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The influence of sex hormones on anterior cruciate ligament ruptures in males

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Abstract
Purpose The purpose of this study is to determine the difference in the concentrations of testosterone, 17-β estradiol and progesterone between male patients with and without ACL rupture, as well as the possible effect of these hormones on generalized joint laxity.
Methods Male subjects with non-contact knee joint injury were included in this study. Two groups were formed: the examined group, consisting of subjects with ACL rupture and the control group consisting of patients without ACL rupture. After this, the patients from these two groups were paired off on the basis of three factors, level of professional involvement in sports (including the type of sports activity), left or right side of the body and the age of the subjects. In the end, there were 29 pairs (58 subjects). The concentration of sex hormones was determined from saliva specimens with the aid of the Salimetrics enzyme immunoassay. The testing of generalized joint laxity was performed with the aid of the "laxity score" according to Beighton et al.
Results Subjects with ACL rupture have highly statistically significantly greater concentrations of testosterone ($p < 0.01$), statistically significantly greater concentrations of 17-β estradiol ($p < 0.05$), and a highly statistically significantly greater generalized joint laxity score than subjects with an intact ACL ($p < 0.01$).
Conclusion Increased concentrations of testosterone or 17-β estradiol may be a risk factor leading to ACL rupture. Also, generalized joint laxity may be a factor leading to ACL rupture, but none of the monitored hormones can be set down as the cause of its existence. Young male athletes with higher concentrations of testosterone and greater hyperelasticity should plan preventive programs of physiotherapy for ACL preservation since they present a vulnerable group susceptible to ACL rupture.
Level of evidence Diagnostic study, Level II.

Keywords Anterior cruciate ligament · Testosterone · 17-β estradiol · Progesterone · Joint laxity

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Introduction

The anterior cruciate ligament is the primary stabilizer of anterior tibial translation and the secondary stabilizer

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of tibial rotation. ACL is involved 85 % in the control of anterior tibial translation [12]. We can say that the injury to this ligament is sex-specific as its frequency amongst the female population is up to ten times greater [3, 10]. The reasons for such incidence are manifold, however, the hormonal status of an individual belongs to one of the three main groups of risk factors leading to ACL rupture (in addition to anatomical and neuromuscular factors). The different hormonal status between men and women regarding sex hormones (testosterone, estrogen and progesterone) has led many researchers to investigate the effect of these hormones on the anterior cruciate ligament [1, 5, 8, 17, 19]. The testosterone receptors are located in the anterior cruciate ligament in men [11] and women [17]. Also, this ligament has been marked as estrogen and progesterone sensitive tissue as the receptors for these hormones are located in it [15].

Testosterone (T) is primarily a male sex hormone but it plays a physiological role in both the sexes. In addition to secondary sexual characteristics, testosterone, in both sexes, also affects the quality of bone tissue [7] as well as the increase of muscle mass [16]. In his in vitro study on a canine cell culture of the anterior cruciate ligament Ohno et al. [18] established that the potent form of testosterone, dihydrotestosterone causes an increased expression of androgen receptors and an increased synthesis of collagen in the ACL fibrocytes, whereas cell division occurs 24 to 48 h after treatment with this hormone. Estrogen is a hormone that is most present in women during the reproductive period, and, in addition to affecting secondary sex characteristics, a greater concentration of estrogen is also connected to the reduction of collagen synthesis and a decrease in fibroblast proliferation [15]. The most potent form of estrogen is 17-β estradiol (E_2) which is synthesized from testosterone, in women in the ovaries and in men in the testicles and via extraglandular conversion of androgens [25]. Liu et al. [14] researched the distribution of estrogen receptors in the human ACL and established their existence in the nuclei of synoviocytes, fibroblasts and the cells of ACL blood vessel walls. In their study on ovariectomized female rabbits, Slauterbeck et al. [23] established that an increase in the concentration of estrogen influences the decrease in the strength of the ACL. Progesterone (P) can be found in the blood of men and women but its role in men is unclear [2]. In women, its main role is in the preparation of the endometrium for the implantation of the fertilized ovum, while in both sexes its secretion is intensified during stress and anxiety.

The concentration of steroid hormones is usually determined from blood samples obtained by vein puncture, which is an invasive method. The greatest part of these hormones in the blood is bound to a certain carrier- protein (SHBG—sex hormone binding globulin), while a smaller (<5 %), unbound, free portion is in fact the potent one that affects tissues. Since such, unbound hormone molecules are able to pass through the membranes of salivary glands and reach the saliva, it is possible, to directly determine the level of a particular steroid hormone in the saliva by applying a noninvasive method and thus indirectly obtain the data related to the concentration of the hormone in the blood.

It is hypothesized in this study that patients with ACL rupture, as opposed to patients with an intact ACL, have different, higher or lower levels of sex hormones and that the concentrations of these hormones may, indirectly, via the level of hyperelasticity, influence ACL rupture. The purpose of this study is to determine the difference in the concentrations of testosterone, 17-β estradiol and progesterone between patients with and without ACL rupture, as well the possible effect of these hormones on generalized joint laxity.

Materials and methods

In this case–control study, a group of male patients from the Clinic for Orthopedic Surgery and Traumatology of the Clinical Center of Serbia was included, who had sought medical attention due to non-contact knee joint injury. The patients were divided into two groups. The examined group (131 subjects) was composed of patients with non-contact knee joint injury and ACL rupture. The control group (156 subjects) was composed of patients with knee injury which did not include ACL rupture. The patients were then paired off according to three factors: level of professional involvement in sports (including the type of sports activity), left or right side of the body, and the age of the subjects (tolerance for up to 5 years difference). Finally, 29 pairs (58 subjects) remained. In 13 pairs, we studied the right and in 16 pairs, the left knee. The average age of the examined group was 26.6 years, while the average age for the control group was 27.1 years. The youngest subject in both groups was 18 years old at the time of data collection, whereas the oldest subject in the examined group was 47 and in the control group 42 years old. The testing did not show a statistically significant difference between these two groups as to the age (student's t test for paired samples; $p = 0.508$; $p > 0.05$). Within this sample, 19 pairs of subjects were professional athletes, while 10 pairs of subjects were amateur athletes, but they worked out/practiced regularly. The subjects of the control group worked out 4.4 times a week, on average while the subjects of the examined group worked out 4.8 times a week on average. The distribution of the clinical groups, according to the type of sports activity was as follows: 19 pairs of subjects were injured while playing soccer, four pairs of subjects were injured playing

basketball, three pairs were injured in one of the athletics disciplines, two pairs were injured playing handball, and one pair was injured playing volleyball.

Exclusion criteria

All of the subjects included in the study signed a form confirming their voluntary consent to be involved in the study. Subjects, involved professionally or for recreational purposes, in sports activities that include running, abrupt change in the direction of movement, jumping and landing, rotating movements (soccer, basketball, handball, volleyball, athletics) who had worked-out two or more times a week prior to injury, were included in the study. Patients receiving any hormonal therapy were excluded from the study. Also, patients who had bleeding from the gums were not included in the study because of an unsuitable saliva specimen.

Method of obtaining and storing saliva

During the testing, the subjects deposited their saliva specimens into sterile stool sample containers. Saliva sampling was unrelated to the injury and was performed at least 3 weeks after it had occurred. For a sample to be valid it was necessary that the subject had not had anything to eat 60 min prior to providing the specimen and no alcoholic beverage to drink 12 h prior to the sampling procedure. The subjects provided saliva specimens into one container three times two specimens within 45 min (a total of six samples were taken because of sex hormone secretion at periodical intervals). These saliva specimens were stored at the temperature of −30 °C until the day of the assay. After all the specimens were collected, the concentrations of sex hormones (testosterone, 17-β estradiol and progesterone) in the saliva were measured applying the ELISA method, with the aid of the "Salimetrics" enzyme immunoassay. The correlation of the concentration of these hormones in the saliva and serum is $r = 0.96$ ($p < 0.001$) for testosterone, $r = 0.80$ ($p \leq 0.001$) for 17-β estradiol and $r = 0.80$ ($p < 0.001$) for progesterone.

On the day of the assay, the specimens were defrosted and centrifuged at 3,000 rpm for 15 min. Purified samples were deposited into test wells with a pipette using a 96-well plate format. All three assays were performed with the aid of commercially available immunoassay kits without any modification to the manufacturer`s recommended protocol (Salimetrics).

Assessment of hormone concentration

A microplate (96-well) coated with rabbit antibodies to the appropriate hormone (testosterone, 17-β estradiol and progesterone) was treated with standard, i.e. unknown hormone concentrations after which the hormone bound to horseradish peroxidase was added. These two hormone samples (the standard sample or a sample of unknown concentration and the one bound to horseradish peroxidase) competed for a place on the antibody and, upon the incubation period, the unbound components were washed away. The hormone bound peroxidase was measured as the reaction of the peroxidase enzyme with teramethylbenzidine. This reaction colored the sample blue. The reaction was stopped by 2-molar sulfuric acid which colored the sample yellow. Optical density was read on a standard plate reader with a 450 nm primary filter and a 570 nm secondary filter. The measured value was inversely proportional to the concentration of the hormone. The hormone concentration value of unknown samples was calculated with the aid of the known standard concentration. The measurement accuracy as set by the manufacturer is equal to the value of 0.1 pg/ml. The variation coefficient for intra-assay precision for testosterone was 2.5 % (12 replicates each): it was 7.0 % (14 replicates) for 17-beta estradiol and 4 % (12 replicates) for progesterone.

Testing generalized joint laxity

The elasticity of connective tissue was tested with the aid of the Beighton, Solomon and Soskolne "laxity score" [4]. This test relies on the existence of joint laxity in the following joints: the fifth metacarpophalangeal joint on both hands (passive extension over 90°), the radiocarpal joints on both hands (passive flexion of the hand with the abduction of the thumb to the forearm), the elbow joints on both arms (active hyperextension over 180°), both knee joints—active hyperextension over 180° with the ability to bend over and touch the floor with the palms of the hands (the knees are extended and the legs are brought together, parallel to each other while the person is standing with the soles of the feet resting full length on the floor). The maximum score for this test is 9 and the authors feel that joint laxity exists if the score is 5 or above [4].

This study was approved by the Ethical Committee of the Faculty of Medicine, University of Belgrade, ID number of approval: 29/11–17.

Statistical analysis

The sample size for the study was determined using a power of 94 % at a p of 0.01 and using a 1:1 case to control ratio. All of the data were processed with the SPSS 11.0 program. In addition to the testosterone, 17-β estradiol and progesterone concentrations, we also monitored the ratio between testosterone and 17-β estradiol (T/E_2) as well as the ratio between testosterone and progesterone (T/P). We

also tested the correlation between the values obtained from the test of generalized joint laxity and the concentrations of 17-β estradiol and progesterone. The differences between the two groups were tested with the student's *t* test for paired samples and with the aid of the two-factor analysis of variance, while the correlation was tested with the Pearson correlation coefficient in the SPSS 11.0 package. The level of statistical significance was set to 0.05.

Results

Standard curves, high and low controls

Our values of the high and low controls were approximately equal to the expected test values although the manufacturer's instructions had been for each laboratory to set its own values due to the differences in the instrumentarium. Table 1 shows the expected and obtained values of the high and low controls.

The highest concentration of testosterone measured in the saliva of men was 258 pg/ml and the lowest was 65 pg/ml. Figure 1 shows the cubic curve which was used to measure the concentration of testosterone in the saliva on the basis of absorbance ($r = 1.000$; $p = -\infty$). The highest measured concentration of 17-β estradiol in the saliva of men was 2.9 pg/ml while the lowest one was 1.4 pg/ml. Figure 2 shows the inverse curve which was used to establish the concentration of 17-β estradiol in the saliva based on absorbance ($r = 0.996$; $p = 0.002$). The highest measured concentration of progesterone in the saliva of men was 73.8 pg/ml while the lowest one was 8.6 pg/ml. Figure 3 shows the cubic curve which was used to measure the concentration of progesterone in the saliva on the basis of absorbance ($r = 1.000$; $p = 0.001$).

Sex hormone concentrations

A highly statistically significant difference between the examined and the control group as to the concentration of testosterone in the saliva (Table 2, $p < 0.01$; $p = 0.004$) was established as well as a statistically significant difference as to the concentration of 17-β estradiol ($p < 0.05$; $p = 0.019$). A highly statistically significant correlation between these two hormones was established within the examined group ($r = 0.738$; $p < 0.01$) while such a correlation did not exist in the control group (n.s.). A statistically significant difference between the examined and the control group as to the concentration of progesterone in the saliva was not established (n.s.). In the same way, a difference between the examined and control group as to the ratio between testosterone and 17-β estradiol (T/E_2; n.s.) and between testosterone and progesterone (T/P; n.s.) was not established either.

Table 1 Expected and obtained concentrations of the high and low controls (pg/ml)

Hormone	High control		Low control	
	Expected	Obtained	Expected	Obtained
Testosterone	226.8 ± 56.7	247.6	16.1 ± 6.4	34.7
17-β estradiol	19.34 ± 4.8	19.5	5.5 ± 2.2	1.2
Progesterone	896.67 ± 224.2	566.8	38.2 ± 15.3	34.1

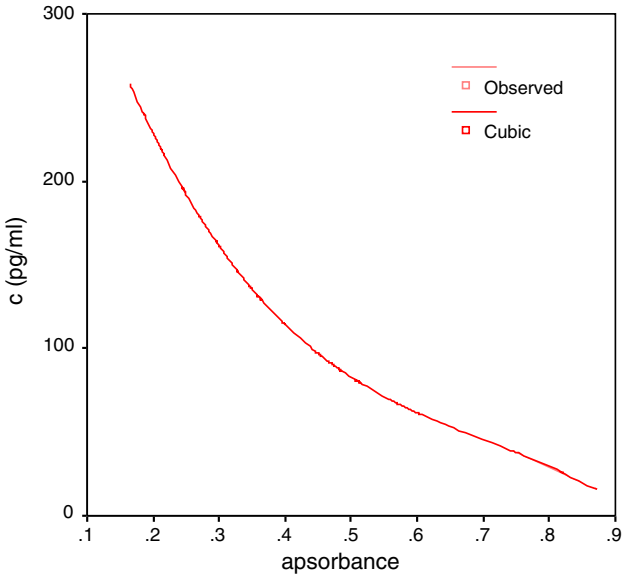

Fig. 1 Four-parameter cubic curve of testosterone concentrations

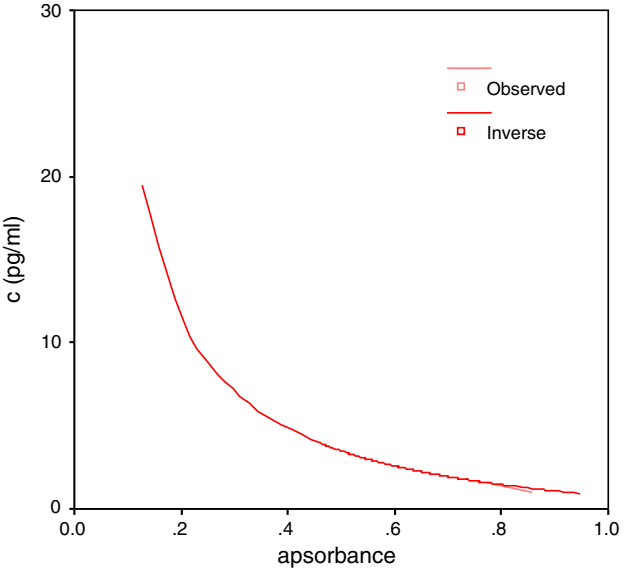

Fig. 2 Four-parameter inverse curve of 17-β estradiol concentrations

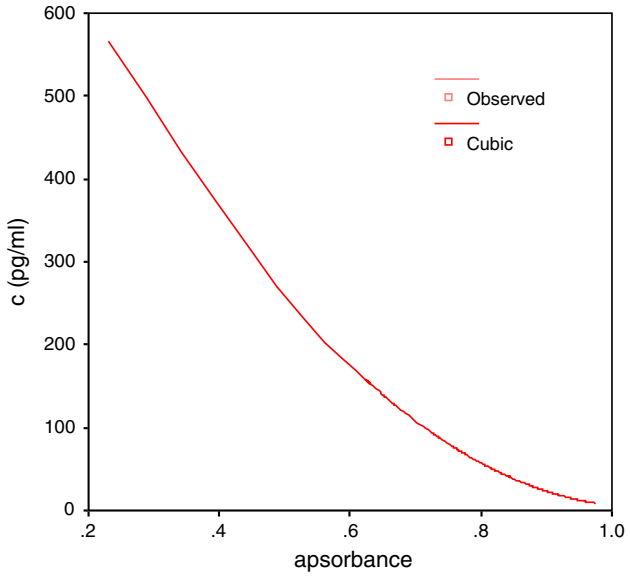

Fig. 3 Four-parameter inverse curve of progesterone concentrations

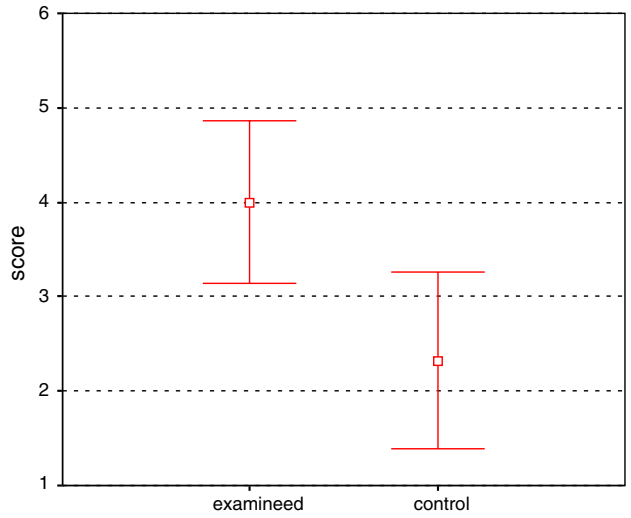

Fig. 4 Generalized joint laxity score. Beighton, Solomon and Soskolne "laxity score"

Table 2 Sex hormone concentrations in saliva

Observed hormone	Examined group	Control group
Testosterone (pg/ml)	182.2 ± 38.9	149.0 ± 49.5
17-β estradiol (pg/ml)	2.2 ± 0.3	2.0 ± 0.4
Progesterone (pg/ml)	33.5 ± 16.6	33.8 ± 18.5
T/E_2	80.5 ± 12.5	84.1 ± 33.4
T/P	6.7 ± 4.1	5.6 ± 3.4

A highly statistically significant difference between the examined and the control group as to the generalized joint laxity score was established (Fig. 4; $p < 0.01$; $p = 0.005$). We did not establish a correlation between the concentrations of 17-β estradiol and progesterone on one hand and the generalized joint laxity score on the other, neither within the examined group (n.s.) nor within the control group (n.s.).

Discussion

The most important finding of the present study is that the concentrations of testosterone in patients with ACL rupture are highly statistically significantly greater than the concentrations of the same hormone in the saliva of their matched pairs. Also, as opposed to female patients, who generally display a high level of hyperelasticity, in male patients with ACL rupture hyperelasticity is highly statistically significantly greater than in male subjects with an intact ACL [24].

The concentration of testosterone in the saliva of men with ACL rupture is highly statistically significantly greater than the concentration of the said hormone in the saliva of men without ACL rupture ($p < 0.01$). If we compare our results with the expected hormone concentration values in saliva, we can say that the concentrations of testosterone in the saliva of both groups are within the expected value range, with the examined group displaying a greater value than the expected average (181:165 pg/ml) and the control group displaying a smaller value than the expected average (149:165 pg/ml).

As men with ACL rupture displayed mildly elevated testosterone values in their saliva we can hypothesize that in men the testosterone level had the effect of increasing muscle strength, thus creating a greater force, which, in turn, probably led to a greater strain of the knee joint, which could not keep up with the increase in muscular activity. In patients with a lower level of testosterone the muscular activity was not excessive, thus the knee joint suffered a lesser degree of force, which is why the injury that had occurred was clinically milder, without ACL rupture, i.e., as can be seen from data obtained from the control group, the injuries were mostly meniscal distortions or tearing.

Testosterone concentrations (163.8 pg/ml) similar to the ones we found were published by Shirtcliff et al. [21] in a study on 20 young men aged 18–23 years. In a study including over 1,400 men Gavrilova and Lindau [9] published testosterone values of 99.5 pg/ml. These values were obtained through saliva analysis and are somewhat lower than the ones we found (149 pg/ml), probably due to the more advanced age of the population monitored by the above cited study (57–84 years of age).

Data on the concentrations of estradiol (2.6 pg/ml) similar to the values from our study were published by Schultheiss et al. [20] in their study on 18 men of an average age of 23 years. We feel that the increased concentration of 17-β estradiol within the examined group is consequential and that it probably occurs through extraglandular conversion of testosterone into 17-β estradiol. This confirms the existence of a direct correlation between these two hormones in the examined group, which has not been established in the control group. However, even though consequential, the increase in 17-β estradiol concentration could lead to a greater laxity of ligaments. In their study of human ACL tissue culture, Yu et al. [28, 29] established that the increase in 17-β estradiol concentration, even within the physiological range, led to the decrease in the proliferation of fibroblasts and in the synthesis of type I procollagen.

Within the male population a nearly equal progesterone concentration was found in the saliva of the subjects with and without ACL rupture ($p > 0.05$; 33.5:33.8 pg/ml). Similar concentrations of this hormone in the saliva were found by Celec et al. [6] in a study on male subjects aged 20–21 years (37.5 pg/ml). Based on these results we cannot say that the progesterone concentration in the saliva affects ACL injury in men. Somewhat higher progesterone concentrations (49.5 pg/ml) were registered in the saliva of older men (57–84 years of age) [9].

The control group demonstrated a highly statistically significantly smaller score of generalized joint laxity ($p < 0.01$) than their matched pairs. Similar results were obtained by Uhorchak et al. [26] in a study on 859 West Point cadets. They found a highly statistically significantly greater score in women than in men ($p < 0.001$), as well as a highly statistically significantly greater score in men with ACL rupture as compared to the subjects from the control group ($p = 0.003$). On the other hand, if only the knee joint is observed, a case–control study on soccer players, basketball players and female gymnasts by Woodford-Rogers et al. [27] suggests that subjects with ACL rupture had greater values for anterior knee joint laxity.

The hormonal milieu of an individual may affect the anterior cruciate ligament and the knee joint in a multifold manner. Sex hormones act on the ACL directly, via their receptors located in the ligament. However, the affinity of a hormone to overlap with the receptors of another hormone should not be overlooked. In this way, the function of the ACL may be affected by numerous products of the adrenal cortex which show a partial affinity for testosterone receptors, as well as other estrogens (estrone, estriol) which are less present but whose effect on estrogen receptors in the ACL cannot be ignored. On the other hand, the indirect effect of these hormones is reflected in their influence on muscular power, i.e. on the muscles where receptors for testosterone and estrogen also exist [13, 22]. In addition, testosterone effects early diaphyseal-epiphyseal fusion in bones before the end of puberty which, in turn, effects the physical constitution of an individual, i.e. the person's height, the width of their pelvis, the valgus knee angle, etc. Finally, the effect of sex hormones on the psychological state of individuals should not be overlooked, as it often contributes to many injuries including ACL injury. Future case–control studies with a larger number of subjects should take into account the above stated considerations.

The results of the present study speak in favor of the fact that male athletes taking exogenous testosterone (or its analogues) for improving performance, place themselves in the group with a higher risk of anterior cruciate ligament injury. For the purpose of preventing ACL rupture, male subjects actively engaged in sports activities should not take exogenous testosterone, especially if they have hyperelastic joints, since this study has also proven that hyperelasticity is another risk factor for ACL rupture.

The limitations of the present study are primarily linked to the time of saliva sampling. Ideally, the sample would be taken directly before the injury (which is basically impossible), while the idea of sampling immediately after injury was abandoned due to the influence of the injury itself on hormone secretion. Also, sampling saliva in a situation similar to the one at the moment of injury would require waiting for the complete recovery of patients, which would affect the length of time for the study. Additionally, while all of the subjects provided saliva samples in the morning hours (between 8 and 12 o'clock), not all were injured in that part of the day. Finally, we theoretically hypothesized that an increased concentration of testosterone in patients with ACL rupture had influenced the increase in muscle strength, although testosterone is not alone in this activity, in fact, a significant role might be attributed to the hormones of the adrenal medulla (catecholamines), which should be the subject of future study.

Conclusion

A higher concentration of testosterone in the saliva of men may be one of the risk factors leading to ACL rupture. The subjects demonstrated a higher concentration of 17-β estradiol but we cannot claim that this hormone can influence rupture, but merely that it is there as the result of a higher testosterone concentration. Also, generalized joint laxity may be a factor leading to ACL rupture but there is no single hormone by itself that can be blamed for its existence, rather it can be surmised that ACL rupture is the result of the effect of a greater number of hormones on connective tissue. Young male athletes with higher concentrations of testosterone and greater hyperelasticity should plan

preventive programs of physiotherapy for ACL preservation since they present a vulnerable group susceptible to ACL rupture.

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