SUPPLEMENT

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Protective Effect of Intravesical Platelet-Rich Plasma on Cyclophosphamide-Induced Hemorrhagic Cystitis

Abstract

Purpose: Hemorrhagic cystitis (HC) is the most common urotoxic side effect of cyclophosphamide (CYP). Platelet rich plasma (PRP) plays an important role in wound healing and inflammatory responses. The aim of this study was to investigate the efficacy of intravesical PRP at treatment of interstitial cystitis (IC).

Material-Methods: Female rats (n=24) were used. IC was induced by intraperitoneal injection of cyclophosphamide (CYP). Rats were randomly allocated to one of four groups (n = 6 per group): a control group; a sham group with saline (75 mg/kg; i.p.) instead of CYP on day 1; a IC group, which was injected with CYP (150 mg/kg; i.p.) on day 1; and, a intravesical PRP-treated group which was injected with CYP (150 mg/kg; i.p.) on day 1. On day 2, the rats in each group were sacrificed under anesthesia.

Results: Histological evaluation showed that bladder inflammation in CYP-treated rats was not suppressed by PRP. CYP administration induced severe IC with marked edema, hemorrhage and inflammation in CYP and CYP+PRP groups, but PRP was not found to be effective to decrease these effects.

Conclusion: The application of PRP could not reverse the histopathological changes in rats that had interstitial cystitis due to the cyclophosphamide injection.

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Interstitial cystitis (IC) is a non-infectious disease that has been known for a century but little is known about its etiology [1]. Symptoms include suprapubic pain, urinary frequency, nocturia and urgency. IC prevalence in the United States in women between ages of 58 – 83 years old is 2.3% [2]. But estimated prevalence with undertreated and underdiagnosed women is between 1-11% [3]. Although it is not a life treating disease, it decreases the quality of life severely, especially in females [4]. It has been shown that, patients suffering from IC have lower quality of life scores than the chronic dialyses patients [5]. There are many theories about the etiology of IC such as autoimmune mechanisms, infections, defective glycosaminoglycan-layer, neurogenic and psychosomatic mechanisms [4]. Although the etiology is still unclear, there are several treatment modalities, including intravesical treatments. Some of these intravesical treatments are effective, but their cost-effectiveness and morbidities should be kept in mind. Studies to develop alternative, cheap and simple solutions for IC are necessary.

Experimental IC was induced by intravesical administration of an irritant or immune stimulant, to induce inflammation so as to investigate IC pathophysiology [6]. The bladder inflammation presents with edema, epithelial damage, inflammatory cell infiltration, fibrosis, venous congestion and hemorrhage in IC models.

Cyclophosphamide (CYP) is a chemotherapeutic alkylating agent that is used in solid tumours, lymphomas and non-neoplastic situations as rheumatic arthritis. It is also used to prevent organ rejection in transplant patients [7]. Its major limitation are the uro-toxic side effects, such as hemorrhagic cystitis (HC), bladder fibrosis, contracture and urgency [8, 9]. CYP is metabolized in liver but eliminated primarily through the kidneys. CYP's major uro-toxic metabolite is acrolein, which causes HC by causing subepithelial edema, hemorrhage and necrosis at the bladder wall [9].

Platelets are a type of blood cell that are reservoirs for factors responsible for tissue repair [10]. Platelet-rich plasma (PRP) is an autologous blood product that has been proposed for wound treatments since 1990 [11]. PRP contains many growth factors and proteins, such as platelet-derived growth factors (PDGF), transforming growth factor beta (TGF- β), fibroblast growth factor (FGF), insulin-like growth factor 1(IGF-1), connective tissue growth factor (CTGF) and epidermal growth factor (EGF) [12,13]. These factors that are present in PRP promote accelerated repair with better tissue differentiation at the area of the injury [15].

In this study, we used CYP-induced cystitis model to examine whether intravesical PRP might be protect the bladder against CYP-induced injury.

Materials and Methods

Animals and environmental conditions

Twenty-four female Sprague–Dawley rats aged 12 weeks weighing 250-300 g, two animals per cage were housed in a room maintained at a temperature of 22-24 °C with artificial lighting from 08:00 to 20:00 and with 13 to 18 air changes per hour. Animals were given rodent chow and tap water *ad libitum*. The Local Animal Care and Use Committee approved the protocols for the animal study.

Rats were randomly divided into four groups of six animals in each group (control (C), sham, CYP only-administered (CYP) and PRP-administered 24 h after CYP administration (CYP+PRP). All groups received intramuscular ketamine (50 mg/kg) and xylazine (4 mg/kg) for anesthesia. Animals in the control group were not given any medications and were sacrificed on the same day. Sham group were given 2 ml of saline one time at the experiment day intravesically. Cyclophosphamide (CYP) (Endoxan ©Sigma Aldrich Co. St. Louis, MO, USA) and CYP+PRP groups were given CYP at a dose of 150 mg/kg dissolved in saline immediately before application (5 mL/kg) by a single intraperitoneal (i.p.) injection to induce chemical cystitis. Fresh prepared 0.3 ml of PRP was given intravesically 24 hours after the CYP injection to the CYP +PRP group. A sterile 18 gauge angiocath was inserted into the bladder through the urethral opening and all treatment agents were administered with 1 mL syringe. PRP was left in the bladder in 1 hr of duration. Sham, CYP groups were sacrificed 24 hours after the saline instillation or CYP injection. CP+PRP group was sacrificed 6 hours after the PRP instillation. Their bladders were removed and stored in 10% formalin solution. The samples were blindly reviewed by a pathologist.

Preparation of PRP

The preparation of PRP by centrifugation was initially completed by a “two-step gradient centrifugation” method immediately after blood collection to avoid undesired non-specific platelet activation. We took 3 ml of blood from the tail veins to a blood tube containing citrate. Samples were centrifuged (1600 rpm- 15 minutes) to separate the erythrocytes from the clotting factors, platelets and leukocytes. The plasma was subjected to a second centrifugation step (2800 rpm- 8 minutes) to harvest the PRP fraction from the

platelets and leukocytes. We took 0.3 ml of the supernatant as PRP. Once the release of biomolecules from platelets is activated, PRP can be used for 3-4 hours.

CYP was dissolved in saline and prepared immediately before treatment. The rats were given a single intraperitoneal dose of CYP (150 mg/kg/day). All animals were sacrificed 24 h after CYP administration.

Tissue preparation

After 24 h of acute hemorrhagic cystitis induction, all female rats were euthanized by decapitation. The bladders were assessed for severity of cystitis then the bladders were fixed for 24 h in 10% buffered formaldehyde for histopathological evaluation and immunohistochemical analysis.

Histopathological examination

The bladders were routinely processed, embedded in paraffin and sectioned at 4- μ m thickness, deparaffinized, and rehydrated using standard techniques according to the protocol provided by the manufacture (Richard-Allan-Scientific, Kalamazoo, MI). The sections were stained with hematoxylin-eosin stain for microscopic examination. All sections were examined with a light microscope by a pathologist blinded to the sample treatments. A pathologist, blinded to the study groups, rated the samples for mean histological damage, including edema, hemorrhage, epithelial degeneration, and inflammation on a scale of 0 (normal) to 3 (severe changes). Mucosal ulceration was scored as 0 (normal), 1 (epithelial denuding), 2 (focal ulceration) or 3 (widespread epithelial ulceration).

Statistical analysis

Statistical analysis was performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Data were expressed as mean $\pm$ standard deviation (SD), unless otherwise stated. The variables were investigated using visual (histograms, probability plots) and analytical methods (Kolmogorov-Smirnov/Shapiro-Wilk's test) to determine whether they are normally distributed. The groups were compared using one-way (ANOVA) analysis of variance for variables that showed a normal distribution, followed by a *post hoc* Tukey multiple comparison test to estimate the significance of differences between groups. P value of <0.05 was considered statistically significant.

Results

Normal histopathological patterns were detected in control and sham groups (Figure 1A-B). Hemorrhages, inflammatory cells with generalized edema at submucosa and lamina propria were seen in CYP group. Because of these changes, submucosa and lamina propria enlarged and the lumen narrowed. Ulcerations with serious degeneration desquamation were observed on epithelial mucosa. It was seen that the exudate collected consisted of erythrocyte, fibrin, desquamated epithelium and inflammatory cells at the lumen (Figure 1C-D). There were similar histopathological lesions observed in CYP+PRP group (Figure 1E-F). There were significant differences between CYP groups compared to the control and sham, but there were no significant differences between CYP and CYP+PRP groups regarding edema, hemorrhage,

FIGURE 1. Histopathologic analysis of rat bladder tissues from different treatment groups.

Normal histologic tissues of rat bladders from control and sham groups (A-B), edema at the submucosa of the rat bladder and exudate on the lumen of CYP group shown by arrows (C-D), submucosal edema, necrosis and hemorrhage at the CYP+PRP group (E-F), (H&E stain; A, C, E; Bar=250 μ m and B, D, F; Bar=50 μ m).

TABLE 1. Comparison of histopathological changes at different groups

	Control	Sham	CYP	CYP+PRP
Hemorrhage	0	0	1.3±0.5	1.0±0.5
Epithelial degeneration	0	0.2±0.2	2.2±0.3	2.2±0.3
Inflammation	0	0	1.0±0.3	1.2±0.3
Edema	0	0	2.5±0.2	2.3±0.2
Ulcer	0	0	1.2±0.5	1.0±0.4

Edema, hemorrhage, epithelial degeneration, and inflammation on a scale of 0 (normal) to 3 (severe changes). Mucosal ulceration was scored as 0 (normal), 1 (epithelial denuding), 2 (focal ulceration), 3 (widespread epithelial ulceration)

epithelial degeneration and inflammation (Figure 1). The histopathological results of all groups are presented in Table 1.

There was a significant difference between control and CYP groups regarding epithelial desquamation, inflammation edema and ulcers ($p=0.001$, $p=0.03$, $p=0.001$, $p=0.003$, respectively), but there were no significant difference between CY and CYP+PRP groups regarding above issues ($p=1$, $p=0.935$, $p=0.868$, $p=0.808$, respectively)

Discussion

Interstitial cystitis (IC) is a chronic non-inflammatory disease especially with irritative urinary symptoms, mostly on women [15]. The Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction (SUFU) defined IC as “an unpleasant sensation (pain, pressure, discomfort) perceived to be related to the urinary bladder, associated with lower urinary tract symptoms of more than 6 weeks duration, in the absence of infection or other identifiable causes” [16]. Even there are many theories including infection, inflammation, autoimmune mechanisms, hypoxia and central neurologic mechanisms the main etiology is still controversial [17]. The most accepted theory is the disruption of bladder lining glycosaminoglycan (GAG) layer [18]. There are many treatment modalities, although there is no cure [19]. The current treatment modalities give only a relief of symptoms.

Cyclophosphamide (CYP) is a nitrogen-mustard alkylating agent that forms cross-links with tumor DNA to show its antineoplastic activity which is widely used as a chemotherapeutic agent [20]. Cyclophosphamide (CYP)-induced bladder inflammation is a well-established pre-clinical model for interstitial cystitis/painful bladder syndrome [8]. CYP is converted in the kidneys to acrolein, which causes cystitis via eliciting an inflammatory response [9]. Heparin, pentosan polysulphate, hyaluronic acid (HA) and chondroitin sulphate are urothelium-restoring agents that are

administered orally or intravesically in order to restore the GAG lining of the bladder. Their efficacy is limited [18] so this directed us to find new intravesical treatments for IC.

The positive potential of platelet-rich plasma (PRP) on cellular anabolism and tissue regeneration has been shown with several *in vitro* and *in vivo* animal studies [21]. PRP is based on obtaining a high concentration of platelets from autologous blood plasma and applying to the damaged area. High levels of VEGF, PDGF-BB, EGF and basic fibroblast growth factor (bFGF) have been detected in PRP, and these components support homeostasis and wound healing [14]. PRP is mostly used for wound healing in orthopedic and plastic surgery clinics. Many studies have shown that PRP preparation increases the platelets count at least two- to six-fold from the baseline value [22].

There are many experimental studies searching for new, effective and safe intravesical agents for the treatment of IC. Kim *et al.* showed a significant decrease in histopathological changes as epithelial ulceration, hemorrhage, submucosal edema, desquamated granular uroepithelium cells, and inflammatory cells infiltration with intravesical diallyl disulfide (DADS) compared to CYP only treated group [23]. In another study, intravesical pentoxifylline showed significant healing on histopathological changes compared to the CYP treated group [20]. In contrast to these studies, Resveratrol did not showed a significant healing when compared with control group in rats with CYP induced cystitis [24]. Our results showed no significant healing with intravesical PRP treatment in CYP+PRP group when compared to CYP only group. And there was a significant difference between CYP+PRP group and control group.

Different concentrations or recurrent instillations of PRP should be investigated as no previous data are available in the literature for its intravesical instillation. The present model is

not identical to chronic human IC as it induces a short-term acute inflammation in the bladder

Conclusion

PRP showed no healing effect on interstitial cystitis when intravesically administered once. It is still a promising agent that requires further studies with different dosages and recurrent applications.

References

- van de Merwe JP, Nordling J, Bouchelouche P, Bouchelouche K, Cervigni M, Daha LK, et al. Diagnostic criteria, classification, and nomenclature for painful bladder syndrome/interstitial cystitis: an ESSIC proposal. *Eur Urol*. 2008;53:60–67
- Lifford KL, Curhan GC. Prevalence of painful bladder syndrome in older women. *J Urol*. 2008; 73:494
- Dyer AJ, Twiss CO. Painful Bladder Syndrome: An Update and Review of Current Management Strategies. *Curr Urol Rep* 2014;15:384
- Dinis S, de Oliveira JT, Pinto R, Cruz F, Buffington CT, Dinis P. From bladder to systemic syndrome: concept and treatment evolution of interstitial cystitis. *Int J Womens Health*. 2015;23(7):735–44
- Chancellor MBA. Multidisciplinary consensus meeting on IC/PBS: outcome of the consensus meeting on interstitial cystitis/painful bladder syndrome. *Rev Urol*. 2007;9:81–83
- Westropp JL, Buffington CA. In vivo models of interstitial cystitis. *J Urol*. 2002;167:694–702
- West NJ. Prevention and treatment of hemorrhagic cystitis. *Pharmacotherapy* 1997;17: 696–706.
- Gray KJ, Engelmann UH, Johnson EH, Fishman IJ. Evaluation of misoprostol cytoprotection of the bladder with cyclophosphamide (Cytosan) therapy. *J Urol*. 1986;136:497–500.
- Levine LA, Richie JP. Urological complications of cyclophosphamide. *J Urol*. 1989;141: 1063–1069.
- Harrison P. Platelet function analysis. *Blood Reviews*, 2005;vol. 19, no. 2, pp. 111–123.
- Quarteiro ML, Tognini JR, de Oliveira EL, Silveira I. The effect of platelet-rich plasma on the repair of muscle injuries in rats. *Rev Bras Ortop*. 2015;50(5):586–595
- Wang W, Zolty E, Falk S, Basava V, Reznikov L, Schrier R. Pentoxifylline protects against endotoxin-induced acute renal failure in mice. *Am J Physiol Renal Physiol* 2006;291:1090–1095
- Marukawa S, Hatake K, Wakabayashi I, Hishida S. Vasorelaxant effects of oxpentifylline and theophylline on rat isolated aorta. *J Pharm Pharmacol* 1994;46:342–345.
- Foster TE, Puskas BL, Mandelbaum BR, Gerhardt MB, Rodeo SA. Platelet-rich plasma: from basic science to clinical applications. *Am J Sports Med*. 2009;37(11):2259–72
- Hanno P, Lin A, Nordling J, Nyberg L, van Ophoven A, Ueda T, et al. Bladder pain syndrome committee of the international consultation on incontinence. *Neurourol Urodyn* 2010;29:191–8.
- Hanno P, Dmochowski R. Status of international consensus on interstitial cystitis/bladder pain syndrome/painful bladder syndrome:2008 snapshot. *Neurourol Urodyn*. 2009;28(4):274–86
- Toft BR, Nordling J. Recent developments of intravesical therapy of painful bladder syndrome/interstitial cystitis: a review. *Curr Opin Urol* 2006;16(4):268–272
- Patel BN, Evans RJ. Overactive bladder and pain: management strategies. *Curr Urol Rep* 2010;11(6):379–384
- Riedl CR, Engelhardt PF, Daha KL, Morakis N, Pflüger H. Hyaluronan treatment of interstitial cystitis/painful bladder syndrome. *Int Urogynecol J Pelvic Floor Dysfunct* 2008;19(5):717–721
- Abo-Salem OM. Uroprotective Effect of Pentoxifylline in Cyclophosphamide-Induced Hemorrhagic Cystitis in Rats. *J Biochem Molecular Toxicology*. 2013;27(7): 343–50
- Yoshioka T, Kanamori A, Washio T, Aoto K, Uemura K, Sakane M, et al. The effects of plasma rich in growth factors (PRGF-Endoret) on healing of medial collateral ligament of the knee. *Knee Surg Sports Traumatol Arthrosc*. 2013;21(8):1763–9
- Gobbi A, Karnatzikos G, Mahajan V, Malchira S. Platelet-rich plasma treatment in symptomatic patients with knee osteoarthritis: preliminary results in a group of active patients. *Sports Health* 2012;4(2):162–72
- Kim SH, Lee IC, Baek HS, Shin IS, Moon C, Bae CS, et al. Mechanism for the protective effect of diallyl disulfide against cyclophosphamide acute urotoxicity in rats. *Food and Chemical Toxicology*. 2014;64:110–118
- Keles I, Bozkurt MF, Cemek M, Karalar M, Hazini A, Alpdagtas S, et al. Prevention of cyclophosphamide-induced hemorrhagic cystitis by resveratrol: a comparative experimental study with mesna. *Int Urol Nephrol*. 2014; 46:2301–10