Effects of testosterone on norepinephrine release in isolated rat heart and the flutamide intervention on testosterone

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Abstract: Aims: To investigate the effects of testosterone on norepinephrine release in the isolated rat hearts and its probable mechanism. **Methods**: Sprague-Dawley male rats (n=280) were randomized to 4 groups. The control group was perfused with modified Krebs-Henseleit (KH) buffer ; the testosterone groups were perfused with modified Krebs-Henseleit buffer containing 4 different concentrations of testosterone (100.0nmol/L, 10.0 nmol/L, 1.0nmol/L, and 0.1nmol/L, respectively); the testosterone with flutamide groups were perfused with modified Krebs-Henseleit buffer containing 4 different concentrations of testosterone plus 100.0nmol/L flutamide (testosterone 0.1nmol/L+ flutamide 100.0nmol/L, testosterone 1.0nmol/L+ flutamide 100.0nmol/L, testosterone 10.0nmol/L+ flutamide 100.0nmol/L, testosterone 100.0nmol/L+ flutamide100.0nmol / L,respectively); the flutamide groups were perfused with KH buffer containing flutamide100.0nmol / L. Observe the norepinephrine release in both case of electrical stimulation and myocardial ischemia. Electrical field stimulation at 5 V (effective voltage) and 6 Hz (pulse width 2 ms) for 1 min, myocardial ischemia was induced by global stopping. **Results**: Electrical stimulation of the ventricle evoked norepinephrine release, and this was diminished by the perfusion with testosterone at a concentration of 1.0, 10.0 and 100.0nmol/L ($P < 0.01$). Following acute ischemia, testosterone (1.0, 10.0 and 100.0nmol/L) significantly reduced norepinephrine release ($P < 0.01$), the norepinephrine overflow was similar between the testosterone group and the testosterone with flutamide ($P > 0.05$). **Conclusions:** It is suggested that testosterone suppresses ischemia and electrical stimulation induced norepinephrine release in the isolated rat hearts and the flutamide could not block this inhibition.

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Norepinephrine (NE), one of the most important neurotransmitter in myocardial tissue, increasing the excitability and triggered activities of the myocardial and promoting the formation of reentrant loops within the myocardium, leading to malignant arrhythmia [1-4]. Studies have found that, the level of testosterone in blood serum was reduced in the male patients with CHF[5]. Testosterone suppresses the inflammatory cell activities, reducing the level of TNF-α, but raising the serum level of IL-10[6-7]. When testosterone was used as a substitution treatment to the ischemic cardiomyopathy, it delays the occurrence of myocardial ischemia and enhances ICM combined sexual dysfunction male patient's exercise tolerance[4]. In recent years, we are interested in the role of testosterone treatment angiocardiopathy. The primary purpose of this study is to investigate whether testosterone is able to influence NE release and ischemia/reperfusion arrhythmias in a rat ex vivo heart and find its probable mechanism.

1. MATERIALS AND METHODS

1.1 Animals and drugs

Male Sprague-Dawley rats (n=280, weight 200-250g) were provided by Experimental Animal Center of Fudan University. Testosterone（C19H28-O2) and flutamide (C11H11F3N2O3) were obtained from Sigma Corp (Shanghai, China).

Krebs-Henseleit solution (NaC1 l25mmol/L, $NaHCO_3$ 16.9mmol/L, KH_2PO_4 0.2mmol/L, $CaCl_2$ 1.85 m-mol/L, $MgC1_2$ 1.0mmol/L, $MgSO_4$ 0.2mmol/L, glucose 11.0mmol/L, and sodium EDTA 0.027mmol/L) was provided by cardiovascular laboratory of Zhongshan Hospital, Fudan University (China).

1.2 Grouping of Experiment

The first study was designed to assess the effect of testosterone on the electricity-induced NE release and whether the flutamide intervention will affect it. Normal male SD rats (140) were randomly divided into the control group and the experimental group (130 rats finally survived). The control group perfused with KH solution without any drugs. In the experimental groups,the testosterone groups were perfused with K-H buffer containing 4 different concentrations of testosterone (100.0nmol/L, 10.0nmol/L, 1.0nmol/L, and 0.1nmol/L, respectively); the testosterone with flutamide groups were perfused with K-H buffer containing four concentrations of testosterone plus 100.0nmol/L flutamide (testosterone 0.1nmol/L+ flutamide 100.0nmol/L, testosterone 1.0nmol/L+ flutamide 100.0nmol/L, testosterone

10.0nmol/L+ flutamide 100.0nmol/L, testosterone 100.0nmol/L+ flutamide 100.0nmol/L,respectively); the flutamide groups were perfused with KH buffer containing flutamide100.0nmol / L.

The second study was to investigate the effect of testosterone on the myocardial ischemia-induced NE release and whether the flutamide intervention will affect it. The grouping of rats was identical to those in the first study (131 rats finally survived).

1.3 Preparation of ischemia-perfusion heart

Under ambient conditions, rats were anesthetized with sodium pentobarbitone (40 mg/kg, ip). Following the anesthesia the peritoneal cavity was opened and 500U heparin was injected into the inferior vena cava. The thorax was open, the heart was rapidly excised and immediately immersed in ice-cold brine and arrest. Ascending aorta separation, isolated heart perfusion with the classic Langendorff method, and perfusion fluid using the modified K-H solution (NaCl 125mmol / L, NaHCO3 16.9mmol / L, Na2HPO4 0.2mmol / L, KCl 4.0mmol / l, CaCl2 1.85mmol / L MgCl2 1.0mmol / L, glucose 11.0mmol / L, EDTA 0.027mmol / L). The perfused before use with 5μm porous filter filtering, The buffer was gassed with 95% O_2, 5% CO_2, was temperature controlled at (37.0±0.3) ℃ by 2 water baths, and the pH was adjusted to 7.30 -7.40.

Within 2 min, the hearts were perfused in a retrograde manner through the aorta at a constant flow rate of 6 ml/min, using a multichannel peristaltic pump for simultaneous perfusion. All hearts were initially perfused for 20 min with the Krebs-Henseleit buffer. The hearts were kept in a glass water-heated jacket, which kept the surrounding air temperature at (37.0±0.5)℃, the basic perfusion pressure was 90 cmH2O, and the low flow perfusion pressure was 15cm H_2O. After the initial perfusion, testosterone or testosterone with flutamide was added to the perfused.

1.4 Electrical field stimulation

Two 10 x 7 mm concavely shaped metal paddles were placed in opposite positions on the surface of the ventricles, in such a manner that the interventricular septum was located between both paddles. Exocytotic norepinephrine release was induced by electrical field stimulation at 5 V (effective voltage) and 6 Hz (pulse width 2 ms) for 1 min.

Balance after 20 minutes of perfusion, followed by the 1st electrical stimulation. Interventions of the testosterone groups (0.1nmol/L, 1.0nmol/L, 10.0nmol/L and 100.0nmol/L) and the control group were started 10 min prior to the second stimulation (S2). Samples for determination of norepinephrine were collected during twice electrical stimulation, and then were stabilized by addition of Na2-EDTA (10mmol/L) and stored at -70 ℃ until being assayed.

Intervention studies on flutamide, balance after 20 minutes of perfusion, followed by the 1st electrical stimulation. Interventions of testosterone (0.1nmol/l, 1.0nmol/l, 10.0nmol/l, 100.0nmol/l), testosteron+flutamide (various concentrations of testosterone were added 100.0nmol/l flutamide) and flutamide (100.0nmol/l) continuous infusion 60 min before the second stimulation. Samples for determination of norepinephrine were collected during twice electrical stimulation, and then were stabilized by addition of Na2-EDTA (10mmol/L) and stored at -70 ℃ until being assayed.

1.5 Myocardial ischemia group

Myocardial ischemia was induced by stopping the perfusion flow. Following an ischemic period of 30 min, the hearts were reperfused at the initial flow rate of 6 ml/min. interventions of testosterone (0.1nmol/L, 1.0nmol/L, 10.0nmol/l, and 100.0nmol/l), testosterone + flutamide (various concentrations of testosterone were added 100.0nmol/l flutamide), flutamide (100.0nmol/l) and the control group were added 60 min before ischemia. Samples were immediately taken before ischemia, and during the first 1 min of the reperfusion period. Cumulative norepinephrine overflow was determined from the reperfusion sampling period. And then were stabilized by addition of Na-2-EDTA (10mmol/L) and stored at -70 ℃ until being assayed.

1.6 Measurement of norepinephrine

Norepinephrine samples were stabilized by addition of Na2-EDTA (10 mmol/L) and stored at -70 ℃ until being assayed. Norepinephrine was determined by high pressure liquid chromategraphy – electrochemistry. The total norepinephrine measured from the perfusate was then divided by the ventricular weight.

1.7 Statistical treatment

SAS 6.12 statistics software package wa-s used to perform the statistical analysis. Data were expressed as mean ± SEM. The significance of differences was assessed by ANOVA. Statistical differences between norepinephrine outflows (S1and S2) were analyzed by a student *t* test. For categorical data, a Fisher calculation of exact probability was used. A P value of less than 0.05 was considered statistically significant.

2. Result

2.1 Effect on electricity stimulation-induced norepinepherine release

There were no significant differences in the norepinephrine release between the S1 and S2 stimulation protocols in the control or testosterone 0.1nmol/L groups (P >0.05).

In the S2 stimulation protocol, the release of norepinephrine in the testosterone 1.0nmol/L, 10.0nmol/L or 100.0nmol/L was lower than the corresponding groups in the S1 protocol (P<0.01) as in

Table1 and Figure 2. Within the S2 stimulation protocol, testosterone (10 and 100nmol/L) significantly reduced norepinephrine release (P <0.01), the norepinephrine overflow was similar between the control and the testosterone group (0.1 nmol/L,1.0nmol/L group) (P >0.05), testosterone group (10nmol/L) and testosterone group (100 nm-ol/L) (P >0.05) (Table1 and Figure 3)

There were no significant differences in the norepinephrine release between the S1 (n = 8,117.0 ± 36.5pmol / g) and S2 (n = 8,113.4 ± 37.9pmol / g) stimulation protocols in the flutamide intervention group (p>0.05, Figure 4).

In the S1 stimulation protocol, there we-re no significant differences in the norepinephrine release between the testosterone groups (0.1nmol/L, 1.0nmol/L, 10nmol/L, 100nmol/L) and the testosterone with flutamide groups (testosterone 0.1nmol/L+ flutamide 100.0nmol/L, testosterone 1.0nmol/L+ flutamide 100.0nmol/L, testosterone 10.0nmol/L+ flutamide 100.0nmol/L, testosterone 100.0nmol/L+ flutamide100.0nmol /L)(P >0.05, Figure 5). In the S2 stimulation protocol, the results are the same. There were no significant differences in the norepinephrine release between the testosterone groups (0.1nmol/L, 1.0nmol/L, 10nmol/L, 100nmol/L) and the testosterone with flutamide groups (testosterone 0.1nmol/L+ flutamide 100.0nmol/L, testosterone 1.0nmol/L+ flutamide 100.0nmol/L, testosterone 10.0nmol/L+ flutamide 100.0nmol/L, testosterone 100.0nmol/L+ flutamide100.0nmol / L) (P >0.05, Figure 6).

2.2 The group of ischemia-perfusion

The effects of testosterone on ischemia-perfusion induced norepinephrine release in the isolated rat heart. Norepinephrine release during ischemia-perfusion:compared with control group (n=10,NE 217.8±17.5pmol/g), group testosterone 1.0nmol/L (n=11,NE 157.0±16.5pmol/g,), 10 n-mol/L （n=12,NE 118.9±10.7pmol/g), 100nmol/L (n=12,NE 116.5±9.8pmol/g) significantly reduced norepinephrine release (P <0.01), the norepinephrine overflow was similar between the control and the testosterone group (0.1nmol/L group, n=9,NE 200.9±13.3pmol/g) (P >0.05), testosterone group (10nmol/L) and testosterone group (100nmol/L) (P >0.05, Figure 7).

There were no significant differences in the norepinephrine release between the flutamide and the control (P >0.05, Figure 8); so as to the testosterone groups and the testosterone with flutamide groups (P >0.05, Figure 9)

3. Discussions

Acute myocardial ischemia (AMI) is often associated with a large number of norepinephrine (NE) and the release of NE is different due to the time. During early period of AMI (<10 minutes), myocardial NE released by exocytosis way. Pain, anxiety can make the activity of cardiac sympathetic nerve increase. In addition, decreased blood pressure and cardiac output can excite the pressure and volume receptors, myocardial acidosis, the gathering of metabolites and wall stretching excited afferent nerves can also increase the sympathetic nerve activity, resulting in myocardial NE release[8]. In physiological conditions, the excited sympathetic nerve endings release NE by exocytosis. This way depends on the presence of the extracellular fluid's Ca^{2+}, the vactivation of protein kinase C and the opening of presynaptic membrane's N-type calcium channel. This process needs energy (consumption of high-energy phosphate bond), with the release of neuropeptide Y which can be used as a symbol of exocytosis. Electrical stimulation of the epicardium releases norepinepherine in a similar way to the direct stimulation of sympathetic ganglion[9]. Therefore, this study survey NE's exocytosis in multi-concentration testosterone by the rat epicardial electrical stimulus. This test shows that the testosterone can reduce myocardial NE content released by the epicardial electrical stimulus when S2-stimulus compares to S1-stimulus in the same group and in Inter-group. Therefore, the testosterone can reduce the release of NE's exocytosis when the sympathetic nervous excites.

β-blockers are widely used in treating heart failure and can improve the prognosis of patients. Excessive long-term stimulation of β-adrenergic receptor (β-AR) is toxic to the heart. Noradrenaline coheres to β-adrenergic receptor is mediated by the protein kinase A, which produces a lot of calcium influx by voltage-dependent calcium channel. As a result, Ca^{2+} accumulation in the cells which can induce cardiac hypertrophy, increase myocardial ischemia, malignant arrhythmias, cardiomyocyte apoptosis and cardiac remodeling [10-12]. By lessening NE exocytosis in the sympathetic nervous exciting, testosterone may reduce the catecholamine's toxic effects on the heart.

The results of this study suggest that testosterone may reduce the release of NE exocytosis excited by the sympathetic nervous and lessen the toxicity of catecholamines on the heart.

Because NE exocytosis requires a lot of energy, with the myocardial ischemia time lasting (>10 minutes), nerve cells occur serious lack of ATP and terminate NE exocytosis. In this time, the release of NE by the intake-vector antiport carrier are become the major route of transmission. After 10-40 min of myocardial ischemia or energy metabolic block, myocardium develops energy depletion and hypoxia. The cytoplasmic Na^+ within the sympathetic nerve is end and the Na^+ gradient of presynaptic membrane transmembrane increases. Accompanied by Na^+ outflow, NE undertakes non-exocytosis release by presynaptic membrane uptake-1 antiport mechanism. In this way, the release amount of NE is very great.

Studies have reported that, the non-exocytosis release of NE in myocardial concentration is up to 100-1000 times than the normal after 15-20 min of myocardial ischemia[13]. Therefore, in our experiment, after 30 min stopping reperfusion, NE of the outflow liquid mostly is delivered by non-exocytosis release.

In isolated heart perfusion model experiments under different concentrations of testosterone environment, ischemia reperfusion reduced myocardial NE released, and with dose increases, the role also significantly enhanced. Therefore, testosterone can be reduced to a non-exocytosis NE release.

Studies have shown that Testosterone (T) can be combined with the androgen receptor (AR) and working in the gene way[14]. In addition, it can also work with signaling and none-gene ways. The gene way needs the transcription of androgen responsive genes, which requires at least 40 min[15] [16]. Testosterone can reduce the release of NE, but whether it works in the gene way?

Flutamide, androgen and androgen receptor competition, blocking cell uptake of androgen, were often used to elaborate the mechanism of the testosterone[17- 18]. The purpose of the study is to investigate the role of testosterone in NE release in myocardial ischemia and explore the mechanism for clinical reference. In order to find whether the gene way is needed in this process, flutamide and testosterone were perfused together. Because the gene pathway requires at least 40 min[15-16], the hearts were perfused for one hour. In this study, we have found that: (1) flutamide itself does not affect the NE release in electric stimulation and ischemic myocardium; (2) flutamide can not block the inhibition of NE release in electric stimulation and ischemic myocardium NE during IR. Working with the membrance-receptor, concern with the second message system, the non-genetic way works quickly, leading to rapid changes in intracellular calcium, protein kinase A, protein kinase C and tear plasminogen activator protein pathway activation, so that different biological effects of androgen to produce smooth muscle relaxation. Recent studies have found that the physiological concentrations of testosterone can improve neonatal rats[19] and adult rats[20] the activity of L-type calcium channels in cardiac myocytes (slow calcium channel); testosterone can increase in the mRNA levels of the cardiac sodium - calcium exchange[21]. Therefore, testosterone may change calcium homeostasis, thereby reducing calcium overload caused by IR injury. Endogenous testosterone can also protect.

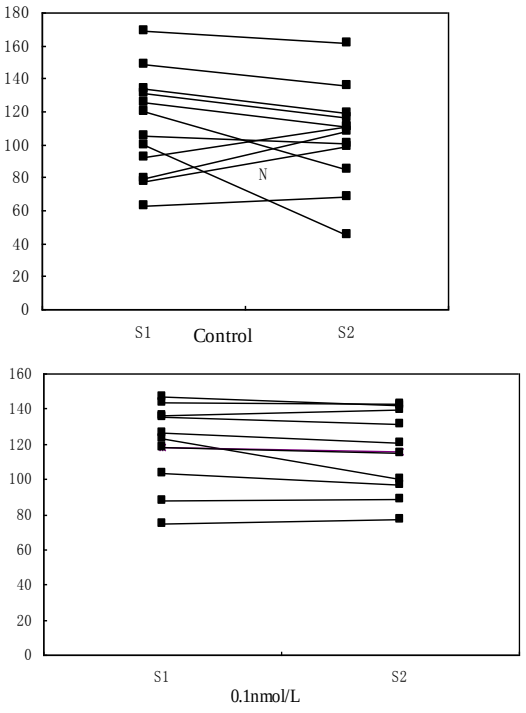
Myocardial by activating the mitochondrial ATP-sensitive potassium channels, which is n-one concern about the androgen receptor[22]. This effects work fast, indicating that they work regardless of the androgen receptor by non-genetic ways. So we concluded that testosterone may inhibit myocardial ischemia NE release through non-receptor pathway, the mechanism may include a direct effect on certain ion channels, such as the mitochondrial ATP-sensitive potassium channel (mitoKATP), the slow calcium channel (L-type calcium), the specific mechanism needs further stu-dy.

Table1. The noradrenaline release during electrical stimulation.

compared S1 and S2 stimulation, $^{\circ}P>0.05$,$^{\bullet}P<0.01$.

Group	S1	S2	S2/S1
Control (n=12)	112.0±31.5	105.0±30.1°	0.9653±0.2481
T 0.1nmol/L (n=11)	119.5±22.9	115.4±22. 5$^{\circ\triangle}$	0.9684±0.0616
T 1.0nmol/L (n=10)	145.0±16.6	88.2±13.6$^{\bullet\triangle\square}$	0.6108±0.0844
T 10nmol/L (n=12)	139.5±18.5	62.6±9.8$^{\bullet\blacktriangle\blacksquare}$	0.4530±0.0750
T 100nmol/L *(n=11)*	146.1±16.9	58.4±4.9$^{\bullet\blacktriangle\blacksquare}$	0.4030±0.0405

$^{\triangle}P>0.05$,$^{\blacktriangle}P<0.01$ compared with control group; $^{\square}P>0.05$, $^{\blacksquare}P<0.01$ compared with T 0.1nmol/L group.



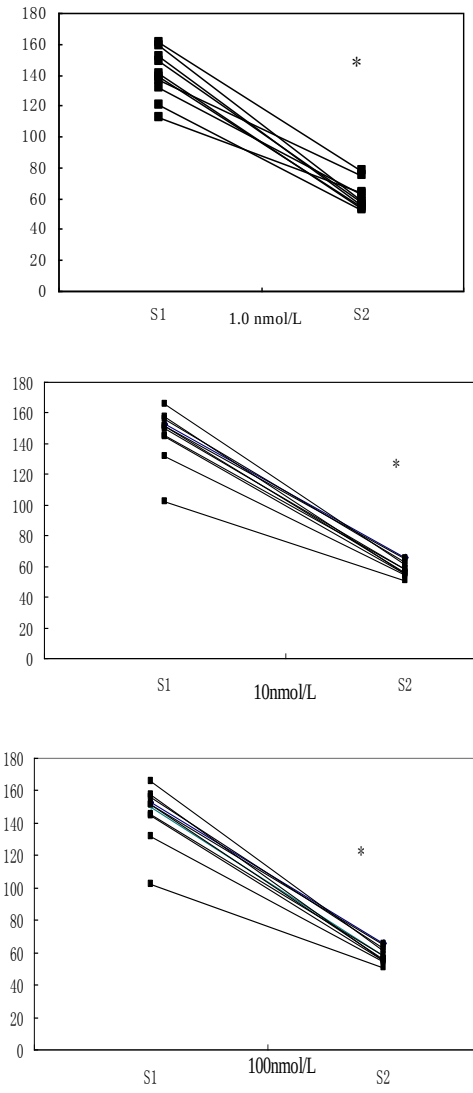


Figure 2 The noradrenaline release in the S1 and S2 stimulation

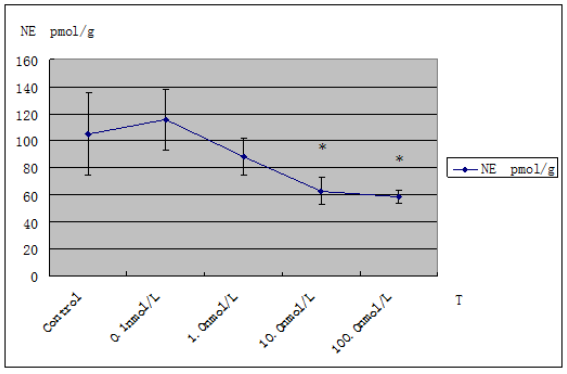


Figure 3 The noradrenaline release during electrical stimulation.

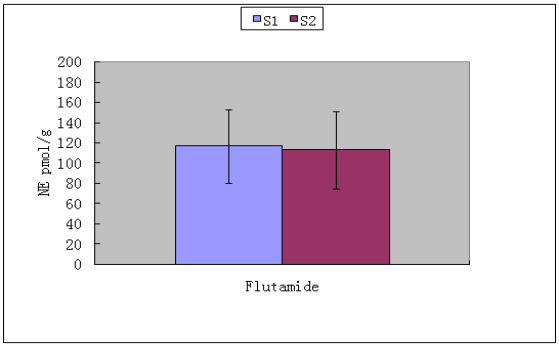


Figure 4 The noradrenaline released during flutamide (S1 VS S2)

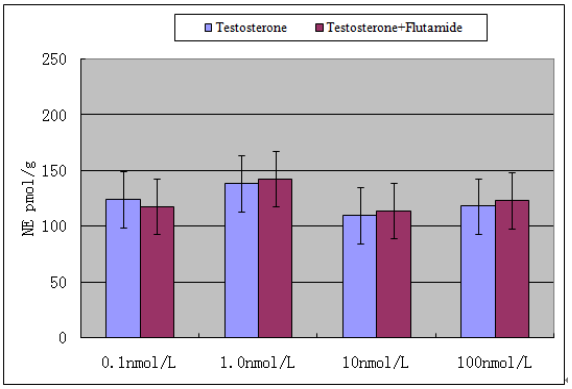


Figure 5 The noradrenaline released during S1 (Testosterone VS Testosterone + Flutamide)

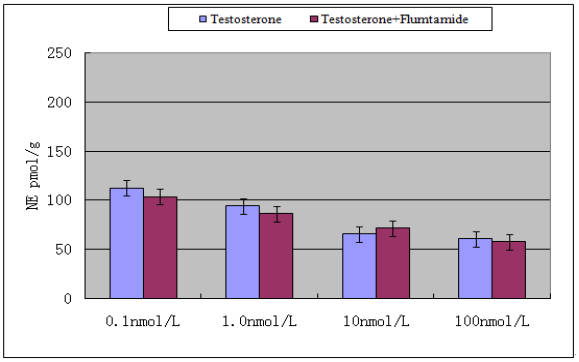


Figure 6 The noradrenaline released during S2 (Testosterone VS Testosterone + Flutamide)

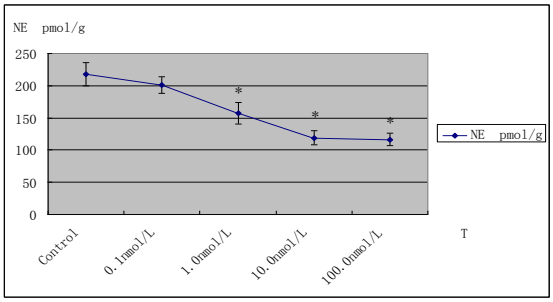


Figure 7 The noradrenaline release during ischemia-reperfusion.

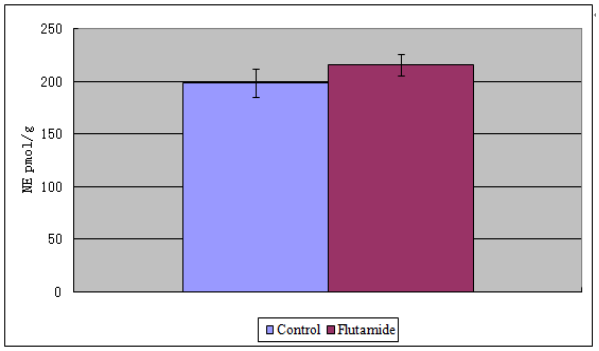


Figure 8 The noradrenaline release during ischemia-reperfusion. (Control VS Flutamide P>0.05)

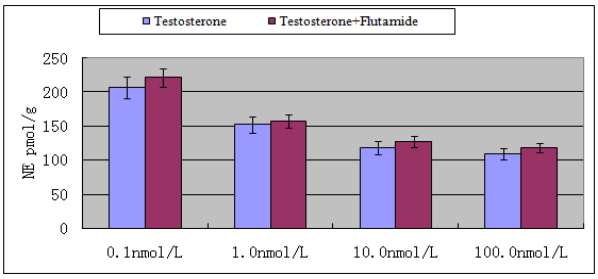


Figure 9 Norepinephrine release during ischemia-reperfusion
(Testosterone VS Testosterone + Flutamide P>0.05)

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