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REGULAR ARTICLE

INFLUENCE OF TNF ALPHA ON TESTOSTERONE INDUCED CARDIAC EFFECTS IN ISOLATED FROG HEART MODEL

A. Kanikkai Raja, A. Babu Vimalanathan, Rajeev Kumar, Margaret Shanthi FX, Naman Hirenkumar Shah, Manoj G. Tyagi*

Department of Pharmacology and Clinical Pharmacology, Christian Medical College and Hospital, Bagayam, Vellore-632 002

SUMMARY

The effect of Tumor necrosis factor-alpha and testosterone was evaluated on the isolated *Rana tigrina* frog heart. The isolated frog heart (n= 10) were used for this study and the effect of drugs on myocardial contractility and heart rate was ascertained. Namely three drugs testosterone (4mg), bicalutamide (8mg) and TNF-alpha antagonist infliximab (0.4mg) were utilized for this study. Testosterone caused an increase in heart rate by 11.28% and decrease in myocardial contractility by 17.67%. Similarly bicalutamide caused a 20.11% decrease heart rate and 41.17% decrease in myocardial contractility. To ascertain the role of TNF-alpha, infliximab was perfused through the heart and it potentiated the inhibitory effect of testosterone on the myocardial contractility and heart rate. The results of this study suggest the importance of TNF-alpha and testosterone hormone in cardiovascular pathophysiology.

Keywords: Tumor necrosis factor-alpha, Testosterone, *Rana tigrina*, heart, infliximab, myocardial contractility

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***Corresponding Author**, *Email*: tyagi239@yahoo.co.in

1. Introduction

Tumor necrosis factor-alpha (TNF-alpha) antagonists have emerged as effective therapy for patients with rheumatoid arthritis (RA), Crohns disease (CD) and other conditions. Early data suggested that they might be a promising treatment for heart failure (HF). However, randomised controlled trails of TNF-alpha antagonists in HF patients were halted due to lack of benefit or trends towards worsened HF in patients receiving higher TNF-alpha antagonist doses[1]. Testosterone depletion (castration) or subcutaneous testosterone blockade 4 wk before I/R improved cardiac functional recovery and decreased myocardial cytokine production, inflammatory signalling and expression of apoptotic-related proteins[2]. Testosterone is the predominant androgenic hormone and has a vast range of biological actions.

The classical effects of testosterone are the induction and maintenance of secondary sexual characteristics and preservation of libido, sense of well-being, lean mass and bone density. The hormone is involved in a negative biofeedback mechanism on the hypothalamic-pituitary axis to inhibit gonadotropin secretion. It has long been recognized that testosterone

has an immune–modulating action. The greater incidence of immune-mediated disease in women and androgen deficient men has attributed to the immunosuppressive effects of androgens compared with estrogens [3]. Circulating and cardiac TNF-alpha levels are elevated in heart diseases such as dilated cardiomyopathy, myocardial infraction and left ventricular (LV) Pressure overload [4, 5]. A high–cholesterol diet and environmental tobacco smoke have detrimental effects on endothelial function of male animals, effects was exacerbated by testosterone at physiological concentration (6).

In 1975, Carswell et al. first identified tumor necrosis factor (TNF), an endotoxin-induced serum factor that caused necrosis of tumors [7].A decade later, investigators isolated a protein from endotoxin treated cells that was named cachectin because of its presumed role in the molecular basis of cachexia [8]. The subsequent cloning of the genes encoding cachectin and TNF alpha confirmed that these two molecules were identical. Produced as a pro-hormone of 233 amino acids, TNF-alpha is anchored in the cell membrane and then processed to a 157 residue mature protein by cleavage of a 76 residue signal peptide. In response to a wide variety of infectious or inflammatory stimuli (e.g. lipopolysaccharide, viruses, fungal or parasitic antigens, interleukin-1 (IL-1), TNF alpha), both transcription and translation of TNF precursor is increased and large amount of mature protein are rapidly released in to the circulation. However, not all of the TNF-alpha is released, as some remains cell –associated in a transmembrane form [9].

Tumor necrosis factor regulates the expression of a variety of peptide regulatory factors including IL-1, IL-6, platelet derived growth factor, transforming growth factor-beta as well as a group of eicosanoids factor and adrenaline [10]. It is produced by a host in response to stress-infection, inflammation, tissue injury and shock and has recently been recognized as myocardial depressant substance.

Since the first observation by Levine noting increased levels of circulating TNF-alpha occurring in patients with severe symptomic heart failure, further clinical studies have supported the potential importance of TNF-alpha in the pathogenesis of this disease process [11]. In an in-vivo experiment by Bozkurt and colleagues, adults were surgically implanted with an osmotic minipump and infused with TNF-alpha in order to reach plasma levels comparable to those reported in clinical heart failure. It showed TNF-alpha mediated myocyte injury and cell loss which may be due to immune-related cytotoxicity. In a rat model of TNF-alpha infusion, Bozkurt and colleagues observed a small increase in the overage LV myocybes-cross sectional area and decrease in the calculated number of myocytes across the transmural thickness of the LV wall[12].

Transgenic mice over-expression myocardial TNF-alpha demonstrated increased soluble myocardial collagen, which is suggestive of reduced collagen cross linking [13]. Human endothelial cells treated with TNF-alpha had increased levels of MMP mRNA and demonstrated increased levels of MMP activation [14]. Furthermore, human smooth muscle cells stimulated with TNF-alpha demonstrated increased MMP activity and de novo synthesis and secretion of MMP-1[15] Adult rat cardiac fibroblasts stimulated with TNF-alpha have demonstrated increased MMP activity [16].

Laboratory studies using animals with experimentally induced inflammatory disease have reported a beneficial result with testosterone [17]. Moreover, there are small clinical trials of testosterone therapy in humans with rheumatoid arthritis in which major improvements of clinical status and inflammatory markers have been recorded [18]. Laboratory evidence has suggested that testosterone suppresses pro-inflammatory cytokines and may up-regulate anti-inflammatory cytokines. Testosterone incubated in cell culture attenuates the production of inflammatory cytokines such as TNF-alpha, IL-1beta, and IL-6 in human

macrophages [19], human monocytes [20], human gingival fibroblasts [21] murine and human osteoblasts [22,23] and human endothelial cells [24]. Furthermore, anti-inflammatory cytokines such as IL-10 are stimulated in the presence of testosterone [25, 26]. Injection of bacterial endotoxin into castrated male mice increased the endogenous production of TNF-alpha, which was abrogated by testosterone replacement [27]. Elderly men with hypogonadism induced with gonadotropin hormone-releasing agonists developed significant increases in serum TNF-alpha and IL-6 [28]. Testosterone replacement induced reductions in TNF-alpha and IL-1beta and an increase in IL-10 with positive co-relation in hypogonadal male [29]

It has been demonstrated that pro-inflammatory cytokines, such as TNF-alpha and IL-1beta, have an inhibitory role in gonadal functions, especially on the steroidogenesis in leydig cells. Elevated TNF-alpha and IL-1 levels have been measured in human patients with critical illness, CHF, burns, and sepsis [30,31, 32], who also experience depressed gonadal functions with low serum testosterone levels [33,34]. Administration of TNF-alpha to healthy men and rats also causes a decrease in serum testosterone levels [35,36] while treatment with TNF-alpha or IL-1 causes an inhibition of steroidogenesis in cultured leydig cells. Pro-inflammatory cytokines TNF-alpha, IL-1, and IL-6 inhibit testicular leydig cell steroidogenesis at the level of gene expression of different steroidogenic enzymes [37].

Steroidogenesis in leydig cells is started with cholesterol transfer into the mitochondria, mediated by the steroidogenic acute regulatory (STAR) protein. Cholesterol is converted to pregnenolone by the cholesterol side chain cleavage enzyme (P450scc) in the mitochondria. Pregnenolone is then converted sequentially to progesterone by 3beta-hydroxysteroid dehydrogenase (3ß-HSD), to 17alpha-hydoxyprogesterone and then to androstenedione by 17alpha-hydroxylase (P450c17), and finally to testosterone by 17beta- hydroxyateroid dehydrogenase. Steroidogenesis in leydig cells is primarily regulated by the pituitary gonadotropin, luteinizing hormone (LH) through the production of the intracellular second messenger cyclic AMP (cAMP) which stimulates steroidogenesis by increasing the expression of steroidogenic-enzyme genes[38] Nur77, which is also known as NGFI-B, TR3, and NAK-1, is a member of the Nur77 gene family. These factors are highly homologous in the zinc finger DNA-binding domain, moderately homologous in the ligand-binding domain, and somewhat divergent in the N terminus[39]NF-κB is also able to repress the activities of other transcription factors, such as steroid receptors[40].

2. Materials and Methods

Materials Testosterone enanthate injections were purchased from German Remedies, Mumbai. Infliximab recombinant, Remicade were purchased from Fulford India Limited, a subsidiary of Schering-Plough Corporation, Mumbai, India. Bicalutamide injections were purchased from Intas Pharmaceuticals Ltd., Matoda, Ahmedabad, India. All other reagents and chemicals were of analytical grade.

Kymograph Starlings Heart lever and Kymograph (Inco, Ambala, India) were used to record the responses of testosterone, bicalutamide and infliximab on suited kymograph papers.

Physiological solution: Frogs Ringer solution was used as a perfusion solution to maintain the rhythm of the isolated tissue. The composition of Ringer solution includes NaCl (6.5g/ L), NaHCO3 (0.2g/ L), KCl (0.14 g/L), NaH2Po4 (0.01 g/L), Dextrose (2.0 g/L) and Cacl2 (0.12 g/L). This frog ringer reagent was diluted with one liter of distilled water and adjusted to pH of 7.3.

Isolated Frog Heart Preparation Using Symes Technique:

Isolation of frog heart was done by standard procedure (41). Briefly, an Indian

frog (*Rana tigrina*) was stunned after ether anesthesia and abdomen was cut and opened. The pectoral girdle was cut using a bone cutter and pericardium was removed carefully. Syme's cannula was connected to the reservoir containing frog Ringers solution and introduced immediately into the Sinus venosus of the frog's heart. The connecting blood vessels were cut and heart was isolated from the animal and mounted on to a stand. Heart was connected to the Starling's heart lever and adjusted for recording the responses of the heart. The level of frog Ringer solution in the Syme's cannula was maintained by fixing a glass tube into the cork fixed to the reservoir (Marriott's bottle) tightly. The heart was allowed to stabilize for 30 minutes before measurement of heart rate and cardiac output. The recordings were made on a slow rotating drum, to which a suited kymograph paper was affixed.

Procedure:

Initially, Frog Ringer solution was perfused in to the isolated frog heart and the results of heart contraction and heart rate was noted. This first frog ringer solution indicating results is normal control values.

First time the testosterone (it's called Testosterone 1) was per fused in to the frog heart and the results of heart contractility and heart rate was predicted.

After perfusion of testosterone 1, we waited for 10 minutes to get baseline, meanwhile the frog ringer solution was perfused.

Likewise bicaluatamide, testosterone 2, ringer 2, infliximab and testosterone 3 were per fused periodically in to frog heart and the results of heart contractility and heart was noted.

The Effect of testosterone was studied by perfusing frog Ringer solution containing testosterone, bicalutamide, infliximab solution to the isolated frog heart preparation. This test solution was perfused in to the heart periodically (10 minutes for each drug). The parameters studied included force of contraction and heart rate (n=10). Then this parameter was compared with control frog ringer solution.

Statistical Analysis

Statistical analysis of parameters of cardiac rate and myocardial contractility were performed by using of Wilcoxon signed Ranks test. Values were expressed as mean ± SEM with values of $p<0.05$ considered significant.

3. Results

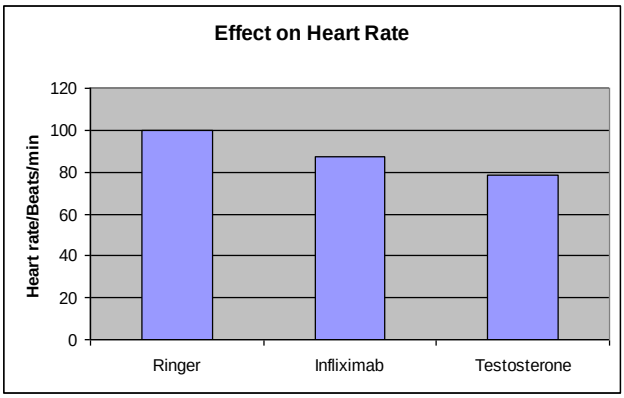
Table: 1. Effects of Testosterone, Bicalutamide, Infliximab on frog heart

Exp.	Heart Rate(Beats/min)	Myocardial Contractility(Amp. of contraction)
Ringer	100	100
Testosterone 1	111.28 ± 9.42	83.33±15.72
Bicalutamide	79.89±6.46	58.83±10.78
Testosterone 2	92.49±2.74	93±10.64

According to results shown in table, testosterone showed increase in heart rate by 11.28% and reduction in myocardial contractility by 17.67% which was significant. Effect of bicalutamide on testosterone induced cardiac effects was not significant. But bicalutamide itself has produced reduction in

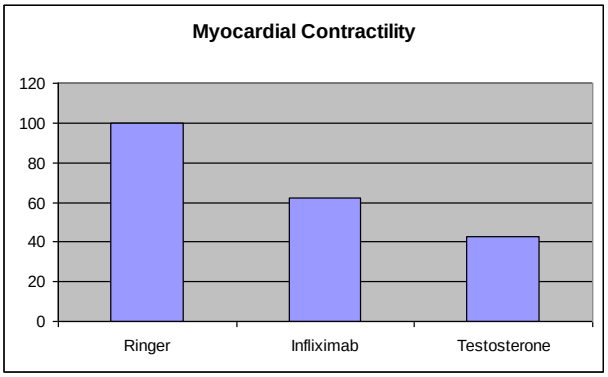
both heart rate and myocardial contractility which was significant statistically.

Figure 1: Heart Rate; Infliximab on Testosterone.



In the figure 1 indicates the effect of Infliximab on testosterone in the heart .In this study the infliximab act as a crucial role,it reduce the heart rate of testosterone treated heart .The 'p' value is less than.0.05 (p<.005).

Figure 2: Myocardial Contractility; Infliximab on Testosterone.



In the figure 2 indicates the effect of Infliximab on testosterone in the heart. The Infliximab decrease the myocardial contractility of testosterone treated heart. The 'p' value is less than 0.05 (p<0.007).

The 'p' values of cardiac rate and myocardial contractility of infliximab and testosterone is 'significant'.

4. Discussion

The main findings of the present study has suggests that the anti-tumor necrosis factor drug, infliximab potentiates the inhibitory effect of testosterone on the myocardial contractility and heart rate.

Cardiovascular disease shows a consistent male to female ratio of 2.2 among different population despite wide variation in absolute rates. Most studies have focused on the role of estrogen in cardiac protection, whereas there is less data which exist regarding the effect of testosterone in cardiac injury and it may be important because the heart can accumulate testosterone at higher concentration than other androgen target organs [42]and functional androgen receptors are present in isolated cardiac myocytes [43]. A vast number of studies have been done to show association between low testosterone and cardiovascular disease or its risk factors. In rats, androgen therapy improves coronary blood flow and increases both fractional shortening and peak myocardial oxygen consumption, thereby improving cardiac function[44].Testosterone therapy has been used to treat men with angina; the beneficial effects on both ischemia and exercise tolerance have been demonstrated in several studies. Numerous reports from animal studies have demonstrated the vasodilator properties of androgens in several vascular beds, both in vitro and in vivo. In humans, testosterone reduces blood pressure and enhances relaxation of brachial arteries; direct injection into coronary arteries produces dilatation and increased coronary blood flow [45-47]. These vasodilatory actions of testosterone observed at supra-physiologic doses are endothelium and androgen receptor independent, and to be mediated via membrane ion channels of smooth muscle, where low-dose testosterone may be acting via endothelium dependant release of vasoactive substance like nitric oxide. TNF-alpha is pro-inflammatory cytokine and is increased in infective and inflammatory disease. Atherosclerosis is acknowledged to be a disease of chronic inflammation. TNF-alpha is both a marker and a mediator of

atherosclerotic plaque development and complications. Serum TNF alpha is greatly increased during acute coronary syndromes but is also increased to a lesser degree in stable chronic angina and is a marker of future events[48].There are no clinical trial data concerning the effects of testosterone on left ventricular function. TNF is produced mainly by macrophages, but also by the myocardium in CHF. It impairs synthesis and promotes catabolism of skeletal muscle, and reduces testosterone production. It causes endothelial dysfunction and impairs production of NO by endothelium [49]. Administration causes left ventricular dysfunction and heart failure in humans; anti-TNF therapy may improve cardiac function[50].

In this study, we have suggested that the increased concentration of testosterone can cause severe cardiovascular problems. The expression of testosterone was inhibited by TNF-α antagonist. Testosterone showed increase in cardiac rate and decrease in myocardial contractility. These results may cause cardiovascular problems or can worsen pre-existing heart condition. Bicalutamide was not able to reverse the testosterone induced cardiac effects as results were insignificant (data not shown here). So, testosterone might be acting via some different pathway other than through androgen receptors. On other hand, increased heart rate due to testosterone was minimized by TNF-α antagonism with infiliximab with statistical significance. So TNF-α antagonism with infiliximab might interfere with multiple pathways known to impair endothelial cell function.

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