Cardiac Depression Produced by L-Arginine and Phosphodiesterase Inhibitor on Isolated Mammalian Rabbit Heart: Function of Cyclic Guanosine Monophosphate (cGMP)

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Abstract: Problem statement: Cyclic guanosine 3',5'-monophosphate cGMP is one of the important second messengers that determines the cardiomyocyte activity and its role in healthy and diseased cardiac muscle is still controversial. We are reporting the effect of adding L-arginine, the NO donor that stimulates cGMP production and Sildenafil citrate (phosphodiestrase inhibitor) that inhibits cGMP hydrolysis on isolated rabbit’s heart to answer: Is it safe to prescribe phosphodiesterase inhibitors for men with low cardiac output?

Approach: Isolated hearts from 6 rabbits were perfused using Langendorff’s apparatus in which the perfusion fluid was ringer-Locke solution, applied at constant flow rate and was continuously bubbled with a mixture of 95% oxygen and 5% carbon dioxide. Each heart served as its own control before infusion of L-arginine in concentration of 3 mmol L\textsuperscript{-1} and Sildenafil citrate 1.5 mg L\textsuperscript{-1} simultaneously. Their effects were recorded after 1, 3, 5 and 10 min. The effluent fluid was collected for cardiac enzymes assay after 5 and 10 min.

Results: Data showed that the infusion of L-arginine and Sildenafil citrate produced negative inotropic and chronotropic effects. Also, the cardiac enzymes were significantly elevated.

Conclusion: The present study, which was carried out on the isolated rabbit’s heart, demonstrated that increased cGMP could produce a cardioprotective role by decreasing the cardiac work, although it might be hazardous to men with depressed cardiac function.

Key words: cGMP, L-arginine, sildenafil citrate, isolated mammalian heart

INTRODUCTION

Cyclic Guanosine monophosphate (cGMP) is a second messenger signaling molecule (Ashman et al., 1963) which is generated from the cytosolic purine nucleotide Guanosine Triphosphate (GTP) by two distinct enzymes: The cytoplasmic heterodimeric haemoprotein s-Guanylate Cyclases (sGC) which is activated by Nitric Oxide (NO) and Carbon monoxide (CO); And the transmembrane receptor particulate Guanylate Cyclases (pGCs, GC or Natriuretic Peptide Receptor (NPR) that act as functional receptors for the natriuretic peptides (Lucas et al., 2000; Feil et al., 2003; Hussain et al., 2001; Kuhn, 2004).

Nitric Oxide (NO) is produced from L-arginine by a family of enzymes known as NO Synthases (NOS). Cardiac muscle fibers and coronary vascular beds express the enzyme endothelial NOS (Balligand et al., 1995). NO activate sGC via different mechanisms: it directly binds the ferrous core of the enzyme, which leads to creation of a protoporphyrin IX-like structure (a potent activator of sGC). Oligomerization and phosphorylation enables the extracellular binding domains of pGC to remain in a high-affinity state, thus priming the cytoplasmic domain to respond to ligand receptor interaction. GTP binds to a single catalytic site on sGC and two catalytic sites on pGC. The α- and β-subunits of the guanylate cyclases cause cleavage of the α-phosphoanhydride bond of GTP yielding cGMP and pyrophosphate (Lucas et al., 2000).

Sildenafil citrate (Viagra) is a selective inhibitor of cGMP-specific Phosphodiesterase type 5 (PDE5) and is
indicated for the treatment of erectile dysfunction. The physiological mechanism responsible for erection of the penis involves the release of NO in the corpus cavernosum in response to sexual stimulation. NO activates the enzyme guanylate cyclase, which results in locally increased levels of cGMP, thereby producing smooth muscle relaxation. By inhibiting PDE5, sildenafil citrate enhances the normal physiological action of NO and cGMP, thereby allowing patients to attain erection adequate for sexual intercourse (Jeremy et al., 1997).

At least three classes of proteins bind cGMP and facilitate its signal transduction roles: cGMP-dependent protein kinases or Protein Kinase-G (PKG), the cGMP-regulated Phosphodiesterases (PDEs) and the CNG (Su et al., 2005).

In the mammalian cardiovascular system, the biological actions of elevated intracellular concentration of cGMP are numerous and diverse. They include vascular smooth muscle relaxation; regulation of ion transport, contributing to electrolyte/ion homeostasis and vascular cell permeability; inhibition of platelet activation mechanisms; regulation of cell growth, differentiation and apoptosis; and cardiac myocyte contractility (inotropy) (Kojda and Kottenberg, 1999; Feil and Kemp-Harper, 2006). These effects in the cardiovascular system are in part attributable to the principal intracellular mediator of cGMP, PKG and in part due to alterations in cAMP and Cyclic Nucleotide Gated (CNG) channel activity (Kuo et al., 1970; Lohmann et al., 1997; Lincoln et al., 2001; Wall et al., 2003). Thus, the aim of the present study is to clarify the role of increased cGMP on isolated mammalian heart.

**MATERIALS AND METHODS**

Adult 6 male rabbits weighing between 2-3 kg were used for the experiments. Approval was obtained from the faculty of medicine ethical committee, King Khalid University, Abha, Saudi Arabia. The animals were obtained from the animal house of the department physiology at King Khalid University where they were fed with standard rabbit pellets and allowed free access to water. They were housed at a controlled ambient temperature of 25±2°C with 50±10% relative humidity and with a 12 h light/12 h dark cycle. All studies were conducted in accordance with the National Institute of Health’s Guide for the Care and Use of Laboratory Animals (National Institute of Health, 1996).

**Preparation of the isolated hearts:** This experiment was carried out in accordance with the Langendorff (1895) procedure. A rabbit was injected with 1000 IU of heparin intravenously through the marginal ear vein. Five minutes later, the rabbit was sacrificed, dissected and the heart with about 1 cm of the aorta attached, was removed as quickly as possible and transferred into a Petri-dish containing Ringer-Locke solution (NaCl; 45.0 g, NaHCO3; 1.0 g, D-glucose; 5.0 g, KCl; 2.1 g, CaCl2.2H2O; 1.6 g, in 5 L of distilled water). The heart preparation was gently squeezed several times to remove as much of residual blood as possible. The aorta was cut just below the carotid bifurcation. The heart was transferred to the perfusion apparatus and tied to the glass canula. The perfusion fluid was Ringer-Locke solution, which was continuously bubbled with a mixture of 95% oxygen and 5% carbon dioxide. The fluid was applied at constant flow rate from a reservoir maintained at 37°C by water circulated through thermostated water bath at rate range of 8-12 ml/minute/g wet weight of tissue.

**Recording of contractility and heart rate:** The response of the drugs was recorded on a student a Narco Bio physiograph (MKIII- S, Narco Bio Systems, USA) by attaching on of end of thread to the apex of the heart by means of palmer clip and the other end of the thread to a force transducer (P- 1000B; Narco Bio Instruments) after passing it through 2 pulleys. The speed of the study of the physiograph was fixed at 0.25 cm sec⁻¹. Contractility was measured as the mean height in mm of 4 cardiac contractions spikes and heart rate as the number of beats per min. During the experiments each heart served as its own control before injection of each solution.

**Protocol of experiment:** Adrenaline was given before the beginning of the experiment procedure to record the sensitivity of the heart. L-arginine in concentration of 3 mmol L⁻¹ and sildenafil citrate in 1.5 mg L⁻¹ was infused to the isolated mammalian heart. The speed of the study of the physiograph was fixed at 0.25 cm sec⁻¹. Contractility was measured as the mean height in mm of 4 cardiac contractions spikes and heart rate as the number of beats per min. During the experiments each heart served as its own control before infusion of each solution.

**Biochemical measurements:** The effluent was collected during the infusion and analyzed for Creatine Kinase (CK), Lactate Dehydrogenase (LDH) and Aspartate Transaminas (AST). The levels of these
enzymes were measured using commercial available kits according to the manufactures instruction (human co.).

**Statistical analysis:** All data obtained were statistically analyzed using students’ t-test. The data were expressed as average mean ± SD and values of p<0.05 were considered significant.

**RESULTS**

Table 1, Fig. 1 and 3 show that continuous infusion of L-arginine and Sildenafil citrate produced a significant decrease in heart rate after 1, 3, 5, 7 and 10 min (p<0.0001) with the percent of inhibition of 47.7, 75.44, 83.04, 88.04 and 94.78% respectively.

![Fig. 1: Negative chronotropic effect produced by combined infusion of L-arginine and Sildenafil citrate. Values are given as mean ± SD for a group of 6 animals](image1)

Table 2, Fig. 2 and 3 show a significant reduction in the amplitude of contraction measured in cm. (p<0.0001) with the continuous infusion of L-arginine and Sildenafil citrate. The percent of decrease in contractility was 48.31, 59.55, 68, 54 and 76.40% after 1, 3, 5 and 10 min respectively.

The level of cardiac enzymes AST, CK and LDH were significantly increased as shown in Table 3 after 10 min of L-arginine and sildenafil citrate infusion when compared to their level in the effluent after 5 min of the infusion. The percent of increase of AST, CK and LDH is 17.46, 12.46 and 14.29% respectively.

![Fig. 2: Negative inotropic effect produced by combined infusion of L-arginine and Sildenafil citrate. Values are given as mean ± SD for a group of 6 animals](image2)

### Table 1: The effect of continuous infusion of L-arginine and Sildenafil citrate on the heart rate (beat min⁻¹), recorded after 1, 3, 5 and 10 min

|          | Control | 1 min | 3 min | 5 min | 7 min | 10 min |
|----------|---------|-------|-------|-------|-------|--------|
| Mean ± SD| 153.33±7.96 | 80.5±5.78* | 37.66±2.94* | 26±2.52* | 18.33±1.96* | 8±2.52* |
| Percentage of decrease | 47.5 | 75.43 | 83.04 | 88.04 | 94.78 |

Values are given as mean ± SD for a group of 6 animals. Values are statistically significant at *p<0.05 when compared with control.

### Table 2: The effect of continuous infusion of L-arginine and Sildenafil citrate on the amplitude of contraction (mm), recorded after 1, 3, 5 and 10 min

|          | Control | 1 min | 3 min | 5 min | 10 min |
|----------|---------|-------|-------|-------|--------|
| Mean ± SD| 14.83±1.72 | 7.66±0.81* | 6.0±0.63* | 4.66±0.51* | 3.5±0.54* |
| Percentage of decrease | 48.31 | 59.55 | 68.53 | 76.40 |

Values are given as mean ± SD for a group of 6 animals. Values are statistically significant at *p<0.05 when compared with control.

### Table 3: The effects of combined infusion of L-arginine and Sildenafil citrate after 5 and 10 min on the level of the cardiac enzymes

|          | AST (U L⁻¹) | CK (U L⁻¹) | LDH (U L⁻¹) |
|----------|-------------|------------|-------------|
| 5 min infusion | 19.30±0.81 | 251.5±9.34 | 27.0±2.82 |
| 10 min infusion | 22.67±0.81* | 287.3±7.28* | 31.5±2.67* |

Values are given as mean ± SD for a group of 6 animals. Values are statistically significant at *p<0.05 when compared with control.
DISCUSSION

L-arginine is the NO precursor which is utilized by the constitutive endothelial NO syntheses to yield NO within the cardiomyocyte. NO stimulates guanylate cyclase, which in turn increases intracellular cGMP levels. Also, the 5’ phosphodiesterase inhibitors produce accumulation of cGMP. At least three classes of proteins...
Surprisingly, NO donor stimulates the cGMP chronotropic effects of B-adrenergic stimulation. Time inhibitors of NO production augments the positive through the induction of endogenous NO. At the same conclude the action of muscarinic agonists is mediated was previously discussed by several studies that production which increased the release of acetyl cholin from the vagal nerve terminals. Yoo Sino-Atrial node. The mechanism of the automatic potential from the slow cardiomyocytes located in the SAN. The diastolic prepotential is mainly caused by the slow inward Na⁺ current through the funny channels and also by the Iₖ1 (inward rectifier). Prolongation of the prepotential slows the heart rate. Also, the heart rate can be slowed by the excess K⁺ efflux through acetyl cholin K⁺ channels producing hyperpolarization of the SAN membrane. The negative chronotropic effects were detected by the gradual decreased heart rate with continuous infusion. Possibly the inhibitory effects of the NO is mediated through cGMP which control ATP sensitive K⁺ channels which increases K⁺ permeability when the ATP is low, cGMP reduces myocardial oxygen consumption (Weiss et al., 1994) and therefore decreases ATP synthesis (Ockaili et al., 1999). Interestingly, cGMP also blocks the L-type Ca²⁺ channels in the membrane of the SAN delaying the process of depolarization (Han et al., 1996). These data was previously discussed by several studies that conclude the action of muscarinic agonists is mediated through the induction of endogenous NO. At the same time inhibitors of NO production augments the positive chronotropic effects of B-adrenergic stimulation. Surprisingly, NO donor stimulates the cGMP production which increased the release of acetyl cholin from the vagal nerve terminals. Yoo et al. (1998) have reported that Sodium Nitroprosside (SNP) may decrease Iᵦ by cGMP dependent stimulation of PDE. The hypothesis was tested that a reduction in Iᵦ during adrenergic stimulation would translate to a decrease in heart rate. Conversely, SNP increased heart rate at concentrations as high as 100 µM transiently. The effect of SNP was abolished by guanyl cyclase inhibition and was mimicked by the membrane permeable analogue of cGMP 8Br-cGMP, suggesting that SNP was acting via release of NO and stimulation of guanyl cyclase (rather than nitrosylation or generation of superoxide radicals caused by some NO donors (Sarkar et al., 2000). Surprisingly, Musialek et al. (1997) concluded that, independent of the autonomic nervous system, application of exogenous NO causes a marked tachycardia due to guanylyl cyclase-cGMP dependent stimulation of Iᵦ in guinea pig SAN cells. Similar results have been observed in the cardiac denervated rabbit (Hogan et al., 1999a) and in human subjects when arterial blood pressure is held constant (Hogan et al., 1999b). NO increases heart rate via cGMP dependent inhibition of Phosphodiesterase 3 (PDE3) to increase cAMP, mobilisation of intracellular calcium and also by a direct action of cGMP itself (Musialek et al., 2000). These well documented pathways all contribute to an increase in Iᵦ (DiFrancesco and Tortora, 1991). However, inhibitors of protein kinase G or Iₖ₁ have no effect on the tachycardia caused by NO. Also, continuous infusion of the L-arginine and sildenafil reduces the force of myocardial contraction. The negative inotropic effect was induced mostly by cGMP generation and consequently protein kinases activation. Most cells contain at least one of three cGMP-dependent protein kinases (cGKs): cGKI, cGKI, or cGKII (Lohmann et al., 1997) that are targeted by their amino termini to distinct substrates and are involved in the regulation of different cellular functions. Mammals have two cGK genes, prkg1 and prkg2, that encode cGKI and cGKII. cGKI is present at high concentrations in all smooth muscles, platelets, cerebellum, hippocampus, dorsal root ganglia, neuromuscular junction end plate and kidney. Low levels have been identified in cardiac muscle, vascular endothelium, granulocytes, chondrocytes, osteoclasts and diverse brain nuclei (Keilbach et al., 1992). The Iₘ isozyme is found in lung, heart, Dorsal Root Ganglia (DRG) and cerebellum. Together with the Iₘ isozyme, the Iᵦ is isozyme is highly expressed in smooth muscle, including uterus, vessels, intestine and trachea (Geiselhoring et al., 2004). This was concluded by Hare and Stamler (2005) which showed that NO modulates cardiac contractility and remodeling. The relative importance of NO effects
mediated by cGMP-dependent and cGMP-independent pathways was discussed with controversial findings through cardiomyocyte-specific cGKI knockout mice study produced by Godecke et al. (2001) and Massion et al. (2003). They demonstrated that cGMP/cGKI contributes to the negative inotropic effect of NO in the juvenile as well as in the adult murine heart. However, the NO/cGMP/cGKI I pathway does not appear to be involved in the negative inotropic action of acetylcholine. Similar findings were reported by Schroder et al. (2003) who claimed that cardiomyocyte-directed overexpression of cGKIα augmented NO/cGMP inhibition but not muscarinic inhibition of L-type Ca2+ channel activity.

Wollert et al. (2003) noted that in cGKI-deficient mice and suggested a mechanism for the negative inotropic action of cGKI, namely, the inhibition of L-type Ca2+ channels. Thus it appears that cardiac contractility is inhibited by NO-stimulated cGMP. Conversely C-naturetic peptide produce positive inotropic effect through GKI-stimulated cGMP and both effects are mediated via cGKI. The opposing effects of cGKI on cardiac contractility might be explained through different subcellular microdomains of NO/cGMP/cGKI versus CNP/ cGMP/ cGKI signaling.

Another possible mechanism of the negative inotropic effect was documented by Geelen et al. (2000), who reported that sildenafil blocks the rapid component of the inward rectifier potassium channel and might prolong cardiac action potential. This could result in a proarrhythmic tendency or, via higher Ca2+ transients, increase myocardial oxygen consumption. This serious effect may require high doses of sildenafil, probably above the therapeutic level (Sugiyama et al. 2002).

Reducing the cardiac workload by reducing the heart rate and contractility may exerts a cardioprotective effects by decreasing myocardial oxygen demand, similar to the effect achieved by B-adrenergic receptors blockade. Several studies have shown that NO, the natriuretic peptides, bradykinin, insulin and adrenomedullin, have infarct-limiting effects via NO generation and subsequent cGMP accumulation (Abdallah et al., 2006; Hamid and Baxter, 2005; 2006; Baxter and Ebrahim, 2002; Cheitlin et al., 1999; Swissa et al., 2002; Paulus and Bronzwaer, 2002; Phillips et al., 2000). Furthermore, recent study concluded that sildenafil citrate pretreatment augments myocardial functional recovery after an ischemic time relevant to clinical cardiac transplantation (Botha et al., 2010). On the other hand, myocardial infarction has been reported following the use of Sildenafil (Velasquez Lopez et al., 2007).

In the recent study, the level of the myocardial enzymes AST, CK and LDH are significantly increased in the cardiac effluent fluid. The enzyme elevation is a sign of cardiomyocyte injury. This happened despite the vasorelaxation of the smooth muscle fibers produced by the cGMP. However, the oxidative stress induces the formation of ROS, which plays a central role in cardiac physiology and Pathophysiology. It is now well established that when the production of ROS exceeds the capacity of antioxidant defenses, oxidative stress will produce a harmful effect on the functional and structural integrity of biological tissues. The biological effects of ROS-mediated reactions are due to their direct interaction with cellular lipids, proteins and DNA (e.g., nicking, base-pair mutations, rearrangements, deletions, insertions and sequence amplification), causing cell damage and death. ROS also causes lipid peroxidation, which results in damage to the cell membrane and the membranes of cellular organelles (Tuteja et al., 2004).

Also, Oldenburg et al. (2004) reviewed an agreement to our data. They concluded that cGMP-elevating interventions may also exhibit negative inotropic effects that could antagonize effects of elevated cAMP (Oldenburg et al., 2004).

To the contrary of our findings, Wallis et al. (1999) reviewed that increased sarcoplasmatic concentrations of cAMP, occurring either as a result of partial PDE 1 inhibition by sildenafil or the cross-talk between PDE 5 and 3, could increase myocardial contractility. Sugiyama et al. (2002) reported an inhibitory effect of sildenafil on the cAMP-hydrolyzing activity of canine and bovine cardiac ventricular membrane preparations, which would support this concept. Also, Senzaki et al. (2001) reported evidence for PDE 5A expression in canine cardiomyocytes, which is still controversial with respect to human cardiomyocytes (Sugiyama et al., 2001). While a lower threshold for ventricular tachycardias was demonstrated in a pacing model of isolated swine right ventricles treated with a high dose of sildenafil combined with a nitric oxide donor decreased the force of contraction as a result of shortening in the diastolic filling period (Hamid et al., 2007).

CONCLUSION

In this study, we found that infusion of L-Arginine and Sildenafil citrate produced both negative
chronotropic and negative inotropic effects which we believe due to the increased cGMP. These effects may exert a cardioprotective actions by reducing the myocardial contractility and the heart rate, thereby reducing the myocardial oxygen demand. This can be helpful to some patients suffering from Ischemic Heart Disease (IHD) but will be detrimental in situations where the systolic cardiac function is depressed.

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