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EFFECT OF OXIDATIVE STRESS AND ANTIOXIDANTS ON HEALING OF COLONIC ANASTOMOSIS EXPOSED TO ISCHAEMIC-REPERFUSION INJURY

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Background: *Research on intestinal ischaemic-reperfusion injury is at fever pitch (related to laparoscopy-intestinal transplant-mesenteric vessels revascularization) and in the past couple of years it becomes clear that its pathogenesis is either hypoxic or cytotoxic and represents an utmost universal step in the impaired intestinal anastomosis. Aim: We try to figure out the pathogenesis of intestinal ischaemic-reperfusion, its impact on the intestinal wound healing from the clinical, physical, biochemical and histological aspects and the role of antioxidants in prevention of its deserve effects. Design: Randomized controlled study. Setting: Research Laboratory Mansoura University. Participants: animal study. Material & methods: Right colonic transection and anastomosis was performed in 45 rats which were divided into 3 groups: GI. The control one, GII rats following one hour ischaemia of right colon and terminal ileum, reperfusion of these tissues was achieved till sacrification and GIII rats together with ischaemic-reperfusion "allopurinol" high dose was administered in the preoperative and postoperative periods. All animals were sacrified on the 5th postoperative day. Main outcome measures: Clinical assessment of anastomotic, physical measurement of anastomosis bursting pressure, biochemical assessment (oxidant MDA-antioxidant GSH-Px) and histological assessment (Ileal injury score-anastomotic healing). Results: clinically 6 cases of anastomotic leak in GII and 3 in GIII with no cases in GI, physically mean anastomotic bursting pressure in mmmHg was 117 in GI, 41 in GII and 65.2 in GIII. Histologically: mean score of intestinal mucosal injury was 0.9 in GI and 4.3 in GII and 2.4 in GIII while the wound healing parameters in GIII were better than in GII. Biochemically: the mean MDA expressed as nanomol/mg and the mean GSH-Px expressed as unit/mg respectively were (5.4-126) in GI, (21.5-150) in GII and (11.2-165) in GIII. Conclusion: The cytotoxic free radicals generation is an utmost universal step in the development of intestinal mucosal injury which has a deleterious effect on the intestinal wound healing (clinical, physical, biochemical, and histological). Lastly although the antioxidants not totally reversing the ischaemic reperfusion effects, the antioxidants seem to improve the intestinal wound healing.*

Key words: oxidative stress, ischaemic reperfusion, intestinal wound healing

INTRODUCTION

Intestinal ischaemic-reperfusion syndrome is the haemorrhagic necrosis of the intestinal mucosa in the absence of extensive vascular damage and the local inflammatory changes prior to haemorrhagic necrosis [1]. It is not a rare event but has been reported in conditions associated with reduced blood flow with consequent low oxygen tension to maintain cell metabolism and integrity [2], that insult has been reported with acute mesenteric occlusion with reperfusion injury from collateral vessels or established reperfusion [3]. After cardiopulmonary bypass [4], secondary to thromboangites obliterans [5] ruptured aortic aneurysm or repair of abdominal aortic aneurysm [6], small intestinal transplant [7], abdominal compression syndrome [8] and in the era of laparoscopy when the intra-abdominal pressure reach 20 mmHg or more [9], with Glypressin therapy [10] and shock [11].

The small intestine is the most sensitive tissue to the ischaemic-reperfusion injury [12], the ileum is the most frequent target [13] and the mucosal layer is the more susceptible layer due to the higher metabolic demands and the existence of counter-current vascular arrangement of the villi [14].

The pathologic events of ischaemic-reperfusion injury include initial increase of the vascular mucosal permeability followed by epithelial shedding [15], severe forms result in disruption of the lamina propia and transmucosal necrosis [16].

The pathogenesis of ischaemic injury is related to either hypoxia per-se or to the cytotoxic free radicals [17], as during hypoxia the mean transit time through the vascular loops is increased resulting in increased extravascular oxygen shunt reducing tissue Po2 that compromise the cellular oxidative metabolism [18], also hypoxia increase the intracellular level of hypoxanthine and activate the xanthine oxidase enzyme converting hypoxanthine into cytotoxic free radicals [19] that cytotoxic free radicals produce lipid peroxidation of the cell membrane and subsequent release of components as lysosomal enzymes producing further tissue damage [20], or degrade the hyaluronic acid which is the principal component of the basement membrane [21].

The mechanism of reperfusion injury include either (i) granulocytes plugging, adherence and priming [12] or (ii) cytotoxic free radicals release [22]. The neutrophil plugging exacerbate ischaemia, while neutrophil adherence create a microenvironment which permit high concentration of injurious agents [23] and release the endothelin-I that reduce the microvessel tone exacerbating ischaemia [24], and neutrophils priming produce proteolytic enzymes and bursts of oxygen free radicals [25].

The cytotoxic free radicals rise the intracellular Ca++ releasing arachidonic acid products [26], producing neutrophil chemotaxis [27], neutrophil priming [28] and impair tissue perfusion [29].

Both ischaemia and reperfusion injuries are semiquantitative and consistent with the view that reperfusion greatly exacerbates the injury produced by ischaemia [30].

Oxidative stress is known to play a role in the pathophysiology of many conditions in the living organism [31] free radicals are inevitably generated and perfectly balanced by the antioxidants, whenever the balance shifts in favor of oxidants, oxidative stress and tissue damage occurs [32]. Previous studies have shown that antioxidants are activated whenever the oxidative stress occurs unless the capacity is attenuated by chronic diseases [33] and that antioxidants provide the first line defense against the cytotoxic free radicals generated during ischaemic reperfusion injury [34].

Intestinal wound healing-nearly similar to cutaneous is a stepwise process including (i) coagulation & inflammation (ii) fibroplasia & matrix deposition (iii) angiogenesis and epithelialization (iv) collagen maturation & wound contraction (35), also it is not a unique process as its few vulnerable spots are (i) tissue perfusion and oxygenation (iii) nutrition and abbreviation of inflammation [36]. Predominantly oxygen is needed for all steps being 20 mmHg for half activity and 200 mmHg for maximal activity [37].

Drug prophylaxis against ischaemic-reperfusion injury has not reached the stage of clinical utility [38] although anoxic reperfusion of the intestine result in very little damage [39] and pretreatment with either superoxide dismutase or allopurinol significantly attenuated the necrosis of the villi and crypt epithelium, and largely prevent disintegration and haemorrhage of the lamina propia [19]. Also interventions administrated at the time of reperfusion are effective in attenuating mucosal injury as agents given before ischaemia [40].

PATIENTS AND METHODS

Right colonic transetion and anastomosis was performed is 45 albino rats which are divided into 3 groups, every group is 15 rats.

Group I: Control group, Group II rats following 1 hour ischaemia of right colon and terminal ileum, reperfusion until sacrification, Group III as group two plus high dose allopurinol [NO-uric tab. from E.I.P.I.Co.]) minced and mixed with the rats pellets (high dose 20 ugm/kg) 5 days pre and postoperative. Anaethesia with intramuscular ketamine (5 mg/kg body weight) all animals are scarified at the end of the 5th postoperative day.

The ilio colic anastomosis was subjected for:-

1. Clinical evaluation for fistulae presence or absence.

2. Physical evaluation of the bursting pressure measured manometrically using 18F Abocath connected to mercury pressure gauge at the anastomotic site with 2 arteries on both sides (colon ileum).

3. Histological evaluation (a) of the terminal ileum segment, frozen section preparation stained with haematoxylin & eosin to score the degree of mucosal injury as Shah et al. (1997) [41] and described by Chiew et al. (1973) [42] as follow:

Grade	Mucosal	
	Epithelium	Lamina propia
0	Normal	Normal
1	Subepith. Spaces of villous tips	Normal
2	Epith. lifting of villous tips	Normal
3	Massive epith. lifting	Occasional haemorrhage
4	Denuded villi	Haemorrhage
5	Denuded villi	Disintegration+haemorrhage

b- The ilio colic anastomosis healing: sections stained with haematoxylin and eosin to grade the epithelialization, inflammatory cell infiltrate, capillary density, fibroblasts deposition & collagen deposition [43] as follow:

Degrees	*Epithelialization*	*Infl. cell infiltration*	*Capillaries*	*Fibroblasts*	*Collagen*
1	1/3 of wound	One/mm^3	1-2/mm^3	Few	Few
2	2/3 of wound	2-4/mm^3	3-6/mm^3	Moderate	Moderate
3	All	$\geq$5/mm^3	$\geq$6/mm^3	Marked	Marked

4- Biochemical assessment:-

Oxidant activity [malonyldialdhyde (MDA)]:. The tissues were homogenized in phosphate buffer 0.1 ml/L (PH 7.4) and the concentration was measured as Mihara and Uehiyama (1978) [34]. 1 ml of 17.5% tricholoric acid + 1 ml of thiobarbituric acid were added to 1 ml of homogenate and boiled for 15 minutes then cooled. The mixture is incubated with 70% tricholoric acid for 20 minutes then centrifuged for 15 minutes at 2000 rpm. Lastly the optical density (at 534 nm) is expressed as nanomoles/milligram protein detected by Lowry method (1951) [45].

Antioxidant activity [glutathion peroxidase (GSH Px)]: using the Paglia and Valentine method, (1967) [46]. The homogenate is centrifuged at 15000 rpm for 60 min, and the supernatant fluid is obtained for the enzyme assay, based on the NADPH oxidation and measured (at 340 nm) and expressed as units per milligrams proteins.

Statistical analysis:-

Data are expressed as a mean + standard deviation. Comparison between groups was done using one way ANOVA test.

RESULTS

I- Clinical assessment:-

The intestinal leak was significantly higher in GII (6 cases) but halfed in GIII and absent in GI as in (Fig. 1).

II- Physical assessment:-

The mean anastomotic bursting pressure as an indicator of hydroxyperoline deposition was significantly better in GIII (65.2+2.5 mmHg) than in group II (41+1.7) but not as group I (117+4.5) as in (Fig. 2).

III- Histologic assessment:

(a) The grade of mucosal injury was severe in GII but amelorated to be moderate in GIII, also the difference between the 3 groups was significant as shown in (Table 1).

(b) As regard the ilio colic anastomotic wound healing there were no difference as regard to inflammatory cell infiltration and epithelialization but the degree of collagen deposition, fibroblasts infiltration and capillaries formation was poor in GII as shown in (Table 2).

IV- Biochemical assessment:-

Levels of MDA, as indicator of oxidant activity were significantly higher in GII and GIII than in the control group, also the difference between GII and GIII was also significant.

Levels of GSH Px as indicator of antioxidant activity were significantly higher in GII and GIII than in the control group but the difference between GII & GIII was insignificant as shown in (Table 3).

Table (1): ***Histologic grade of intestinal mucosal injury of terminal ileum expressed as median value***

Group	Grade	P value
I	0.9	
II	4.3	<0.05
III	2.4	

P1 <0.05 P2 <0.05 P3 <0.05

Table (2): ***Histologic assessment of ilio colic anastomotic wound healing***

	Epithelialization	Infla. cell	Capillaries	Fibroblasts	Collagen
	1 2 3	1 2 3	1 2 3	1 2 3	1 2 3
GI	0 7 8	0 13 2	0 2 13	0 0 15	2 9 4
GII	9 6 0	0 1 14	2 13 0	3 8 4	2 13 0
GIII	4 8 3	1 6 8	1 7 7	0 3 12	2 12 1
P value	>0.05	>0.05	<0.05	<0.05	<0.05

Table (3): ***Biochemical assessment***

	Oxidant		Antioxidant	
	Mean	±SD	Mean	±SD
GI	5.4	+0.4	126	+31
GII	21.5	+1.2	150	+37
GIII	11.2	+0.9	165	+41
P value	<0.05		<0.05	
P1	<0.05		<0.05	
P2	<0.05		<0.05	
P3	<0.05		>0.05	

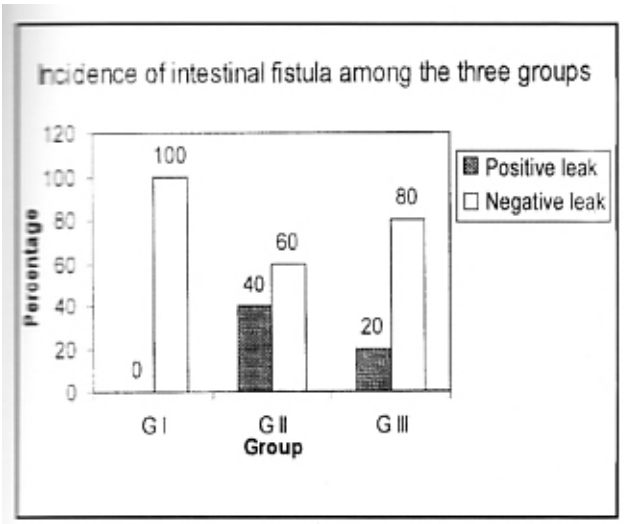

Fig (1)

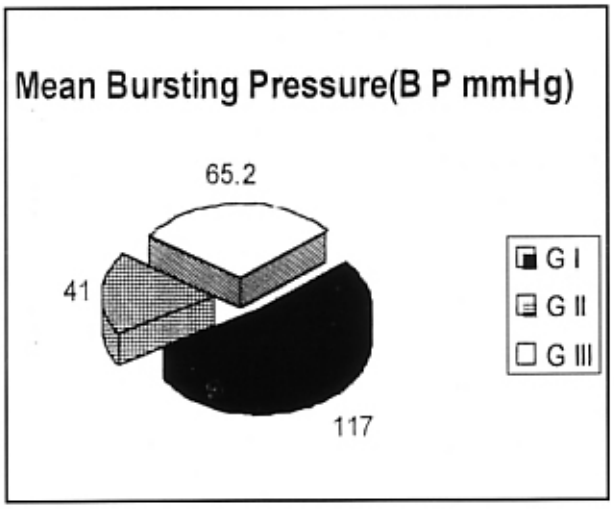

Fig (2)

Fig. (3): Villi from GII showing marked necrosis and ulceration of villous epithelium, subepithelial spaces and marked cellular infiltration of lamina propia (H&E x 400).

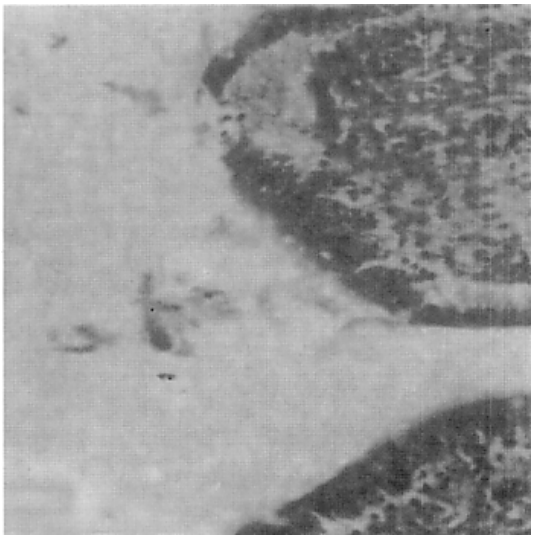

Fig. (4): Villi from GII showing denuded epithelium, wide subsepithelial space and cellular infiltration (H&E x 250).

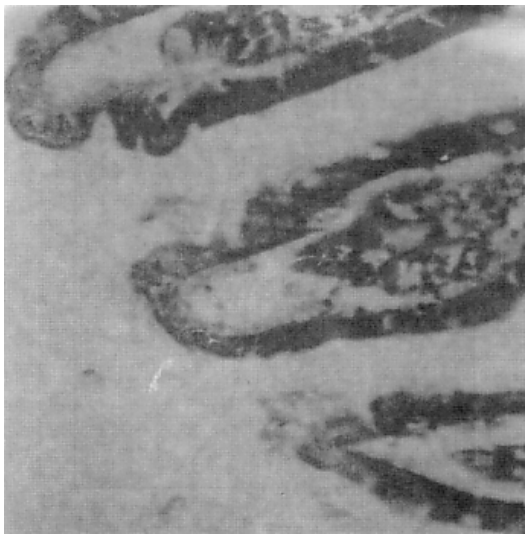

Fig. (5): Villi from GII showing denuded epithelium at tips and sides, subepithelial spaces, marked cellular infiltration and hemorrhage in the lamina propia (H&E x 400).

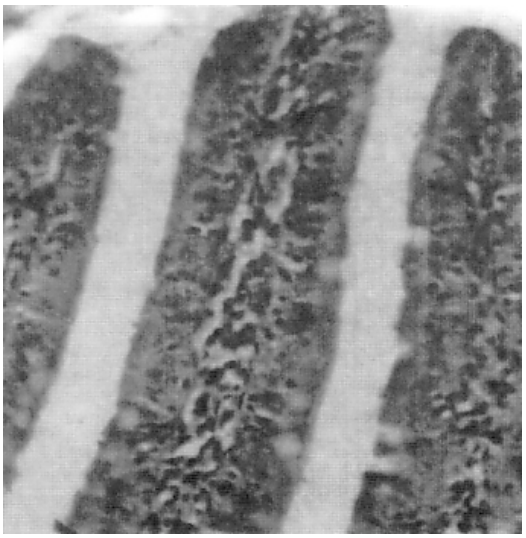

Fig. (6): Villi from GIII showing healing epithelium, still cellular infiltration and reformed lamina (H&E x 400).

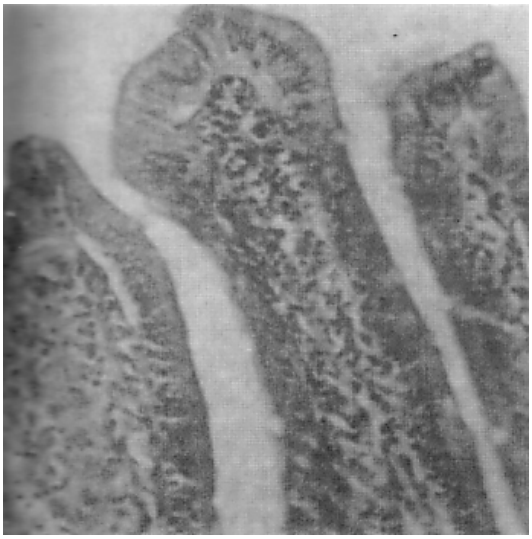

Fig. (7): Villi from GIII showing healing epithelium and reformed lamina propia (H&E x 250).

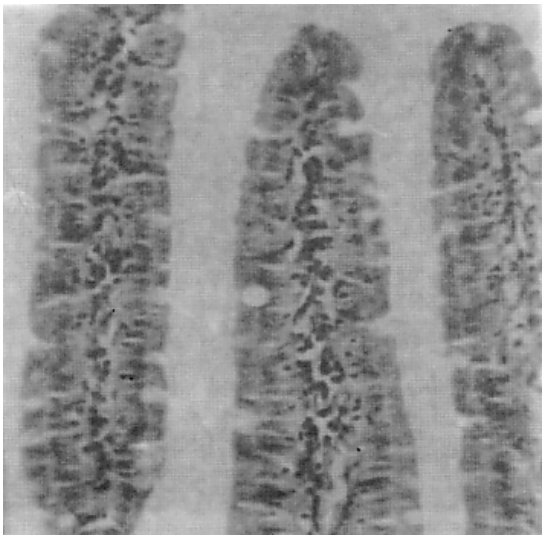

Fig. (8): Villi from GI. Normal one (H&E x 400).

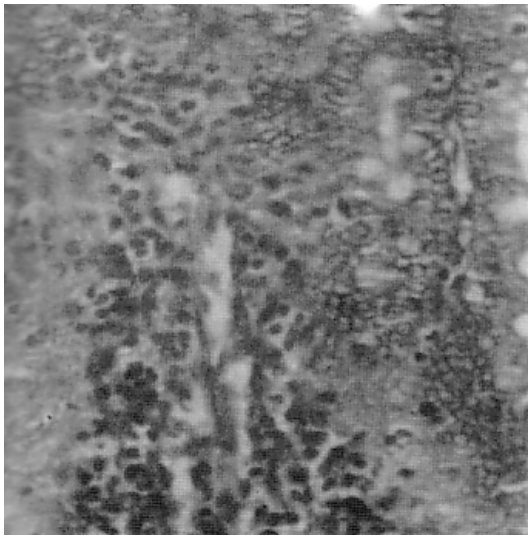

Fig. (9): The cryps from GIII are heavily studied with capillaries and cellular infiltration (H&E x 400).

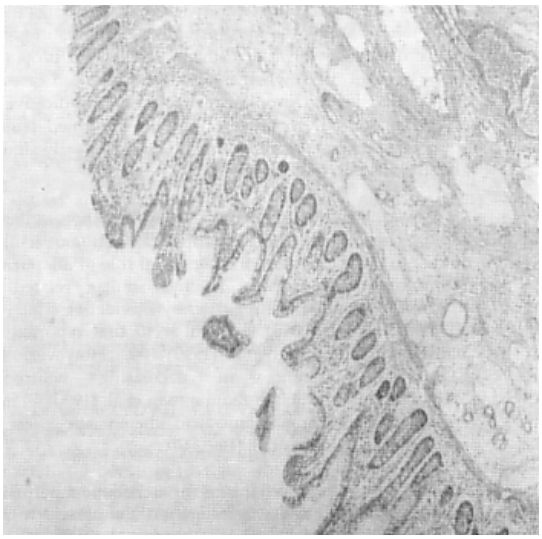

Fig. (10): Nearly healed ilio colic anastomosis from GIII (H&E x 250)

DISCUSSION

Although intestinal musocal ischaemic reperfusion syndrome has been known since Cumin, 1823 and Dupuytren, 1833, this syndrome has been recently the subject of intensive clinical and experimental analysis. That's although many studies had defined the role of free radicals in its pathogenesis [17, 12, 37], its deleterious effect on the intestinal wound healing [48], but still drug prophylaxis against ischaemic reperfusion injury has not reached the stage of clinical utility [38].

Fear of anastomotic leakage often deters surgeons from making a primary anastomosis specially in equivocal cases (infection and anaemia) [49], and in this study anastomotic leakage was prominent in the G2 nearly doubling G3 which is related to the role of antioxidant in improving the outcome as reported by [48].

Also, the colonic bursting pressure which reflect the mean hydroxyperoline concentration [50] was improved n G3 suggesting the role of antioxidant in reversing the effects of the ischaemic reperfusion injury as reported by Friedl et al. (1990) [40] which may be related to increased fibroblasts number and activity producing more collagen deposition.

Obviously damage that can be detected microscopically would be of greater value than macroscopic findings [51] in this histologic study, the most prominent features in group II were denuded villi and disintegrated lamina propia as shown in (Figs. 3,4,5) whereas in GIII no reported cases of disintegrated lamina propia or haemorrhage indicative of severe damage but reported cases with reformed lamina propia and healed epithelium as in (Figs. 6,7) nearly similar to control cases in (Fig. 8).

Nevertheless the higher capillaries formation, collagen deposition and fibroblasts in group III as in (Figs. 9&10) in contrast to group II reflect the beneficial role of allopurinol in improving anastomotic healing similar related to improved oxygenation as reported by Davidet al. (2003) [35] and Sheikh et al. (2000) [37], similar to that reported by Zimmerman and Granger, (1992) [23]. However, the insignificant difference in decreasing neutrophil accumulation in contrast to Zimmerman et al. (1990) [52] may be related to the arachidonic acid chemotactic effect as reported by Wellbourn et al. (1991) [22].

The molecular mechanisms of ischaemic-reperfusion mucosal injury still under investigation, the present results show that both oxidants and antioxidants are stimulated, suggesting the role of oxidative stress in this process as membrane lipid peroxidation yields MDA, which is accepted as an early indicator of oxidative damage and cell death [53]. Also the observation of increased GSH-Px activity indicates that the antioxidants system is also activated and points indirectly to the involvement of oxidative stress in this process.

That disturbed oxidant-antioxidant level is related to the neutrophil infiltration producing superoxide-hydroxyl-hydrogen peroxide [54, 55].

Significantly the level of oxidants was decreased in GIII as reported by Morris et al. (1987) [56] and Moorhouse et al. (1987) [57] but no statistically difference is noted in the level of GSH-Px and this is related to the beneficial role of allopurinol in preventing oxidative stress but not a scavenger of free radicals already formed as reported by Girsham et al. (1986) [58] and Zimmerman et al. (1988) [59].

Clinically the lower incidence of anastomotic leak, physically the better bursting pressure, histologically the low grade mucosal injury and higher healing score and biochemically the lower MDA in GIII than in GII appear to be related to the significant role of antioxidants in amelorating the adverse effects of intestinal ischaemic-reperfusion injury.

CONCLUSION

The intestinal ischaemic-reperfusion injury is not a rare entity, its pathogenesis is related to the disturbed oxidants, antioxidants balance caused by neutrophilic infiltration and the antioxidant allopurinol has a beneficial effect in reversing its sequences.

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