Protective effect of simvastatin in the cyclophosphamide-induced hemohrragic cystitis in rats[1]

Efeito protetor da sinvastatina na cistite hemorrágica induzida pela ciclofosfamida em ratos

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ABSTRACT

Purpose: Cyclophosphamide (CYP) is an antineoplastic agent used for the treatment of many neoplastic and inflammatory diseases. Hemorrhagic cystitis is a frequent side effect of CYP. Several studies show that simvastatin has important pleiotropic (anti-inflammatory and immunomodulatory) effects. The purpose of the study was to investigate the effect of simvastatin on bladder, ureter and kidney injury caused by CYP. **Methods:** Adult male Wistar rats were randomly divided into three groups. The CYP/SIM group received simvastatin microemulsion by gavage during 7 days (10 mg/kg body wt) before the administration of CYP and the CYP/SAL group rats received saline 0.9%. The control rats were not treated. After that, all rats were treated with a single dose of CYP 200 mg/kg body wt intraperitoneally. The rats were killed 24 h after CYP administration. Plasma cytokines (TNF-α, IL-1β, IL-6) were measured by ELISA. Macro and light microscopic study was performed in the bladder, kidney and ureter. **Results:** In the bladders of CYP/SIMV treated rats edema of lamina propria with epithelial and sub-epithelial hemorrhage were lower than in CYP/SAL treated rats. The scores for macroscopic and microscopic evaluation of bladder and ureter were significantly lower in CYP/SIMV rats than in CYP/SAL rats. The kidney was not affected. The expression of TNF-α, IL-1β and IL-6 was significatly lower in CF/SINV rats (164.8±22, 44.8±8 and 52.4±13) than in CF/SAL rats (378.5±66, 122.9±26 e 123.6±18), respectively. **Conclusion:** The results of the current study suggest that simvastatin pretreatment attenuated CYP-induced urotelium inflammation and decreased the activities of cytokines.

Key words: Cyclophosphamide. Cystitis. Simvastatin. Inflammation. Cytokine. Rats.

RESUMO

Objetivo: Ciclofosfamida (CF) é um agente antineoplásico frequente implicado na etiologia da cistite hemorrágica. Vários estudos mostram que a sinvastatina tem importantes efeitos pleiotrópicos (anti-inflamatórios e imunomoduladores). O objetivo do trabalho foi estudar os efeitos da sinvastatina na prevenção de cistite hemorrágica e lesões uroteliais induzidas por CF em modelo experimental. **Métodos:** Doze ratos Wistar foram distribuídos de forma randomizada em três grupos: nos ratos do grupo experimental (CF/SINV), foi administrada microemulsão de sinvastatina 10mg/Kg, por via oral (gavagem), durante 7 dias antes da administração de CF e os ratos do grupo controle (CF/SAL), foram tratados com solução salina 0,9% nas mesmas doses e prazos. O grupo controle não foi tratado. Todos os ratos foram tratados com CF 200mg/Kg intraperitonial (dose única) e 24 horas após foram sacrificados. Exame macro e microscópico foi feito na bexiga e os rins e ureteres foram avaliados microscopia. Foram realizadas dosagens plasmáticas de TNF-α, IL-1β, IL-6 (ELISA). **Resultados:** O escore para avaliação macroscópica do dano à bexiga e o escore para avaliação do dano histológico na bexiga e nos ureteres mostraram-se significativamente menores no grupo CF/SINV em comparação ao grupo CF/SAL ($p<0,05$). Os rins não foram afetados. A expressão de TNF-α, IL-1β, IL-6 também foi significativamente menor ($p<0,05$) no grupo CF/SINV (164,8±22, 44,8±8 e 52,4±13) em comparação ao grupo CF/SAL (378,5±66, 122,9±26 e 123,6±18), respectivamente. **Conclusão:** O estudo demonstrou eficácia da sinvastatina na atenuação da cistite hemorrágica e lesão ureteral induzidas por CF em ratos Wistar, através da interferência nas citocinas plasmáticas e nas lesões uroteliais.

Descritores: Ciclofosfamida. Cistite. Sinvastatina. Inflamação. Citocina. Ratos.

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Introduction

In the last decade, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) have become the drug of choice for the treatment of dyslipidemia[1]. Statins have been shown to be especially effective in reducing low-density lipoprotein cholesterol (LDL) and, to a lesser extent, triglyceride levels[2]. Statins' antiinflammatory effects have been proposed not only to reduce cardiovascular disease, but also to help improve other conditions that are not influenced by lipid levels, including rheumatoid arthritis[3], stroke[4], graft rejection[5] and infection[6,7].

Cyclophosphamide (CYP) is a nitrogen mustard alkylating agent that imparts its antineoplastic activity by forming cross links with tumor DNA. It is metabolized in the liver to its active form and eliminated primarily via renal excretion. A small portion of the cyclophosphamide is converted to a metabolite, acrolein, the causative agent responsible for CYP induced hemohrragic cystitis (HC)[8]. Histologically, acrolein imparts a great inflammatory response characterized by subepithelial edema, neutrophil infiltration, hemorrhage and necrosis. Some investigators have demonstrated increases in proinflammatory mediators such as tumor necrosis factor-α (TNF-α), interleukin-1ß (IL-β) and type 2 cyclooxygenase within the bladder epithelium in response to cyclophosphamide therapy[9,10]. Systemic or intraperitoneal injection of CYP induces a reproducible dose-dependant chemical cystitis in both mice and rats and therefore has been used as an experimental model of cystitis[11]. Although statins are not employed in the treatment of HC, we hypothesized that their use may be useful due to their pleiotropic anti-inflammatory effects[12]. We did not find reports in the literature on the use of statins in clinical and experimental studies involving HC induced by CYP. The aim of this work is to investigate the cholesterol-independent effects of simvastatin in the prevention of HC, ureter and kidney damage induced by CYP and in the expression of plasma pro-inflammatory cytokines.

Methods

Twelve Wistar rats, weighing 185±23g, were housed in polypropylene cages and kept under controlled conditions of temperature in a clear-dark cycle of 12 hours and allowed *ad libitum* access to food and water. All experimental procedures in animals were conducted according to the code of ethics for animal experimentation of the Council for International Organization of Medical Sciences and the Brazilian Law on the Scientific use of Animals (Law No. 11794). The protocol was approved by the Institutional Research Ethics Committee.

*Experimental design and procedures*The animals were randomly distributed into three groups with six rats each: the cyclophosphamide/saline group (CYP/SAL) and the experimental cyclophosphamide/simvastatin group (CYP/SIMV) and control. All animals were given a single dose of CYP 200mg/kg intraperitoneally (IP). In the CYP/SIMV rats, a microemulsion of simvastatin 10 mg/kg was administered orally (gavage), during 7 days, prior to the administration of CYP. In CYP/SAL rats, 0.9% saline solution was administered orally for seven days. The control rats did not use CYP neither SIMV. Twenty four hours after treatment with CYP, blood was collected from all animals by cardiac puncture and then the rats were killed with overdose of anesthetic. Through a median laparotomy, the whole bladder, ureter and kidney were removed for macroscopic and microscopic examinations to search for inflammation, congestion and hemorrhage. The blood was treated with EDTA, centrifuged and the plasma separated for determination of interleukine-1b (IL-1β), Interleukine-6 (IL-6) and tumor necrosis factor-α (TNF-α) by ELISA, using kits Peprotec, USA.

Macroscopic examination of bladder

Bladders were examined macroscopically for edema and hemorrhage according to pre-established criteria[13]. Edema was considered severe (3+) when fluid was seen externally and internally in the bladder wall; moderate (2+) when confined to the mucosa; mild (1+) between normal and moderate, and none (0) as normal. Hemorrhage was scored as follows: (3+), intravesical clots; (2+), mucosal hematomas; (1+), telangiectasia or dilatation of the bladder vessels and (0) normal. The scores obtained by the observation of the bladder of each animal were summed up to obtain the total score for each group.

Microscopy of bladder and ureter

Tissues were fixed in 10% formaline, embedded in paraffin, sectioned (5µ) and stained with hematoxylin and eosin. Histological damage was scored on a scale of (0) - normal epithelium, (1)- mild changes involving a decrease in epithelial cells, flattening with submucosal edema, mild hemorrhage and few ulcerations, and (2)- severe changes, including mucosal erosion, inflammatory cell infiltration, fibrin deposition, hemorrhage and multiple ulcerations[13]. The slides were evaluated in a blind fashion.

Microscopic analysis of kidney

The histopathological data (edema, congestion, inflammation, cell degeneration and necrosis) were graded according to the following scale: absent (0), mild (1+), moderate (2+) and severe (3+). The scores obtained by the observation of 3 random microscopic fields of each histological kidney section were summed up to obtain the total score for each group.

Statistical analysis

The results were treated by analysis of variance (ANOVA) followed by the Student t test. The results of the scores for histological evaluation were treated by the non-parametric Mann-Whitney test. Differences between groups were considered significant when $p<0.05$.

Results

Macroscopy

After analysis of the quantitative criteria used for the macroscopic evaluation of bladders, we observed that the cumulative amount of scores in the simvastatin treated rats (CYP/SIMV) was 26, lower than in the CYP/SAL rats (score 48). The bladders of control rats were normal. By using the Mann-Whitney test, the difference was significant ($p<0.05$).

Histopathology

CYP administration resulted in severe bladder damage, including acute inflammation with vascular congestion, severe edema, hemorrhage, inflammatory cell infiltration in the lamina propria and epithelial denudation. These findings in bladder and ureter resulted in less representative histopathologic scores in rats treated with CYP and simvastatin. The scores are summarized on Table 1. In kidney the histopathologic findings were not statistically different comparing the CYP/SAL and CYP/SIMV rats ($p>0.05$). So, the pretreatment with simvastatin ameliorated CYP induced inflammatory changes only in bladder and ureter. No histologic

abnormality was observed in the organs of control rats.

TABLE 1 - Values of histologic scores for bladder, ureter and kidney of rats submmited to treatment with cyclophasphamide, saline 0.9% and simvastatin

Organs \ Groups	CYP/SAL	CYP/SIMV	CONTROL
Bladder	23	14*	0
Ureter	18	7*	0
Kidney	10	7	0

* $p<0.05$ comparing the histologic scores from CYP/SAL (cyclophosphamide and saline) treated rats and CYP/SIMV (cyclophosphamide/simvastatin) treated rats, by using the Mann-Whitney test.

Cytokines analysis

All 3 cytokines assayed were detected in the plasma of saline and CYP-treated rats, as well as in controls. TNF- α plasma expression was significantly lower in rats treated with simvastatin (164.8±22 pg/ml) than in rats treated with 0.9% saline (378.5±66 pg/ml). IL-1β plasma levels showed the most drastic decrease (44.8±8 pg/ml) and were 3-fold lower in CYP/SIMV rats, compared with the (CYP/SAL) group rats (122.9±26 pg/ml). The plasma levels of IL-6 in the CYP/SAL-treated rats (123.6±18) were elevated at least 2.3-fold compared with the CYP/SIMV-treated rats (52.4±13). In control rats the plasma cytokines were significantly less expressive than in the other groups. These data are summarized in Table 2.

TABLE 2 – Plasma cytokine values from rats treated with cyclophasphamide, saline 0.9% and simvastatin

Cytokines \ Groups	CYP/SAL	CYP/SIMV	CONTROL
TNF-α (pg/mL)	378.5±66	164.8±22*	24.8±5
IL-1β (pg/mL)	122.9±26	44.8±8*	13.2±4
IL-6 (pg/mL)	123.6±18	52.4±13*	17.1±5

* $p<0.05$ comparing the plasma cytokines from CYP/SAL (cyclophosphamide and saline) treated rats and CYP/SIMV (cyclophosphamide/simvastatin) treated rats, and controls, by using the t test.

Discussion

Cyclophosphamide (CYP) has remained a mainstay in the treatment of several common malignancies including breast cancer, lymphoma and leukemia. In addition, it has immunosuppression effects in autoimmune disorders such as systemic lupus erythematosis[14]. While patients with these diseases have derived profound clinical benefits, the use of CYP has been associated with severe urological side effects, most notably hemorrhagic cystitis (HC). While continuous bladder irrigation and avoidance of future exposure to CYP can ameliorate the acute issues, the treatment of HC is costly. Clearly prevention of this urological morbidity is the preferred course[15].

CYP is a nitrogen mustard alkylating agent that imparts its antineoplastic activity by forming cross links with tumor DNA. It is metabolized in the liver to its active form and eliminated primarily via renal excretion. A portion of CYP is converted to a metabolite, acrolein, the causative agent responsible for CYP induced HC[16]. Multiple investigators have demonstrated increases in serum proinflammatory mediators such as TNF-a, IL-1b and type 2 cyclooxygenase, which are directly involved in the pathogenesis of cystitis, since individual inhibition of these molecules has been shown to decrease CYP induced bladder damage[9,10].

An exogenous mediator of bladder inflammation includes the pharmaceutical use of CYP to treat cancer. The toxic CYP metabolite, acrolein, can accumulate in the bladder to cause acute inflammation and hemorrhagic cystitis[16]. CYP mediates a robust inflammatory response, resulting histologically in subepithelial edema, neutrophil infiltration, hemorrhage and frank tissue destruction. This was observed in the current study since CYP led to higher histological inflammation scores on comparative analysis. Additionally, we observed similar damage in ureter of rats treated with CYP.

Cytokines, including IL-6, have been implicated in the pathophysiology of HC with increased levels identified in patients with HC. Lamale *et al*.[17] reported that IL-6 levels were increased in patients with HC, compared with controls[17]. Furthermore, Lotz *et al*.[18] confirmed that the IL-6 high levels in HC have its origin in urothelial cells, since increased levels of this molecular marker were present in spontaneously voided urine but not in urine obtained from urethral washings[18]. Another study confirmed this observed correlation between IL-6 and HC, further establishing an association between IL-6 and symptom severity[19]. In fact, in the current study the expression of TNF-a, IL-1b and IL-6 were high in all the rats treated with CYP, and affected with HC. However, it was significantly lower in simvastatin treated rats than in rats treated with 0.9% saline, meaning that simvastatin exerted a protective effect to the urotelium.

Despite the various options for preventive measures for the occurrence of CH after the use of CYP, such side effect remains without adequate treatment. Some investigators reported a reduction in severe cyclophosphamide HC with the institution of a hyperhydration and forced diuresis regimen[20]. In addition to forced diuresis, have been studied the benefits of administering sodium-

2-mercaptoethansulfonate (MESNA) in conjunction with cyclophosphamide. In patients treated with MESNA, the HC persists in 10-40%[21]. Thus, the results obtained in the current study may represent a new approach for prevention of HC, due to the demonstration of decreased inflammation and urothelial lesions in the animals that made use of simvastatin. Although no literature reports on the use of statins for the prevention and treatment of CH, there is increasing evidence to support the suggestion that simvastatin acts as a powerful antiinflammatory with a wide spectrum of effects[22-24]. The use of different statins *in vivo* and *in vitro* showed a decreased production of chemokines involved in leukocyte migration[25]. It has been also demonstrated that statin decreases the expression of cytokines[26]. Moreover, recent experimental studies have demonstrated the antiinflammatory action of statins in improving the healing of infected wounds in rats[6], as well as in abdominal sepsis[7].

Furthermore, we suggest that simvastatin supplementation may offer a simple and inexpensive method that can be used in conjunction with CYP therapy to decrease its side effects. Further functional studies may provide important insights and relevant information on the roles of simvastatin in urotelium protection. In conclusion, simvastatin was effective in preventing hemorrhagic cystitis induced by CYP in an experimental model, by decreasing inflammation and urothelial injury.

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