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The effects of estradiol on cardiac muscle electrophysiology in orchiectomized rat model: a new insight to side effects caused by castration

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Abstract. – OBJCTIVE: Although the testosterone has a protective effect on heart, patients having maximal androgen blockade due to prostate cancer resembles endothelial dysfunction and cardiac problems when compared to normal population. We aimed to test the effect of 17 beta estradiol on the orchiectomized male rat heart electrophysiology and ion channel expression levels.

MATERIALS AND METHODS: This study was conducted on 27 male rats with 4 groups (healthy, orchiectomized, orchiectomized+17 beta estradiol treated and orchiectomized+vehicle treated). Action potentials and contractions were recorded simultaneously, while expressions of the calcium and potassium ion channels were measured.

RESULTS: Testosterone depletion for 4 weeks has caused a significant prolongation in the action potential durations and decrease in maximal contraction force as well as a deceleration. While this depletion suppressed expression of potassium channels, it increased the expression of calcium ion channels. Application of estradiol on the other hand, except for the calcium ion channel expression, had no positive effect on the tested parameters.

CONCLUSIONS: Testosterone has a markedly important and protective effect on male cardiac muscle preparations while estrogen does not have any. It is predicted that testosterone has showed this effect by means of modulation of some key points of excitation-contraction pairing of cardiac muscle.

Key Words:
Electrophysiology, Orchiectomy, Rat, 17 beta estradiol, Androgen depletion.

Introduction

Recent researches have shown that gender is the main determinant in nearly all of the physiological system parameters. Moreover, under stress conditions both the degree of damage and responses from male and female hearts are different[1]. Results of the clinical and the epidemiological researches have shown that gender dictates the heart's distinct adaptation to traumatic conditions. Subsequently to the heart failure diagnosis, females live longer than males and following the myocardial infarcts females resemble much more heart failure compared to male patients[2].

Same age male body and heart weights are greater than the ones obtained from females. The differences seen in the cardiac excitation-contraction coupling start to appear following puberty indicates possible role of sex hormones on this system[3].

Androgen receptors have a wide spread expressions in male and female ventricular cells[4]. Increased risk for a cardiovascular disease for females by the decreased estrogen levels during menopausal period indicates a possible protective effect of estrogen[5-8]. Furthermore, it was shown that the occurrence of atrial fibrillation and sudden death was greater in males[9]. In addition to be a sex hormone, estrogen can be a potent agent for the cardiovascular system[10-12]. Current research aims to test the protective effect of ovarian hormone 17 beta estradiol on the orchiectomized male heart excitation-contraction coupling.

Material and Methods

Design of Experimental Groups and Induction of Orchiectomy

This study was approved by the Ethical Committee of Selcuk University Experimental Medicine Research and Application Center (project number: 2010/005). Only adult male (n=27) Wistar-Albino rats were used for the experiments. After birth, 5 rats were housed per cage at ambi-

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ent temperature and humidity on a 12/12 h light/dark cycle at 22 ± 2°C. All animals received food and water *ad libitum*. Unless otherwise stated all chemicals used were purchased from Sigma (Sigma-Aldrich, Munich, Germany).

Induction of orchiectomy was done like in elsewhere[13]. Briefly, under the anesthesia [ketamine (0.15 ml/100 g) and rompun (0.07 ml/100 g), i.p.] and aseptic conditions. Approximately 1 cm skin incision was made at the tip of the scrotum; then a 5 mm incision was made into each sac at the tip of each testis. Then the cauda epididymis was pulled out, accompanied by the testis and followed by the caput epididymis. The vas deferens and spermatic blood vessels were ligated, and the testes were removed. Sham operation was performed in which the testis and epididymis were only pulled out and then replaced and the blood vessels were left intact.

In order to test the effects of estradiol on the male heart electrophysiology four groups were designed. Control group (Con, n = 5) was sham operated. To assess the effect of absence of testosterone, rats were castrated and received vehicle till the end of experimental period (M-, n = 8). To observe the effects of estradiol on male rats, the third group received 5 μg/100 mg/day estradiol injections starting from the day after surgery (MX, n = 8). To test the effect of vehicle on the castrated group, rats were orchiectomized and received sesame oil (MT, n = 6). These doses of estradiol were shown to supply normal serum hormone levels[14]. Estradiol injections were prepared inside vehicle (sesame oil, 0.1 ml/100 g/day) and were injected intraperitoneally. Experiments were carried out for 30 days. No infection was observed during follow-up.

Action Potential and Contraction Recordings

Under the intraperitoneal (ip) ketamine (0.15 ml/100 g) and rompun (0.07 ml/100 g) anesthesia, hearts were excised rapidly. Papillary muscles of the left ventricle were excised under stereo microscope (Labomed Microscope, Burhington, NC, USA), placed in a recording chamber, and pinned down at one end with a stimulating electrode while the second end was connected to a force-displacement transducer (MAY FDT 05). The dimensions of the papillary muscle strips were similar in between the groups (data not shown here). The recording chamber was super fused with Krebs solution (gassed with 95% O_2-5% CO_2 and maintained at 37°C): 119 mM NaCl, 4.8 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 18 mM $NaHCO_3$, and 10 mM glucose (pH:7.4). Intracellular action potential recordings were performed with glass microelectrodes, which were manufactured from glass capillaries (Clark Electromedical Instruments, Reading, UK) with a puller (PT 30, Sutter Instrument Co, Novato, CA, USA). The microelectrodes filled with 3 M KCl had 15-20 MΩ resistance and were connected to a microelectrode amplifier (IE-251A, Warner Instrument Co, Hamden, CT, USA).

The muscle strips were stimulated with rectangular electrical pulses 3 ms in duration at a frequency of 0.2 Hz (MP35, Biopac Systems Instruments, Goleta, CA, USA). Simultaneous recordings of the action potential and contractions were performed 20 min after the equilibration period. The action potential and contraction data were then transferred to a PC through an MP35 data acquisition system for further analysis.

Immunohistochemical Examination of Potassium and Calcium Ion Channels

At the end of the experimental period, the heart tissues were fixed in 10% buffered formaldehyde solution and subsequently embedded in paraffin following standard procedures. Serial sections with a 4-5 μm thickness were taken from paraffin blocks. The sections were then deparaffinized and rehydrated. Immunohistochemical staining was performed with primary antibodies for calcium channel (CACNA2D1; Novusbio, Littleton, CO, USA) NB120-2864, mouse monoclonal) and potassium channel (Kv1.4; Novusbio, NBP1-48304, mouse monoclonal). Briefly, sections were subjected to heat-mediated antigen retrieval and incubated with 3% hydrogen peroxide to block endogenous peroxidase activity. Sections were then treated with protein blocking solution to block nonspecific staining. Subsequently, the sections were incubated with primary antibodies (CACNA2D1; dilution: 1:500 and Kv1.4; dilution: 1:1000) overnight at 4°C and were then treated with biotinylated secondary antibodies followed by incubation with peroxidase-conjugated streptavidin. All steps were followed by washing in phosphate buffer solution. Immunoreaction was visualized by incubating the sections with 3, 3'-diaminobenzidine (DAB) chromogen. Finally, the sections were counterstained with hematoxylin, dehydrated and mounted. Negative con-

trols were carried out without primary antibodies. The results of the immunohistochemical staining were independently evaluated by two observers under the light microscopy (Olympus Tokyo, Japan; BX51) and digital images (Olympus; DP72) were recorded. The expressions of calcium and potassium channels were scored according to the intensity of the staining, as follows: negative staining; 0, weakly positive staining; 1+, moderately positive staining; 2+, strongly positive staining; 3+.

Statistical Analysis

Results were expressed as mean ± standard deviation. Comparisons between multiple groups were analyzed by Friedman test. Wilcoxon test was applied for comparisons between two groups. Results were considered significant with a probability level of less than 0.05.

Results

General Characteristics of Experimental Group of Animals

Mean body weights, blood glucose and papillary muscle weights of the animal constituents of the experimental groups on the 1st and 4th week were summarized in Table I. At the end of the experimental period, except for the control group, all of the group animals lost weight compared to their initial values. The mean blood glucose levels of M- and MX groups were found be increased. As for the papillary weight measurements, no difference was found between the Con and M-group but the application of estradiol for four weeks resulted in a significant decrement in this parameter.

Action Potential and Contraction Parameters

The measured resting membrane potentials (RMP) from the recorded single cell action potentials were found to be affected. Four weeks of orchiectomization resulted in depolarized RMP values (RMP_{Con} = –86.43 ± 0.74 mV and RMP_{M-} = –73.58 ± 1.41 mV). Moreover, application of estradiol for four weeks to orchiectomized animals resulted in a further depolarization (RMP_{MX} = –64.22 ± 1.24 mV).

The mean maximum depolarization (MD) values of M- and MX groups were also found to be affected from the experimental period. Castration resulted in a significant decrement (MD_{Con} = 19.92 ± 0.37 mV and MD_{M-} = 15.70 ± 0.62 mV) and cross hormone application here again produced additional decrease (MD_{MX} = 10.46 ± 0.46 mV) in the measured parameter. In agreement with the MD results, time required to reach the MD has also been affected from the castration and estradiol treatment (TP_{Con} = 2.44 ± 0.46 ms; TP_{M-}= 3.52 ± 0.64 ms and TP_{MX} = 2.98 ± 0.30 ms).

The effect of castration and estradiol treatment was also observed in the repolarization phase. Figure 1 summarizes the time course of repolarization. The time required to reach 25%, 50%, 75%, and 90% of the maximum repolarization was increased by orchiectomy. Those changes were not prevented by estradiol treatment for four weeks.

Figure 2 shows the effect of castration and estradiol treatment on the mechanical activities of the heart muscle. Figure 2a shows the amplitudes of the peak tension (PT) measured in mg. The maximum contractile performances of the papillary muscle strips were significantly lower in the orchiectomized group. The estradiol treatment for four weeks had no effects on the mea-

Table I. General characteristics of the experimental group of animals.

	Body weights (g)		Blood glucose (mg/dL)		Papillary muscle weights (mg)
	I	F	I	F	
Con N=5	326.80 ± 23.41	330.40 ± 17.13	103.60 ± 3.75	115.00 ± 7,90	17.35 ± 0.57
M- N=8	353.78 ± 6.76	285.33 ± 13.11*	95.22 ± 1.74	138.17 ± 10.62*	16.33 ± 1.34
MX N=8	358.20 ± 11.54	293.00 ± 18.40*	93.40 ± 3.14	129.25 ± 2.64*	11.97 ± 1.16†,¥

In the table Control (Con), gonadectomized male group (M-), gonadectomized and 17 beta estradiol treated group (MX). Body weights and blood glucose represented as BW and BG respectively. *Represents the degree of significance ($p < 0.05$) compared to mean corresponding initial values. †Represents the degree of significance ($p < 0.05$) compared to mean corresponding control group animal values. ¥Represents the degree of significance ($p < 0.05$) compared to mean corresponding M- group values. Values are presented as the mean ± SEM.

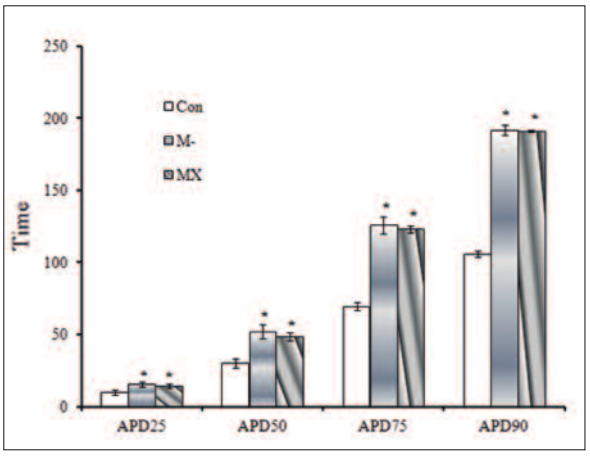

Figure 1. Action potential parameters of the experimental group of animals. Control (Con), orchiectomized male group (M-), orchiectomized and 17 beta estradiol treated group (MX). APD 25; 50; 75; 90 represents the time required to reach the 25, 50, 75, and 90% of the repolarization values respectively. Values are presented as the mean ± SEM. *represents the degree of significance ($p < 0.05$) compared to control group animals.

sured PT values (Figure 2a). Together with the decreased PT, the time required to reach the PT was greater in the orchiectomized group (Figure 2b). Here, again, the estradiol treated group was unaffected form the period. Kinetic measurements for the contraction phase were found to be significantly higher in orchiectomized and unaffected from the estradiol application for four weeks (Figure 2b). As for the relaxation kinetics of the contractions, both for the 50% (RT50) and 90% (RT90) have been found to be increased. Unlike the contraction phase, estradiol treatment for four weeks normalized these parameters to age matched control group animal values (Figure 2c).

Immunohistochemical Examination of Potassium and Calcium Ion Channels

Immunohistochemical results on the experimental groups were summarized in Figures 3 and 4. The expression of potassium channel protein decreased in M- group compared to control

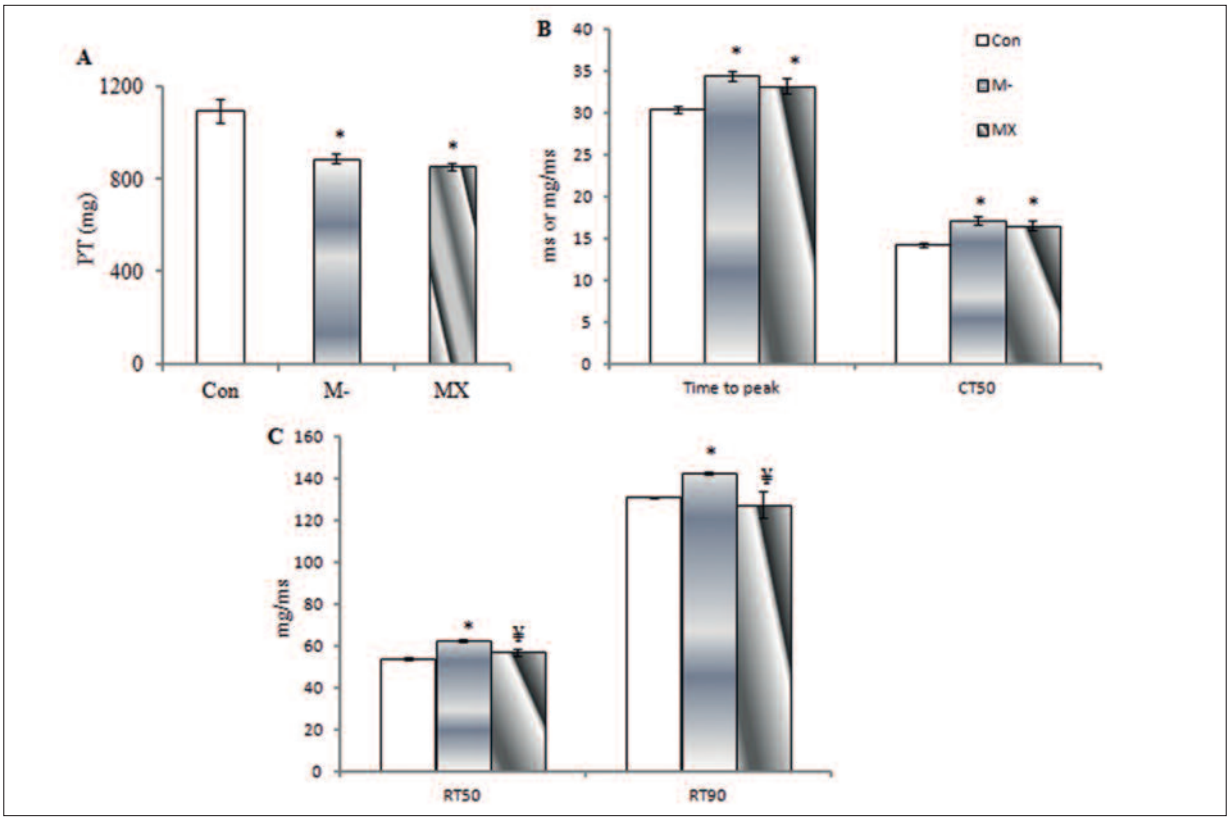

Figure 2. Contraction parameters of the experimental group of animals. Control (Con), orchiectomized male group (M-), orchiectomized and 17 beta estradiol treated group (MX). ***A,*** Show the peak tensions (PT) of the experimental group of animals. ***B,*** Shows the average values of the time to peak and the average contraction kinetics of the experimental groups. ***C,*** Shows two points mean relaxation kinetic values (50 and 90%). Values are presented as the mean ± SEM. *Represents the degree of significance ($p < 0.05$) compared to control group animals while ¥ represents the degree of significance ($I < 0.05$) compared to M-group animals.

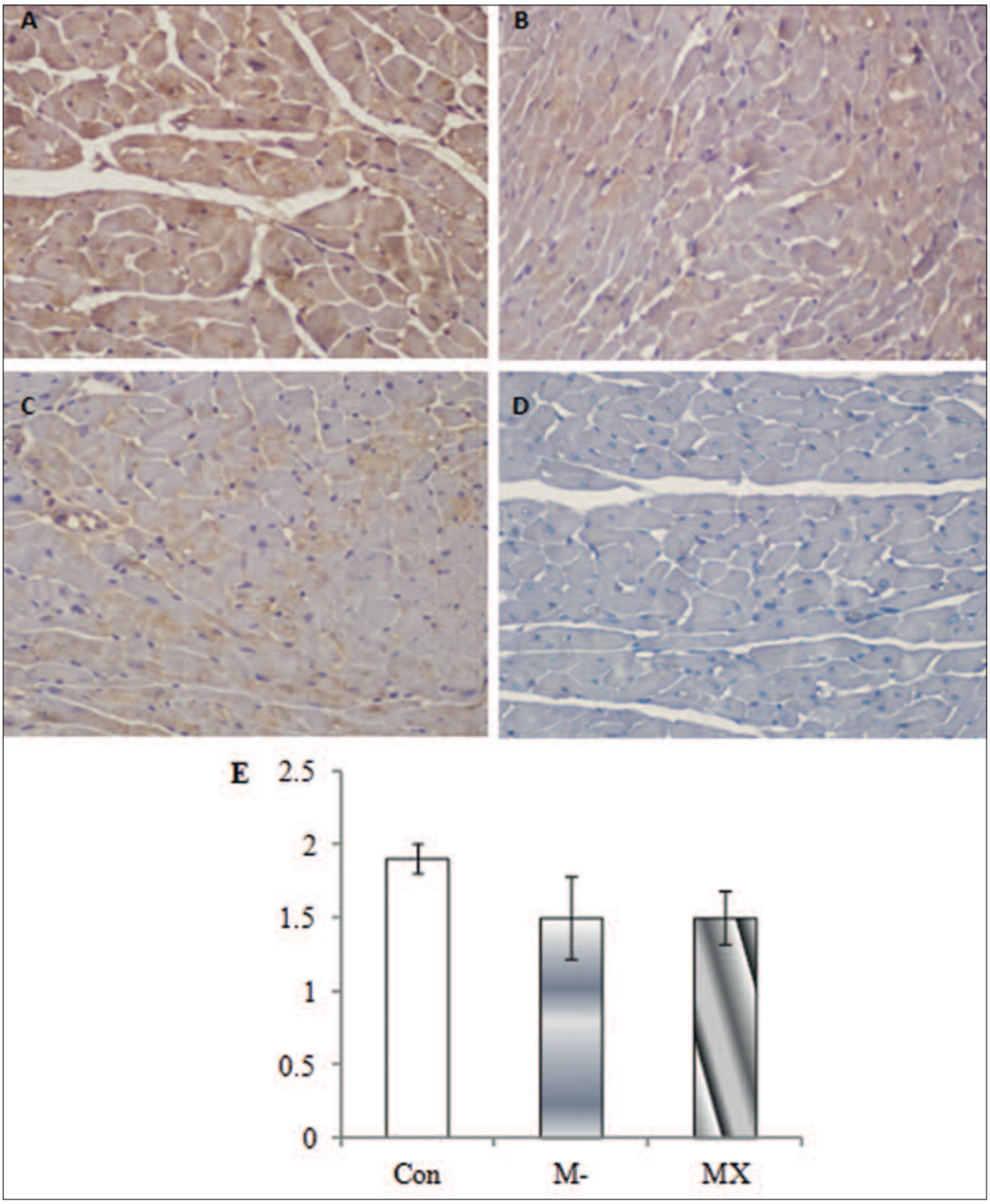

Figure 3. Immunohistochemical detection of potassium ion channel expressions of the experimental group of animals. ***A,*** Control; ***B,*** Orchiectomized male group (M-). ***C,*** Orchiectomized and 17 beta estradiol treated group (MX). ***D,*** Negative control and ***E,*** Mean average values expressed as mean ± SEM. *Represents the degree of significance ($p < 0.05$) compared to control group animals. Positive staining for ion channel was seen brown in color. Magnification: ×40.

group. Potassium channel proteins increased in MT group while did not change in MX group. The differences between groups for potassium channel expression did not reach the level of statistical significance (Figure 3). The expression of calcium channel protein increased in M- group compared to control group ($p < 0.05$). Calcium channel protein was detected at similar levels in the M- and MT groups. Protein expression decreased in MX group compared to M-group; however, the difference was not statistically significant (Figure 4).

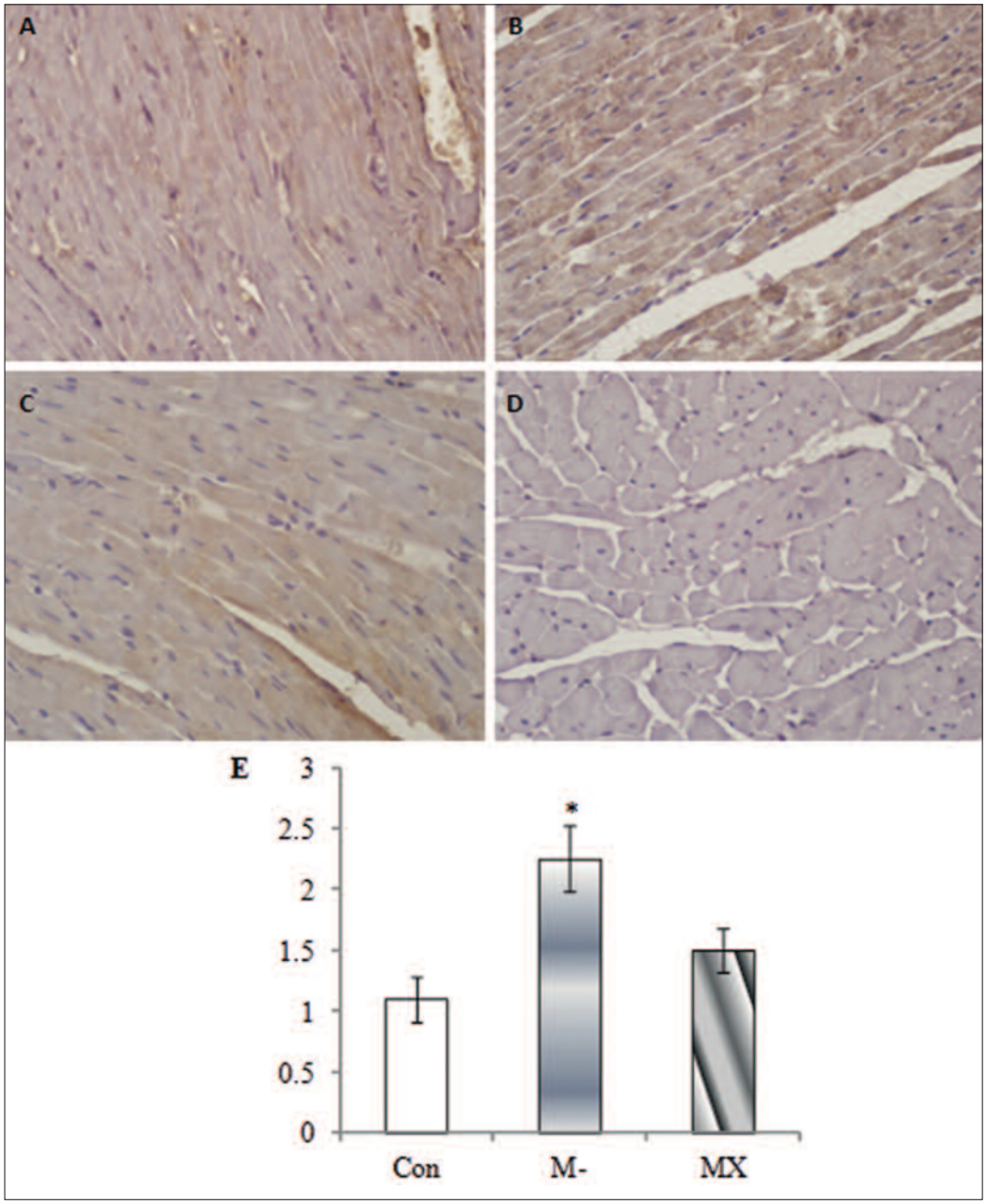

Figure 4. Immunohistochemical detection of calcium ion channel expressions of the experimental group of animals. ***A,*** Control. ***B,*** Orchiectomized male group (M-). ***C,*** Orchiectomized and 17 beta estradiol treated group (MX). ***D,*** Negative control and E. Mean average values expressed as mean ± SEM. *Represents the degree of significance ($p < 0.05$) compared to control group animals. Positive staining for ion channel was seen brown in color. Magnification: ×40.

Discussion

Morphological differences among sexes have made a sensation throughout the mankind history and have been the subject of many researches. The conspicuous difference in mean lifetime between the both sexes has been the basic factor that has made the subject attractive for the researches. Today, while the difference in mean life time in favor of females is 4.2 years, it is estimated to be 4.8 years in 2050[15].

Nowadays, sex hormones are not used only for determining the sex, but also for treatment purposes. For this reason, with the idea of making

Table II. Measured action potential parameters of the experimental group of animals.

	RMP (mV)	MD (mV)	TP (ms)
Con N=5	-86.43 ± 0.74	19.92 ± 0.37	2.44 ± 0.46
M- N=8	-73.58 ± 1.41*	15.70 ± 0.62*	3.52 ± 0.64*
MX N=8	-64.22 ± 1.24*,¥	10.46 ± 0.46*,¥	2.98 ± 0.30

In the table Control (Con), gonadectomized male group (M-), gonadectomized and 17 beta estradiol treated group (MX). Mean resting membrane potential (RMP), maximum depolarization value (MD) and time required to reach the maximum depolarization (TP) were expressed as mean ± SEM. *Represents the degree of significance ($p < 0.05$) compared to mean corresponding control group values. ¥Represents the degree of significance ($p < 0.05$) compared to mean corresponding M-group values.

regression in the opposite sex genital organs, estrogens have been used in prostate cancer treatment and it has been showed that they have a suppressing effect in tumor growth in prostate cancer[16]. With the increasing number of new prostate cancer diagnoses more men receive hormone ablation treatment. In accordance with the increasing number of patients' data regarding benefit to side effect ratio of castration show that the overall survival rate of these patients does not improve as expected. This has been attributed to increased risk factors effecting general health such as diabetes mellitus, atherosclerosis, metabolic syndrome and osteoporosis.

QT intervals in ECG have been found to be longer when adult females were compared with adult males. This difference has risen from electrical re-modeling occurred during depolarization and repolarization phases of the ventricles[17]. This elongation in QT interval generally comes from the alterations observed during the phases 0, 1, 2 and 3 of the action potential[18]. The researches defending the positive and protective effects of estrogen on cardiac muscle have concluded that this hormone has preventive effects against left ventricle hypertrophy and heart failure[19].

In this study, aiming to find out whether 17-beta estradiol has positive effects on male cardiac muscle, it has been observed that castration has caused a significant decrease in live animal weight during the following 4-weeks period. Treating with 17-beta estradiol for four weeks has not made any additional difference on this parameter. Our measurements of papillary muscle weight used for electrophysiological records have showed a significant decrease in this tissue weight during 4-weeks period. This decrease has been more markedly with the effect of estradiol. When papillary muscle weight measurements are interpreted with body weights, it can be estimated that a general decrease can occur in whole body muscle mass. It has been observed that while castration has increased blood sugar significantly, estradiol application has not caused any further changes in this parameter.

Contraction and intracellular action potential measurements have been recorded in order to exhibit the effects of sex hormones on cardiac muscle (in a more open fashion) and to determine the electrophysiological properties.

It has been stated in the literature that cardiac performances of castrated male and female rats have been decreased, and this decrease has been reverted with replacement treatment. Referring to these results, sex hormones have an active role in the arrangement of cardiac performance[20].

In a normal cardiac cycle, first the excitation and then the chain of events observed during contraction play an important role for the heart to perform its routine function. In the control of these events, action potential recordings for excitation and contraction recordings, which are the direct indicators of intracellular free calcium homeostasis for contraction, are used. According to the results of the studies searching for the effects of sex steroids on this issue, it has been observed that the gene expressions of L-type calcium channel, $Na+/Ca^{+2}$ exchanger and β1-adrenergic receptors in myocardium have been decreased in castrated male rats[21]. The acute and chronic effects of the sex steroids in both sexes have been studied, and it has been observed that castration results in alterations in currents of various potassium channels and L-type calcium channels in both sexes when the mechanisms responsible for the non-genomic modulation of the cardiac ion channels by sex steroids have been researched. Various reports have showed that 17-beta estradiol can impede ICaL, IKl, Ito, IKs and IKr currents[22].

We found a significant decrease in peak values of action potentials and a correlate prolongation in the time to reach the peak after 4-weeks castration, which are compatible with the current literature. This result has showed us that testosterone depletion for 4 weeks has caused a significant depression in Na channel currents. This depression can be explained by either the paucity of the numbers of the channels expressed on the membrane or the modification observed in current magnitudes and/or kinetics. Our recordings have showed that castration has caused a significant prolongation in almost every phase of the repolarization of action potential[23]. When the currents responsible for the repolarization were evaluated, it has been shown that L-type calcium channel currents increased and/or all repolarizating potassium channel currents had tendency for decrement. Application of estrogen for 4 weeks after castration did not have a positive effect on the observed changes in action potential.

When our contraction recordings were evaluated, testosterone depletion for 4 weeks has caused a significant decrease in maximal contraction force as well as a deceleration in the kinetics of calcium secreted from intracellular storages. For the relaxation period, the measured values indicate that the pathways (calcium pomp making clearance from sarcolemma to the outside of the cell, calcium pumps based on sarcoplasmic reticulum and mitochondrial reuptake) taking role in the regulation of intracellular free calcium may have been affected.

The clinical papers in the literature have proved that testosterone has a protective effect on heart. Testosterone is reported to be the preferred ligand of the human androgen receptor in the myocardium and directly modulates transcription, translation and enzyme function[24]. Besides this, in the patients having maximal androgen blockade because of prostate cancer, endothelial dysfunction and cardiac problems due to this have been increased when compared to normal population[25]. Additionally, endothelial nitric oxide excretion decreases, and contraction and relaxation mechanisms of vascular structures deteriorate in hypogonadism[26]. In epidemiologic studies, the risk of cardiovascular diseases has been found to be increased in patients with hypogonadism. Moreover, when these patients are treated with testosterone replacement therapy, it is reached to the cardiovascular risk rate for normal people[27].

Conclusions

Our study has showed that testosterone has an extremely important and protective effect on male cardiac muscle preparations while estrogen does not have any. It is predicted that testosterone has showed this effect by means of modulation of some key points of excitation-contraction pairing of cardiac muscle. Further studies are needed to evaluate the molecular mechanisms of the effect of testosterone.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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