Effects of Tadalafil, a Phosphodiesterase-5 Inhibitor, on Renal Ischemia/Reperfusion Injury: An Experimental Study in a Porcine Model

Tadalafil and Renal Ischemia/Reperfusion Injury

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Abstract

To evaluate the potentially beneficial effects of tadalafil, a phosphodiesterase type 5 inhibitor, on oxidative stress, pathology and renal function in a porcine model of kidney ischemia-reperfusion injury.

A total of 12 male right uninephrectomized Large White pigs was divided into two groups of 6 animals each. Group 1: Normothermic right kidney ischemia and Group 2: Tadalafil-treated right kidney ischemia. Tadalafil pretreatment consisted of 20 mg/kg Tadalafil. Renal function, histology and lipid peroxidation were analyzed. Statistical significance was set at P <0.05.

Baseline levels of serum creatinine were similar in both groups (P=0.114). There was a statistical significant increase in its levels 24 and 36 hours after the surgical procedure in both groups (P<0.001). After that, creatinine levels decreased and remained stable. Tadalafil treatment did not affect serum creatinine levels (P=0.501). It was not observed any statistical significant difference between the groups regarding serum urea levels, neither in terms of tadalafil treatment (P=0.563) nor throughout time (P=0.122). Tadalafil treatment decreased renal malondialdehyde (MDA) levels after 50 min of ischemia (P=0.019). After that, renal levels of MDA were similar in both groups. No significant difference was observed in acute tubular necrosis, vascular congestion and glomerulitis. Vacuolization (P<0.016) as well as interstitial polymorphonuclear infiltration (PMN) (P<0.011) scores were significantly higher in Group 1 when compared to Group 2 in the biopsy made at 50 minutes of ischemia.

Tadalafil has a protective effect on renal ischemia-reperfusion injury in swines, as evidenced by histological and lipid peroxidation results.

Keywords
Ischemia-reperfusion Injury; Tadalafil; Malondialdehyde; Oxidative Stress; Kidney

Introduction

Renal ischemia-reperfusion (RIR) injury is characterized by a sequence of interrelated mechanisms including renal vasoconstriction, tubular damage and severe glomerular injury (Bird et al., 1988). In daily clinical and surgical practice, these events are present in kidney transplantation, renal artery revascularization, treatment of aortic aneurysms, partial nephrectomy and nephrolithotomies (Bird et al., 1988).

It is well known that both renal ischemia and renal reperfusion can cause tissue damage (Oruc et al., 2010), which involves many mediators, such as reactive oxygen species (ROS) formation, activation of vasoactive substances and imbalance in nitric oxide (NO) levels (Oruc et al., 2010; Waz et al., 1998; Choi et al., 2009). NO, an important endogenous vasodilator, has been seriously implicated in the pathophysiology of RIR injury (Waz et al., 1998; Choi et al., 2009), by either increasing renal vascular resistance(Hanssen et
Phosphodiesterase type 5 (PDE5) inhibitors, such as sildenafil, tadalafil and vardenafil, comprise the first-line therapy for treating erectile dysfunction (ED) in male (Burnett, 2008). Initially, PDE5 inhibitors were just used for this purpose (Burnett, 2008; Verit et al., 2010). Nowadays, they are also used in the treatment of pulmonary hypertension, type 2 diabetes mellitus, depression, chronic heart failure and renal insufficiency (Burnett, 2008; Verit et al., 2010; Rodriguez-Iturbe et al., 2005). Besides that, there are overwhelming evidences in the literature supporting their protective role against ischemia-reperfusion injury in many tissues such as myocardium (Bremer et al., 2005; Ahmad et al., 2009; Salloum et al., 2009), kidney (Oruc et al., 2010; Choi et al., 2009), ovary (Arikan et al., 2010) and fetal brain (Ozdegirmenci et al., 2011). However, most of these studies were performed in rabbits (Bremer et al., 2005), mice (Ahmad et al., 2009; Salloum et al., 2009) and rats (Oruc et al., 2010; Choi et al., 2009; Arikan et al., 2010; Ozdegirmenci et al., 2011). Currently, there are no studies published in the literature that evaluated the protective role of PDE5 inhibitors, especially tadalafil, against renal oxidative stress (OS) damage in a porcine model of RIR injury.

Therefore, the aim of the present study is to evaluate, for the first time in the literature, the possible protective effects of tadalafil on renal OS during RIR injury in a porcine model.

**Materials and Methods**

**Animals**

A total of 12 male uninephrectomized Large White pigs, 3 months old (± 30kg), was used in the present study. The animals were kept for seven days under standard conditions, fed a standard swine diet and had water available ad libitum. All the experimental procedures were performed according to NIH Guidelines for Use of Laboratory Animals and were approved by our local Ethical Committee for Research (179/2007). All the efforts were made to minimize discomfort, distress and pain in the animals.

**Experimental Design**

The animals were randomly divided into two groups of six animals each: Normothermic renal ischemia group (Group 1) and Tadalafil-treated renal ischemia group (Group 2). Tadalafil treatment consisted of 20 mg/kg Tadalafil (Cialis®, Eli Lilly do Brasil, São Paulo, SP, Brazil), administered orally once 8 hours before surgical procedure and at 48, 96 and 144 hours after the surgery.

**Surgical Procedure**

All surgical procedures were carried out in the Surgery Unit at the Veterinary Hospital of the Medical Veterinary School of Universidade of Passo Fundo, RS, Brazil, under sterile conditions. All animals were fasted 12 hours before surgery in order to avoid any adverse effects associated with anesthesia. The animals were premedicated with 0.5 mg/kg Midazolan (Dormire®, Cristália, Itapira, SP, Brazil) and 0.5 mg/kg Morphine (Dimorf®, Cristália, Itapira, SP, Brazil) both by intramuscular injection (IM). Anesthesia was induced with a mixture of 0.5 mg/kg Diazepan (Diazepam® UniãoQuímica-Brasília, DF, Brazil) and 2 mg/kg 2,6-diisopropilphenol (Propofol®, Cristália, Itapira, SP, Brazil) both by intravenous access (IV). Controlled mechanical ventilation was provided and anesthesia was maintained by continuous intravenous infusion of 20 mL/kg/min Isoflurane (Isoforine®, Cristália, Itapira, SP, Brazil) under pure oxygen in combination with 20 µg/kg/h Sufentanil (Fastfen®, Cristália, Itapira, SP, Brazil). As an antibiotic prophylaxis, 22 mg/kg Amphicilin (Ampicilina®, Sandoz, Cambé, PR, Brazil), IV, was administered before surgical operation and every 1.5 h until the end of the surgery. As an analgesic agent, we have used 2 mg/kg tramadol, IM, (Dorless®, UniãoQuimica-Brasília, DF, Brazil) every 8 hours.

Right nephrectomy was performed using the classic videolaparoscopic approach. Briefly, three punctures were made: the first 10-mm trocar was positioned 2 cm above umbilical scar, in which the 0º optics was introduced and the inspection of the peritoneal cavity was allowed. Pneumoperitoneum was established using CO2 in allow rate of 3 L/min until the pressure insufflation reached 15 mmHg. Pneumoperitoneum was maintained stable until the end of the procedure. The other two trocars were positioned as follows: one 10-mm to the right and the second 5-mm to the left, both of them laterally to the first, forming a triangle. After dissection of the right kidney and ligature and section of its renal vessels and ureter, this renal unit was removed from the abdominal cavity through the first 10-mm trocar. The incisions were closed with nonabsorbable monofilament wire 2.0(Prolene,
ETHICON, São José dos Campos, São Paulo, Brazil) sutures and the skin was sutured with monofilament 3.0 (Mononylon, ETHICON, São José dos Campos, São Paulo, Brazil).

After this procedure, the animals were repositioned, now in the right lateral decubitus position and the trocars were placed in the same arrangement as described above, to access the left kidney laparoscopically. Renal vessels were dissected and carefully isolated using the same technique as described above. At this point (Time zero), two renal fragments were removed with a laparoscopic forceps: one fragment was immediately frozen in liquid nitrogen for malondialdehyde (MDA) determination and the other fragment was immediately fixed in 10% buffered formalin solution for pathologic examination. After that, the renal artery was clamped by applying a laparoscopic bulldog vascular clamp (Bulldog Dieffenbach, Erwin Gut, Barueri, São Paulo, Brazil) during 50 minutes (period of normothermic ischemia). During this period, the animals were maintained anesthetized and with intra-abdominal pressure as previously described. Before the removal of the clamp, a new biopsy was performed, with the removal of two new fragments which were stored as described above (Time 50). The renal artery flow was released and kept under the same conditions of intra-abdominal pressure during 60 minutes. By this moment, two other fragments were obtained and stored as previously described (Time 110). Right after this procedure, anesthetic recovery was performed and the animals were kept under standard conditions of convalescence. On the seventh day after surgery, the animals were anesthetized again, according to the technique described above, and new kidney biopsies were obtained (final biopsy – FB). All biopsies were performed in the cranial poles of the kidneys in a standard technique.

The experimental protocol used can be observed in Figure 1.

**Biochemical Analysis of Renal Function**

Blood samples were taken through puncture of the marginal ear vein for creatinine and urea levels determination immediately after the recovery of the anesthesia (time 0), 24, 36, 48, 60, 72, 84 and 96 hours after surgical procedure. Creatinine and urea levels were measured by colorimetric end-point assay (Cobas Mira Plus, RocheDiagnostic Systems, Indianapolis, IN, USA).

**Thiobarbituric acid reactive substances (TBARS)**

Kidney tissue was homogenized in 9 volumes of 120 mM KCl, 30 mM sodium phosphate buffer (pH 7.2) for 1 min at 0-2°C. The suspension was centrifuged at 1000 g for 10 min at 0-4°C to remove nuclei and cell debris. The pellets were discarded and the supernatants were used as homogenates. The thiobarbituric acid reactive substances (TBARS) method (Buege et al., 1978) was used to determine lipid peroxidation (LP), a measurement of tissue oxidative stress. Briefly, the homogenates were precipitated with 10% trichloroacetic acid (TCA), centrifuged, and incubated with 0.67% thiobarbituric acid (TBA) (Sigma Chem. Co., St. Louis, USA) for 15 minutes at 100°C. TBARS were extracted using butanol (1:1; v/v) and the absorbance of the butanol layer was measured at 535 nm. The results were expressed in nmol MDA/mg of total proteins. MDA standard was prepared from 1.1.3.3-tetramethoxypropane (Gonzales-Flecha et al., 1991).

Protein concentration was measured using the Bradford Protein Assay method at 595 nm using bovine serum albumin as standard (Bradford, 1976).

All oxidative stress analyses were performed using a spectrophotometer (Lambda35 UV/Vis, Perkin Elmer, Norwalk, Connecticut, USA).

**Histopathologic Evaluation**

Renal tissues were fixed in 10% buffered formalin solution, embedded in paraffin and stained with hematoxylin-eosin (H&E) for light microscopy. The sections were examined for the presence of vacuolization of tubular cells, interstitial polymorphonuclear infiltration (PMN), glomerular polymorphonuclear infiltration (featuring glomerulitis, GLO), vascular congestion and acute tubular necrosis (ATN). Morphological changes such as eosinophilia, cell disruption, loss of architecture and nuclear changes with pyknosis and karyolysis were used to assess necrosis.

The abovementioned characteristics were scored from 0 to 3 according to their severity, except for vascular congestion which was scored from 0 to 2. The...
following scores were used: vacuolization: 0=absent, 1=focal, 2=multifocal, 3=diffuse; PMN infiltration: 0=absent, 1=focal, 2=multifocal, 3=diffuse; glomerulitis (GLO): 0=absent, 1=focal (rare glomeruli), 2=multifocal (various glomeruli), 3=diffuse (most or all glomeruli); vascular congestion (score 0-2): 0=absent, 1=focal, 2=diffuse; ATN: 0=absent; 1=focal (rare tubules), 2=multifocal (various tubules), 3=diffuse (most or all tubules). All histopathologic analyses were performed under a microscope (Axiophot 2; Zeiss, Munich, Germany), photographed (Pixelink PLA 662; Pixelink, Ottawa, Canada) and observed by the same pathologist blind to the treatment groups.

Statistical Analysis
Data are expressed as mean ± standard error of the mean (SEM), unless otherwise stated.

Repeated-measures Two-Way Analysis of Variance (Two-Way RM ANOVA) was used to evaluate quantitative variables (MDA, urea and creatinine concentrations) followed by Bonferroni test in multiple comparisons. Mann-Whitney U test was used to analyze categorical variables (histopathologic scores).

All statistical analyses were performed using GraphPad Prism statistical software package version 5.0 (La Jolla, CA, USA). Statistical significance was set at P < 0.05.

Results
As it can be demonstrated in Figure 2A, baseline levels of serum creatinine were similar in both groups (P=0.114). However, there was a statistical significant increase in its levels 24 and 36 hours after the surgical procedure in both groups (P=0.001). After that, creatinine levels decreased and remained stable. Tadalafil treatment did not affect serum creatinine levels, once creatinine levels remained similar in both groups throughout time (P=0.501).

No statistical significant difference was observed in acute tubular necrosis, vascular congestion and
glomerulitis between the groups. On the other hand, vacuolization (P=0.016) as well as interstitial polymorphonuclear infiltration (PMN) (P=0.011) scores were significantly higher in Group 1 when compared to Group 2 in the biopsy made at 50 minutes of normothermic ischemia (Figures 4 and 5).

**FIG. 4 HISTOLOGY OF NORMAL RENAL GLOMERULI (LEFT) AND NORMAL RENAL TUBULES (RIGHT) OF THE GROUP TREATED WITH TADALAFIL (G2), BIOPSY MADE BEFORE THE BEGINNING OF THE NORMOTHERMIC ISCHEMIA (TIME 0 min) (HE 200x)**

**FIG. 5 HISTOPATHOLOGICAL RESULTS: INTERSTICIAL POLYMORPHONUCLEAR INFILTRATION IN RENAL TISSUE OF SWINE OF THE GROUP NOT TREATED WITH TADALAFIL (G1), BIOPSY AFTER 50 MIN OF NORMOTHERMIC ISCHEMIA (TIME 50 min) (LEFT); DIFUSE ACUTE TUBULAR NECROSIS IN RENAL TISSUE OF SWINE OF THE GROUP NOT TREATED WITH TADALAFIL (G1), BIOPSY AFTER 60 MIN OF REPERFUSION (TIME 110 min) (MIDDLE); INTENSE VACUOLIZATION IN RENAL TISSUE OF SWINE OF THE GROUP NOT TREATED WITH TADALAFIL (G1), BIOPSY AFTER 50 MIN OF NORMOTHERMIC ISCHEMIA (TIME 50 min) (RIGHT) (HE 200x)**

**Discussion**

The need for temporary interruption of arterial blood flow to certain organs and tissues has been widely observed in daily clinical practice. In addition, to understand the mechanisms involved in ischemic-reperfusion phenomenon, as well as to develop interventions that reduce the deleterious effects of transient ischemia is of paramount importance in this context, which can certainly result in a significant impact in terms of therapeutic outcomes.

The present study shows that tadalafil administration before the induction of renal normothermic ischemia in uninephrectomized swines and maintained every 48 hours for 7 days, significantly reduced the lipid peroxidation of renal cells as evidenced by lower tissue concentrations of MDA. Moreover, vacuolization and interstitial polymorphonuclear infiltrate were severer in those animals not treated with tadalafil, although the effects on renal function were not significantly different between the groups.

To our knowledge, this is the first study that evaluates the protective effect of tadalafil, a long-acting PDE5 inhibitor, on renal oxidative stress in a porcine model of ischemia-reperfusion injury. Most studies published so far involve the protective effect of PDE5 inhibitors, usually sildenafil, against ischemia-reperfusion injury in several cell lines and tissues, such as cardiomyocytes (Bremer et al., 2005; Ahmad et al., 2009; Salloum et al., 2009), kidney (Oruc et al., 2010; Choi et al., 2009), ovary (Arikan et al., 2010) and fetal brain (Ozdegirmenci et al., 2011). Therefore, the comparison of our results to others may be compromised due to the lack of data in the literature regarding the protective effect of tadalafil against oxidative stress injury in pig’s kidney.

It is well known that ischemia and reperfusion of organs and tissues are a multi-factorial phenomenon which involves a series of interrelated physiological mechanisms, such as anoxia, neutrophil accumulation and release of lytic enzymes (Toledo-Pereyra et al., 2004). In addition, ROS formation which can occur both in ischemic as well as in reperfusion conditions, is strongly associated to ischemia-reperfusion injury (Toledo-Pereyra et al., 2004). Besides that, NO has also been implicated in the pathophysiology of these injuries (Waz et al., 1998; Choi et al., 2009), by either increasing renal vascular resistance (Hasson et al., 1997) or interaction with hydroxyl free radical (-OH) to form peroxynitrite free radical (-ONOO-) (Gross et al., 1995). In these aspects, PDE5 inhibitors could certainly ameliorate the deleterious effects promoted by RIR, due to their mechanisms of action. PDE5 inhibitors are known to prevent the enzymatic degradation of cGMP by PDE5, leading to reduced intracellular availability of Ca²⁺ and increased NO bioavailability (Carson et al., 2005).

In the present study, a significant decrease in MDA concentration is clearly demonstrated in Tadalafil-treated animals (Group 2) after 50 min of ischemia. MDA is a lipid peroxidation product of cell membranes and its concentration in tissues can directly reflect the extent of ischemic reperfusion injury (Lledó-Garcia E et al., 2009; Li et al., 2009). It has recently been demonstrated that PDE5 inhibitors are capable of protecting tissues against oxidative stress damage by inhibiting ROS formation, due to increasing cGMP levels (Verit et al., 2010). Increased cGMP levels lead to increasing NO bioavailability (Salloum et al., 2009). Furthermore, it has already been demonstrated that increased cGMP levels can prevent
lipid peroxidation by blocking the expression and activity of NADPH oxidase (a well-known substance that forms O$_2^-$), reducing, therefore, the levels of ROS (Verit et al., 2010; Arikan et al., 2013). Besides that, NO inhibits NADPH oxidase, which, in turn, leads to a more pronounced decrease in O$_2^-$ levels (Arikan et al., 2013). On the other hand, NO can act as a scavenger of O$_2^-$ and produce substantial amounts of peroxynitrite with potential free radical effects (Rhoden et al., 2001; Noiri et al., 2001; Schnackenberg, 2002). The low MDA levels after 50 min of ischemia followed by a slightly increase in its levels after reperfusion can possibly be explained by the phenomena described above.

Our results are in agreement with some studies published so far (Verit et al., 2010; Arikan et al., 2013). Arikan et al (2010), analyzing ischemia-reperfusion injury in rat ovary, have also found that tadalafil pretreatment significantly reduced tissue MDA levels (Arikan et al., 2013). In addition, Verit et al (2010) observed that tadalafil administration reduced serum levels of oxidative stress in male, exerting a beneficial effect on the cardiovascular system (Verit et al., 2010).

In this regard, Lledó-Gracia et al (2009) who used a model of renal transplant in swines, have demonstrated that sildenafil pretreatment significantly increased arterial blood flow and decreased renal vascular resistance (Lledó-Garcia et al., 2009). Furthermore, many studies have demonstrated the protective effect of PDE5 inhibitors on cardiac tissue, (Verit et al., 2010; Bremer et al., 2005; Ahmad et al., 2009) by either reducing apoptosis (Ahmad et al., 2009) or limiting myocardial infarction area (Verit et al., 2010; Ahmad et al., 2009), supporting the positive effects of PDE5 inhibitors on cardiac and vascular systems.

Despite the decrease in MDA levels observed in Tadalafil-treated ischemia group (Group 2), serum creatinine and urea levels were not statistically different between groups (Figure 2). Although renal function is commonly assessed using creatinine and urea levels, it is well known that these tests have some important limitations due to their lack of ability to better evaluate glomerular filtration, which is commonly associated with acute renal failure, an important condition found in normothermic ischemia (Rhoden et al., 2001). On the other hand, tests that better assess glomerular filtration, such as endogenous creatinine clearance, were not available and practical in animals of medium and large size.

One of the most interesting aspects of this study was that vacuolization and interstitial PMN infiltration scores, phenomena of significant relevance in this context, were significantly higher in the group of animals that were not treated with tadalafil (Group 1) when compared to the animals treated with tadalafil (Group 2) in the biopsy performed after 50 min of ischemia. Vacuolization, also called hydropic degeneration, is caused by a failure in the sodium-potassium pump due to ischemia and it would happen prior to acute tubular necrosis (Tirapelli et al., 2009).

Tadalafil treatment demonstrated to be effective in preventing this event. Furthermore, it has been well demonstrated that endogenous activation of neutrophils is an important factor in tissue damage during the ischemic-reperfusion phenomenon (Kinsey et al., 2008; Xiong et al., 2010). In this regard, tadalafil treatment decreased interstitial PMN infiltration score after 50 min of ischemia, reinforcing the possible protective role of tadalafil in preventing endothelial damage. Our results are consistent with the study published by Lledó-Garcia et al. (2009) who demonstrated that endothelial cell structure was better preserved in the animals pretreated with sildenafil in a model of renal transplant in swines. Likewise, tadalafil pretreatment, in a model of ischemia-reperfusion injury in rat ovary, significantly decreased ovarian tissue damage scores, which included the evaluation of vascular congestion, hemorrhage and interstitial edema (Arikan et al., 2010). Similarly, Choi et al (2009) as well as Oruc et al (2010), studying ischemia-reperfusion renal injury in a rat model, have also demonstrated that pretreatment with sildenafil decreased renal tubular injuries (Choi et al., 2009), histological damage and leukocyte infiltration (Oruc et al., 2010).

Although the experimental design of our study has significantly reduced potential biases, some limitations should be taken into account. First of all, this experimental study was performed in swines, so attention should be paid when the results are extended to a larger clinic context. Secondly, the small sample size after analyzing MDA, creatinine and urea levels may have weaken the statistical power. However, Two-Way RM ANOVA can not be performed when there are missing values, so some animals had to be excluded from analysis. Thirdly, the oral administration of tadalafil may have irregular absorption, but all animals followed the protocol in a similar way, reducing potential bias related to this aspect.

Conclusions

In summary, this study demonstrated the possible
protective role of tadalafil, a long-term PDE5 inhibitor, against RIR injury, evidenced by reduced renal tissues of MDA levels as well as reducing vacuolization and interstitial PMN infiltration in kidney tissue samples. Further studies with increased sample size are required to better evaluate the protective effect of tadalafil against renal OS in a swine model of ischemia-reperfusion.

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REFERENCES


