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Research Article

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# The effect of pentoxifylline on leukocyte accumulation and angiogenesis in an air pouch model in rat

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**ABSTRACT**

*Inflammation is a complex biological response of a tissue to harmful stimuli, such as pathogens, damaged cells, and irritants. Inflammation is characterized by marked vascular changes including angiogenesis and increased permeability induced by various inflammatory mediators. Findings have shown that pentoxifyllineexerts inhibitory effect on vascular permeability by TNF-α inhibition. In this study, we investigated the effect of pentoxifylline on inflammation process such as leukocyte infiltration and angiogenesis in an air pouch model in rat. Twenty-four male wistar rats (160–190 g) were divided randomly into a control and three treated groups. Inflammation was produced by injection of carrageenan into a newly formed air pouch. Treatment groups were orally administrated for five days with 10, 20 and 40 mg/kg/day of pentoxifylline following injection of carrageenan. Oral administration of pentoxifylline at a dose of 40 mg/kg suppressed leukocyte infiltration into the air pouches by reducing the number of cells in the pouches from 11±1.6 × $10^7$ in control to 5.5±1.1 × $10^7$ ($p<0.05$). Meanwhile, 10 mg/kg of pentoxifylline inhibited the inflammatory induced angiogenesis by 43% ($p<0.001$). The anti-angiogenic action of pentoxifylline was reversed by increasing the dose. Pentoxifylline dosedepentely inhibits PMN recruitment anddecreases inflammatory reaction in the air pouch model, but suppresses angiogenesis only with a low dose. Pentoxifylline might be a candidate for use as an anti-inflammatory agent that with low doses produces an anti-angiogenic activity as well.*

**Keywords**: Pentoxifylline, Inflammation, Leukocyte Infiltration, angiogenesis, air pouch.

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## INTRODUCTION

Pentoxipfylline (PTX) is a non-selective inhibitor of cyclic-3′, 5′-phosphodiesterase (PDE), which is used in the treatment of peripheral vascular diseases. Pentoxifylline (PTX) is a methylxanthine derivative which possesses important vasodilator and rheologic properties, altering erythrocyte deformability, erythrocyte oxygen delivery, decreasing platelet and red cell aggregation, decreasing fibrinogen and plasma viscosity, which account for its effect in increasing local tissue perfusion and haemodynamics [1]. Recent studies demonstrated that pentoxifylline displays some important immuno-regulatory properties that might affect the anticancer responses [1]. PTX reduces secretion of some cytokines such as, IL10, IL-12, TNF and IFN-gamma and thus it could exert immunomodulatory activity. Inhibition of TNF has been shown to increase leucocytes adhesion and to disrupt intercellular junctions of postcapillary venular endothelium, leading to plasma extravasation [2]. Furthermore, PTX blocks lymphocyte cytotoxicity affecting the perforin-dependent pathway [2]. TNF-alpha is an important mediator in inflammation [3] and its inhibition is considered the likely mechanism underlying the anti-inflammatory effects of PTX [4]. Several studies have described the effect of PTX on inflammatory process [5-8]. PTX given after initiation of inflammation was effective in reducing the progression of edemas. Administration of the PTX to rats before intraplantar injection of carrageenan has also been reported to reduce paw edema [7]. In addition, PTX has venodilator, antioxidant properties, inhibits platelet aggregation and prevents adherence of leucocytes in post-capillary and later venules [1].

In this way, PTX could be reducing inflammation by alleviating the consequent generation of oxidant stress by neutrophils. Angiogenesis, the process of growth of new vessels and capillary networks, is tightly regulated by angiogenic factors. A large body of literature indicates that many angiogenic factors including VEGF signal in part through a common pathway that involves activation of Akt and/or eNOS derived NO production [9]. Pentoxifylline may cause suppression of endometriotic lesions by suppressing angiogenesis through VEGF-C and flk-1 expression [9]. Herein, we have explored the regulatory effect of this drug on accumulation, of leukocytes, the components of inflammatory processes and angiogenesis. PTX exhibited a down-modulatory effect on these phenomena. This study was designed to further analyze the effects of PTX treatment on inflammation in air pouch model and inflammatory angiogenesis.

## EXPERIMENTAL SECTION

**Chemicals**: We used pentoxifylline 400mg tablets from Apotex Pharmaceutical Company (Ontario, Canada). Carrageenan was obtained from Sigma Company (Germany). All other chemicals were of highest grade commercially available.

**Animals**: Male wistar rats (170-190 g) specific pathogen-free, were used in this study. The animals obtained from Animal House of Tabriz University of Medical Sciences at a controlled ambient temperature of 25 ± 2 °C with 40 ± 10% relative humidity and with a 12 h light / 12 h dark cycle condition. They were feed with standard laboratory chow and tap water ad libitum. All animals and their care were conducted in accordance with Tabriz University of medical sciences guidelines for the care and use of laboratory animals.

**Carrageenan-induced Air pouch model of inflammation**

Air cavities were produced under light diethyl ether anesthesia by subcutaneous injection of 8 ml of sterile air into the intra scapular area of dorsal part of rats to open an oval in shape space and Twenty-four hours later, 4 ml of 1% (w/v) carrageenan dissolved in saline was injected into the air pouch under light diethyl ether anesthesia. The carrageenan solution had been sterilized by autoclaving at 121°C for 15 min and supplemented with antibiotics [0.1 mg of penicillin G potassium (Daana Pharm, Tabriz, Iran) and 0.1 mg of dihydrostreptomycin sulfate (Daana Pharm, Tabriz, Iran) per milliliter of the solution] after cooling to 35–40°C.

Pentoxifylline tablets dissolved were crushed with a mortar and pestle and added to distilled water with 1% CMC (w/v). Stock solutions were diluted with distilled water and 0.2 ml of the diluted solution containing the indicated amount of drugs was gavaged to animals. Animals were divided into 3 dose groups of 7 rats per group and then vehicle as control group, pentoxifylline 10, 20 and 40 mg/kg were given orally by gavages to pouch-bearing rats 30 minutes before injection of carrageenan and every twenty four hours until the $6^{th}$ day. Control rats received the same amount of distilled water containing CMC at 1% (W/V).

**Determination of pouch fluid volume, leukocyte infiltration, and granulation tissue weight**

6 days after the injection of carrageenan solution, the Rats were sacrificed by cervical dislocation under anesthesia by intra peritoneal injection of ketamine (60 mg/kg); xylazine (10 mg/kg) and acepromazine (10 mg/kg). Total pouch fluid was collected and its volume measured. The exudate samples collected in the EDTA-treated glass tubes, and they were refrigerated at 4°C until to be counted. The pouch fluid was diluted 2-fold with saline and the leukocytes in the fluid were enumerated using a hemocytometer. Granulation tissue that formed was also dissected and weighed.

**Determination of angiogenesis in granulation tissue**

Measurement and visualization of angiogenesis in granulation tissue were carried out using carmine red dye (Sigma, Germany) according to the method described by Ghosh et al with slight modifications [10]. 6 days after the injection of carrageenan solution, 3 ml of 5% (w/v) carmine dye in 5% (w/v) gelatin (Sigma, Germany) in saline at 37°C was injected into jugular vein of each rat anesthetized by intra peritoneal injection of Ketamine (60 mg/kg); Xylazine (10 mg/kg) and Acepromazine (10 mg/kg). The carcasses were chilled in 4 °C refrigerator for 3 h, and the whole granulation tissue was dissected and after drying by Whatman paper (Germany), it was weighted accurately. After being washed with PBS (pH 7.4), granulation tissue was homogenized in 2 volumes of 0.5 mM NaOH solution using a homogenizer for 4 min at scale 40 on an ice bed. The tissue homogenate was centrifuged at 10,000*g* and 4°C for 30 min. Five hundred microliters of the supernatant was diluted 2-fold with 0.5 mM sodium hydroxide and centrifuged again at 14,000*g* and 4°C for 30 min. The dye content in 200 ml of the supernatant was determined spectrophotometrically by measuring absorbance at 490 nm. For the standard curve, known amounts of carmine dye were added to the final supernatant of granulation tissue of control rats that were injected with 3 ml of a 10% (w/v) gelatin solution in saline without carmine dye, and the absorbance determined. The amount of carmine dye in the whole granulation tissue was then calculated [10].

**Statistical analysis**

Data were presented as mean ± SEM. Statistical comparisons were made by oneway analysis of variance (ANOVA) as appropriate. If ANOVA analysis indicated significant differences, a Student Newman Keuls post test was performed to compare mean values between treatment groups and control. Differences between groups with a *p* value <0.05 were considered significant. The database and the various tests mentioned were done with the programme Instat 2.

## RESULTS

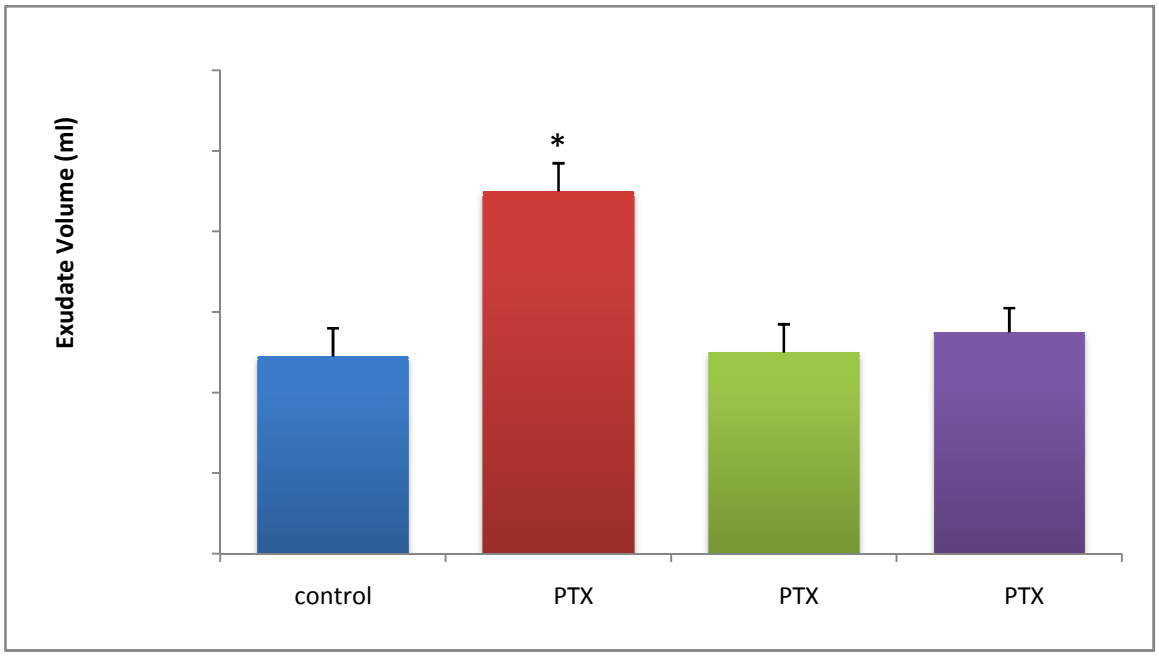


**Figure 1. Effect of pentoxifylline on exudate volume in rats' air pouch. Data are presented as mean± SEM of at least 6 animals per group. This experiment was repeated in triplicate. Pentoxifylline with 10 mg/kg increased exudate volume, but other doses did not make significant difference in comparison with control. *, *p* < 0.05 compared with control group.**

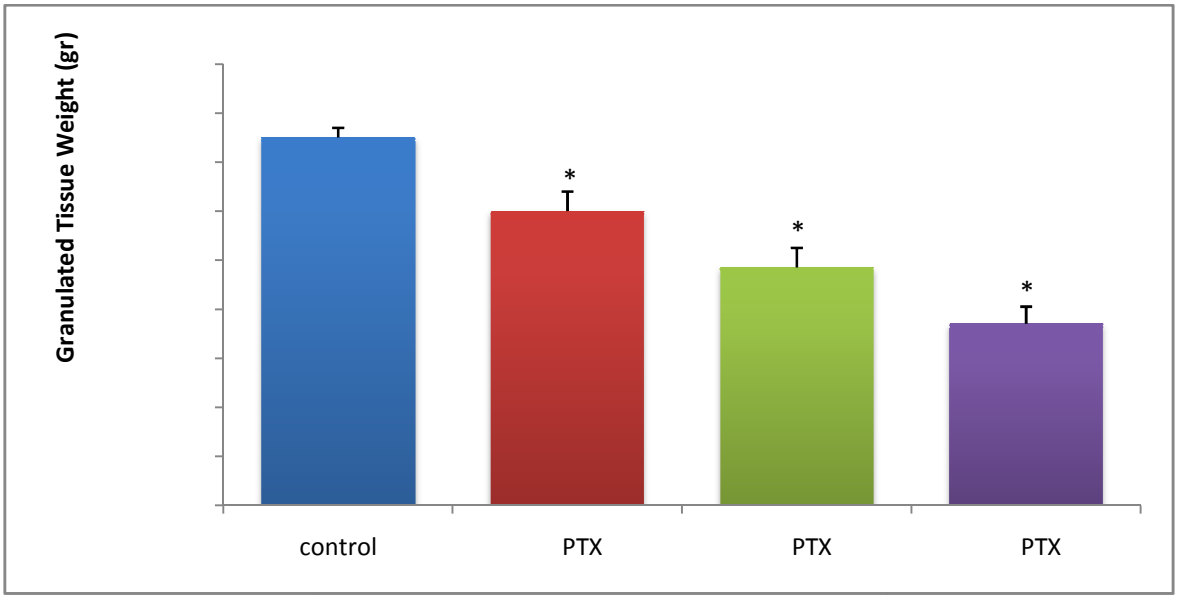


**Figure 2. Effect of pentoxifylline on granulated tissue weight in rats' air pouch. Graphs show the mean± SEM of at least 6 animals per group. This experiment was repeated in triplicate. Granulated tissue weights were expressed in grams. The control group was treated only with 1% CMC in saline. *, *p* < 0.05 compared with control group.**

**Effect of pentoxifylline on pouch fluid volume, number of infiltrating leukocytes and granulation tissue weight in carrageenin-induced inflammation in rats**

The degree of inflammation induced by carrageenan, as measured by exudate volume, leukocyte count in the air pouch exudates and granulation tissue weight was determined in pentoxifylline-treated and untreated rats. The anti-inflammatory action of pentoxifylline was evaluated by determination of its effect on the number of leukocytes recruited into the pouch. Oral administration of PTX (10 mg/kg bodyweight) evoked slight changes in mean the pouch fluid accumulation, but the other doses (20 mg/kg, 40 mg/kg bodyweight) did not make an apparent difference (Figure 1). The leukocyte infiltration into the pouch fluid was determined by hemocytometer and total leukocytes were calculated. PTX (10, 20, 40 mg/kg bodyweight) reduced total leukocytes, in dose depended manner significantly in comparison to control group (Figure 2). The effect of PTX (10, 20 and 40 mg/kg) on granulomatous tissue weight assessed (Figure 3) 6 days after carrageenan injection. All of treatment groups reduced granulation tissue weight significantly, in comparison with control group.

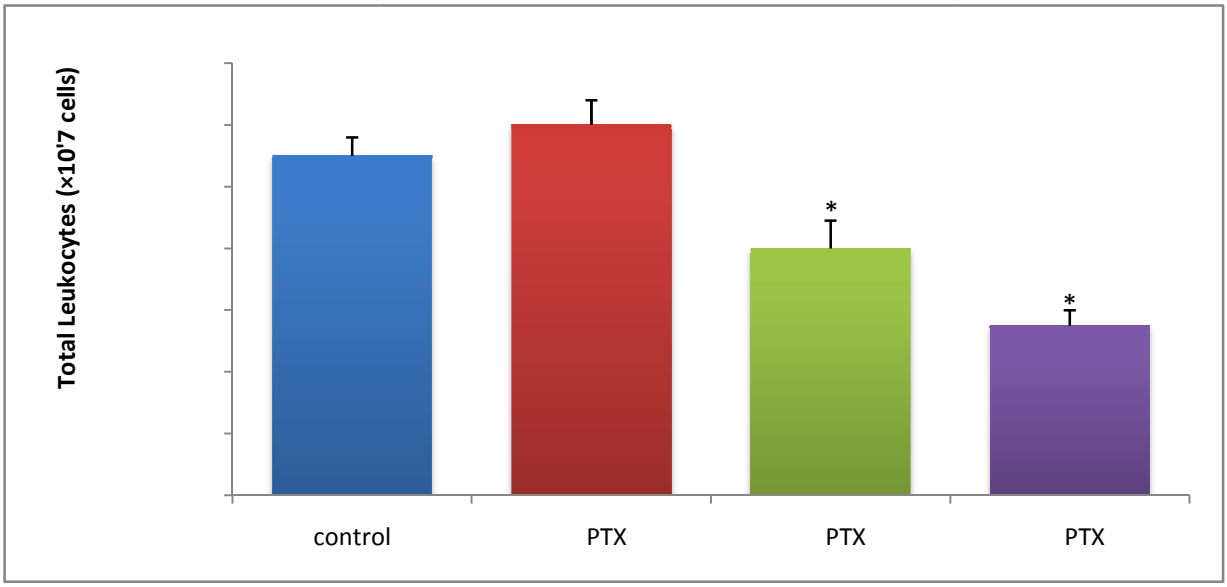


**Figure 3. Effect of pentoxifylline on leukocytes recruitment in rats' air pouch. Data are presented as mean± SEM of at least 6 animals per group. This experiment was repeated in triplicate. The control group was treated only with 1% CMC in saline. *, $p < 0.05$ compared with control group**.

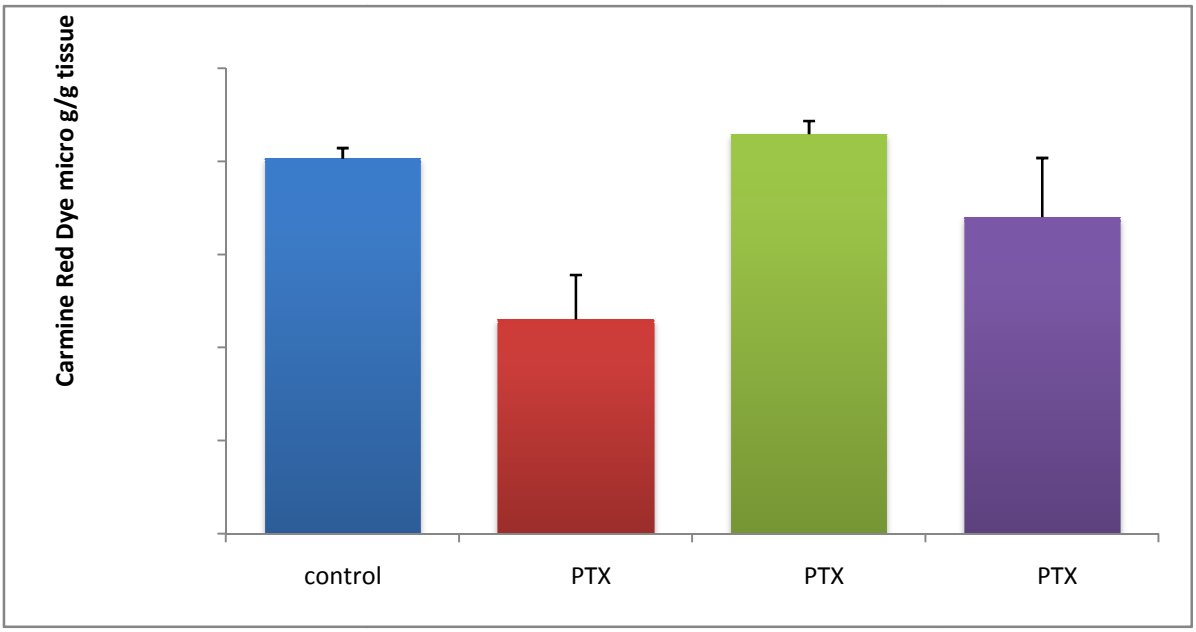


**Figure 4. Effect of pentoxifylline on angiogenesis in rats' air pouch. Data are presented as mean± SEM of at least 6 animals per group. Results have been shown as micrograms of carmine dye per milligram weight of tissue. This experiment was repeated in triplicate. The control group was treated only with 1% CMC in saline. *, $p < 0.05$ compared with control group.**

**Effect of pentoxifylline on angiogenesis in air pouch model**

New vessels formations during chronic inflammatory processes are required not only for the maintenance of tissue perfusion but also to allow increased cellular traffic [11]. Therefore, angiogenesis in the granulation tissue was assessed by the carmine dye method. Oral administration of pentoxifylline (10 mg/kg), lowered angiogenesis by 43% ($p < 0.001$) and with other dose (40 mg/kg) showed less potent anti- angiogenic effects by 15% ($p < 0.05$) but with 20 mg/kg induced angiogenesis 6% with no significance (Figure 4).

**Histopathological examination**

A separate group of treated rats with pentoxifylline (10, 20, and 40 mg/kg) was prepared for the histopathological examination. The results are compared to those of control group rats. The tissues were embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin (H&E) for evaluation of histology (Figure 5).

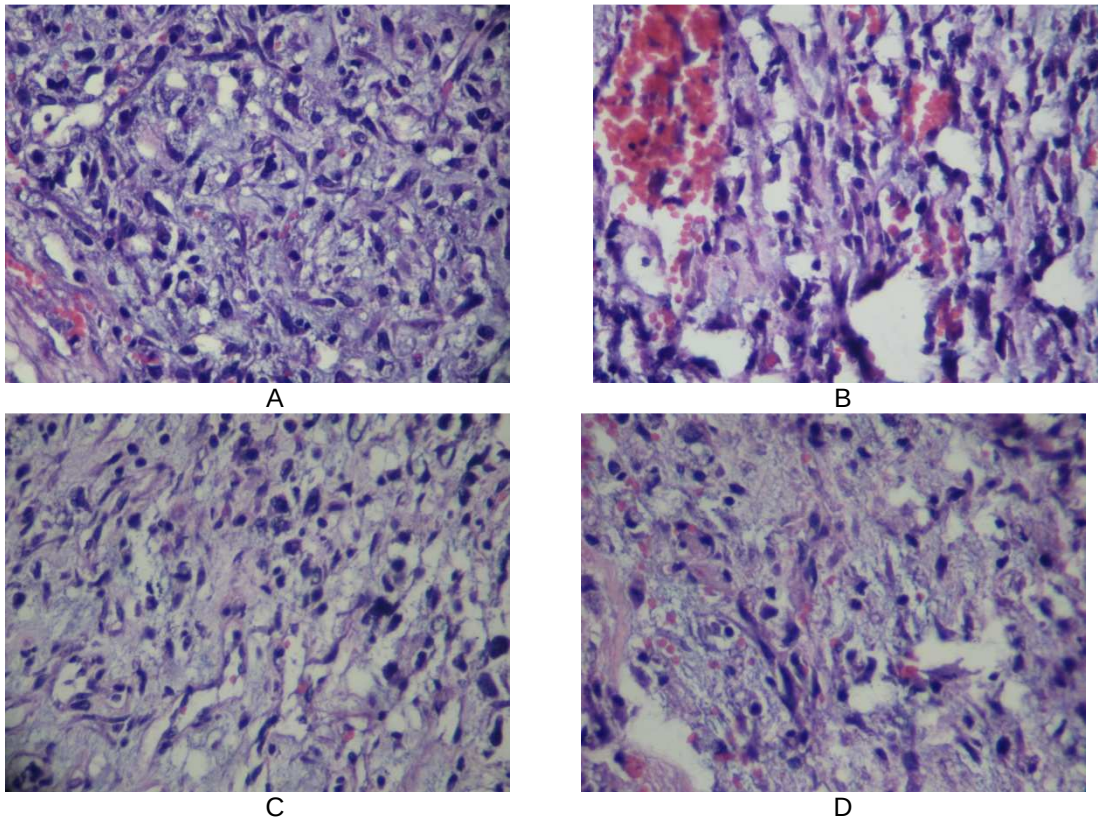


**Figure 5. Histological sections of skin biopsies obtained from rats with air pouches granuloma. Hematoxylin and eosin-stained sections from rat hypodermal granuloma. *A)* Panel *A* at 40X magnification. Note that the majority of the cells comprising the newly formed granuloma are polymorph nuclear leukocytes, indicative of the acute inflammation. *B)* Granuloma from PTX 10 mg/kg treated rats, 40 x magnifications. The cellular makeup has shifted to less macrophage/monocytes, suggesting that inflammation and angiogenesis has reduced in comparison to control. *C)* Granuloma from PTX 20 mg/kg treated rats, 40 x magnifications; leukocyte infiltration has been reduced while angiogenesis increased in comparison with control. Note the numerous fine capillaries that branch off the larger vessel. *D)* Granuloma from PTX 40 mg/kg treated rats, 40 x magnifications. Leukocyte infiltration and angiogenesis both are reduced significantly.**

## DISCUSSION

This study provides evidence for an anti-inflammatory effect of PTX. Oral administration of PTX was effective in reducing the progression of inflammation at doses comparable to that used in man for the treatment of circulatory disorders (40 mg/ kg). A number of investigators have also described an anti-inflammatory effect of PTX [6–9]. PTX at low dose (10 mg/ kg) elevated exudate volume in air pouch model of inflammation but higher doses did not make any significant change in exudate volume in rats. PTX demonstrated reduction in granulated tissue weight and total leukocytes in dose manner behaivior. PTX alongside its well-known hemorheological properties, has been found attenuated the secretion of some inflammatory cytokines such as TNF, IL-12 and IFN-y. Lazarczyke and

colleagues demonstrated that pentoxifylline inhibits leukocyte infiltration on colon adenocarinoma in rats [12]. Pentoxifylline prevents lymphocyte [1] and neutrophil cytotoxicity [13, 14]. The mechanism of PTX in reducing inflammation is thought to be due to inhibition of TNF [8,13], a cytokine that has been shown to increase leucocyte adhesion and to disrupt intercellular junctions of postcapillary venular endothelium, leading to plasma extravasation [18]. Other studies showed that in the carrageenan-induced paw oedema model, PTX reduced the oedema response, while injection of a monoclonal anti-TNF antibody reproduced the inhibitory effect [8]. Other phosphodiesterase inhibitors such as rolipram also significantly inhibited paw oedema caused by carrageenan as well as localized TNF levels in the paw [18]. In addition, PTX has venodilator, antioxidant properties, inhibits platelet aggregation and prevents adherence of leucocytes in post-capillary and later venules [2]. In this way, PTX could be reducing inflammation by alleviating the consequent generation of oxidant stress by neutrophils. PTX (20, 40 mg/kg) reduced leukocytes infiltration into the pouch [15]. Iskesen and colleagues mentioned that pentoxifylline infusion during cardiac surgery inhibits the pro-inflammatory cytokine release caused by cardiopulmonary bypass [17]. Edema is an important part of vascular responses to inflammatory processes. Exudate has high content of protein and cellular debris which has evoked from blood. Abdel salam et al find out that PTX given with %1 carrageenan into the rat hind paw reduced the edema response [2]. Our results showed that PTX reduced inflammation and edema that it seems to be dose dependent.

Pretreatment with PTX attenuate the levels of inflammatory cytokine release such as TNF-α, and some studies have demonstrated that PTX reduces the hemodynamic equilibrium disruption observed in cases such as hypovolemic shock, endotoxaemic shock, gastric aspiration and TNF-induced pulmonary [18]. It is thus possible that the observed reduction in anti-inflammatory effects seen with the higher doses of the drug are a consequence of marked or sustained vasodilator activity leading to increased functional lumen (intraluminal volume), intravscular pressure and microvascular permeability. In addition, release of prostacyclin [19,20] or nitric oxide [20] following the higher doses of the drug could have worked to induce inflammatory exudation, thereby apparently attenuating the anti-inflammatory effect seen in the 1-h experimental time period.

PTX at doses 10 and 40 mg/kg reduced Carmine red concentration in granulated tissue. This pheromone was because of suppression of inflammation in tissues.

In the present study, we observed reduction of angiogenesis beside the anti inflammatory properties due to pentoxifylline treatment. Vhalos et al suggested that pentoxifylline suppresses angiogenesis in the rat endometriosis model, our studies showed this drug reduced angiogenesis significantly by 10 and 40 mg/kg [21].

## CONCLUSION

In conclusion, the data presented suggest that pentoxifylline modulates the production/release of a pro-inflammatory cytokine (TNF-a) and inflammatory cells and reduced angiogenesis.

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