Evaluation of the effects of omega-3 & interferon alpha-2b administration on partial bladder outlet obstruction in a rat model

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Background & objectives: In bladder outlet obstruction-induced rat models, the transforming growth factor-beta (TGF-β) and collagen ratios have been shown to be increased. Increased TGF-β leads to fibrosis. In this study, the effect of omega-3 and interferon alpha-2b (IFN α-2b) was investigated on oxidative stress, inflammation and fibrosis in bladder structure in a partial bladder outlet obstruction (PBOO) rat model.

Methods: A total of 35 male Wistar albino rats, weighing 300-350 g, were used in the study. The rats were randomly divided into five groups. At the end of the experimental period, bladders were harvested from all the rats, and pathological analysis of the rat bladder tissues was performed. In addition, investigations were carried out with enzymatic and non-enzymatic antioxidant systems to study the antioxidant properties of omega-3 fatty acid and IFN alpha-2b.

Results: Increased bladder weight in the PBOO group, in comparison to the control group, was decreased by the administration of omega-3 and IFN α-2b ($P=0.002$). Significantly higher superoxide dismutase (SOD) levels were detected in group 2 in comparison to the control group. It was also detected that serum SOD, glutathione peroxidase and nitric oxide (NO) levels were significantly higher in group 2 when compared to the control group ($P<0.05$). In the pathologic evaluation, group 2 showed significantly increased inflammation and fibrosis compared to the control group. Omega-3 treatment significantly decreased inflammation. It was shown that IFN α-2b application partially decreased inflammation.

Interpretation & conclusions: The results of the present study showed that in addition to the standard primary approaches to prevent the damage to the upper urinary tract secondary to PBOO, omega-3 fatty acid and IFN α-2b could be beneficial as adjunct treatment in clinical practice. However, this needs to be further investigated with prospective, randomized clinical trials with larger sample sizes.

Urinary obstruction is defined as the structural and functional changes associated with the impairment of normal urine flow. It may occur in any part of the urinary tract[1]. The level of obstruction, duration and cause of illness, the presence and severity of infection and presence of other pathologies are parameters that affect

the patient's overall clinical condition. Bilateral upper urinary tract obstructions and bladder and infravesical obstructions can cause chronic renal failure. On the other hand, supravesical obstructions generally result in unilateral loss of renal function. Obstructive uropathy is often reversible and treatable. Increased pressure in the urinary system leads to pathological changes at the vesical and supravesical level, such as cellular atrophy, necrosis and infection. In bladder outlet obstruction, many histological changes have been reported such as smooth muscle hyperplasia, extracellular matrix storage growth, degradation of the collagen structure, increased activity of the growth factor and deterioration in the ratio balance of matrix metalloproteinases (MMPs) to tissue inhibitors of MMPs[2-4]. In bladder outlet obstruction-induced rat models, the transforming growth factor-beta (TGF-β) and collagen ratios have been reported to be increased[5]. As a result of these changes, detrusor hypertrophy, increased voiding pressure and detrusor instability can occur, and these changes can result in decompensated detrusor activity clinically and pathologically[5]. It has been suggested that bladder outlet obstruction may lead to ischaemic changes in the bladder. Ischaemia-induced reactive oxygen species (ROS) may lead to lipid peroxidation in the cell and consequently is thought to cause tissue damage[6]. The most important strategy to reduce the effect of ROS is the use of antioxidants.

Omega-3 is an essential fatty acid required for many physiological functions. It is one of the most important components of the cell membrane structure. Omega-3 fatty acid plays a critical role in the prevention and treatment of atherosclerosis, coronary heart disease, mild hypertension, breast, colon and prostate cancer as well as chronic diseases, inflammatory diseases and even behavioural disorders[7]. In addition, omega-3 has been reported to have antioxidant properties[8]. Many agents, other than antioxidants, have been shown to reduce damage due to obstruction. Interferon (IFN) alpha-2b is known to inhibit the proliferation of fibroblasts. In addition, it increases collagen production in collagen-producing cells and production of collagenase in fibroblasts[9]. Retinoic acid and INF α-2b synergistically lead to apoptosis and inhibition of cell growth of human carcinoma cells *in vivo*[10,11]. In bladder outlet obstruction, TGF-β is thought to increase and lead to fibrosis. Hence, it is logical to hypothesize the use of IFN to inhibit fibrosis. This study was aimed to investigate the effects of omega-3 and IFN α-2b on oxidative damage and fibrosis in partial bladder outlet obstruction (PBOO) in rat model.

Material & Methods

This study was conducted in the department of Urology, Gaziosmanpasa University, Tokat, Turkey. A total of 35 male Wistar Albino rats (weight 300-350 g) were used. The rats were accommodated in standard rat cages. Each cage accommodated a maximum of three rats. Rats were fed with rodent special standard feed and water. The study protocol was approved by the Ethics Committee for Animal Experiments of the University.

The rats were divided equally into five groups of seven rats each by simple randomization. The rats were anaesthetized with 50 mg/kg ketamine. Group 1 underwent sham surgery and was evaluated as a control group. Partial bladder outlet obstruction (PBOO) was created in group 2. In group 3, after the creation of PBOO, 5 ml/kg/day omega-3 was administered by gavage for four weeks. After the creation of PBOO, group 4 received daily 100,000 IU/rat subcutaneous IFN α-2b for a total period of four weeks[9]. After the creation of PBOO, group 5 was given 5 ml/kg/day omega-3 by gavage for a total of 4-week duration along with subcutaneous 100,000 IU/rat IFN α-2b administered three times within 48 h for a total of four weeks.

To create the PBOO, the surgical field was first prepared by shaving the lower abdomen and perineum followed by applying sterile povidone-iodine in the supine position. A perineal incision was used to expose the bulbar urethra followed by separation of the urethra muscles to reveal the urethra. Blunt and sharp dissection was used to enter between the urethra and the corpus cavernosum, and the urethra hanged (Fig. 1A). Non-absorbable 4-0 suture was passed under the urethra to include the urethra, and a catheter (1.70 mm) was placed on the urethra and finally both were tied (Fig. 1B). The catheter was then pulled, and urethra was completely closed. With this method, urethral lumen width became approximately two-thirds of the normal size, and the incision was closed[12].

In groups 2, 3, 4 and 5, PBOO was created, followed by cystectomy under general anaesthesia four weeks later, following which blood samples were taken from systemic venous circulation. The harvested bladders were evenly divided into two parts, one placed into formaldehyde for pathologic analysis and the other specimen prepared for the biochemical analysis.

Biochemical analysis

<u>Serum antioxidant enzyme analysis</u>: Total superoxide dismutase (SOD) activity was determined and was expressed as U/mg protein[13]. Glutathione peroxidase (GSH-Px) activity was measured by the method of Paglia and Valentine[14]. The enzymatic reaction in the tube containing NADPH-reduced GSH, sodium azide and GSH reductase was initiated by the addition of H_2O_2, and the change in absorbance at 340 nm was monitored by a spectrophotometer. Activity was expressed as U/g protein. Catalase (CAT) activity was determined as described elsewhere[15]. Results were expressed as k/g protein. All samples were assayed in duplicate.

The carbonyl contents were determined spectrophotometrically (Cintra 10 E, Austria) based on the reaction of carbonyl group dinitrophenylhydrazine to form 2,4-dinitrophenylhydrazone. The results were

Fig. 1. (A) Blunt dissection between the urethra and the corpus cavernosum with hanging of urethra (arrow). **(B)** The process of narrowing the urethral lumen over a catheter (arrow).

reported as nanomoles of carbonyl per milligram of protein[16].

Tissue nitrite (NO_2^-) and nitrate (NO_3^-) were estimated as an index of NO production. Samples were initially deproteinized with Somogyi reagent. Total nitrite (nitrite + nitrate) was measured after conversion of nitrate into nitrite by copperized cadmium granules in a spectrophotometer at 545 nm. A standard curve was established with a set of serial dilutions (10-8-10-3 mol/l) of sodium nitrite. Linear regression was carried out using the point from the nitrite standard. The resulting equation was used to calculate the unknown sample concentrations. Results were expressed as nmol/g wet tissue. The thiobarbituric acid (TBA)-reactive substance level was determined by a method based on reaction with TBA at 90-1000°C. In the TBA test reaction, malondialdehyde (MDA) or MDA-like substances and TBA react to produce a pink pigment with an absorption maximum at 532 nm. The reaction was performed at a pH of 2-3 and 900°C for 15 min. The sample was mixed with two volumes of cold 10 per cent (w/v) trichloroacetic acid to precipitate the protein. The precipitate was pelleted by centrifugation, and an aliquot of the supernatant was reacted with an equal volume of 0.67 per cent (w/v) TBA in a boiling water bath for 10 min. After cooling, the absorbance was read at 532 nm. Results were expressed as nmol wet tissue, according to the standard graph prepared from a standard solution (1, 1, 3, 3-tetramethoxypropane)[17]. All chemicals used were obtained from Sigma-Aldrich, USA.
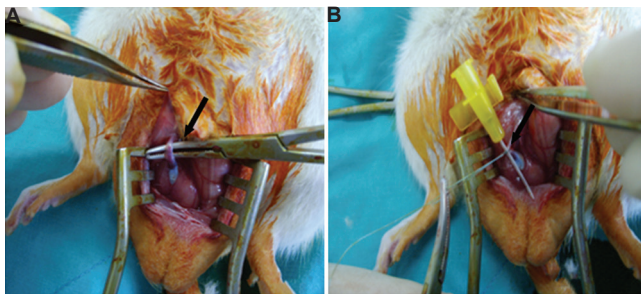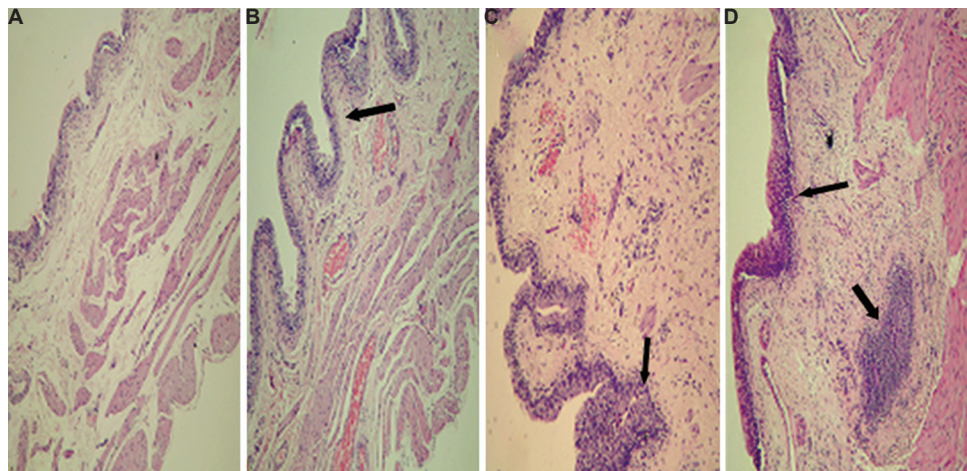

Fig. 2. (A) Normal bladder mucosa, no inflammation (inflammation grade 0) (H and E, ×200). **(B)** A few scattered inflammatory cells (arrow) in bladder mucosa (inflammation grade 1) (H and E, ×200). **(C)** Small foci of inflammatory cells (arrow) in bladder mucosa (inflammation grade 2) (H and E, ×200). **(D)** Diffuse inflammation with lymphoid follicle (arrows) in bladder mucosa (inflammation grade 3) (H and E, ×200).

Pathologic analysis: Formalin-fixed and paraffin-embedded bladder tissues were cut into 5-μm thick sections which were stained with haematoxylin-eosin (HE) and Masson's trichrome (MTC). Chronic inflammation was evaluated, and lymphoplasmositer cells were counted. HE staining was used to evaluate inflammation[18]. The grading for inflammation was 0: no inflammatory cell, 1: a few scattered inflammatory cells, 2: small localized multiple foci of inflammatory cells involving more than one area, and 4: diffuse severe inflammatory infiltrates. MTC staining was used to determine fibrosis and the ratio of thickness of the muscularis propria to whole full thickness of the bladder wall. According to the amount of collagen fibres within the lamina propria and muscularis propria, the grading of fibrosis was 0: no fibrosis, 1: thin fibrous bands in small foci, 2: thick fibrous bands in multiple foci, and 3: diffuse fibrosis (Fig. 2).

Statistical analysis: Kruskal-Wallis test was used for the comparison of continuous data among groups. For multiple comparisons, Mann-Whitney U-test with Bonferroni adjustment was used. The continuous data were presented as mean ± standard deviation. For comparing categorical data, Chi-square test was used. Categorical data were expressed as count (n) and percentages (%). Analyses were performed using commercial software (IBM SPSS Statistics 19, SPSS Inc., an IBM Co., Somers, NY, USA).

Results

Significant difference in the weight of bladder tissue was found between the control group and group 2 ($P<0.05$). Tissue CAT activity was significantly different among the groups ($P=0.002$). Significant difference was found as a result of the paired comparison between control group and groups 3 and 4 ($P<0.05$) (Table I). Significant difference was found between control group and groups 2 and 5 and between group 2 and groups 3 and 4 for SOD activity ($P<0.05$). Similarly, significant difference was detected between control group and groups 2, 3 and 4 and between group 2 and groups 3 and 4 ($P<0.05$) for the level of

Table I. Comparison of tissue weights and biochemical test results between the groups						
Groups	Sham (Group 1)	PBOO (Group 2)	PBOO + omega-3 (Group 3)	PBOO + IFN alpha-2b (Group 4)	PBOO + omega-3 + IFN α-2b (Group 5)	*P*
Tissue weight (g)	0.05±0.02	0.26±0.04*	0.07±0.01§	0.07±0.01§	0.18±0.17	0.002
tCAT (k/g protein)	0.12±0.06	0.17±0.03	0.27±0.08*	0.28±0.08*	0.19±0.08	0.002
tSOD (U/mg protein)	0.06±0.01	0.11±0.02*	0.06±0.00§	0.06±0.01§	0.05±0.01*§	<0.001
tMDA (nmol/g protein)	11.81±5.71	13.10±3.13	32.74±10.01*§	29.41±12.68	13.63±7.52	<0.001
tGSPHx (U/g protein)	2.13±1.98	3.39±1.77	2.24±0.62	2.66±2.08	3.82±3.66	0.423
tNO (µmol/g protein)	0.75±0.06	0.41±0.07*	0.59±0.09*§	0.53±0.06*§	0.56±0.22	0.001
tPC (nmol/mg protein)	4.41±1.02	5.17±1.15	3.65±1.02	3.55±0.43	3.32±0.60§	0.008

Data are expressed as mean±SD. *$P<0.05$ compared to sham group; §$P<0.05$ compared to PBOO group. PBOO, partial bladder outlet obstruction; tSOD, tissue superoxide dismutase; tMDA, tissue malondialdehyde; tGSHPx, tissue glutathione peroxidase; tNO, tissue nitric oxide; tPC, tissue protein carbonylation; tCAT, tissue catalase; IFN, interferon

Table II. Biochemical blood test results in various groups						
Groups	Sham (Group 1)	PBOO (Group 2)	PBOO + omega-3 (Group 3)	PBOO + IFN α-2b (Group 4)	PBOO + omega-3 + IFN α-2b (Group 5)	*P*
sSOD (U/ml)	3.69±0.23	10.91±0.77*	4.08±0.26§	4.08±0.24§	4.38±0.08*§	<0.001
sMDA (µmol/l)	1.90±0.18	2.42±0.37	1.97±0.16	1.82±0.31§	1.93±0.36	0.026
sGSPHx (U/l)	601.63±185.26	1501.09±320.24*	589.31±95.53§	688.87±171.81§	905.47±348.93*	0.001
sNO (µmol/l)	56.07±4.07	75.93±7.69*	59.65±4.68§	62.69±8.82	70.51±10.61	0.001
sPC (nmol/ml)	1081.74±431.06	490.13±84.61	1339.49±371.53§	1053.92±163.68§	1168.21±219.27§	0.001

Data are expressed as mean±SD. *$P<0.05$ compared to sham group; §$P<0.05$ compared to PBOO group. PBOO, partial bladder outlet obstruction; SD, standard deviation; sSOD, serum superoxide dismutase; sMDA, serum malondialdehyde; sGSHPx, serum glutathione peroxidase; sNO, serum nitric oxide; sPC, serum protein carbonylation; IFN, interferon

tissue NO. In terms of the level of MDA, a significant difference was detected between control group and group 3 and between groups 2 and 3 ($P<0.05$). Tissue protein carbonylation (PC) levels were significantly different between groups 2 and 5 ($P<0.05$) (Table I).

Serum SOD levels were significantly different between control group and groups 2 and 5, and also between group 2 and groups 3, 4 and 5 ($P<0.05$). Serum MDA levels were significantly ($P<0.05$) different between group 2 and group 4 ($P<0.05$) (Table II). Serum GSPHx and NO levels were significantly different between control group and group 2 and also between group 2 and groups 3 and 4 ($P<0.05$). For PC levels, significant difference was detected between group 2 and groups 3, 4 and 5 ($P<0.05$) (Table II).

According to the pathological evaluations, significant difference was detected between control group and all the other groups based on inflammation status ($P<0.001$). When the groups were evaluated individually, no inflammation was detected in control group and grade 1 inflammation was detected in other groups such as groups 2, 3, 4 and 5. The inflammation ratios were 28.6, 85.7, 42.9 and 71.4 per cent, in groups 2, 3, 4 and 5, respectively. In addition, grade 2 inflammation ratios of groups 2, 4 and 5 were 57.1, 28.6 and 28.6 per cent, respectively. Grade 2 inflammation was not detected in group 3. Finally, grade 3 inflammation rates of groups 2 and 4 were 14.3 and 28.6 per cent, respectively. Grade 3 inflammation was not detected in groups 3 and 5 (Fig. 3, Table III).
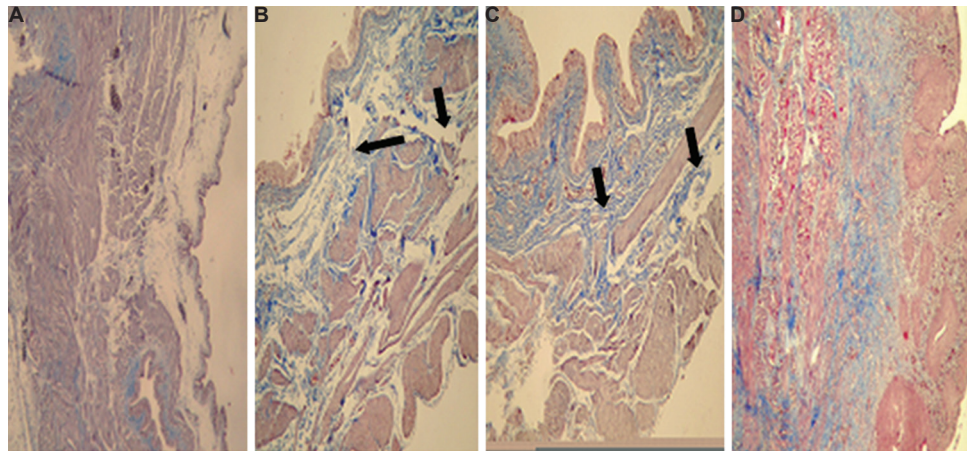

Fig. 3. (A) Collagen (not increased) in the lamina propria and muscularis propria (fibrosis grade 0) (MTC, ×200). **(B)** The photomicrography shows the increased thin bands (arrows) of collagen (fibrosis grade 1) (MTC, ×200). **(C)** Increased thick bands (arrows) of collagen (fibrosis grade 2) (MTC, ×200). **(D)** The photomicrography shows diffuse increased collagenous tissue (fibrosis grade 3) (MTC, ×200).

Table III. Tissue inflammation and fibrosis rates of different groups

Groups	Sham, n (%) (Group 1)	PBOO, n (%) (Group 2)	PBOO + omega-3, n (%) (Group 3)	PBOO + IFN α-2b, n (%) (Group 4)	PBOO + omega-3 + IFN α-2b, n (%) (Group 5)	P
Inflammation						
0	7 (100.0)	0	1 (14.3)	0	0	<0.001
1.00	0	2 (28.6)	6 (85.7)	3 (42.9)	5 (71.4)	
2.00	0	4 (57.1)	0	2 (28.6)	2 (28.6)	
3.00	0	1 (14.3)	0	2 (28.6)	0	
Fibrosis						
0	3 (42.9)	0	0	0	0	0.019
1.00	4 (57.1)	2 (28.6)	4 (57.1)	1 (14.3)	3 (42.9)	
2.00	0	3 (42.9)	3 (42.9)	4 (57.1)	4 (57.1)	
3.00	0	2 (28.6)	0	2 (28.6)	0	
PBOO, partial bladder outlet obstruction						

Fibrosis was only different between control group and group 2 (*P*=0.019). Grade 1 fibrosis was detected in all groups, and the degree and rate of fibrosis in groups 1, 2, 3, 4 and 5 were 57.1, 28.6, 57.1, 14.3 and 42.9 per cent, respectively. Grade 2 fibrosis rates in groups 2, 3, 4 and 5 were 42.9, 42.9, 57.1 and 57.1 per cent, respectively. Grade 2 fibrosis was not detected in the control group. Grade 3 fibrosis rates in groups 2 and 4 were 28.6 per cent each (Table III).

Discussion

A bladder outlet obstruction model has been applied in several experimental studies, and many changes have been reported including increased bladder wall thickness and weight, changes in extracellular matrix, increase in the amount of collagen in lamina propria and interfascicular space, urothelial hyperplasia, increased inflammatory infiltration, neuronal function deterioration of bladder (denervation), decrease of total antioxidant capacity, increased pro-inflammatory cytokines [interleukin-1 (IL-1) beta, tumour necrosis factor-alpha (TNF-α), IL-8], increased apoptosis, deterioration in the regulation of Bcl-2 and Bax expression and impaired contractile function[6,19-21]. In an experimental study reported by Metcalfe *et al*[22], rats with PBOO were compared with a sham group and it was found that the total bladder capacity significantly increased at the end of 2-, 4- and 8-wk periods, bladder pressure significantly increased at the end of 4-, 8- and 13-wk periods, while there were no bladder pressure changes at the end of a 2-wk period. It was also found that bladder wall thickness and weight significantly increased in all groups over time, inflammatory infiltration began histologically in the 2-wk period and increase in muscle mass was detected in a 4-wk period while maintaining the basic structure. In another experimental study with rabbits conducted by Matsumoto *et al*[23], while the average bladder weight in the control group was 2.85 g, it was 13.05 g after a 3-week PBOO and this increase was related to the structural changes after bladder obstruction. In our study, there was a significant increase in bladder wall thickness, weight, fibrosis and inflammation in obstructed bladder group compared to the control group. This suggests that in bladder outlet obstruction, detrusor muscle and nerve damage can occur partly due to inflammation caused by ischaemia-reperfusion.

It is well known that ischaemia-reperfusion and inflammation can cause the production of reactive nitrogen species and ROS[24]. Physiologically, there are antioxidant enzymes and various antioxidants available as an endogenous defence mechanism against ROS. In some cases when excessive free radicals produce ischaemia-reperfusion and inflammation, the antioxidant defence mechanism becomes insufficient. This phenomenon is called oxidative stress. Normally, there is a balance between oxidants and antioxidants in the body. However, in oxidative stress, superoxide, hydrogen peroxide and hydroxyl radicals increase[25]. It is thought that antioxidative capacity is decreased in the obstructed bladder tissue[26]. In the present study, there was a significant increase in SOD activity of tissue in the PBOO group compared to control group. CAT, MDA, GSH-Px and PC levels are also increased compared to control group, but this increase was not significant. Serum SOD, GSH-Px and NO levels were also found to be significantly different when compared to the control group.

Although the main approach in the treatment of obstruction is the elimination of mechanical obstruction, in some cases, symptoms persist after treatment. There are several studies that have attempted to minimize histological damage caused by obstruction. In a study done by Beamon *et al*[27], sildenafil administered to rats with PBOO for six weeks prevented an increase in detrusor activity, voiding pressure, detrusor muscle hypertrophy and collagen deposition. Similarly, long-term and high-dose vardenafil administration to PBOO rats significantly increased bladder contraction, and thus it has been reported to have a protective effect on the bladder[28]. The use of natural products has been reported to decrease oxidative stress in the bladder and maintain bladder function in PBOO treatment[26]. In a study, reported by Matsumoto *et al*[23], Eviprostat, an antioxidant, was administered in PBOO rabbit models for a period of four weeks and they showed an increase in antioxidant activity. In another experimental study by Li *et al*[29], coenzyme Q 10 and alpha-lipoic acid administered to a PBOO rat model were reported to have protective effects against ischaemia-reperfusion injury. Omega-3 diet has been reported to have antioxidant activity and anti-inflammatory properties. Eicosapentaenoic acid and docosahexaenoic acid, metabolites of omega-3 fatty acids, have strong anti-inflammatory and antioxidant properties[8]. In the present study, there was a significant decrease in SOD

activity of tissue in the PBOO group treated with omega-3, and GSH-Px and PC levels also decreased but not significantly. In serum, SOD, GSH-Px, and NO levels significantly decreased in omege-3-treated PBOO group compared to PBOO controls. MDA levels also decreased but it was not significant. Data from our study support that ischaemia in the bladder neck as a result of PBOO application leads to excessive production of free radicals. Omega-3 fatty acid and IFN α-2b administration decreased the free radicals caused by PBOO to levels comparable to the sham group. Antioxidants also prevent tissue inflammation and fibrosis. Pathologically, there was a significant increase in inflammation and fibrosis after establishing PBOO. In the omege-3-treated PBOO group, there was a significant decrease in inflammation while fibrosis did not decrease significantly. It is known that all ischaemic conditions such as testicular torsion and renal artery occlusion may cause oxidative stress secondary to hypoxia[25]. Oxidative stress leads to cell injury and fibrosis. Antioxidants prevent these harmful effects.

In a study done by Kim et al[30], following the administration of IFN-gamma to PBOO rats, IFN-γ was reported to reduce collagen deposition in the bladder. In the present study, pathologically, there was a significant increase in inflammation and fibrosis in the PBOO group compared to the sham group, while in the IFN-treated PBOO group inflammation and fibrosis did not decrease significantly. In the combined omega-3 and IFN-treated PBOO group, the rate and degree of inflammation significantly decreased, while fibrosis did not. There was also a significant decrease in the level of tissue SOD activity and PC level compared to PBOO alone. This study, though was performed in an animal model, in the treatment of partial urethral obstruction frequently encountered in daily practice, IFN and omega-3 could have a potential role in clinical practice to prevent or reduce damage secondary to obstruction. However, prospective randomized clinical trials with larger samples would be necessary to power future studies.

Conflicts of Interest: None.

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