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Thymosin Beta 4 Improves the Intestinal Ischemia/Reperfusion Injury in Rats

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Abstract

Background and purpose: Intestinal ischemia-reperfusion (I/R) injury is a common clinical issue involving sepsis, shock, necrotizing enterocolitis, mesenteric thrombosis. Inflammatory reactions and cellular apoptosis are mainly involved processes in intestinal I/R injury. Data regarding the effect of thymosin beta 4 (T β 4) on intestinal I/R injury are scarce. It was designed to evaluate the potential effects of T β 4 on intestinal IR injury.

Methods: The study was conducted on 3 groups of adult male albino rats: each containing ten rats: group I (I/R group), group II (Sham- operated group), and group III (T β 4-treated group). After one hour of intestinal ischemia, reperfusion was performed by releasing the clamp. Thirty minutes before reperfusion, the rats in group III received subcutaneous injections of 30 mg T β 4 per kg and 0.1 ml physiologic saline. Rats in groups I and II received only 0.1 ml physiologic saline. Intestinal histopathologic examination and scoring the degree of injury were done according to chiu's score. Biochemical analyses of malondialdehyde (MDA), super oxide dismutase (SOD) and glutathione peroxidase (Gpx) levels in addition to TNF- α , IL-6 and caspase-3 levels in the rat intestinal tissues were determined. Serum and tissue nitric oxide (NO) levels were also measured.

Results: Intestinal I/R injury was confirmed by intestinal histopathologic examination. In the I/R group, tissue MDA, TNF- α , IL-6 and caspase-3 levels were significantly elevated, however, tissue SOD, Gpx, serum and tissue NO levels were significantly declined when compared to the sham group. On receiving T β 4, the findings demonstrated significant reductions of tissue MDA, TNF- α , IL-6 and caspase-3 levels and significant elevations of serum and tissue NO, tissue SOD and Gpx levels compared to non-treated one. Further, rats treated with T β 4 showed amelioration of the degree of intestinal I/R lesions and improvement of intestinal score injury compared to non-treated group.

Conclusions: T β 4 administration significantly protected the intestinal tissues against the intestinal I/R injuries. T β 4 treatment appears to be a promising protective and therapeutic approach for intestinal I/R injury.

Keywords: Thymosin beta 4, Intestine, Ischemia, Reperfusion

1. Introduction

Intestinal ischemia, created by intestinal blood supply disruption, results in initial intestinal injury further worsened by reperfusion in a paradoxical manner [1]. Intestinal ischemia-reperfusion (I/R) injury may be induced by the enhanced oxygen radicals and the activated phospholipase [2]. Moreover, intestinal I/R damage may be associated with shock, cardiopulmonary bypass, transplantation of small intestine, thrombosis of the mesenteric artery, vascular surgery and trauma [3,4]. Inflammation and apoptosis are central players in the induction of the intestinal I/R injury [5,6].

Thymosin beta 4 (T β 4) is a polypeptide composed of 43 amino acids and initially isolated from the thymus of calf [7]. It may have a promising role in regenerative medicine as it acts as an immune-modulator [8]. It may protect against inflammation and fibrosis by suppressing the inflammatory cytokines secretion and blocking the activation of NF κ B [9]. Further, it may improve the wound healing especially in the eye, heart and skin [10, 11, 12, 13, 14]. Topically, it may improve the corneal injury [10]. Moreover, it maintains the cardiac function after myocardial infarction and prevents the myocardial rupture and scar formation by enhancing the survival of myocardial and endothelial cells [8, 11, 15]. In addition, T β 4 may protect against acute liver injury induced by carbon tetrachloride in rats [16]. Taken together, these data may suggest a potential protective and therapeutic role for T β 4 as anti-inflammatory and antioxidant agent. Therefore, this study aims to evaluate the potential impact of T β 4 on intestinal I/R damage via investigation of biochemical measurements and intestinal histopathologic examinations.

2. Materials and Methods

2.1 Animal experiment

This study was conducted in the scientific and medical research center (ZSMRC) in Faculty of Medicine, Zagazig University in the period from 9th January to 27th March 2019 and involved thirty healthy adult male albino rats of local strain weighing 210-260 gm obtained from the animal house of Zagazig Veterinary Medicine Faculty. Rats were kept under hygienic conditions in steel wire cages (5/cage) at room temperature, maintained on a natural light/dark cycle with free access to water and adapted to the new environment for one week before the experiments going on. This study protocol was approved by the Institutional Research Board and Ethics Committee of Medicine Faculty, Zagazig University. The experimental procedures were performed in accordance with the guide for the use and care of laboratory animals, published by the Institute of Laboratory Animal Resources. The rats were randomly allocated into three groups according to the procedures performed, each group containing ten rats: Group (I) (I/R group): Surgery was performed to establish the intestinal I/R injury model. Rats underwent superior mesenteric artery occlusion. Group (II) (Sham- operated group): laparotomy only, no ischemia, no superior mesenteric artery ligation. Rats in groups (I and II) received subcutaneous injection of 0.1 mL of physiologic saline thirty minutes before the reperfusion [17]. Group (III) (T β 4-treated group): Thirty minutes before reperfusion, the rats received subcutaneous injections of thymosin β 4 TB-500 Thymosin Beta 4 (10 mg vial in powder form) (Sigma-Aldrich, CAS# NO.77591-33-4) in a dose of 30 mg/kg , the selected dosage of T β 4 was based on previous studies [18, 19, 20].

2.2 Intestinal I/R injury model

After a 12-hour fasting, under sterile techniques, the procedures were performed as previously described by [17, 21, 22]. Before the surgery, rats were anesthetized with intraperitoneal injection of thiopental sodium (25 mg/kg). We utilized the morbidity score with a maximum of 7 (including weight changes: 3 points, behavioral alterations: 3 points, and stool existence: 1 point). After anesthesia induction, if the score was above 3, the protocol included euthanasia [23]. None of the included rats had to be euthanized. Then, hair was removed and the abdomen was prepped in a sterile manner. Preoperatively, 1 mL of subcutaneous 0.9% saline was injected to compensate for fluid loss. The superior mesenteric artery was obstructed with an a traumatic vascular clamp through a midline incision. The intestine was replaced into the peritoneal cavity and the abdomen was temporarily closed to prevent fluid loss with 5/0 silk suturing (Silk, Ethicon, UK). The rats were placed on heating pads at 37℃ throughout the experiments. For resuscitation, all rats received 25 ml/kg of 0.9% saline subcutaneously after superior mesenteric artery occlusion. Notably, all rats received their medications and vehicles thirty minutes before the reperfusion. Reperfusion was performed one hour after the intestinal ischemia by the abdominal reopening and clamp removal. Reperfusion was continued for 60 minutes. Superior mesenteric arteries reperfusion was determined by pulsation and color return. Intestinal I/R injury occur after ischemic injury and reperfusion injury. Ischemic injury is due to hypoxemia induced by microcirculatory flow disruption. This is followed by reperfusion injury as blood flow resumption after the ischemic phase worsens the intestinal damage; however, it is required for intestinal epithelium survival [24, 25]. One hours after the reperfusion, thoracotomy was performed on all rats to collect the intestinal tissues for histopathologic and biochemical analyses. The terminal ileum was harvested and placed on ice. As the terminal ileum was mostly affected, it was the preferred site to evaluate the intestinal I/R injury consequences [26]. It was fully rinsed with physiologic saline and equally divided into two portions: one piece was immediately put at -80 ℃ and used for biochemical assay and the other one was placed in 10% formalin solution for histopathologic examinations.

2.3 Intestinal histopathological examinations

Each intestinal sample was fixed in 10% formalin solution for 48 hours, dehydrated in alcohol, then embedded in paraffin wax. They were serially sectioned and stained with Hematoxylin and Eosin (H & E) staining. The samples were evaluated with light microscopy. Intestinal mucosal tissue injury was assessed as defined by Chiu’s classification [27]. Briefly, mucosal injury scoring was as follows: 0, Normal mucosal villi; 1, Capillary congestion and subepithelial Gruenenhagen gap; 2, Moderate elevation of epithelial layer from lamina propria; 3, Massive epithelial elevation extending down sides of villi; 4, Denuded villi with lamina propria exposed and dilated capillaries; and 5, Disintegrated lamina propria, hemorrhage and ulceration.

2.4 Biochemical analyses

2.4.1 Tissue superoxide dismutase (SOD) activity determination

According to the method described by Sun et al. [28], it was measured using the xanthine-oxidase-cytochrome c method, which reacts with Nitrobluetetrazolium (NBT) to form a formazan dye. Then, NBT was reduced to blue formazan by O_2-. Absorbance was detected at 560 nm. One unit of SOD (U) is defined as the protein amount inhibiting NBT reduction rate by 50%.

2.4.2 Tissue malondialdehyde (MDA) determination

It reflects the tissue lipid peroxidation using thiobarbiturate test [29]. The excised intestinal samples were rinsed with cold saline, weighed and homogenized in 10 mL of 100 g/L KCl. We add the homogenate (0.5 mL) to a specific solution consisted of 0.2 mL of 80 g/L sodium lauryl sulfate, 1.5 mL of 200 g/L acetic acid, 1.5 mL of 8 g/L 2-thiobarbiturate, and 0.3 mL of distilled water. Then, it was incubated at 98◦C for one hour. Five mL of n-butanol/pyridine (15:1) was added upon cooling. It was centrifuged at 4,000 rpm for 30 minutes. The absorbance was recorded at 532 nm and a standard

curve using 1,1,3,3- tetramethoxypropane was obtained. The recovery was over 90% [21].

2.4.3 Tissue glutathione peroxidase (GPx) determination

It was measured using Sedlak and Lindsay's method [30]. The intestinal sample was homogenized in 2 mL of 50 mMTris – HCl buffer containing 20 mM EDTA and 0.2 M sucrose, pH 7.5. The homogenate was precipitated with 0.1 mL of 25% trichloroacetic acid, and the precipitate was removed after centrifugation at 4,200 rpm for forty minutes at 4℃. Using 5,5--dithiobis (2-nitrobenzoic acid), the supernatant was used to determine tissue GPx. Absorbance at 412 nm was detected by the spectrophotometer [21].

2.4.4 Serum and tissue nitric oxide (NO) measurement

The intestinal sample was homogenized and then centrifuged at 4,000 × g for five minutes. Supernatant was assayed by a modification of cadmium reduction method [31]. The results were expressed as micrometer per liter of protein for the serum and micrometer per milligram of protein for the tissue levels.

2.4.5 Tissue TNF- α and IL-6 levels

Intestinal TNF- α content was assessed using Enzyme Linked Immunosorbent Assay (ELISA) using a microplate reader. Tissue IL-6 levels were measured using a commercially available ELISA kit according to the manufacturer's instructions.

2.4.6 Tissue Caspase-3 levels

Tissue caspase-3 content was measured using a rat caspase-3 ELISA kit (Cusabio Biotech Co., China). The concentration of caspase-3 was determined from a standard curve constructed from a set of serial dilutions of the standard [32,33].

2.5 Statistical Analysis

Results were presented as mean ± SD and analyzed using version 18 SPSS program (SPSS Inc. Chicago, IL, USA). One way analysis of variance (ANOVA) was used followed by student- least significant differences (LSD) test to compare statistical differences between groups. P value less than 0.05 was considered to be significant.

3. Results

3.1 Histopathological studies

As shown in Fig. 1, histopathological analyses in the intestinal mucosa of sham animals (Fig. 1A) revealed a normal mucosal pattern. Group I/R animals exhibited severe mucosal damage (Fig. 1B, C, D), including villous edema, vascular congestion, and hemorrhage, compared to the sham group. However, the intestinal mucosa was preserved in I/R + T β 4 (Fig.1E, F) and the injury was much less severe compared to the intestinal I/R animals.

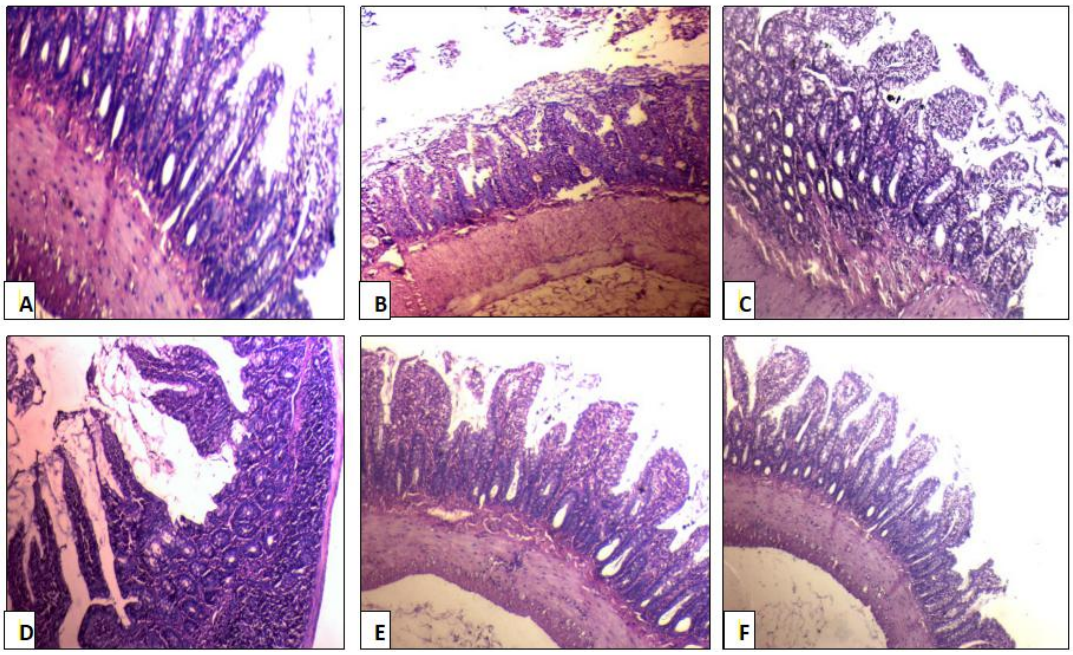

Figure1: Histopathological changes in the intestinal mucosa: Sham group (Fig. 1A) show tall villi with equal thickness and normal crypts (G0), significant enhancement in histopathological scores was noted in I/R group compared to sham group. I/R group animals showed severe mucosal damage, denudation of villi, shortened villi, complete loss of villi, inflammatory cell infiltration and eventually complete mucosal necrosis. In this group, the most sections were graded G3 to G5 (Fig. 1B, 1C, and 1D respectively). Tβ4 treated group showed less severe lesions in compared to I/R group and the major histopathological lesions were only increasing subepithelial (Gruenhagen's) space, epithelial lifting and villi denudation that ranged G1 to G2 and (Fig. 1E, and 1F). Tissue sections of small intestinal mucosa stained with H&E in different groups (Original magnification ×100)

Injury to the intestinal mucosa was quantified using the Chiu's score. The intestinal I/R group showed significant increase in the intestinal injury score (P< 0.001) in comparison to sham animals. However, T β 4 treatment significantly decreases the intestinal injury score when compared with I/R group (P< 0.01) (Fig. 2)

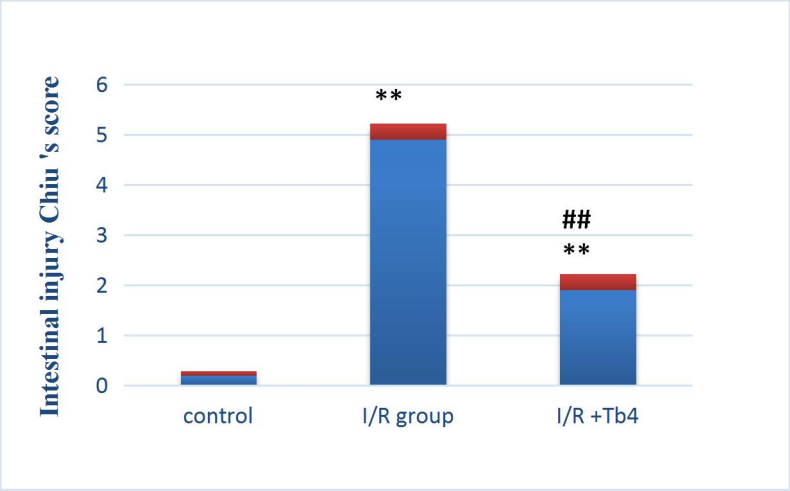

Figure 2: Chiu's scores of the intestinal mucosa of the control, I/R, Tβ4 +I/R groups, data are the means ± SD. **P< 0.001 compared to the control group; ##P< 0.01 compared to the intestinal I/R group

3.2 Effect of T β 4 treatment and I/R injury on oxidative stress markers in the intestinal tissue

The levels of small intestinal MDA and GPx and SOD are good indicators of lipid peroxidation and ischemic damage. Intestinal tissue MDA has been increased significantly, while GPx and SOD levels have been decreased significantly in the intestinal I/R group compared to the control group (Fig.3). T β 4 significantly decreased intestinal tissue MDA, and elevate GPx and SOD to significant levels when comparable with I/R group ($P< 0.01$).

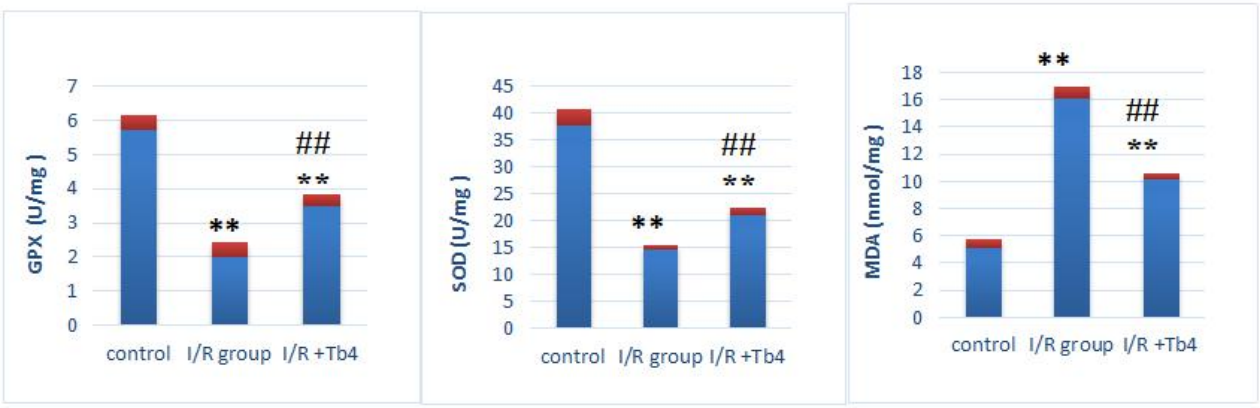

Figure 3: Effects of Tβ4 on tissue MDA, GPx and SOD in the ileum after intestinal I/R. MDA levels in the intestinal tissue were elevated while GPx and SOD were decreased significantly after 1 h of reperfusion in the intestinal I/R groups compared to sham rats, Tβ4 significantly reduced MDA, elevated GPx and SOD levels. Data are the means ± SD. **P< 0.001 compared to the control group; ##P< 0.001 compared to I/R group.

3.3 Effect of T β 4 treatment and I/R injury on intestinal tissue cytokine levels

TNF- α and IL-6 levels in the intestinal tissue of the intestinal I/R group increased significantly compared to the control group ($p < 0.01$), whereas T β 4 treatment significantly decreased TNF- α and IL-6 levels in intestinal tissue compared to the intestinal I/R group as indicated in Fig.4.

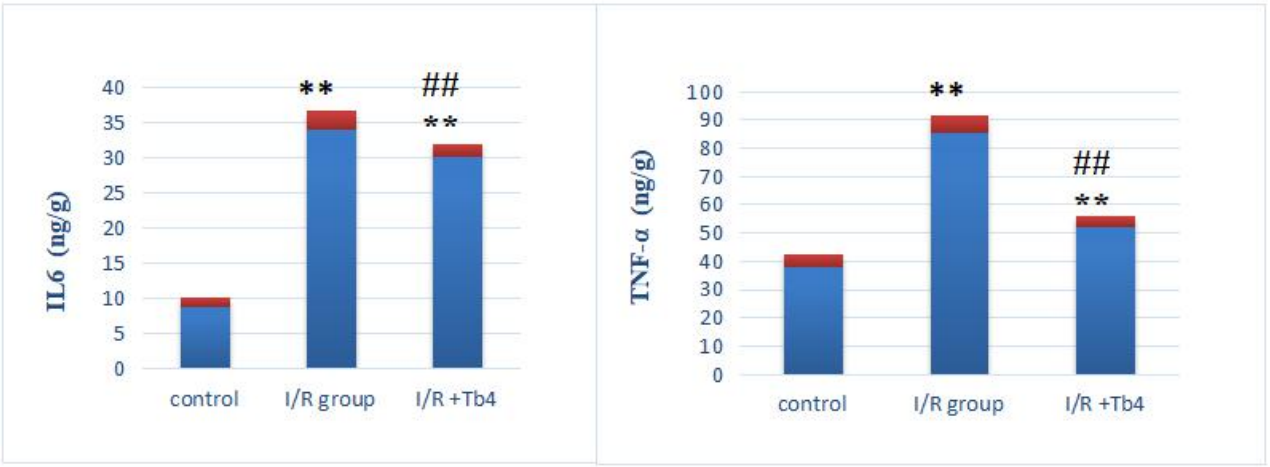

Figure 4: Effects of Tβ4 treatment and the intestinal I/R injury on cytokine levels in the ileum. TNF-α and IL-6 levels in intestinal tissue were elevated significantly after 1 h of reperfusion in the I/R groups compared to sham group, and Tβ4 significantly reduced TNF-α and IL-6 levels. Data are the means ± SD. **P< 0.001 compared to the control group; ##P< 0.001 compared to the intestinal I/R group.

3.4 Effect of T β 4 treatment and I/R injury on serum and tissue nitric oxide levels

NO levels in the serum and tissue of the intestinal I/R group were decreased significantly compared to the sham group ($p < 0.01$), and in T β 4-treated group showed a significant increase in NO levels when compared to the intestinal I/R group (Fig.5).

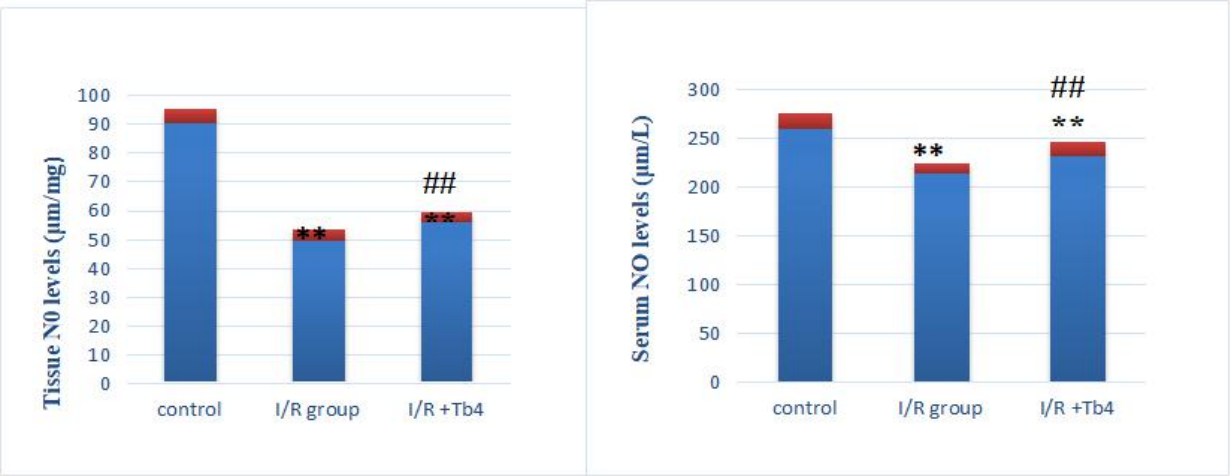

Figure 5: Effects of Tβ4 treatment and the intestinal I/R injury on tissue and serum NO levels. NO levels were decreased significantly in both tissue and serum after 1 h of reperfusion in the I/R groups compared to sham group, and Tβ4 significantly increase NO levels, data are the means ± SD. **P< 0.01 compared to the control group; ##P< 0.01 compared to the I/R group.

3.5 Effects of T β 4 treatment and I/R injury on intestinal tissue caspase-3 levels

Caspase 3 is an important marker of apoptosis, the levels of caspase-3 in intestinal tissue (fig.6) was high in the intestinal I/R group when compared to sham group ($P<0.01$). In contrast, the level of caspase-3 in the T β 4-treated group was significantly lower than the intestinal I/R group ($P<0.01$).

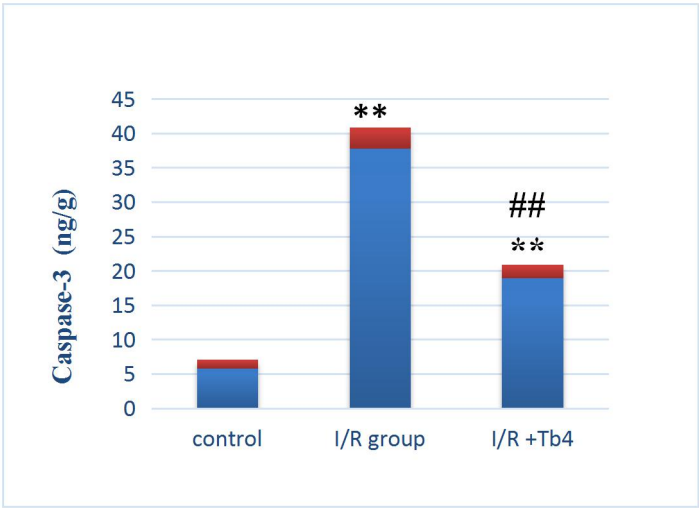

Figure 6: Effects of Tβ4 treatment and the intestinal I/R injury on caspase 3 levels in the ileum. Caspase 3 levels in intestinal tissue were elevated significantly after 1 h of reperfusion in the intestinal I/R groups compared to sham group, and Tβ4 significantly reduced caspase 3 levels, data are the means ± SD. **P< 0.001 compared to the control group; ##P< 0.001 compared to the intestinal I/R group.

4. Discussion

The intestine is aggressively affected during stressful conditions as in shock, sepsis and mesenteric thrombosis [34, 35]. Compared to other organs, intestinal I/R is principally harmful as injury to the mucosal barrier and bacterial translocation resulting in sepsis, organ failure and subsequently death [36]. Intestinal mucosal cell injury is induced by hypoxia in the ischemic phase of intestinal I/R injury. However, reoxygenations of the hypoxic area results in more ROS production [17]. Neutrophils are activated by intestinal I/R injury and migrate to the area of inflammation. Intestinal I/R injury might be induced by ROS, proinflammatory mediators, leukocytes infiltration, dysfunction of intestinal barrier and bacterial translocation [37-39]. Moreover, an inflammatory cascade is initiated triggering I/R injury [40,41]. Therefore, oxidative stress is a cardinal player in the intestinal I/R injury [42]. Herein, we have done an experimental model of intestinal I/R injury in rat to study the possible factors associated with the condition and the possible role for T β 4 treatment in this model.

In this study, the intestinal I/R injury is evidenced in the rats by the histopathological examination that showed marked damage of mucosal barrier including mucosal erosion, necrosis, edema, submucosal invasion of inflammatory cells, shortening of the villi vascular congestion and hemorrhage compared to the sham group. The intestinal tissue sections in I/R group were graded from G3 to G5 according to Chiu's scores of the intestinal mucosa. These findings are in accordance with other previous studies [43, 44].

The histopathological injury reflected the oxidative stress degree. The oxidative stress could be biochemically detected. Tissue MDA levels were markedly increased in the intestinal I/R injury group compared to the sham group reflecting marked lipid peroxidation and oxidative tissue injury. In addition, tissue SOD and GPX levels were markedly reduced indicating more free radical production in the intestinal I/R injury group compared to the sham one. Elevated tissue MDA and reduced SOD and GPX in intestinal I/R injury group are in agreement with the findings of previous works [44-47]. Moreover, the intestinal I/R injury development depends on ROS production and protective mechanisms including NO production. The current study demonstrated that serum and tissue NO levels significantly declined in the intestinal I/R injury group compared to the laparotomy performed group. In accordance to our findings, Sarsu et al [17] demonstrated that intestinal I/R injury was reduced by elevating NO production resulting in mucosal blood flow rearrangement, and polymorphonuclear leukocyte infiltration suppression. Hence, reduced NO production in the mesenteric endothelium resulted in progressive microvascular deterioration in intestinal I/R injury [48,49]. Moreover, administering L-arginine, NO substrate, before reperfusion significantly elevated the serum NO and reduced the serum MDA in I/R damage [50]. Furthermore,in line with previous studied [39,51] we found that tissue TNF- α and IL-6 levels were significantly elevated in the tissue of the terminal ileum indicating worsening of the inflammatory burden in the intestinal I/R injury group compared to the laparotomy performed group. Moreover, the tissue caspase 3 as a cell death marker was elevated in the intestinal I/R injury group as well.

T β 4, G-actin-sequestering protein, is involved in tissue development and regeneration [52]. It protects against cardiac, kidney and corneal injury [53]. Herein, in agreement with the previous studies in which systemic T β 4 administration decreased the TNF- α level in mice with sepsis and with experimental colitis as well [54, 55], the present study demonstrated that T β 4 significantly reduced the elevated tissue TNF- α and IL-6 levels in the intestinal I/R injury group suggesting restoration of the balance of proinflammatory cytokines confirming the potential anti-inflammatory effect of T β 4 in attenuating the intestinal I/R injury.

In addition, intestinal I/R injury may be resulted from oxidants and antioxidants imbalance. The present study showed that T β 4 treatment significantly reduced tissue MDA levels and increased tissue SOD and GPX levels in the intestinal I/R injury group demonstrating T β 4 efficacy as a ROS scavenger and an antioxidant in the intestinal I/R injury. The inflammatory cells are the main inducers of ROS. Therefore, the anti-oxidative effect of T β 4 in the intestinal tissue following injury may be attributable to its anti-

inflammatory role. In consistent, previous studies showed that ROS levels were reduced under the effect of T β 4 in corneal and cardiac injury via enhancing SOD activity [56,57]. We also demonstrated that T β 4 treatment significantly elevated serum and tissue NO levels in the rats with intestinal I/R injury group.

Apoptosis in the intestinal epithelium is an indicator of the mucosal injury [58,59]. This study revealed that the administered T β 4 decreased caspase-3 activity in the ileum suggesting a potential anti-apoptotic property of T β 4 in intestinal I/R injury. The suppressing actions of T β 4 on inflammatory process and oxidative stress may protect against mucosal epithelial cell apoptosis in I/R damage group. This finding is evidenced by the anti-apoptotic effect of T β 4 on various locations as the cardiomyocytes, corneal, normal intestinal epithelia, neurons and colorectal cancer cells by regulating various mechanisms as Bcl-2 phosphorylation, Akt activation, c-Jun phosphorylation and reduced caspase-3 activity [60-64]. Further analysis is required to clarify other potential mechanisms by which T β 4 could inhibit the intestinal epithelial apoptosis in intestinal I/R injury. The degree of the oxidative stress in the intestinal tissue determines the levels of intestinal injury scores. Histopathological analysis showed that administration of T β 4 ameliorated the degree of intestinal I/R injury. The tissue sections include increasing sub epithelial space, epithelial lifting and villi denudation ranged from G1 to G2 confirming that the intestinal tissue injury score was significantly lower in T β 4-treated group compared to the untreated I/R group.

Further studies are required to confirm these preliminary findings and evaluate the underlying mechanisms. Further, clinical studies should be performed to evaluate the efficacy of T β 4 in management of intestinal I/R injury. Some discrepancies in the present findings and other studies might be due to the variations of the animal models and duration of treatment.

5. Conclusion

Collectively, these data have verified -for the first time- that T β 4 has a potential protective role against the intestinal I/R injury in rats possibly through its antioxidant, anti-inflammatory and anti-apoptotic properties. Therefore, it may be used as a novel approach for managing intestinal I/R injury.

Declaration of interest statement

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Ethical approval

All animals received care according to the guide and ethical regulations for the care and use of laboratory animals according to Institute of Laboratory Animal Resources, all experimental procedures were approved and in accordance with the ethical standards of the Institutional Research Board of Zagazig University.

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