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RESEARCH ARTICLE

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Effects of Lonidamine-Loaded Lipid-Polymer Hybrid Nanoparticles on Cardiac Fibrosis Induced By High-Dose Testosterone Propionate In Adult and Neutered Male Rats[#]

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ABSTRACT

In recent years, the usage of exogenous testosterone has tripled in humans. Although, mechanism has not yet been fully elucidated, there is a correlation between the increased risk of heart failure and testosterone usage. The aim of this study was to demonstrate cardiac fibrosis, which a main finding of hearth failure, in testosterone propionate-applied male rats and to show its regression by Lonidamine as an anti-hyperplastic agent. A total of 72 adult Wistar albino and neutralized male rats were divided into 4 groups (n=18 in each groups). Animals in all groups were received to testosterone propionate until 2nd, 4th, and 12th weeks (n=6 in each groups). Subsequently, group I and II also received the pure solution of Lonidamine hydrochloride and its lipid-polymer hybrid nanoparticulate formulation via intraprostatic injection. Group III, blank lipid-polymer hybrid nanoparticle formulation were solely administrated via same way. The control group or group IV were received only testosterone. At the end of the experiment period, hearts were collected and fibrocytic changes were confirmed by histochemical and immunohistochemical methods. Histopathologically, fibrosis were lower in group I and II when comprared to that of group III and IV. Imunohistochemically, bFGF, cyclin D1 and p16 protein expressions were evaluated. bFGF and cyclinD1 epxressions correspondingly to increasing fibrosis were found higher in last two groups during the experiment. But, p16 expressions were lower in Lonidamine treated-group I and II. In conclusion, results of this study supported that testosterone propionate may promote cardiac fibrosis. Lonidamine hydrochloride may be used in its prevention of fibrosis.
Keywords: Cardiac fibrosis, Lonidamine hydrochloride, Testosterone, Rat.

Yetişkin ve Kastre Edilmiş Erkek Ratlarda Yüksek Doz Testosteron Propiyonatın İndüklediği Kardiyak Fibrozis Üzerine Lonidamin Yüklü Lipit-Polimer Hibrit Nanopartiküllerin Etkisi

ÖZ

Son yıllarda, insanlarda ekzojen testosteron kullanımı üç katına çıkmıştır. Mekanizma henüz tam olarak aydınlatılmamış olmasına rağmen kalp yetmezliği riskini arttırdığına dair bir korelasyon vardır. Bu çalışmanın amacı, erkek sıçanlarda testosteron propiyonat kullanımının kalp yetmezliğinin temel bulgusu olan fibrozise yol açtığını ve bir anti-hiperplastik ajan olan Lonidaminin bunu geriletici etkisini göstermekti. Çalışmada, toplam 72 adet kastre edilmiş, yetişkin Wistar albino erkek sıçan kullanıldı ve hayvanlar 4 ana gruba ayrıldı (her bir grupta için n= 18). Bu ana gruplar 3 alt gruba ayrılarak (n=6 her bir grup için) hayvanlara, 2., 4. ve 12. haftalara kadar testosteron propiyonat uygulandı. Group I ve II için, Lonidamin hidroklorür, saf çözelti içinde ve bunun lipit-polimer hibrit nanopartikül formülasyonu halinde prostatlara enjekte edildi. Grup III için sadece lipit-polimer hibrit nanopartikül formülasyonu uygulandı. Kontrol grubu olarak grup IV'de sadece testesteron uygulandı. Çalışmanın sonunda kalpler toplandı. Histopatolojik olarak fibrosis grup I ve II'de grup III ve IV'dekiyle karşılaştırıldığında daha düşüktü. İmmünohistokimyasal olarak bFGF, siklin D1 ve p16 protein ekspresyonları değerlendirildi. Son iki grupta, artan fibrozise karşılık gelen bFGF ve siklinD1 ifadeleri, deney sırasında daha yüksek bulundu. Ancak, p16 ifadeleri Lonidamin uygulanan grup I ve grup II'de daha düşüktü. Sonuç olarak, bu sonuçlar testosteron propiyonatın kardiyak fibrozisi etkileyebileceğini desteklemektedir ve fibrozisin önlenmesinde Lonidamin hidroklorür kullanılabilir.
Anahtar Kelimeler: Kardiyak fibrozis, Lonidamin hidroklorür, Testosteron, Sıçan.

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INTRODUCTION

Heart failure is predominant problem overall the world. The incidence between men and women can be changed dependently to sex differences (Weidner 2000; Cleland et al. 2001). The usage of exogenous testosterone or other anabolic steroids on human cardiac health is still controversial. Particularly, the risk in men cardiac failure can be increased when used supplementally. This risk is higher in men when compared in that of women at every age (Thurm and Borlak 2002; Grunewald and Matsumoto 2003).

Myocardial tissues of left ventricles may be more affected and therefore systolic and diastolic functions may be easily detoriated as result of continuously usage of testesterone supplying in men (D'Andrea et al. 2007; Hassan et al. 2009). Testosterone may cause hyperthrophy in cardiac myocyte because they have functional androgen receptors. These receptors have been indicated in humans, dogs, and rats (Marsh et al. 1998). In this context, the rat is a good and usefull model for understanding human cardiac health (Doggrel and Brown, 1998).

Endogenouse testosterone also effects cardiac failures and is likely to modulate the fibrosis and collagen deposits also it is reported that castration decreased the cardiac fibrosis when compared to intact males (Hori et al. 2008; Yang et al. 2017). Related with the cardiac fibrosis progression of hypertension, coronary heart disease, heart failure, and other cardiovascular diseases, and it may initiate myocardial remodeling (Hori et al. 2008; Porter and Turner, 2009).

It is believed that several biomolecules, including growth factors and cytokines, activate proliferation of cardiac fibroblasts (CFs) and modulate the expression of extra cellular matrix (ECM) (Frangogiannis, 2012). Cyclin D1, which is a protein of cycline dependent kinases, plays an important role in the development of proliferative disease and oncogenesis as a cell cycle regulator (Fu et al. 2004). It is reported that overexpressed cyclin D1 by rodent fibroblasts enhanced cooperation in response of basic fibroblast growth factor (bFGF) and thereby stimulation in fibroblastic cell cycle progression and anchorage-independent growth of cells (Tashiro et al. 2003). P16, which is also known as cyclin-dependent kinase inhibitor 2A, plays an reversal role in cell cycle regulation because it prevents proliferation and provide cellular senescence (Serrano et al. 1993; Rayess et al. 2012). It is reported that p16 takes a role in fibroblast senescence. For p16, such antifibrotic activity is thought because of fibroblast senescence is duty on prevention of myocardial fibrosis by antifibrotic activity (Zhu et al. 2013; Meyer et al. 2016; Xie et al. 2017). For prevention and treatment of cardiac fibrosis, no effective remedy could not still be found. Main interest has focused on suppress the symptoms. However, it should take a more necessary and factual steps on treatment of fibrosis-attenuating mechanism. It is reported that current pleiotropic drugs could fortunately solve the problem of developing cardiac fibrosis out. In this process, it has stated that biochemical microenvironment focused therapies taking under control the development of fibrosis should be paid attention and such therapies should be developed (Bronnum and Kalluri 2012).

Lonidamine (LND) which is known since over 30 years has been known to have anti-neoplastic and antiproliferative effects (Caputo and Silvestrini 1992). LND make an effects on neoplastic cells inhibiting lactate export by the proton-linked monocarboxylate transporter(s) (MCT) and pyruvate uptake into mitochondria. Furthermore, LND also inhibit indirectly hexokinase activity there (Floridi et al. 1981a; Floridi et al. 1981b). As result of this enzymatic blokade, cytosolic and extracelluler pH decrease in neoplastic cells by increasing lactate (Nath et al. 2015a; Nath et al. 2015b). But, according to reports, this effect of LND is selective on normal tissues if provided dose are under approximately 400 mg/m2 (oral or i.v. doses) (Price et al 1995; Price et al. 1996). And, it does not affect cell progression (Caputo and Silvestrini 1992). In the current study, it was aimed that the relationship between some molecular factors providing and attenuating cardiac fibrosis and overdose testesterone in adult rat modelling has been emphasized and the usefull effect of Lonidamine as a lipid-polymer hybrid nanoparticle formulation has been proven for prevention of cardiac fibrosis inducing high testesterone.

METHODS

Preparing of Lonidamine nanoparticles

To investigate the effect of Lonidamine on myocardial cells and fibrosis, pure Lonidamine and Lonidamine-loaded lipid-polymer of hybrid nanoparticles were applied to separate groups. Lonidamine encapsulated lipid-polymer hybrid nanoparticles were prepared through one-step self-assembly approach technique. Thirteen hybrid nanoparticle formulations were produced based on a Design of Experiment (DoE) approach and the optimized one was selected (Sengel-Turk and Hascicek 2017). Composition of the optimum nanoparticle formulation are tabulated in Table-1. Blank nanoparticles were prepared in the same way except Lonidamine.

Table 1. Content of the Lonidamine-loaded optimum lipid-polymer hybrid nanoparticles.

Fomulation content	Amount
Organic Phase	
Poly-D,L-lactide-co-glycolide (PLGA)	4000 µg
Lonidamine	600 µg
Acetonitrile	1600 µl
Aqueous Lipid Phase	
1,2-distearoyl-sn-glycero-3-phosphoetanolamine-N-carboxy(poly(ethyleneglycol) (DSPE-PEG-COOH) 2000	800 µg
Lecithin	50 µg
Ethanol solution	850 µl
Water (enough amount)	16000 µl

Animal exposure

Approval for the study was granted by the Local Ethics Committee of Animal Experiments, Ankara University (date of 15.11.2017 decision no. 2017-23-185). Male Wistar rats were mated in the Animal Research Laboratory, Faculty of Medicine, Ankara University. The animals were maintained at a temperature of 22°C-24 °C and 55% humidity with a 12-hr light/l2-hr dark cycle. Detailed descriptions of the experimental procedures are given below. All procedures were performed according to the guidelines stated in the National Institute of Health Guide for the Care and Use of Laboratory Animals.

The neutralizing procedure was performed by incising the scrotal sac under general anesthesia by species dosage of xylazine and ketamine hydrochloride combination. Seven days after this manupulations, animals were divided into 4 groups (n=18). Testosterone propionate applications, at the 150 mg/kg dose, were applied at twice to all animals via intraperitoneal route at the 2nd and12th weeks of the experiment. Animals were sacrified (n=6 for the dates of 2nd, 6th and 12th weeks) using high dose of xylazine-ketamine combination of at the end of experiment and the hearts of rats were collected for pathomorphological and immunohistochemical examinations.

Group I (pure Lonidamine+testosterone): After testosterone propionate administration according to procedure, 2 mg/kg/day dose of Lonidamine hydrochloride was dissolved into PBS was applied to prostate of rats in this group (n=18). For this aim, in this group, the lower abdomen was opened 1.5 cm under the anesthesia of the animals and 200 µl sterile samples were injected with the insulin injector into ventral prostate loops and the animals were awakened by suturing the abdominal membranes, muscles and skin of the animals after injection. Group II (Lonidamine-loaded nanoparticles+testosterone): After testosterone propionate administration using same method, lipid-polymer hybrid nanoparticles containing 2 mg/kg/day dose of Lonidamine hydrochloride was applied to the prostate of rats (n=18).

Group III (blank nanoparticles+testosterone): After testosterone propionate administration using same method, lipid-polymer hybrid nanoparticles at same dose was applied to the prostate of rats (n=18).

Group IV (testesterone): Only testosterone propionate using same method was applied to prostate of rats in this group (n=18).

Macroscopic and Histopathological examination

Heart samples were collected from all animals. Sizes (cm) of left-right atriums and ventricles at outside measurements were measured using digital compass and mean ± standard deviation was calculated for each determined dates of experiment in all groups. Then, the tissues were fixed in 10% buffered formalin. After fixation, the tissues were processed through degraded alcohol and xylene series and embedded in paraffin wax. Sections of 4µm thickness were cut from the paraffin blocks. The sections were stained with haematoxylin-eosin (H&E) and Masson's Trichrome stainings (by being followed to instructions of manual Bioptica, Italy) and evaluated under a light microscope (Olympus BX51 digital microscope) and illuminated using camera attachment (Olympus DP25 camera). A total of 10 High Power Fields in 400x magnification (10 HPFs) was counted for scoring of histopathological findings. For calculation of mean ± standard deviation, the mean scores were entered in each column established for all groups on the Excel spreadsheet and their standard deviations were calculated (Microsoft Excel Program).

Immunohistochemical method

In the study, in harmony with its procedure, the strep Avidin-Biotin Complex Peroxidase (strep ABC-P) kit (Peroxidase Detection System, RE7110-K, Leica, Novocastra) was used. The sections taken from the paraffin blocks were used. Deparaffinized and rehydrated sections were digested with trypsin for 10 min under 37º C.

Then, for revealing of antigenic determinats, sections placed in citrate buffer (pH 6.0) were kept in a microwave oven at 600 W for 15 minutes. To eliminate endogenous peroxidase activity, the tissues were kept in 3% hydrogen-peroxide (H2O2)-methanol solution for 15 minutes. Non-specific protein activity was prevented with the use of blocking serum (Novocastra, Leica). Incubation with primary antibodies (Cyclin D1, ABIN782606, in 1:400 dilution), (bFGF, ABIN726425, in 1:500 dilution), (p16, Santa Cruz, sc-1661, in 1:200 dilution) was left overnight at +4°C. The rest of the procedure was executed in accordance with the streptavidin biotin complex peroxidase (Strept ABC-P) staining method (Novocastra, Leica). In this process, the tissue slices were rinsed twice for 5 minutes, using PBS at the end of each phase, except in the the protein blocking phase. For the control sections, PBS was used instead of primary antibody as the negative control. Diaminobenzidine (DAB) was used as chromogen, while Gill's hematoxylin was used as ground staining. The slices were covered using Entellan® which is a non-aqueous mounting medium.

RESULTS

Macroscopical findings

In terms of lenghts and widths of atrium and ventricles for left and right sites, measurements in Group I were lower than in that of Group II. However, when compared to Groups I and II which were administrated by different Lonidamine forms, there were much higher measurements in Groups III and IV. As compared between Group III and Group IV, identical measurements were obtained. The atrial dimensions were slightly increased at 4th and 12th weeks when compared to initial week of the experiment. There was no significant increase in atrial measurements of Group I and II when compared to that of other groups. In terms of ventricle dimension for left and right sites, the situation amongst groups was like to be in atrial measurements. The mean digits were lower in first two groups when compared to other groups. The situation was found differently during experimental procedure. The mean digits followed stable without any significant change for all groups. All results are illustrated in Table-2.

Histopathological and histochemical findings

Cytoplasms and nuclei of cardiomyocytes were found more hypertrophic in some areas of left ventricles of Group III and IV. However, in Groups I and II, cardiomyocytes were often normal in appearence. Additionally, a number of fibrocytes and fibroblast were accorded to the interstitium of cardiomyocytes in Groups III and IV. Especially, significant increasement were detected in Group III when compared to Group IV during the experiment. The mean digits for 2nd and 12th weeks of the experiment were found identical in Group IV. The cells in affected areas were easily differentiated in special staining. However, the number of these connective tissue cells were much lower in Groups I and II, which were administrated with pure Lonidamine and nanoparticulate form of Lonidamine, during the experiment process (Figures-1 and 2).

Basic Fibroblast Growth Factor (bFGF) expressions

The expressions in all experimental groups showed great parallelism to each other. The positive reactions were observed in cytoplasm of the fibrocyte and fibroblast as brownish color. These expressions during ongoing time were become more increased in all groups. However, the increasement was slighter in Groups I and II when compared to Groups III and IV at each euthanasied day (Figure-3).

Cyclin D1 expressions

The positive reactions were seen in cytoplasm and lesser degree of nuclei as brownish color. The expressions were in low level in Groups I and II. Particularly, those expressions were slightly-higher in 12th week of the experiment when compared to be in 2nd and 4th week. The level of expressions was much higher in Groups III and IV at each euthanasied day. But, cyclin D1 expressions were slightly higher in 12 th week of the experiment. The expression levels between 4th and 12th weeks of experiment were similar to be in histopathological results and bFGF expressions (Figure-4).

P16 expressions

The positive reactions were seen in cytoplasm and nuclei, previously. In general, the expressions were more decreased when compared to previous expressions. The level of expressions was again in low during experiment in 2nd week of Groups I and II. In the ongoing period of the experiment, the level of expression kept in stable. However, in Groups III and IV, the expressions were higher in 2nd week when compared to other euthanasied day of experiment. The level of expresssion were getting more decreased until 12th week in Groups III and IV. In particular, prominent decreasing were observed in 12 th week of Group IV. When a comparison of 12th week of experiment between Groups III and IV, the expression was lower in Group IV than in that of Group III (Figure-5). All histopathological scores and immunohistochemicals expressions obtained in the study are illustrated in Graph-1.

Table-2: Measurements of atrium and ventricle according to groups

Duration of experiments / Groups	2nd week				4th week				12th week			
	GI	GII	GIII	GIV	GI	GII	GIII	GIV	GI	GII	GIII	GIV
Lenght												
Right atrium	2.40±0.83	3.25±0.38	4.25±0.90	4.16±0.37	2.91±0.60	3.00±0.57	4.21±0.73	3.75±0.62	3.00±0.28	3.50±0.28	4.00±0.50	3.50±0.28
Left atrium	3.08±0.93	3.50±0.28	4.41±0.73	4.75±0.55	3.58±1.01	3.58±0.73	4.36±0.37	4.83±0.23	3.75±1.34	4.08±0.53	4.08±0.18	4.50±0.40
Right ventricle	15.80±2.48	14.91±0.83	13.66±3.14	13.5±2.06	12.5±2.06	11.93±0.60	13.41±1.78	14.16±0.74	11.83±3.84	12.66±1.49	13.66±1.34	14.25±0.80
Left ventricle	10.58±1.96	11.75±0.90	12.33±1.79	13.16±2.06	11.5±2.75	10.41±0.93	13.33±1.49	13.75±1.57	11.5±1.97	10.66±1.14	13.08±1.09	13.66±0.84
Width												
Right atrium	1.16±0.2	1.66±0.37	1.80±0.47	1.58±0.18	1.00±0.50	1.75±0.38	1.83±0.23	2.33±0.23	2.33±1.46	1.40±1.8	2.66±0.37	2.33±0.23
Left atrium	1.91±0.83	1.58±0.44	1.50±0.50	1.75±0.25	1.33±0.37	1.82±0.44	2.91±0.34	4.15±0.40	2.00±1.00	1.41±0.34	3.91±0.18	4.75±0.47
Right ventricle	1.40±0.20	1.91±0.34	2.16±0.47	2.58±0.34	1.50±0.40	1.75±0.25	2.16±0.47	2.58±0.34	1.25±0.25	1.58±0.34	2.50±0.28	3.00±0.28
Left ventricle	2.91±1.01	2.83±0.23	4.50±0.76	4.33±0.37	2.66±0.47	3.58±0.73	3.75±0.25	4.66±0.37	3.00±0.00	3.08±0.18	3.75±0.25	4.50±0.40

The scoring was given as mean±standard deviation (SD) by calculation of total animals in each group. Experimental groups were coded from GI to GIV. (GI: Group I; GII: Group II; GIII: Group III; GIV: Group IV). The results were given on the basis of centimeter (cm).

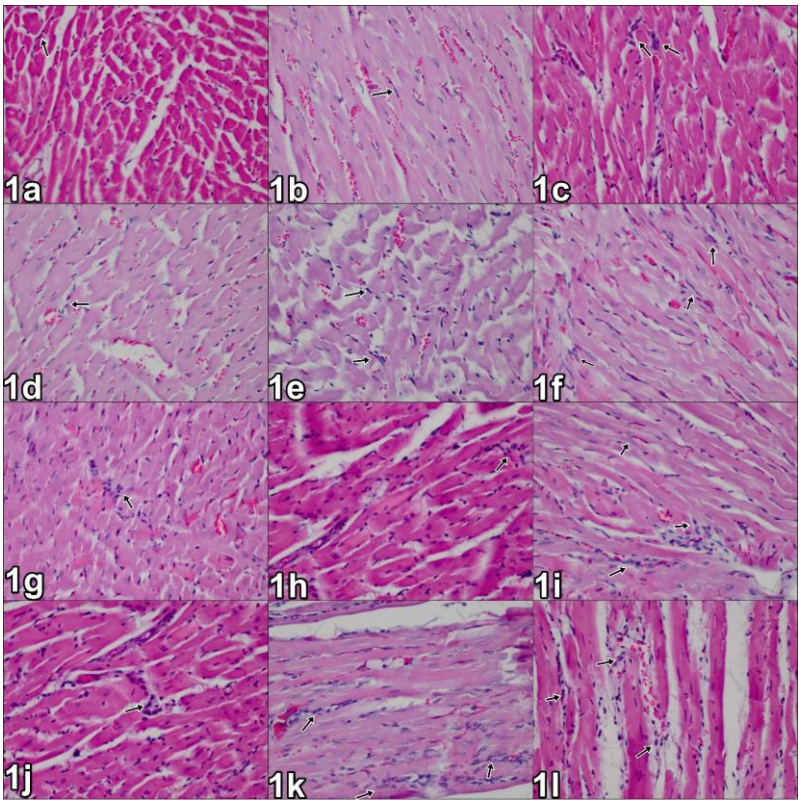


Figure 1. Progress of cardiac fibrosis in all groups during 2nd to 12 th week. Fibrocytes were marked with arrows. Group I (a-c), Group II (d-f), Group III (g-i), and Group IV (j-l), x400, HXE staining.

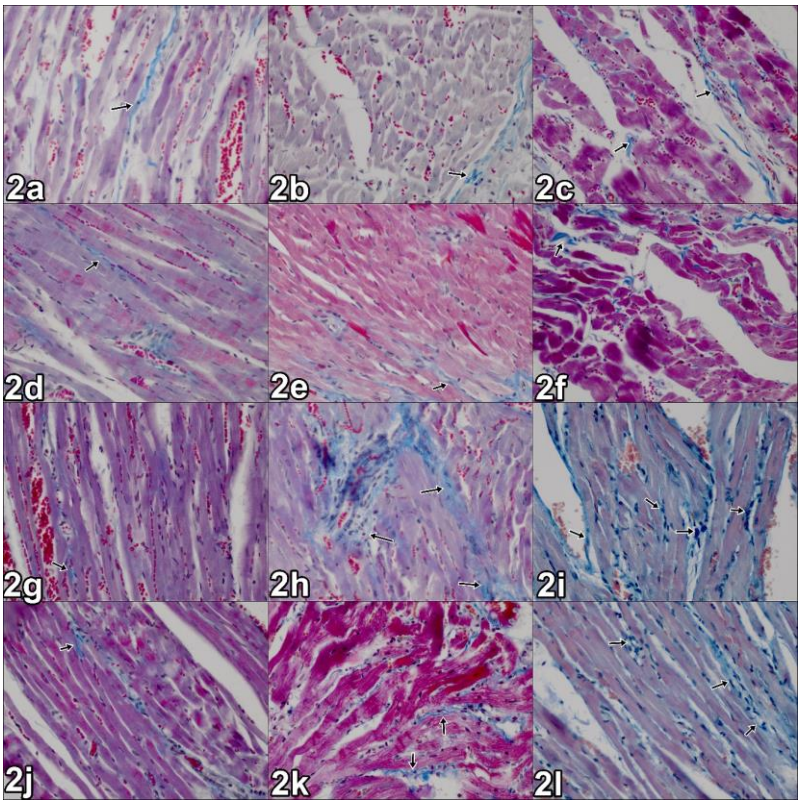


Figure 2. Progression of cardiac fibrosis in all groups during 2nd to 12 th week by Masson's trichrome stain. Group I (a-c), Group II (d-f), Group III (g-i), Group IV (j-l), x400, Masson's Trichrome stain.

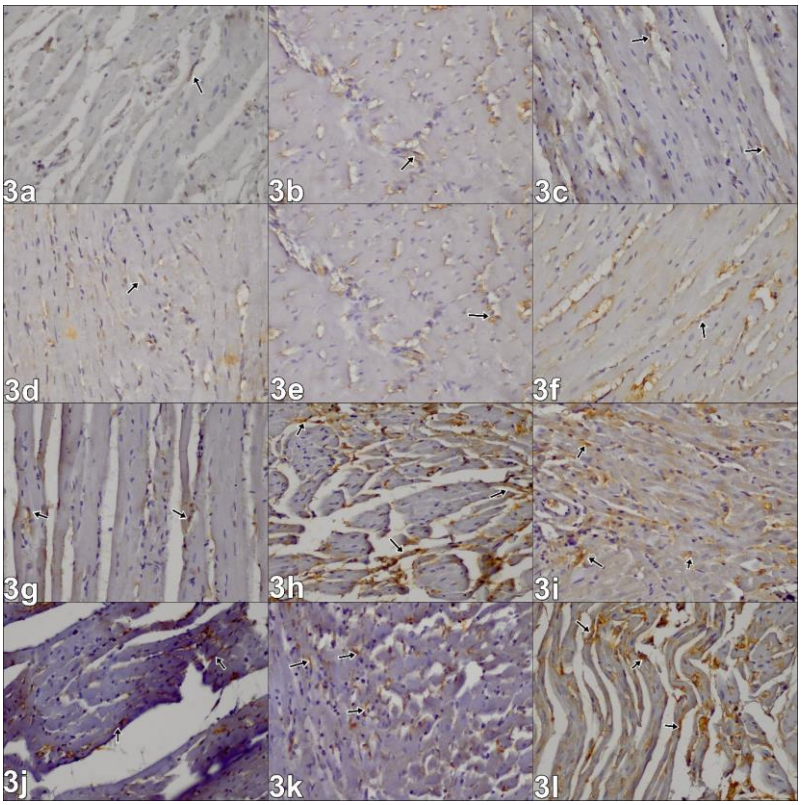


Figure 3. bFGF expressions in cardiac tissue in all groups during 2nd 12th week. Group I (a-c), Group II (d-f), Group III (g-i), Group IV (j-l), x400, Strept ABC-P immunostaining with Gill's hematoxylin and DAB Chromogen.

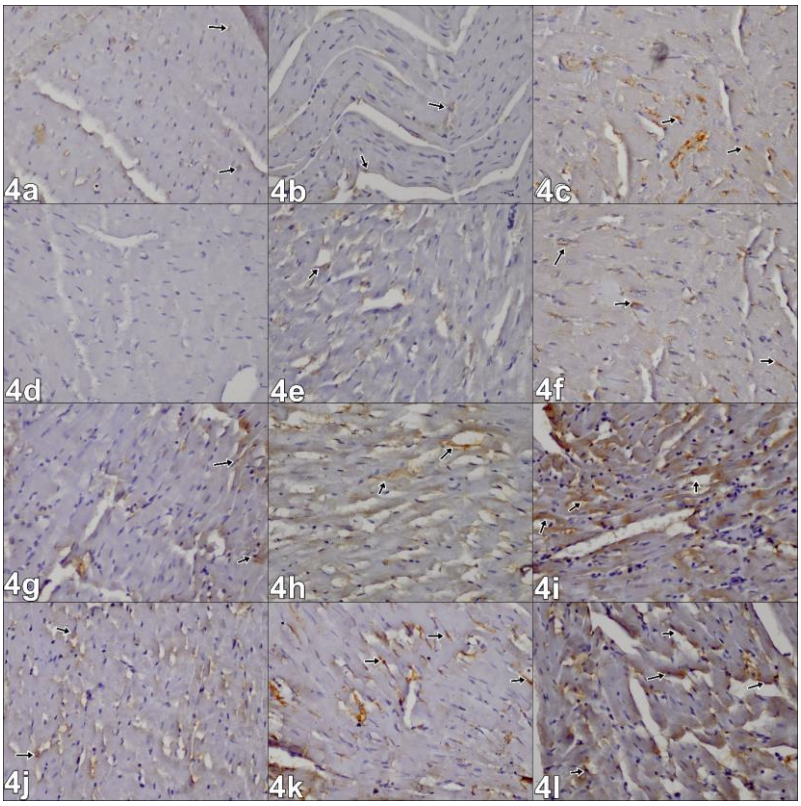


Figure-4. Cyclin D1 expressions in cardiac tissue in all groups during 2nd 12th week. Group I (a-c), Group II (d-f), Group III (g-i), Group IV (j-l), x400, Strept ABC-P immunostaining with Gill's hematoxylin and DAB Chromogen.

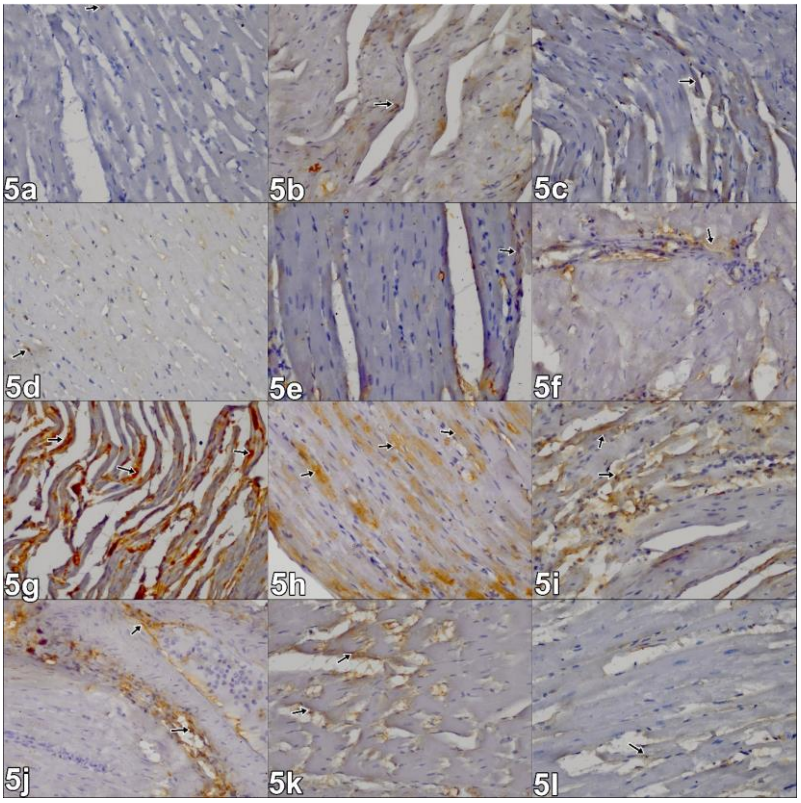
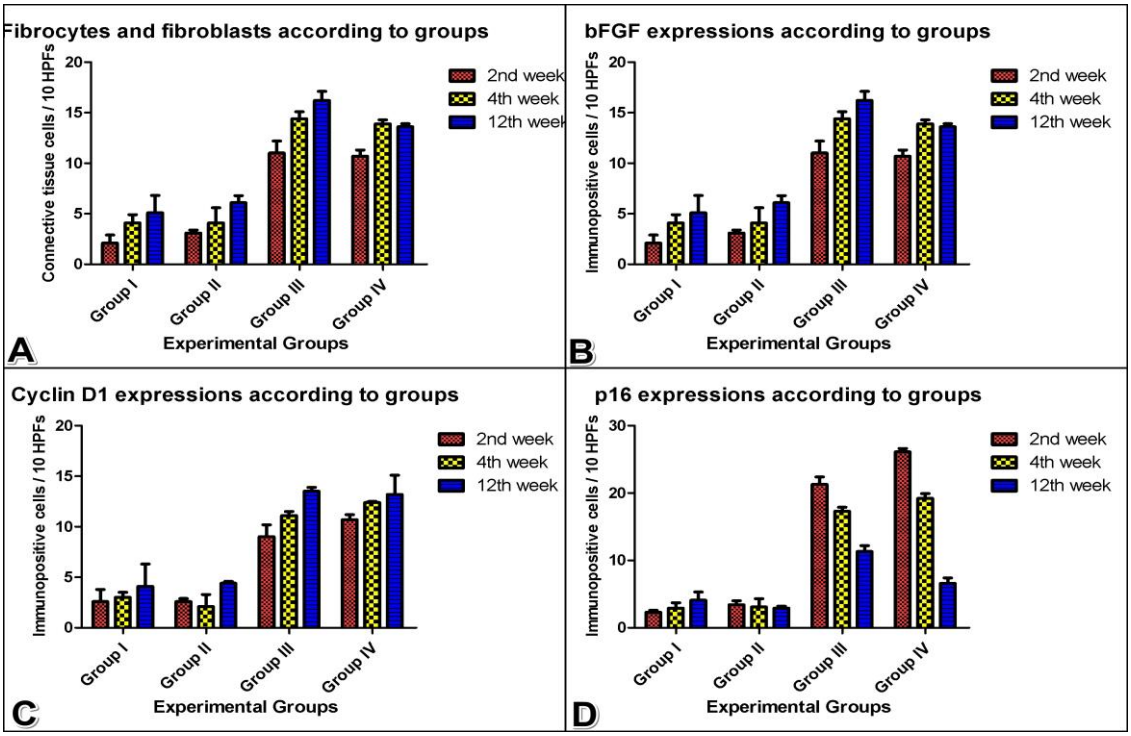


Figure-5. P16 expressions in cardiac tissue in all groups during 2nd 12th week. Group I (a-c), Group II (d-f), Group III (g-i), Group IV (j-l), x400, Strept ABC-P immunostaining with Gill's hematoxylin and DAB Chromogen.



Graph 1. Graphical comparison of histological and immunohistological data between groups.

DISCUSSION

In this study, development of cardiac fibrosis induced by high-dose testesterone and the reversal effect of Lonidamine in nanoparticle and pure form has been investigated in adult and neutered rats.

Testosterone which is known as anabolic steroid and its supplementation has a negative effect onto cardiovascular functions after chronic misuse (Toma et al. 2012, Culic 2015). In general, systolic and diastolic dysfunction are associated with its usage in the presence of myocardial hypertrophy (Hassan et al. 2009, Angel et al. 2012, Pirompol et al. 2016). Pirompol et al. (2016) has studied the development of cardiac hypertrophy in the early and late phases of testosterone administration in spite of experienced in 4th, 8th and 12th weeks of experiment. However, they observed most prominent findings in relation to the hypertrophic development in cardiac muscles after long-term treatment by testosterone. In our study, we also observed the cardiac hypertrophy both in early and late phase (12th week). However, the findings were increased toward 12th week of experiment. Macroscopical findings supported these results. Both atrial and ventricle dimensions were increased in Groups III and IV when compared to

Lonidamine treated groups or Groups I and II. In this point, we have found a strong correlation between cardiac hypertrophy and testosterone administration. Particularly, we observed most outstanding hypertrophic changes in solely testesterone group or Group IV and free nanoparticle form of Lonidamine group or Group III. In spite of that we found more decreased in cardiac hypertrophy in group administrated by pure form of Lonidamine group or Group I and Lonidamine-loaded hybrid nanoparticles or Group II. As different above, we encountered also different histopathological findings in association with cardiac fibrosis in the interstitial areas by using special stain. Cardiac fibrosis was more common and outstanding when compared to hypertrophy in cardiac muscles. These lesions and others such as disorganized muscle fibers, misshapen nuclei and increasing apoptosis have also been described in previous studies (Belhani et al. 2009, Papamitsou et al. 2011, Angell et al. 2012, Angell et al. 2014).

Although there are several postulates resulting in cardiac failures, molecular mechanism is not fully understood. In an investigation, which studied in heart of adult transgenic mice, cyclin D2 overexpression has been resulted in significant cardiomyocyte proliferation (Pasumarthi et al. 2005). Because cyclin D1 as cell cycle regulator is an important factor in the development of proliferative disorders, there have been found some studies in relation to the role of cyclins in development of fibrosis at different organs except for cardiomyocytes (Fu et al. 2004, Kato et al. 2005, Watts et al. 2006). In our study, we described an increased cyclin D1 expression in especially testosterone induced group and Lonidamine nanoparticle free group during the experiment. In other groups, which treated with Lonidamine, we observed significantly lower expressions when compared to other groups during the 2nd, 4th and 12th weeks of experiment. We attributed this situation to both development of cardiac fibrosis and cardiac hypertrophy in heart muscle under testosterone activity.

In contrast to this information, cell cycle inhibitors have a potential effect on recession of cardiac lesions. In another investigation which studied in neonatal and adult hearts, it is reported that there are low levels of cell cycle inhibitors such as cyclindependent kinase inhibitors (CDKIs) due to an increased cardiomyocyte number (Di Stefano et al. 2011). In our study, we also encountered with a serious down-regulation of p16 for Groups III and IV, which is another cyclin dependent kinase inhibitor. In these groups, the expressions were higher in 2nd week. However, the expressions were decreased in ongoing times. On the other side, in Group I and II, the expressions were very low during 2nd, 4th and 12th weeks of experiment when compared to Group III and IV. According to recent literatures (Zhu et al. 2013, Meyer et al. 2016, Xie et al. 2017), it is stated that p16 might have antifibrotic role on progression of fibroblast senescence. For this situation in terms of literature searches, we postulate that there is a possible association between p16 as an inhibitor of cycline dependent kinase and cardiac fibrosis. In this point, Lonidamine known as anti-hyperplastic agent might have been related likely to inhibit the function of p16 because it is encountered to a possible relation between low p16 expression and fibrosis or fibrocyte proliferation.

On the other side, Lonidamine (LND) has antiproliferative activity for long time (Caputo and Silvestrini 1992). Particularly, this effect is produced by inhibiting hexokinase activity and consequently disturbing mitochondrial process (Floridi et al. 1981a; Floridi et al. 1981b). In previous reports regarding dose dependent effectivity of LDN, it is mentioned that it has selective on normal tissues in presence of optimal dose and it has no disadventageous on normal cell progression (Caputo and Silvestrini 1992; Price et al 1995; Price et al. 1996). However, there have been documented that LND might also affect negatively normal cells in contrast to previous knowledges. Especially, cell death, fibrosis and also

decreasement in vascularization might develop after blokade of hexokinase activity in cardiac tissue during experimental studies (Wu et al. 2011; Pasdois et al. 2012; Nederlof et al. 2016). Hence, several hekzokinase blockers like LND might not make an positive effect on intact cells at every time. In this study, we did not observe any negative effects on healthy cardiomyocytes in contrast to recent knowledges. We detected only anti-fibrocytic activity on cardiac tissues. But, to the best of author knowledges, this situation might be resourced from dose-dependent response. Some intact cardiomyocytes as well as hyperplastic cells would be negatively affected from overdose LND if proper dose for body weight was not selected. However, this knowledge obtained from our study should be supported by new in-vivo studies considering dose-dependent response.

In conclusion, we believe that testosterone administration can create cardiac hypertrophy and fibrosis for long duration as much as to effect macroscopical measurements of atrium and ventricles. In development of this process, cyclin dependent kinase and its inhibitors may have a potential role according to results of the experiment. Lonidamine may be usefull preventive medicine for cardiogenic failures originated from excess hormon usage. But, to show exact effectiveness of Lonidamine, it needs to be supported by new investigations in this way and to be experimented by different dosage in rat modelling. We believe that only when the results to be obtained from new phase investigations, a proper dosage can give to the patients with cardiac problems.

Conflict of Interest
There is no any conflict of interests with other authors, sources and the funding program.

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