
Fitness Score (points)	75.1 ± 2.93*	69.3 ± 5.51
Basal Metabolic Rate (kcal)		
- **Menstruation**	1284 ± 75.8*	1160 ± 96.6
- **Follicular phase**	1272 ± 75*	1167 ± 90.9
- **Luteal phase**	1275 ± 77.2*	1170 ± 94.7

Differences between body compartment in athletes and non-athletes at different phases of the menstrual cycle are shown in Table 3. Body composition measured by bioelectrical impedance analysis (BIA) method. The average total body water, total protein, total mineral, skeletal muscle mass, body fat mass, body mass index, waist hip ratio, fitness score and basal metabolic rate in athletes and non-athletes population were presented. Data showed the mean and standard deviation for the impedance measurements. Measurement was performed twice and the average values entered was the final measurement. The Levene's equality of variable assumption stated that there were no significant difference between the variables (Cohen, 1988).

Our findings indicate that athletes mean weight were generally higher than non-athletes although the differences were not statistically significant. The weight was higher in the luteal phase and menstruation as compared to the follicular phase. Athletes were in general have higher total body water than non-athletes. There were no significant difference in total body water between phases of the menstrual cycle. Meanwhile, total protein content was significantly higher in non-athletes as compared to athletes ($p<0.05$) and no significant difference was noted between phases of the menstrual cycle. Total mineral content in athletes was significantly higher than non-athletes and in both groups, total mineral content was the highest in the menstruation phase of the cycle.

Skeletal muscle mass was found to be higher in athletes as compared to non-athletes ($p<0.05$) and was not significantly differ between phases of the menstrual cycle. Body fat mass was higher in non-athletes than athletes ($p<0.05$) which also not significantly differ at different cycle phases. Body mass index was slightly lower in athletes than non-athletes although no significant difference was noted between the two groups. Waist hip ratio was

significantly lower in athletes than non-athletes, while fitness score index was much higher in athletes than non-athletes ($p<0.05$). Finally, athletes have higher basal metabolic rate (BMR) than non-athletes with no significant difference was noted between phases of the menstrual cycle.

Table 8-2 Knee joint varus/valgus angles on 0 and 30°

	Athletes Mean(SD)	Non-Athletes Mean (SD)	T value	P value	Effect size
Varus 0°					
- **Menstruation**	3.75 (1.13)	5.33(1.02)	4.63	<0.05	1.47
- **Follicular phase**	1.80 (0.43)	3.44(0.95)	7.00	<0.05	2.38
- **Luteal phase**	5.45 (1.01)	7.19(1.01)	5.46	<0.05	1.72
Valgus 0°					
- **Menstruation**	3.09 (1.21)	4.76(.89)	4.99	<0.05	1.60
- **Follicular phase**	1.39 (0.51)	3.44(.56)	12.09	<0.05	3.83
- **Luteal phase**	4.76 (1.32)	5.56(.72)	2.37	<0.05	0.78
Varus 30°					
- **Menstruation**	11.48(1.21)	12.80(1.03)	3.74	<0.05	1.18
- **Follicular phase**	9.35(0.75)	10.95(0.72)	6.89	<0.05	2.18
- **Luteal phase**	14.25(1.18)	15.11(1.05)	2.44	<0.05	0.77
Valgus 30°					
- **Menstruation**	9.58(1.56)	11.71(1.51)	4.40	<0.05	1.39
- **Follicular phase**	7.63(1.00)	10.11(0.88)	8.36	<0.05	2.64
- **Luteal phase**	11.64(1.29)	12.98(1.79)	2.73	<0.05	0.87

Table 8-2 shows the degree of knee angles at 0° and 30^oflexion in varus and valgus stress tests in athletes and non-athletes. In varus 0° test, non-athletes have higher knee angle as compared to athletes at all phases of the cycle. In both groups, knee angle was the highest in the luteal phase, followed by menstruation phase and the lowest in the follicular phase. In varus 30° test, non-athletes have higher knee angle than athletes at all phases of the cycle ($p<0.05$). Similarly, in both groups, the highest knee angle was noted in the luteal phase, followed by menstruation and the lowest in the follicular phase.

In valgus 0° test, non-athletes appear to have greater knee angle in all phases of the cycle as compared to athletes ($p<0.05$). The highest angle was noted in the luteal phase, followed by menstruation and follicular phases. In valgus 30° test, non-athletes was still

found to have greater knee angle as compared to athletes at all phases of the cycle with the highest noted in the luteal phase.

8.2.2 Changes in Serum Sex-Steroids and Relaxin Levels at Different Phases of the Menstrual Cycle

Table 10 shows the values of serum sex-steroids and relaxin levels in athletes and non-athletes females at different phases of the menstrual cycle. Our findings indicate that the highest oestrogen level was observed in the follicular phase in both groups. There were no significant differences in oestrogen levels between athletes and non-athletes. Meanwhile, progesterone levels were the highest in luteal phase with athletes have a significantly lower progesterone level than non-athletes. Similarly, progesterone level was also higher in non-athletes as compared to athletes at menstruation, although this level was approximately 12 times lower than in the luteal phase of the cycle. No significant difference in the level was observed in the follicular phase between the two groups.

Meanwhile, testosterone level, which was far lower than oestrogen and progesterone, was found to be higher in non-athletes than athletes. In the non-athletes, testosterone level was the highest in the follicular phase followed by luteal phase. The lowest level was noted in athletes at menstruation. Serum level of relaxin was the highest in athletes as compared to the non-athletes, particularly during the follicular and luteal phases of the cycle. No significant difference in relaxin level was noted between the two groups during the menstruation phase.

Table 8-3 Sex-steroids and relaxin levels at different phases

	Athletes Mean(SD)	Non-Athletes Mean(SD)	T value	P value	Effect size
Oestrogen (pg/ml)					
- **Menstruation**	170.20(39.86)	197.65(71.97)	1.49	>0.05	0.50
- **Follicular phase**	507.35(161.52)	492.30(276.96)	0.21	>0.05	0.07
- **Luteal phase**	439.50(150.54)	409.45(163.89)	0.60	>0.05	0.19
Progesterone (ng/ml)					
- **Menstruation**	2.28(1.00)	4.19(2.53)	3.14	<0.05**	1.08
- **Follicular phase**	1.80(1.24)	1.74(0.60)	0.20	>0.05	0.07
- **Luteal phase**	24.74(11.34)	31.33(8.09)	2.12	<0.05**	0.68
Testosterone (ng/ml)					
- **Menstruation**	0.82(0.38)	1.24(0.47)	3.08	<0.05**	0.99
- **Follicular phase**	1.21(0.54)	1.39(0.48)	1.12	>0.05	0.36
- **Luteal phase**	1.22(0.61)	1.37(0.63)	0.76	>0.05	0.24
Relaxin (pg/ml)					
- **Menstruation**	2.10(0.56)	1.69(1.27)	1.33	>0.05	0.45
- **Follicular phase**	1.38(0.87)	0.34(0.45)	4.73	<0.05**	1.58
- **Luteal phase**	15.58(5.36)	10.35(2.96)	3.82	<0.05**	1.26

8.2.3 Correlations between Sex-Steroids and Relaxin Levels with Knee Joint Angles

Table 8-4 Correlation of sex steroid and knee angles in total polulation

	0 º		**30 º**	
	varus	**valgus**	**varus**	**valgus**
Oestrogen				
- **Pearson**	-0.15	-0.17	-0.09	-0.14
Progesterone				
- **Spearman**	0.70**	0.62**	0.76**	0.62**
Testosterone				
- **Pearson**	0.07	0.12	0.12	0.07
Relaxin				
- **Spearman**	0.58**	0.50**	0.65**	0.47**

Table 8-5 Correlation of sex steroid and Knee angles in athletes

	0 º		**30 º**	
	varus	**valgus**	**varus**	**valgus**
Oestrogen				
- **Pearson**	-0.13	-0.15	-0.04	-0.14
Progesterone				
- **Spearman**	0.66**	0.58**	0.70**	0.59**
Testosterone				
- **Pearson**	0.05	0.03	0.06	-0.06
Relaxin				
- **Spearman**	0.69**	0.65**	0.74**	0.64**

Table 8-6 Correlation of sex steroid and Knee angle in non-athletes

	0 °		30 °	
	varus	**valgus**	**varus**	**valgus**
Oestrogen				
- **Pearson**	-0.17	-0.26*	-0.13	-0.17
Progesterone				
Spearman	0.72**	0.65**	0.80**	0.60**
Testosterone				
- **Pearson**	-0.10	0.01	0.01	0.00
Relaxin				
- **Spearman**	0.81**	0.73**	0.80**	0.62**

* Correlation is significant at the 0.05 level
** Correlation is significant at the 0.01 level

Table 11, 12 and 13 show correlation between sex-steroid and knee joint angle in all subjects, in athletes and non-athletes respectively. In general, there were strong correlations between serum progesterone and relaxin levels with knee joint angles as determined by varus and valgus tests (both at 0° and 30°). In athletes, a significant correlation between progesterone and relaxin levels with knee joint angles in both tests were also observed. Similar observation was noted in non-athlete subjects. The highest correlation was observedbetween serum progesterone and relaxin levels with knee angle at 30^0 in varus test in athletes and non-athletes.

8.3 Discussion

Our findings revealed the followings: (i) athletes have higher total body water, total mineral content, skeletal muscle mass, fitness score and BMR however lower total protein content, body fat mass and waist to hip ratio as compared to non-athletes, (ii) athletes have lower medial and lateral knee angles than non-athletes, (iii) both athletes and non-athletes have the highest medial and lateral knee angles in the luteal phase of the cycle, (iv) in both athletes and non-athletes, the levels of progesterone and relaxin were the highest in the luteal phase while oestrogen was the highest in the follicular phase, (v) progesterone levels

in athletes was lower than non-athletes in the luteal and menstruation phases of the cycle and (vi) there was a strong correlation between serum progesterone and relaxin levels and knee joint angles (both in varus and valgus tests) in athletes and non-athletes.

Our findings which indicate that medial and lateral knee laxities were the highest in the luteal phase of the cycle correlate with higher serum progesterone and relaxin levels and were supported by several previous observations. Higher incidence of anterior cruciate ligament tear and non-contact knee injury in the post-ovulatory (Zazulak *et al.*, 2006) and luteal (Hicks-Little CA, 2007 ; Park *et al.*, 2009a) phases have been reported. This increase could be attributed to higher plasma progesterone and relaxin levels in the luteal phase as both hormones have been implicated in the increase in knee laxity. Recent evidence suggested that anterior laxity and valgus movement of the knee correlate with blood progesterone level (Bell DR, 2014). Progesterone, which levels is high during pregnancy has been implicated in the increase in medial collateral ligamentlaxity in rabbits (Hart *et al.*, 2000). Meanwhile, serum biomarkers of collagen degradation (ICTP) was reported to be high in early luteal phase in eumenorrhoeic women which correlates with increased anterior knee laxity (Shultz *et al.*, 2012), pointing towards progesterone influence. Heitz et al (Heitz *et al.*, 1999)reported that a significant increase in ACL laxitywas observed with peak levels of progesterone in physically active females.

Relaxin levels also correlated with the increase in knee laxity. Our findings indicate that the level of relaxin was the highest in the luteal phase and correlate with the increase in serum progesterone level. This finding is consistent with the report that serum relaxin levels positively correlates with progesterone (Dragoo *et al.*, 2011b). Futhermore, Dragoo et al, (Dragoo *et al.*, 2011a) reported a strong correlation between serum relaxin levels and

incidence of ACL tear in elite collegiate female athletes .Relaxin administration to guinea pigs also resulted in weaker ACL laxity (Dragoo *et al.*, 2009). It has been proposed that relaxin acts via several mechanisms to cause increase in the laxity of the ligaments. Relaxin has been reported to up-regulate matrix metalloproteases (MMPs) (Naqvi *et al.*, 2005) which cause increase collagen breakdown, therefore increase ligament laxity (Pearson *et al.*, 2011). Progesterone may enhance relaxin action which could result in a further decrease in the laxity of the ligaments. Progesterone has been reported to up-regulates the expression of relaxin receptor isoforms RXFP1 and RXFP2 in rat's knee joint (Dehghan *et al.*, 2014). Besides the rats' knee, relaxin receptors have also been found in human's knee (Dragoo *et al.*, 2003)and carpometacarpal (Wolf *et al.*, 2013) joints.

We have shown that medial and lateral knee laxities in non-athletes as measured by varus and valgus tests at 0° and 30° were higher than athlete females with the highest laxities in the luteal phase of the cycle. Progesterone levels were higher in non-athletes than athletes, which could be one of the reason for the increase in laxity as previously discussed. There were no significant differences in oestrogen and testosterone levels between athletes and non-athletes in the luteal and follicular phases of the cycle although testosterone level is slightly lower in athlete during menstruation phase. Although oestrogen level was high in the follicular phase, laxity appears to be the lowest. Our finding is in contrast with several studies which reported increased incidence of non-contact knee injury during ovulatory phases of the cycle (Park *et al.*, 2009b; Romani *et al.*, 2003; Wojtys *et al.*, 2002), correlates with high levels of oestrogen. We concluded that oestrogen could confer protection against non-contact knee injury in female. In addition, our findings also revealed that serum progesterone and relaxin levels were the lowest in the follicular phase, which suggest that these hormones may have little influence on knee laxity during this phase. Our finding was

supported by the observation by Wojtys et al, (Wojtys *et al.*, 1998) who reported significantly fewer injuries in the follicular phase as compared to post-ovulatory phase. Meanwhile, in this study, the influence of testosterone on medial and lateral knee laxities were rather vague. No significant difference in testosterone levels were noted between athletes and non-athletes females during the follicular and luteal phases of the cycle. A recent finding by O'Leary et al, (O'Leary *et al.*, 2013) indicated that prolonged aerobic exercise in women with normal menstrual cycle can induce a short-term elevation in plasma testosterone level, however this effect was not seen in our study. Shultz et al, (Shultz *et al.*, 2004; Shultz *et al.*, 2005) reported that testosterone effect on knee laxity could be masked by the effect of other predominant hormones namely oestrogen and progesterone.

Our finding revealed that serum relaxin level in athletes is approximately 4 and 1.5 times higher during the follicular phase and luteal phase respectively than non-athletes. In athletes, relaxin level is the highest in the luteal phase (15.58±5.36pg/ml) which suggest that relaxin may not have a role in determining knee laxity in humans. The increase in medial and lateral knee laxity in the luteal phase could be due to higher serum progesterone. The lower knee laxity in athletes could also be contributed by a greater muscular control in view that athletes have greater skeletal muscle mass and greater fitness ratio which could help to exert greater tone that resist an increase in knee joint movement. In this study, varus and valgus tests were performed by three (3) experience examiners and were found to have high intra-rater reliability and adequate inter-rater reliability. Wiertsema et al, (Wiertsema *et al.*, 2008) reported that clinical examination such as theLachman test is a reliable measure to determine anterior–posterior laxity of the ACL while arthrometer was used mainly to determine anterior tibiofemoral movement with limited application in determining medial and lateral laxities of the knee.

In conclusion, our study has demonstrated for the first time changes in medial and lateral knee laxities in athletes and non-athletes females at different phases of the menstrual cycle. We have shown that serum sex-hormones in particular progesterone has a strong association with the increase in medial and lateral laxities of the knee. Although relaxin levels is increased in parallel to the increase in progesterone level, this hormone however may have little influence on laxity. In contrast to the popular belief, oestrogen may not have role in the increase in laxity particularly in the follicular phase of the cycle.

CHAPTER 9: CONCLUSION

In this study, we investigated the effect of sex-steroids (oestrogen, progesterone, testosterone and relaxin) and oestrous cycle stages/menstrual cycle phases on knee joint laxity. The key findings of this thesis are as follows:

1. RXFP1 is the main isoform expressed in the hamstring muscle, patellar tendon and collateral ligaments of the knee joint.
2. RXFP1 is up regulated by oestrogen and progesterone, but down-regulated by testosterone.
3. RXFP2 expression is up regulated by oestrogen at doses exceeding 2 μg/ml and progesterone in the hamstring muscle, patellar tendon and collateral ligament, but there is no significant effect of 0.2 μg/ml oestrogen on this receptor expression.
4. RXFP1 and RXFP2 are up regulated in the hamstring muscle as compared with patellar tendon and collateral ligaments.
5. RXFP1 is up regulated in the hamstring muscle treated by oestrogen, but no significant changes in RXFP2 expression.
6. RXFP1 and RXFP2 up regulated by progesterone, but down regulated by testosterone.
7. Higher expression of RXFP1 in the patellar tendon under oestrogen or progesterone treatments than in the ligament, but no significant changes in RXFP2 expression in tendon and ligament, except following 50 μg/ml oestrogen and progesterone treatment.
8. Knee range of motion is increased under the effect of 20 and 50 μg/ml oestrogen and progesterone and decreased with presence of testosterone.

9. Oestrogen antagonists (ICI 182/780, MPP, PHTPP) administration blocked oestrogen influence on RXFP1 and RXFP2 expressions in hamstring muscle, patellar tendon, and collateral ligament.
10. Knee range of motion is reduced following treatment with 0.2 μg/ml dose oestrogen + ICI 182/780/ MPP/ PHTPP
11. Decreased knee range of motion and RXFP1 and RXFP2 expressions in presence of mifepristone + progesterone in all hamstring muscle, patellar tendon and collateral ligaments tissues.
12. RXFP1andRXFP2expressionsare increased in presence of flutamide and finastride in hamstring muscle, patellar tendon and collateral ligament.
13. Knee range of motion in increased in the presence of flutamide and finastride.
14. The correlations of RXFP1andRXFP2 expressions with knee range of motion are observed at proestrus and diestrus stages of the oestrous cycle.
15. The mean of varus-valgus angle is higher in the non-athletes group compared to athletes group throughout the menstrual cycle phases.
16. Greater varus-valgus angle is observed in luteal phase than follicular phase in athletes and non-athletes population
17. There is a significant correlation between progesterone/relaxin and knee laxity among athletes and non-athletes groups in different phases of the menstrual cycle
18. There is no significant correlation between oestrogen/testosterone between both athletes and non-athletes groups in different phases of the menstrual cycle.

In conclusion, investigation of complex relationship between several hormones and expression of relaxin receptor isoforms on rat knee tissues indicated that RXFP1 is the main isoform in these tissues and expressed higher in the hamstring muscle more than patellar tendon and collateral ligaments. Progesterone and oestrogen up regulated and testosterone down regulated the RXFP1 and RXFP2 expressions in the rat knee tissues.This effect could be due to a decrease of collagen content in these tissues and extracellular matrix in the presence of relaxin hormone. Hence, the effects of reproductive hormones on knee surrounding tissues suggested that these hormones influence knee laxity. We postulated that increase in plasma testosterone may result in down-regulation of relaxin receptors in these knee structures, thus could help to reduce knee laxity in exercising women. The highest level of progesterone in the luteal phase can cause an increase in knee laxity, rendering the knee to be more susceptible towards a non-contact injury. In addition to this, testosterone has also been reported to increase the strength of the muscles that control knee joint movement.

Moreover, rat knee range of motion increased under treatment of oestrogen and progesterone and decreased under testosterone. Progesterone treatment resulted in the highest increase in the knee range of motion correlated to both relaxin receptor isoforms' expressions in patellar tendon and lateral collateral ligaments. Increased expression of RXFP1, as compared with RXFP2, mediates increased knee joint laxity in response to high doses of oestrogen and physiological dose of progesterone. Our findings thus could explain higher vulnerability of female than male towards non-traumatic knee with oestrogen and progesterone fluctuation at various phases of reproductive cycle.

Our results also illustrated that testosterone by interceding relaxin receptors decreased knee laxity and thereby range of motion. However, flutamide and finasteride antagonists showed suppressor effect on testosterone and consequently altered RXFP1 and RXFP2 expressions in hamstring muscle, patellar tendon, and lateral collateral ligaments, inequitably.Taken together, these findings may explain increased knee joint stiffness in female following testosterone administration. These findings provide better understanding of the underlying mechanism of sex-steroid modulation of relaxin receptors expression and its influence on the knee laxity. Further studies should investigate the direct and indirect mechanisms underlying these associations.

In this study, we also examined the expression of relaxin receptor during oestrous cycle. Higher expressions of RXFP1and RXFP2 observed in proestrous and diestrous stages correlated to higher knee range of motion. Progesterone and relaxin levels were at their highest in the same stage as greater knee range of motion was observed. Relaxin hormone can stimulates degradation of collagen content and prevents fibrogenesis (Samuel, 2005). Ligamentous and tendonous laxity can partially be influenced by relaxin hormone during proestrous and diestrous stages.

We evaluated serum oestrogen, progesterone, testosterone, relaxin hormones, and varus-valgus assessments in female athletes and non-athletes. In line with our previous findings, we found there was positive correlation between progesterone/relaxin and knee rotational movement during different phases of a menstrual cycle. The knee rotational movement for athletes and non-athletes did not show any relationship with oestrogen/testosterone. The highest level of progesterone/relaxin and the greatest angle of knee were observed in luteal phase in both groups and lowest level observed in follicular

phase. Since progesterone and relaxin are involved in collagen metabolism, these hormones may influence knee joint laxity via this mechanism.

This study provided insight into the effect of sex-steroids on RXFP1 and RXFP2 expressions on the hamstring muscle, patellar tendon, and collateral ligament, and relationship between oestrogen, progesterone, testosterone, and relaxin with knee rotational movement during reproductive cycles. Increased expression of RXFP1, as compared with RXFP2, mediates increased knee joint laxity in response to high doses oestrogen and progesterone and at proestrous and diestrous stages of the oestrous cycle. Our findings thus could explain higher vulnerability of female than male towards non-traumatic knee with oestrogen and progesterone fluctuation at various phases of menstrual cycle. Finally, these findings provide better understanding of the underlying mechanism of sex steroid modulation and relaxin hormone interaction and its influence on the knee laxity.Future studies on fluctuation of sex steroid hormones interaction in both gender are warranted to elucidate the physiological cause of this selectivity in the impact of relaxin and endocrine system on the phenotype of various tissue.

The human body joints' appears to react to menstruation hormones levels (oestrogen, progesterone, testosterone, and relaxin) by causing a change at the cellular level of the knee tissue thus resulting in changing of knee laxity. Endogenous and exogenous female sex steroids have effect on many metabolic parameters but these agents do not restrict the female athletes to compete in sports, recreationally or professionally. It is important to recognize all components of the knee injury mechanism in order to address the potential for prevention and screening programs that considers for risk factors and multifaceted interplay of these factors. More studies is needed to investigate the influence of hormones on joint

laxity as well as the influence of other factors that are associated with the female menstrual cycle, such as weight, age, altered reaction times, the circulation of hormones, shoe-surface, athletes level, fatigue, environmental conditions, water retention, to find an explanation for the disproportional distribution of knee injuries.

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Appendix A: reagent preparation

SDS-page

1. ***Acrylamide*** (Acrylamide, MW: 71.08 kd and bisacrylamide, MW 154.17 kda)

   Stock concentration of acrylamide: 30%
   Stock concentration of bisacrylamide: 0.8%
   Acrylamide (30g) and bisacrylamide (0.8g) was dissolved in double distilled water. The solutions were topped up to 100ml with double distilled water and stored in dark at 4 ºC.

2. ***4 x resolving buffer*** (ph 8.8) (Tris, MW: 121.1 kda)

   Stock solution (1.5M): 18.17g of tris was dissolved in double distilled water and volume topped up to 100ml. The solution was stored at 4ºC.

3. ***4 x stacking buffer*** (ph 6.8) (Tris, MW: 121.1 kdh)

   Stock solution (0.5M): 3.03g of tris was dissolved in double distilled water and volume topped up to 50ml. The solution was stored at 4ºC.

4. ***Sodium dodoceyl sulphate*** (SDS) (MW: 288.38 kdh)

   Stock solution (10%): 5g of sodium dodoceyl sulphate was dissolved in double distilled water and volume topped up to 50ml. The solution was stored at 4 ºC.

5. ***Ammonium persulphate*** (APS) (MW : 228.2 kda)

   Stock solution (10%): 0.1g of ammonium persulphate was dissolved in double distilled water and volume topped up to 1ml. The solution was stored at 4 ºC.

6. ***2 x treatment buffer***

   Stock concentration of tris: 0.125M (ph 6.8)
   Stock concentration of SDS: 4%
   Stock concentration of glycerol: 20%
   Stock concentration of dithiothreitol: 0.2M
   Stock concentration of bromophenol blue: 0.02%
   Glycerol (2ml), dithiothreitol (0.31g), bromophenol blue (0.002g), SDS (4ml of 10% SDS) and tris (2.5ml of 0.5M tris) was dissolved in double distilled water. The solution were topped up to 10ml with double distilled water and stored at 20ºC.

7. ***Tank buffer*** (Tris,MW: 121.1 kd and glycine, MW 75.07 kda)

   Stock concentration of tris: 0.025M (ph 8.3)
   Stock concentration of glycine: 0.192M
   Stock concentration of SDS: 0.1%
   Tris (3.03g), glycine (14.41 g) and SDS (1g) was dissolved in double distilled water.
   The solution were topped up to 1000L with double distilled water and stored at RTºC.

8. ***Recipe of 2 mini resolving SDS-page gel (gel percentage: 12%)***

Composition	volume
Acrylamide	8.3ml
4 x resolving buffer	4.0ml
Sodium dodoceyl sulphate	0.2ml
Ammonium persulphate	100µl
Double distilled water	6.4ml
TEMED	6.75µl

9. ***Recipe of 2 mini stacking SDS-page gel***

Composition	volume
Acrylamide	0.665ml
4 x stacking buffer	1.25ml
Sodium dodoceyl sulphate	50µl
Ammonium persulphate	25µl
Double distilled water	3ml
TEMED	2.5µ

Reagent used for **Coomassie blue staining**

1. ***Coomassie blue solution***

   Stock concentration of coomassie: 0.025%
   Stock concentration of methanol: 40%
   Stock concentration of acetic acid: 7%
   Coomassie (0.05g), methanol (80ml) and acetic acid (14ml) was topped up to 200ml with double distilled water and stored at RTºC.

2. ***Destain solution*** Ⅰ

Stock concentration of methanol: 40%
Stock concentration of acetic acid: 7%
Methanol (80ml) and acetic acid (14ml) was topped up to 200ml with double distilled water and stored at RTºC.

3. ***Destain solution*** Ⅱ

Stock concentration of methanol: 40%
Stock concentration of acetic acid: 7%
Methanol (10ml) and acetic acid (14ml) was topped up to 200ml with double distilled water and stored at RTºC.

Western-blot

1. ***Towbin buffer***

Stock concentration of tris: 25mM
Stock concentration of glycine: 192mM
Stock concentration of glycine: 192mM
Tris (3.03g) and glycine (14.4g) was dissolved in double distilled water. The solution were topped up to 1L with double distilled water and stored at RT.

2. ***Phosphate buffer saline-tween (TBST)***

Stock concentration of bovine serum albumin (BSA): 1%
Stock concentration of tween: 0.1%
Bovine serum albumin (2.5ml) and tween (250µl) was dissolved in double distilled water. The solution were topped up to 250ml with double distilled water and stored at 4ºC.

3. ***Phosphate buffer saline***

Stock concentration of potassium phosphate (Kh_2PO_4):
Stock concentration of sodium phosphate (NA_2HPO_4):
Stock concentration of sodium chloride ($NACl_2$):
Stock concentration of potassium chloride (KCl):
Potassium phosphate (0.12g), sodium phosphate (0.72g), sodium chloride (4.0g) and potassium chloride (0.1g) was dissolved in double distilled water. The solution were topped up to 500ml with double distilled water and stored at 4ºC.

Appendix B: research outcomes

Publications:

1. **Firouzeh Dehghan,** Sekaran Muniandy, Ashril Yusof and Naguib Salleh*.2013. Sex-Steroid Regulation of Relaxin Receptor Isoforms (RXFP1 & RXFP2) Expression in the Patellar Tendon and Lateral Collateral Ligament of Female WKY Rats. International J Medical Sciences- **Accepted** 26 Nov 13

2. **Firouzeh Dehghan**, Batoul Sadat Haerian,Sekaran Muniandy,Ashril Yusof, Jason L Dragoo,Naguib Salleh* 2013. The effect of Relaxin on the Musculoskeletal System.Scandinavian Journal of Medicine and Science in Sports.**Accepted** 2 Oct 2013

3. **Firouzeh Dehghan**, Sekaran Muniandy, Ashril Yusof & Naguib Salleh* 2014. Testosterone Decreases Knee Passive Range of Motion and Down-Regulates Relaxin Receptor Isoforms Expression in the Patellar Tendon and Lateral Collateral Ligament of Female Rats via 5 - Dihydrotestosterone and involves Androgen Receptor binding. Int Journal Molecular Sciences.**Accepted** 14 Jan 2014

4. **Firouzeh Dehghan**, Sekaran Muniandy, Ashril Yusof & Naguib Salleh.Differential Effects of Oestrogen and Progesterone on Knee Range of Motion and Relaxin Receptor Isoforms (RXFP1 & RXFP2) Expression in the presence of Sex-Steroid Antagonists.Under review

5. **Firouzeh Dehghan**, Sekaran Muniandy, Ashril Yusof & Naguib Salleh. Relaxin Receptor Isoforms (RXFP1 & RXFP2) Expression and Knee Range of Motion throughout the rat oestrous cycle stages. Under review

6. **Firouzeh Dehghan**, Sekaran Muniandy, Ashril Yusof & Naguib Salleh.Investigating the effect of menstrual cycle phases on knee joint laxity in the female athletes and non-athletes. In preparation for submission

Presentation:

1. **Firouzeh Dehghan**, Sekaran Muniandy, Ashril Yusof & Naguib Salleh. 27th Annual Scientific Meeting in Kuantan, Pahang, Malaysia, 6-8 Sept 2013 (Oral presentation)

2. **Firouzeh Dehghan**, Sekaran Muniandy, Ashril Yusof & Naguib Salleh.International Conference on Biological and Chemical Sciences, Dubai, UAE, April, 8-9, 2014 (Oral presentation)

3. **Firouzeh Dehghan**, Sekaran Muniandy, Ashril Yusof & Naguib Salleh. Experimental Biology 2014, April 26-30, San Diego, California (poster presentation)