

# The protective effect of roflumilast and ibuprofen on testicular ischemia reperfusion injury: An experimental study

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## ABSTRACT

**BACKGROUND:** The aim of the present study is to investigate the efficiency of roflumilast and ibuprofen in an experimental rat testicular ischemia reperfusion injury model in the light of histological and biochemical data.

**METHODS:** A total of 32 prepubertal male rats were randomly divided into four groups as G1: Control Group (testicular torsion/detorsion + saline (0.9% of 2 ml) was applied). G2: Sham Group only right scrotal incision was performed; G3: Ibuprofen Group (testicular torsion/detorsion + ibuprofen administration); and G4 Roflumilast Group (testicular torsion/detorsion + roflumilast administration). Oxidative markers such as malondialdehyde (MDA), myeloperoxidase (MPO), total sulfhydryl (TSH), and nitrite (NO) levels as well as histopathological changes were analyzed.

**RESULTS:** Tissue MPO, MDA, and NO levels were significantly higher and TSH levels significantly lower in control group compared to sham group ( $p<0.001$ ). The histopathologic scores of drug groups (Groups 3 and 4) were significantly lower than group 1 ( $p<0.001$ ). In comparison of Group 3 and Group 4 with each other, the mean values of MPO and MDA were statistically significantly lower in Group 4 ( $p<0.001$ ). A higher mean value of TSH was found in Group 3 without statistical significance ( $p=0.32$ ). There was also an insignificant decrease in mean NO values of Group 3 compared to Group 4 ( $p=0.44$ ). In comparison of drug groups, Group 4 had statistically insignificant better scores.

**CONCLUSION:** Our results indicate that administering ibuprofen and roflumilast reduced testicular ischemia reperfusion injury in rat testis torsion model. In comparison, roflumilast is found to be more beneficial.

**Keywords:** Ibuprofen; ischaemia reperfusion; oxidative stress; roflumilast; testis.

## INTRODUCTION

Testicular torsion is one of the most significant and common emergency in children and adolescents. The incidence is around 4.5/100.00 between 1 and 25 years, males.<sup>[1]</sup> The torsion is formed by the rotation of the spermatic cord in the longitudinal axle, first venous and then arterial block occurs. It was demonstrated that the chance of recovery of testicular tissue without a histological damage if intervention for tes-

ticular torsion is performed within the first 6 h is more than 90%, but it is <10% within 12–24 h.<sup>[2]</sup> When testicular torsion is not treated urgently and appropriately, it causes germinal cells and seminiferous tubule damage. The severity of the damage caused by testicular torsion depends on the degree of rotation and duration, but despite a timely restoration, the oxygenated blood begins a reaction causing more damage than ischemia.<sup>[3]</sup> In experimental testicular torsion and detorsion models, it has been demonstrated that, reperfusion

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causes increased lipid peroxidation, myeloperoxidase (MPO) activity, nitric oxide production, and apoptosis in germ cells.<sup>[4,5]</sup> To reduce this reperfusion injury different agents might have an effect beside detorsioning the testis.

Ibuprofen is a widely used analgesic and anti-inflammatory drug. The fundamental mechanism of action is to inhibit the enzyme cyclooxygenase, which has a catalyst effect on the conversion of arachidonic acid to prostaglandins.<sup>[6]</sup> It was previously found to have a protective effect of ibuprofen on ischemia reperfusion injuries.<sup>[7]</sup>

Roflumilast is a second generation selective phosphodiesterase type 4 (PDE4) inhibitor and has an anti-antioxidant effect.<sup>[8]</sup> It holds a unique mechanism of activity on the inflammation by eosinophils, neutrophils and also remodeling. We hypothesized that this selective PDE4 inhibitor can prevent the oxidative injury due to reperfusion. In the present experimental study, we investigated and compared the effect the protective effects of these two widely used drugs on testicular ischemia-reperfusion injury by assessing oxidative markers such as malondialdehyde (MDA), MPO, total sulfhydryl (TSH), and nitrite (NO) levels as well as histopathological changes.

## MATERIALS AND METHODS

Thirty-two prepubertal (175–205 g) Wistar albino rats were used in this study. Rats were maintained for 2 weeks on a standard diet and had access to the food and water. They were kept in the animal room at a constant temperature (25±2°C) at 30–70% humidity with 12-h light/12-h dark cycle.

Animals were randomly divided into four groups as follows:

- G1: Control Group (testicular torsion/detorsion+ saline (0.9% of 2 ml) was applied)
- G2: Sham Group only right scrotal incision was performed
- G3: Ibuprofen Group (testicular torsion/detorsion + ibuprofen intraperitoneal and oral administration)
- G4 Roflumilast Group (testicular torsion/detorsion + roflumilast intraperitoneal and oral administration).

All of the procedures of this experimental study were approved by the local ethics committee (Approval No.632020). Anesthesia was administered as 5 mg/kg ketamine and 2 mg/kg xylazine, and rats were observed to be in spontaneous respiration during the surgical procedure. Surgical procedures were performed under sterile conditions. After right scrotal incision was performed, tunica vaginalis was opened, and the testis was delivered out the scrotum. The testes were rotated clockwise 720° and fixed with 3/0 polyglactin suture. The testes of the torsion groups (G1, G3, and G4) were detorsioned after 3 hours. The first doses of the drugs of the groups were administered (20 mg/kg/day for ibuprofen and 1 mg/kg/day for roflumilast) intraperitoneally 60 min after torsion (2 h before detorsion). In Group 2, the right scrotum

was insized. The testes were removed and replaced through the incision and a 3/0 polyglactin suture was placed through the tunica albuginea.

Maintenance doses of the drugs were administered for 7 days, 70 mg/kg/day for ibuprofen, and 1.5 mg/kg/day for roflumilast, respectively.<sup>[9,10]</sup> All the tablets were crushed and diluted with 0.9% NaCl and homogenized and administered with a single dose of orogastric lavage per day. After 24 h of the administration of the last dose of drugs, the rats were sacrificed. Orchiectomy of the detorsioned testes was performed. Testicular tissues were cut in half from the vertical axis in a standard way; one half was preserved for biochemical analyses and the other half was sent for histological analyses.

## Biochemical Analysis

Tissues and serums were stored in a deep freezer at –80°C until assays. MDA levels were calculated by the fluorometric method, as described by Wasowicz et al.<sup>[11]</sup> For total NO, the reduction of nitrate to NO by nitrate reductase and measurement of the occurred color in the end of the reaction of NO with Griess reagent; NO alone was then measured without enzymatic reaction and nitrate was calculated by subtraction.<sup>[12]</sup> MPO activity was assayed spectrophotometrically as previously described.<sup>[13]</sup> TSH groups were also measured spectrophotometrically using the Sedlak and Lindsay method.<sup>[14]</sup>

## Histopathological Examination

The testis was fixed in Bouin fixative. The tissues were sectioned into 5 µm thickness stained with hematoxylin-eosin and evaluated by an experienced genitourinary pathologist. The histopathological changes were scored according to Cosentino's classification as stated in Table 1.<sup>[15]</sup>

## Statistical Analysis

Statistical Package for the Social Science, IL CHICAGO version 20 was used for statistical analysis. In biochemical evaluation, 1- way analysis of variance followed by *post hoc* analysis using Bonferroni was performed. Chi-square test and *post*

**Table 1.** Grades of changes in the testicular architecture

Grade 1	Normal testicular architecture with an orderly, arrangement of germinal cells.
Grade 2	Less orderly, non cohesive germinal cells and closely packed seminiferous tubules.
Grade 3	Disordered sloughed germinal cells with shrunken pyknotic nuclei andless distinct seminiferous tubule borders.
Grade 4	Seminiferous tubules closely packed with coagulative necrosis of the germinal cells.

**Table 2.** Effect of ibuprofen and roflumilast on post torsion testis tissue. (Data were expressed mean±SD, n=8)

	Groups						
	Group 1* (Control)	Group 2 (Sham)	p	Group 3 (Ibuprofen)	p	Group 4 (Roflumilast)	p
Myeloperoxidase	0.404±0.98	0.036±0.19	0.000	0.245±0.03	0.000	0.144±0.02	0.000
Malondialdehyde	27.22±3.17	3.78±0.62	0.000	14.36±1.89	0.000	9.50±1.25	0.000
Total Sulfhydryl	0.67±0.13	1.77±0.23	0.000	1.29±0.17	0.000	1.11±0.18	0.000
Nitrite	1.36±0.24	0.38±0.12	0.000	0.55±0.18	0.000	0.72±0.18	0.000

\*P: Significance by way ANOVA among the 3 studied groups.

**Table 3.** Scores of the comparative histologic damage

Histologic Damage Score	Group 1* (Control)	Group 2* (Sham)	Group 3 (Ibuprofen)	Group 4 (Roflumilast)
Grade-1		8		
Grade-2			1	3
Grade-3			7	5
Grade-4	8			

\*P<0.01 for group 1 vs 2, 3, 4 and group 2 vs 3, 4.

*hoc* analyses of Bonferroni were applied in the evaluation of pathology results. Biochemical data were expressed as mean and standard deviation. A p-value has been considered significant when it is <0.05.

## RESULTS

### Oxidative Stress Parameters in Testicular Tissue

The average values and comparison of oxidative stress parameters for both groups are shown in Table 2. In comparison of Group 3 and Group 4 with each other, the mean values of MPO and MDA were statistically significantly lower in Group 4 (p<0.001). A higher mean value of TSH was found in Group 3 without statistically significance (p=0.32). There was also an insignificant decrease in mean NO values of Group 3 compared to Group 4 (p=0.44).

### Histological Parameters

Histological damage scores of the groups are shown in Table 3. Both drug groups had statistically significantly better scores than Group 1 (p<0.05). In comparison of drug groups, Group 4 had better scores, but was not statistically significant (Figs. 1–4).

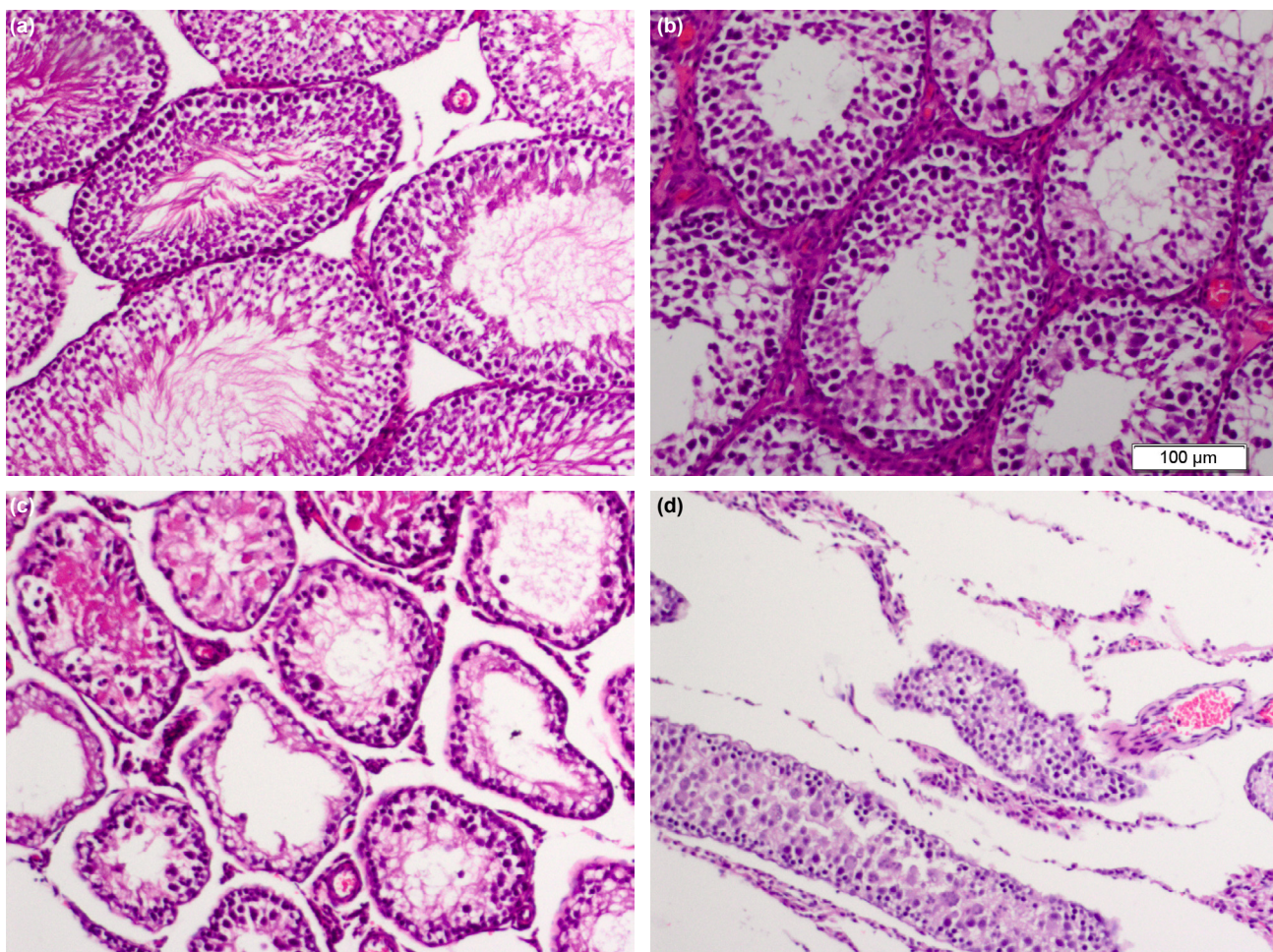
## DISCUSSION

Testicular torsion is characterized by rotation of the spermatic cord, microcirculation is first disrupted.<sup>[16]</sup> It was

demonstrated that for testicular parenchymal damage resulting in necrosis, 2 h of arterial blockade and 6 h of venous blockade are an enough time and usually both systems are occluded.<sup>[17,18]</sup> Prompt diagnosis and surgical detorsion are the most important point of preventing tissue damage. However, after a correct detorsion, reperfusion process participates in the event. Reperfusion is necessary as soon as possible to maintain the function of ischemic tissue, but pathophysiological consequences of testicular detorsion (reperfusion) inevitably occur. An increase in neutrophil accumulation, nitric oxide production, lipid peroxidation, MPO activity, is observed in germ cells.<sup>[19]</sup> This stressful situation causes intense tissue damage and occurs due to an excessive production of reactive oxygen species that are not adequately eliminated by the natural antioxidant defense mechanisms in the body.<sup>[20]</sup> Antioxidant treatment has been proposed to prevent ischemia reperfusion injury at that point. Various agents have been used in experimental models to date.<sup>[4,7,9]</sup>

MPO plays a fundamental role throughout the oxidative process created by the immune system. As a result of neutrophil accumulation and activation, MPO levels are detected at high levels after reperfused tissues.<sup>[21]</sup> It is used as a specific biomarker reflecting the status of the relevant disease in terms of the oxidative system.<sup>[22]</sup> In our study, MPO levels are significantly higher in controls (Group 1). We also detected a significant decrease in drug groups. When roflumilast and ibuprofen were compared, there was a significant decrease in MPO levels in favor of roflumilast.

High neutrophil accumulation may also cause lipid peroxidation, carrying damage risk to cells with a membrane of unsaturated fatty acids. It was clearly demonstrated that the marker of lipid peroxidation was MDA and its level increases significantly torsion and detorsion periods.<sup>[23,24]</sup> In the present study, a difference was found between the control and sham groups. It was also shown that there was a statistically significant decrease in both drug groups compared to the control group (p<0.001) and summarized in Table 2. A significant decrease in favor of roflumilast was found in comparison between two drug groups (p<0.001).



**Figure 1.** (a) In the sham group, the seminiferous tubules, the germ cells in regular form (Cosentino Grade 1), H&E, reduced from  $\times 100$ . (b) In ibuprofen and roflumilast group, reduced edema, irregularities, and vacuolization were observed in the seminiferous tubules but well organized germ cells with almost a normal architecture (Cosentino Grade 2) H&E, reduced from  $\times 100$ . (c) In ibuprofen and roflumilast, reduced edema, irregularities, and vacuolization were observed in the seminiferous tubules, moderate organized germ cells (Cosentino Grade 3), H&E, reduced from  $\times 100$ . (d) In the control group, interstitial edema, intensive vacuolization, and irregularities in tubule borders (Cosentino Grade 4), H&E, reduced from  $\times 100$ .

Sulfhydryl (SH) groups are important components various cell membrane activities and reperfusion injury results a decrease in T-SH levels. The loss of groups inhibits an important enzyme called superoxide dismutase. This enzyme disintegrates superoxide radicals and the inhibition of this enzyme causes an increase of superoxide radicals.<sup>[25]</sup> In our study, T-SH values are significantly higher in sham and drug groups than the control group and similar to the findings reported by other investigators.<sup>[4,23]</sup> In addition, T-SH in the ibuprofen group was higher than the roflumilast group, without statistical significance.

NO levels reflect levels of nitric oxide and reactive substrates. Nitric oxide is an important physiological regulator that regulates blood flow. Some groups suggest the beneficial effects such as vasodilation after I/R, inhibiting platelet aggregation, anti-inflammatory effects while others thought that accumulated NO synthesis damages testis through its pathway.<sup>[26,27]</sup> In comparison with the sham group, a significant increase

was found in the control group while there was a significant decrease in roflumilast and ibuprofen group. In comparison, of the roflumilast and ibuprofen groups with each other, no statistically significant difference was found in our study. This result again supports the notion that each agent sufficiently prevents the cells after reperfusion injuries.

Cosentino scoring was used for histopathological evaluation. This scoring consists of four different grades. Grade I shows normal testicular tissue, while Grade 4 shows necrosis in the testicular parenchyma.<sup>[28]</sup> In our study, the scores of both drug groups had significantly better scores than the control group. Although no statistically significant difference was found between the scores of the roflumilast and the ibuprofen groups, the best pathological scores are detected in the roflumilast group.

In the previous I/R studies, it has been shown that the duration of ischemia of 3 h is sufficient for testicular tissue, and

in our study, period of ischemia was applied as that period.<sup>[29,30]</sup> Our torsion period may represent a rationale for the need to seek the benefits of different ischemic periods since the time from torsion to surgery varies due to the admission time of patients. Although there are 360° of spermatic cord rotation studies in the literature, it was clearly shown that 720° rotation angle is sufficient for the expected ischemia to occur.<sup>[31]</sup> Therefore, to create complete ischemia a 720° torsion model was used in the current study. Furthermore, as it does not make sense to try a prophylactic (pre-torsion) agent, the possible application time that can be started at hospital admission and can be continued for a while (1 week) after discharge have been tried under the guidance of previously published important publications.<sup>[4,8-10]</sup>

The PDE 4 inhibitors reduce oxidative stress and improve cellular functions.<sup>[10]</sup> The first PDE4 inhibitors are limited in their practical application due to side effects especially gastrointestinal system. Roflumilast is a second-generation oral PDE4 inhibitor, its tolerability is higher because side effects are less and has recently been granted marketing authorization both in the European Union and United States for the treatment of multiple inflammatory conditions such as psoriatic arthritis and inflammatory airway disease.<sup>[32]</sup> By inhibition of PDE4, the intracellular level of cyclic AMP increases and anti-inflammatory response initiates. The results of the present study revealed that roflumilast suppressed the inflammation and improved histopathological parameters which support the hypothesis mentioned above.

Ibuprofen has been used in experimental studies and its beneficial effect has been shown in oxidative stress parameters and also histopathologically.<sup>[19]</sup> In that study, Keseroglu et al. concluded that the main difference is that other drugs did not have any anti-inflammatory properties. However, in the present study, the beneficial effects of the different anti-inflammatory drugs were detected and compared. The evaluation in our study is based entirely on both the objective biochemical and pathological criteria and it was demonstrated that both ibuprofen and roflumilast has protective effects. Furthermore, when these two drugs are compared due to their antioxidant features roflumilast seems more beneficial than ibuprofen. These findings should be contextualized considering that studies on different doses of ibuprofen and roflumilast may alter the outcomes.

Our study is not devoid of limitations. First, there is lack of long-term results on drugs. Second, although very experienced, the evaluation of histopathology is made by a single pathologist. Third, the absence of evaluation of spermatogenesis and finally, since to study with high numbers is contrary to the animal rights and power analysis was performed, our sample size is small. The duration of many testicular torsion studies in the literature is limited to 24 h. In our study, 8 days of medication appears long enough compared to previous studies and it might be expressed as a strength of the present

model. But to us, it may be more accurate to detect effects longer than the mentioned time also.

## Conclusions

This study demonstrates the protective effect of both ibuprofen and roflumilast as an antioxidant treatment in testicular ischemia reperfusion injury with the obtained objective criteria. In comparison, roflumilast is found to be more beneficial. Future studies using different doses of PDE4 inhibitors administered after different torsion/detorsion times are expected.

**Ethics Committee Approval:** This study was approved by the Ankara Training and Research Hospital Animal Experiments Local Ethics Committee (Date: 26.11.2020, Decision No: 0063).

**Peer-review:** Internally peer-reviewed.

**Authorship Contributions:** Concept: B.C.Ö., C.N.Y., T.K.; Design: B.C.Ö., H.S., E.Ö.; Supervision: B.C.Ö., T.K.; Resource: B.C.Ö., H.S.; Data: B.C.Ö., H.S., C.N.Y., E.Ö., E.Öğ.; Analysis: T.K., E.Ö.; Literature search: B.C.Ö., C.N.Y., T.K.; Writing: B.C.Ö., H.S., E.Ö.; Critical revision: C.N.Y., T.K., E.Ö.

**Conflict of Interest:** None declared.

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## DENEYSSEL ÇALIŞMA - ÖZ

### Roflumilast ve ibuprofenin testis iskemisi reperfüzyon hasarına etkisi: Deneysel bir çalışma

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**AMAÇ:** Bu çalışmanın amacı ibuprofen ve roflumilastın testis iskemisi reperfüzyon hasarına olan etkilerini bir sıçan modelinde histolojik ve biyokimyasal veriler ışığında araştırmaktır.

**GEREÇ VE YÖNTEM:** Toplam 32 puberte öncesi erkek sıçan rastgele şu şekilde gruba ayrıldı: G1: Kontrol Grubu (testis torsiyonu / detorsiyonu + salin (2 ml %0.9) uygulandı) G2: Sham Group sadece sağ skrotal insizyon yapıldı; G3: İbuprofen Grubu (testis torsiyonu / detorsiyonu + ibuprofen intraperitoneal ve oral uygulama); G4: Roflumilast Grubu (testiküler torsiyon / detorsiyon + roflumilast intraperitoneal ve oral uygulama). Her grupta malondialdehit (MDA), miyeloperoksidaz (MPO), total sülfhidril (TSH) ve nitrit seviyeleri gibi oksidatif belirteçler ile histopatolojik değişiklikler analiz edildi.

**BULGULAR:** Kontrol grubunda sham grubuna göre doku MPO, MDA ve nitrit düzeyleri anlamlı olarak yüksek ve TSH düzeyleri anlamlı olarak düşüktü ( $p < 0.001$ ). Grup 1 ile karşılaştırıldığında tüm bu parametreler ilaç gruplarında anlamlı farklıydı. İlaç gruplarının (grup 3 ve 4) histopatolojik skorları, grup 1'den anlamlı olarak düşüktü ( $p < 0.001$ ). Grup 3 ve Grup 4 birbirleriyle karşılaştırıldığında, MPO ve MDA ortalamaları Grup 4'te anlamlı derecede düşüktü ( $p$  değerleri;  $< 0.001$ ). Grup 3'te anlamlı olmayan daha yüksek bir TSH ortalama değeri bulundu ( $p = 0.32$ ). Grup 4'e göre grup 3'ün ortalama nitrit değerlerinde de anlamsız bir düşüklük mevcuttu ( $p = 0.44$ ). İlaç grupları karşılaştırıldığında, Grup 4 anlamsız daha iyi histopatolojik puanlara sahipti.

**TARTIŞMA:** Sonuçlarımız, ibuprofen ve roflumilast uygulamasının sıçan testis torsiyon modelinde iskemisi reperfüzyon hasarını azalttığını göstermektedir. Her iki ajan karşılaştırıldığında, roflumilast'ın daha faydalı olduğu bulunmuştur.

**Anahtar sözcükler:** İbuprofen; iskemisi reperfüzyon; oksidatif stres; roflumilast; testis.

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