Sildenafil in the prevention of hepatic ischemia/reperfusion injury in rats.

Sildenafil na prevenção da lesão induzida por isquemia/reperfusão hepática em ratos.

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ABSTRACT

Purpose: This study aimed to determine the effect of sildenafil on the liver, when administered prior to the episode of liver ischemia/reperfusion**. Method:** We used 12 Wistar rats. In both IR group (n=6) and IR sildenafil group (n=6), hepatic ischemia was induced for 45 minutes by occlusion of blood vessels supplying the median and lateral lobes of the liver using a vascular clip (bulldog). IR group animals were treated with sildenafil citrate at a dose of 0.7 mg/kg by gavage, 30 minutes before ischemia. During the observation period of ischemia/reperfusion, the abdomen was temporarily closed. After 45 min the vascular clamp was removed, and reperfusion occurred by 60 min. Liver biodistribution of Tc-99m-phytate, histopathology and serum AST, ALT, LDH were analyzed. **Results**: In animals treated with sildenafil the right lobe of the liver (without ischemia) had significantly higher radioactivity uptake than in the untreated rats ($p < 0.001$). After ischemia/reperfusion, sildenafil failed to improve the radioactive uptake in the left lobe ($p = 0.01$). Comparing the right and left lobes, the radioactive uptake was lower in lobe left than in right lobe ($p = 0.005$) of untreated rats (controls). The liver function tests were significantly more impaired in the group treated with sildenafil than in controls. **Conclusion:** Sildenafil contributed to the deterioration of liver function in a model of ischemia/reperfusion.

Keywords: Sildenafil. Liver injury. Ischemia. Reperfusion. Rats.

RESUMO

Objetivo: Determinar se o sildenafil exerce proteção hepática, quando administrado previamente ao episódio de isquemia hepática e reperfusão. **Método:** Foram usados 12 ratos Wistar. No grupo IR (n=6) e no grupo IR sildenafil (n=6), a isquemia hepática foi induzida por 45 minutos por oclusão dos vasos que suprem os lobos mediano e lateral esquerdo do fígado usando *clamp* vascular (bulldog). Durante os períodos de observação da isquemia/reperfusão, o abdome foi fechado temporariamente. Após a retirada do *clamp* vascular, a reperfusão ocorreu por 60 min. Os animais do grupo IR sildenafil foram tratados com citrato de sildenafil na dose de 0.7 mg/kg v.o., 30 minutos antes da isquemia. Biodistribuição de Fitato-Tc-99m para fígado, histopatologia e dosagens de AST, ALT e DHL foram analisadas. **Resultados:** Nos animais tratados com sildenafil o lobo direito do fígado (sem isquemia) teve captação radioativa significantemente maior que nos ratos não tratados (p<0,001). Após isquemia, o sildenafil não foi capaz de melhorar a captação radioativa no lobo esquerdo (p=0,01). Comparando-se os lobos D e E, a captação radioativa foi signitivamente menor no lobo E (p=0,005) nos ratos não tratados (controles). As provas de função hepática foram significativamente mais comprometidas nos ratos do grupo tratado com sildenafil. **Conclusão:** O sildenafil contribuiu para o agravamento da função hepática em modelo de isquemia e reperfusão.

Descritores: Sildenafil. Lesão hepática. Isquemia. Reperfusão. Ratos.

Introduction

Liver injury induced by ischemia/reperfusion (I/R) is a phenomenon frequently observed in the immediate postoperative period and early in liver transplantation and partial hepatectomy, when the organ undergoes a period of partial or total ischemia at various times. Much progress has been made in understanding the basic mechanisms of cell injury by hypoxia, considered the main factor involved in I/R.[1,2] A fact of particular importance for hepatectomy and liver transplantation is the inevitable period of ischemia during organ removal in the donor or during the partial hepatectomy, since the interruption of blood flow promotes anoxia, the depletion of ATP, decreasing gradients of calcium and electrolytes, leading to the activation of several proteases and fospholipases[3,4]. Anaerobic metabolism is then triggered by increasing the formation of lactate and a consequent decrease in pH, causing instability and enzymatic changes in the lysossomal membranes[5,6]. In the process of reperfusion, mitochondria are important targets, because the intracellular changes promote increased permeability of mitochondrial membrane, triggering apoptosis[4].

Not only the liver suffers with I/R. A large amount of biochemical metabolites is released after ischemia. There is a complex interaction between microvascular changes, release of inflammatory mediators, oxygen free radicals, activation of neutrophils, platelets, Kupffer cells and sinusoidal endothelial cells. The activation of these cells can lead to the release of tumor necrosis factor (TNF-α), leukotrienes, thromboxanes, prostaglandins, endothelins, platelet activating factor, causing damage to the cell membrane, mitochondrial membrane and endothelium, leading to disturbances in the microcirculation. Nitric oxide, endothelin and possibly leukotrienes are released after ischemia. These agents can act as important regulators of regional blood flow or of the metabolic tubular activity, which can either be beneficial but also contribute to liver failure[3, 7, 8].

Several maneuvers have been performed to protect the liver from ischemia/reperfusion. Among them is the use of drugs, hypothermia and ischemic preconditioning with successive intermittent I/R episodes, at different times [8,9]. The relationship between warm ischemia and onset of hepatic ischemia/reperfusion has been studied experimentally with interesting results. Studies in rats subjected to warm ischemia showed the recovery of liver function after ischemia time of 90 minutes with the use of substances with anti-inflammatory action, such as D-allose[1].

It has also observed the efficacy of drugs to prevent or reduce I/R injury. Recently, there was considerable interest in investigating the role of sildenafil in the protection I/R injury in animal models. This drug belongs to a new class of vasoactive agents, developed for the treatment of erectile dysfunction. Its mechanism of action involves the active inhibition of the enzyme phosphodiesterase type 5 (PDE5), resulting in increased cGMP, nitric oxide (NO) and improvement of endothelial dysfunction [10,11]. Some studies have demonstrated the effect of sildenafil as inducing a cardioprotective effect against I/R injury in dogs, rabbits, rats and mice. The effect is attributed to a pre-conditioning pharmacological mechanism [12]. Similarly, it has been observed beneficial effect of sildenafil after warm ischemia in kidneys in an experimental study that determined the hemodynamic, biochemical, and histological effects when administered before ischemia[13].

In the liver, studies show that in patients with Child A liver cirrhosis, sildenafil caused a decrease in portal sinusoidal resistance. The drug is being studied as a potential modality of treatment for portal hypertension[12]. It is estimated that porto-pulmonary hypertension has a prevalence of 3-6% in patients undergoing liver transplantation[14]. It is caused by an increase in vascular resistance, resulting in disturbance in liver architecture and peri-sinusoidal fibrosis[15]. This factor is associated with cirrhosis and carries a high mortality to these patients[16]. However, studies show that sildenafil may stabilize or improve hemodynamics of transplanted patients, in order to facilitate the hepatic transplantation[14-16]. Study in Brazil has demonstrated success in using this drug in the treatment of the association of cardiac and pulmonary disorders in both adults and children and adolescents with pulmonary hypertension[17].
Moreover, no study has been carried out to test the protective effect of sildenafil in pre-

warm ischemia associated with the use of radiopharmaceutical biodistribution as a method to measure liver function after I/R.

The use of scintigraphy is achieving success in experimental studies. This method is based on the administration of a radioactive compound that has metabolic specific affinity to the hepatic parenchyma, and at the same time continuously emits some form of radiation that allows recording of quantifiable images by a detector (scintillation camera). The ^{99m}Tc is the radionuclide most often used to evaluate liver function, associated with the radiotracer phytate, as demonstrated in a previous experimental study from our laboratory[18]. Thus, this study aimed to determine the biodistribution of a radiopharmaceutical, as well as the hemodynamic, biochemical, and histopathological effects of sildenafil administered before hepatic I/R.

Methods

We used 12 male Wistar rats supplied by the Center for Health Sciences-UFRN, which were handled in accordance with ethical rules of the National Ethics Committee on Animal Research-Brazil. The animals were selected and randomly distributed into 2 groups of 06 rats each: I/R group and I/R sildenafil group. The I/R group rats were treated with sildenafil citrate (0.7 mg / kg) p.o. (gavage), 30 minutes before the ischemia and reperfusion. Anesthesia was induced using intramuscular ketamine 50 mg/kg, associated with sodium thiopental (20 mg/kg) intraperitoneal. The operations were performed under aseptic technique and the animals were observed in individual cages with food and water *ad libitum*.

The surgical procedure began with 5 cm median laparotomy, and identification of the hepatic pedicle. In the I/R group rats (n = 06) and the I/R sildenafil rats (n = 06) hepatic ischemia was induced by occlusion of the blood vessels supplying the median and lateral lobes of the liver, using a vascular clip, for 45 minutes. During observation of I/R, the abdomen was temporarily closed with 4-0 nylon thread. After removal of the vascular clamp, reperfusion occurred for 60 minutes. During the surgery were injected 05 ml of saline 0.9% per 100g of body weight subcutaneously for hydration.

Hepatic biodistribution of ^{99m}Tc-phytate and dosages

The biodistribution of ^{99m}Tc-phytate was determined in all animals after 60 minutes of reperfusion to evaluate the liver response to ischemia and reperfusion in order to compare the radio-uptake of the median and left lobes, with the right lobe (not subjected to ischemia). About 0.1 ml of ^{99m}Tc-phytate was injected in femoral vein and after 30 min. the rats were anesthetized. Samples of the right lobe and left lobes (ischemic) were harvested from all animals to study the biodistribution and uptake of the radiopharmaceutical. The samples were washed with saline 09% and weighed. The biodistribution of ^{99m}Tc-phytate per gram of tissue was performed by percentage

of radioactivity/g (% ATI/g), by using the Automatic Gamma Counter/Wizard, PerkinElmer-Finland.

Before the radioactive uptake examination, blood samples were harvested by cardiac puncture from all rts. The blood was centrifuged at 3000 rpm for 10 min and serum samples were stored at -40°C until analysis. Aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) were measured in autoanalyzer (Konelab, Software Version, 60i, Finland).

Histopathologic analysis

The animals were killed by an overdose of anesthetic (thiopental 100mg/Kg ip), and samples of liver (left lobe and right lobe separately) were harvested, fixed in 10% formalin and later included in paraffin blocks. Sections of 5μ were stained with hematoxylin and eosin. Histopathologic analysis was performed according to the following criteria and findings: infiltration of neutrophils, sinusoidal congestion, degree of necrosis and vacuolization of cytoplasm. These changes were quantified and converted into scores ranging from 0 to 4. Statistical analysis was performed using the Mann-Whitney and Student t tests, considering differences significant when $p<0.05$.

Results

Table 1 shows the descriptive results found on the biodistribution of ^{99m}Tc-phytate in both hepatic lobes, as well as tests to investigate the existence of statistically significant differences between sildenafil and control groups.

Table 1 - Descriptive statistics and test regarding the percentage of radioactivity per gram of liver tissue (% ATI/g).

Hepatic lobe	*%ATI/g*		p-value[(1)]
	Control	Sildenafil	
Right	12.96 ± 1,91	4.47 ± 1,47	<0.001
Left	6.31 ± 1,97	3.04 ± 1,56	0.01
P-value[(2)]	0.005	0.086	

Mean ±standard deviation
1. P-value after analysis by Student t test for independent samples, comparing the treatments.
2. P-value after analysis by Student t test for dependent samples, comparing the right and left hepatic lobes.

Table 1 shows that in both the right and the left hepatic lobes there was a higher uptake of radioactivity in the control group rats compared with the sildenafil group rats, and these differences were statistically significant ($p \leq 0.01$). When comparing the hepatic lobes of the same group rats, it was observed that in the control group, the left lobe - which suffered ischemia - showed a lower uptake of ^{99m}Tc-phytate ($p=0.005$) when compared with the right lobe. In the group receiving sildenafil we did

not observe statistical significance between the uptake of ^{99m}Tc-phytate comparing the right and left lobes (p=0.086).

Table 2 – Biochemical laboratory data and statistical test analysis.

Biochemical Parameters	*Group*		p-value(1)
	Control	Sildenafil	
AST (U/L)	26.50 ± 6.83	121.83 ± 9.37	<0.001
ALT (U/L)	33.00 ± 7.43	376.17 ± 40.53	<0.001
LDH (U/L)	35.50 ± 9.85	71.00 ± 11.52	<0.001

Mean ± Standard deviation

1. P-value after analysis by Student t test for independent samples.

Com relação à tabela 2, pode-se verificar que houve aumento significativo na concentração sérica das enzimas AST, ALT e LDH nos animais do grupo sildenafil, quando comparado com o controle (p<0,01), indicando injúria do parênquima hepático para os animais tratados com o sildenafil.

Regarding table 2, it is shown a significant increase in serum concentrations of AST, ALT and LDH in the sildenafil group rats, compared with controls (p<0.01), indicating injury to the liver and its function in animals treated with sildenafil.

Table 3 – Descriptive statistics and respective interpretation of data from hepatic histological analysis.

Histological Parameters	Right lobe			Left lobe		
	Control	Sildenafil	p-values(1)	Control	Sildenafil	p-values(1)
NI	0.17 (32)	0.67 (45)	0.298	1.33 (24)	2,50 (54)	0,016
SC	0.00 (24)	1.00 (54)	0.016	2.17 (32)	2,83 (46)	0,262
NG	0.00 (39)	0.00 (39)	1.000	1.67 (29)	2,67 (48)	0,128
CV	0.17 (36)	0.33 (42)	0.631	1,50 (26)	2,67 (51)	0,045

Mean of scores (Sum of points)

1 - P-values analysis of non-parametric Mann-Whitney test for independent samples.

NI – Neutrophil infiltration; SC –Sinusoid congestion; NG – Necrosis grade; CV – Cytoplasm vacuolization.

Looking at Table 3, it appears that in the right liver lobe (not subjected to ischemia), we did not observe significant difference in most histological parameters, when compared to the control and sildenafil groups, i.e. the p values were always higher than 0.05. Histological images about this may be seen in figures 1 and 2. Sildenafil caused a significant increase in congestion of sinusoids in the right lobe, meaning that the drug must have caused great sinusoid vasodilation compared with controls (p=0.016). However, in the left lobe (underwent 60 minutes of I/R), sildenafil has contributed to a significant increase in neutrophil infiltration, and vacuolization of the hepatocytes cytoplasm, when compared with the control group (p<0.05). There was an increase in congestion of sinusoids (p=0.262) and necrosis (p= 0.128) in

ischemic left lobe of rats using sildenafil (Figures 3 and 4) but the difference in scores was not significant compared with controls.

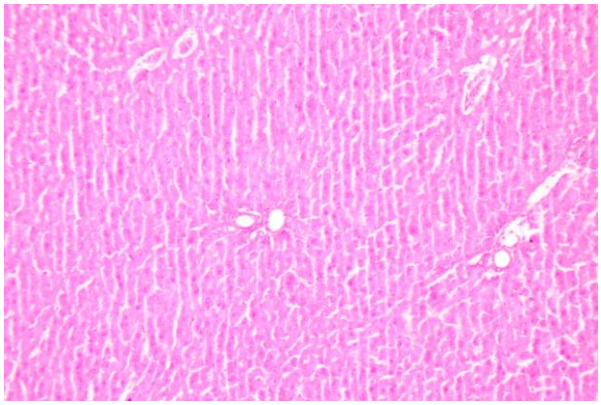

Figure 1. Normal liver. Right lobe of a control rat. HE, 200x

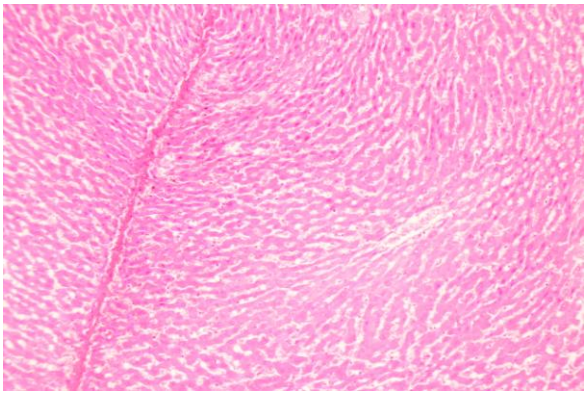

Figure 2. Sinusoid congestion and short neutrophil infiltration. Right hepatic lobe, control group rat. HE, 200x

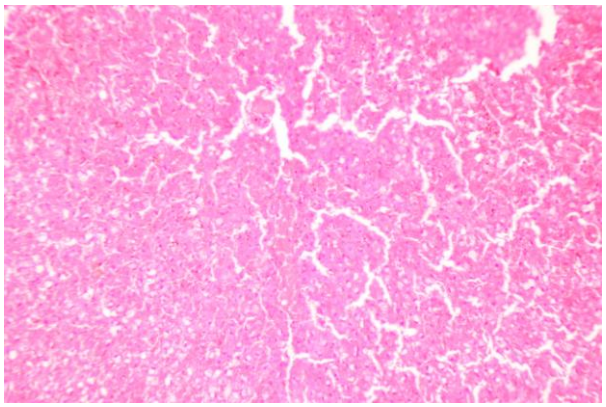

Figure 3. Sinusoids congestion and cytoplasm vacuolization in left hepatic lobe from a sildenafil group rat. HE, 200x

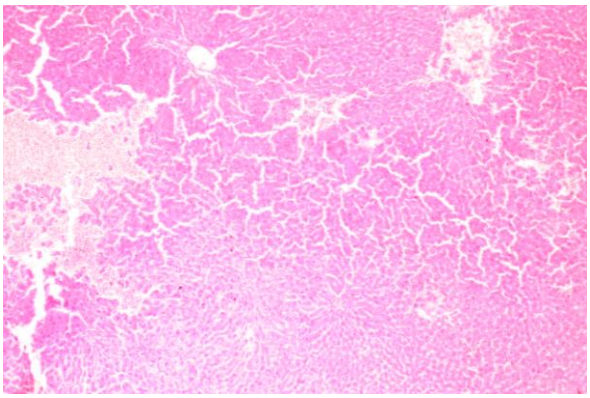

Figure 4. Focal áreas of necrosis and neutrophil infiltration in hepatic left lobe from a sildenafil group rat. HE, 200x.

Discussion

Sildenafil, an inhibitor of phosphodiesterase-5 (PDE5), have had great influence on the world market by offering treatment for erectil disfunction with favorable pharmacokinetic characteristics in relation to previously tested methods. PDE5 enzyme is expressed not only in the corpus cavernosum of penis, but also in peripheral vessels, lung, nasal mucosa and tracheobronchial muscles. So, its clinical use has become wide-ranging. It has been successful in cardiac and pulmonary disorders. Moreover, it has significantly protective effect against ischemia and reperfusion inherent to surgical techniques of transplantation[19].

However, despite the scarcity of studies on the hepatic effects of sildenafil, reports have suggested hepatotoxicity related to its use. Frank et al[20] described a rare

immune-mediated hepatotoxicity case attributed to routine use of silfenafil for 3 months, with elevated liver transaminases in a 59 old male patient. Two French studies show the same in female patients[21,22]. Our study confirms these observations, since significant increases were detected ($p<0.001$) in serum transaminases (AST and ALT) and the enzyme lactate dehydrogenase (LDH) in rats treated with sildenafil.

Continuing the analysis of the effects of sildenafil, it was found that there was a higher liver uptake of ^{99m}Tc-phytate in the control group rats compared with the group receiving the drug. The analysis of this finding parallels the observed biochemical disorder, corroborating the hypothesis that hepatic disfunction altered the biodistribution of the radiotracer to the liver. This was not tested in previous experimental studies involving biodistribution of ^{99m}Tc-phytate.

With regard to the histopathological analysis, we detected significant ($p<0.05$) neutrophil infiltration and cytoplasmic vacuolation in the liver of sildenafil group rats, compared to controls. Histopathologic analysis confirmed the tests conducted primarily in which there was evidence of hepatocytes dysfunction. However, previous studies showed opposite effect on the use of this drug prior to renal ischemia. Experimental study detected a structural improvement in the renal endothelium of animals treated with sildenafil, as well as significant decreases in renal arterial resistance and perfusion[23].

Analogous fact was observed with the use of sildenafil for the treatment of porto-pulmonary hypertension in previously transplanted patients with liver failure[24,25]. Concerning the cardiovascular protection, it was shown that the drug limits the tecidual apoptosis and necrosis[26]. Such contradictions suggest that the mechanisms of action in the liver vasculature have yet unknown pathophysiological features. This fact could explain the harmful hepatic effects as opposed to the benefits observed in other organs, as described in the literature.

Few existing studies show conflicting results. Halverscheid et al[14] in an experimental model to study hepatic hemodynamics under effect of sildenafil, conducted in rats, observed constancy of portal pressure with a slight tendency to decrease. Lee et al.[27] showed that a standard dose of 50mg of sildenafil reduced significantly the sinusoid resistance. This fact corroborates the findings of our study, mainly the presence ($p=0.016$) of sinusoidal congestion in the non-ischemic right lobe. Moreover, Jiao et al[26] reported an increase in portal pressure after hepatic reperfusion in pigs. Such results are often contradictory and more studies are needed to explore and define the safety of the clinical use of sildenafil. Its indiscriminate use without medical oversight may trigger the onset of hepatic disorders, causing significant damage to patients. This facts justify the need for further exploration.

Conclusion

In conclusion, this study showed that pre-treatment with sildenafil in rats resulted in increased damage to hepatocytes demonstrated by metabolic,

histopathological and radiopharmaceutical biodistribution in a model of hepatic ischemia/reperfusion. Future studies are needed to elucidate the injuries mechanisms.

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