

# Pterygium: Glutathione, Nitric Oxide Production and N-Acetylcysteine Treatment

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## Research article

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## Abstract

Background There was not investigated the participation of glutathione (GSH), endothelial nitric oxide synthase (eNOS) and nitric oxide (NO) in pterygium pathogenesis and the effects of the precursor of GSH synthesis N-acetylcysteine in treatment prior to surgery.

Methods The levels of eNOS, NO, 3-nitrotyrosine, reduced and oxidized GSH (GSSG) and catalase were measured in tissue homogenates of 120 patients with primary or recurrent pterygium, and systemic or topical pretreatment with N-acetylcysteine, compared to control group.

Results A decrease in eNOS, NO, 3-nitrotyrosine, and GSH oxidation degree (GSSG%) was observed in primary pterygium, which remained decreased in other pathological groups in case of GSSG% and 3-nitrotyrosine. The levels of GSH and catalase increased in recurrent pterygium, even more, they increased in the group with systemic treatment, while topical treatment did not affect them, compared with control or primary pterygium groups. High positive correlation in groups of the study was observed between behavior of eNOS with NO or GSSG%, while GSH showed it with GSSG or catalase. Women showed a higher level of GSH and catalase in primary pterygium group, lower its level and a higher level of NO in recurrent pterygium. It was observed in women positive correlation in groups of study of GSSG% with 3-nitrotyrosine alone, while in men with NO.

Conclusions Study data do not contradict the assumption about the following possible sequence of events in pathogenesis of the primary pterygium: decrease in the activity of eNOS and consequently of NO, decrease in S-nitrosation of GSH, possible modulation of the intracellular level of GSH through synthesis and/or mobilization from other tissues.

## Background

Pterygium is a degenerative process that involves the conjunctiva and cornea of the eye and results in the growth of fibrous tissue on the surface of the cornea. Surgery is the principal pterygium treatment with some frequency of disease recurrence. Mechanisms of the pathogenesis of this disease were discussed in several reviews [1–3]. The main factor in the etiology of pterygium is considered chronic exposure to UV radiation [4,5]. UV radiation induces oxidative stress that, in the long term, can cause damage to DNA and other critical biological molecules, cell membrane structure, participating in the pathogenesis of different ocular diseases.

Nitric oxide (NO) in high concentrations and its derivatives such as peroxynitrite contribute to oxidative stress, but they are also important signaling molecules involved in processes of functional adaptation in the multitude of tissues to stress, including glutathione (GSH) synthesis, and can modulate effects of oxidative stress. Their effect then results from the balance between negative for oxidative stress and positive for their physiological effects. Data on NO levels in pterygium tissue are contradictory, from high, which was probably determined by high levels in women [6], to decreased [7]. There are data on the close relationship between NO and metabolism and/or transport of GSH, which synthesizes in all tissues but large quantities, mainly in the liver. Synthesis of GSH involves three constitutive amino acids (cysteine,

glutamate, and glycine), but three, the limiting factor in its synthesis is cysteine. In healthy ocular tissues, GSH concentration reaches millimolar levels in cornea and micromolar in other tissues and fluids of the eye [8, 9]. Micromolar concentrations of reduced GSH and oxidized GSH (GSSG) were also observed in "retinoblastoma" [10]. We have found no data on levels of GSH in pterygium.

NO affected the concentration of GSH in the liver during endotoxemia in the animal model [11], in primary cultural rabbit conjunctival epithelial cell layers [12] and retinal pigment epithelial cells [13]. GSH has a high affinity to NO, and S-nitrosoglutathione is the primary intermediary in the production of other metabolic regulating nitrosothiols [14, 15]. This suggests that the role of NO in pterygium pathogenesis may be through synthesis and/or transport of GSH in ocular tissues. N-acetylcysteine (NAC) is derived from the amino acid L-cysteine and is used as a precursor of GSH synthesis in respiratory tract diseases [16]. Modulating oxidative stress, this drug indirectly expresses antiviral effect, anti-inflammatory, inhibits angiogenesis, and decreases cancer cell growth in different diseases, including ocular [17, 18].

## Methods

The idea of this study was to evaluate levels of GSH, GSSG, NO, 3-nitrotyrosine, the activity of CAT and eNOS, in tissues of primary pterygium, recurrent pterygium, and primary pterygium with preoperative systemic or topical treatment with NAC.

### Study population

The study lasted three years and finally involved a total of 120 patients (54 men and 66 women) and included six patient groups: control group (C), primary pterygium (P), recurrent pterygium (R), primary pterygium with systemic treatment with NAC (Ps), and topical (Pt). The following were criteria for non-inclusion: 1) history of disease or trauma to the ocular surface involving the sampling area; 2) ocular surgery during the three months prior to surgery; 3) oral or topical immunosuppressive treatment during the four weeks prior to surgery; 4) systemic diseases such as diabetes, rheumatoid arthritis or other autoimmune disease or neoplasia; 5) chronic addictions such as tobacco or alcohol.

The number of patients by group and their age was presented in Table 1.

Table 1. Patient's population

Groups	C	P	R	Ps	Pt
all patients age	$61.3 \pm 6.0$	$56.9 \pm 10.5$	$46.1 \pm 6.3^{**}$	$53.0 \pm 10.3$	$55.4 \pm 9.3^*$
N	19	36	19	17	29
men age	$61.9 \pm 5.9$	$53.8 \pm 8.7^*$	$45.6 \pm 7.1^{**\&}$	$50.1 \pm 8.0^{**}$	$56.5 \pm 13.4^{\&}$
N	7	18	10	8	11
women age	$60.8 \pm 5.4$	$62.1 \pm 13.0$	$46.8 \pm 4.9^{**}$	$55.5 \pm 8.9$	$52.5 \pm 10.3^{*\&}$
N	12	18	9	9	18

C - control group, P - primary pterygium group, R - recurrent pterygium group, Ps - systemic and Pt - topic pretreatment with NAC of patients with primary pterygium. \* - p<0.05 compared to the control group; & - p<0.05 compared primary pterygium to other pathological groups.

Compared to control group of all patients, a decreased age shown patients with recurrent pterygium. Compared to primary pterygium other pathological groups (&) shown moderate differences in men principally.

### **Tissue samples obtaining**

The pterygium samples were obtained by the author of the article from the patients of the Hospital "Nuestra Señora de la Luz" with a clinical diagnosis of primary or recurrent pterygium, who came for pterygium resection surgery, during the period from 2015 to 2018. The control sample of the normal conjunctiva was taken from patients who underwent cataract or dacryocystorhinostomy surgery, removing it from the nasal area of the ocular limb and in the same proportion as the pterygium tissue. The surgery was performed by dissecting the head of the pterygium of the cornea, followed by resection of the body in a block. During the process, the conjunctiva was taken together with the subconjunctival fibrovascular tissue. The surgery was performed by dissecting the head of the pterygium of the cornea, followed by resection of the body in a block. During the process, the conjunctiva was taken together with the subconjunctival fibrovascular tissue. A conjunctival autograft covered the bare sclera from the upper or lower conjunctiva and fixed with nylon 10.0 sutures with separate stitches. The tissue obtained was stored in 2 ml Eppendorf tubes with flat bottom, and immediately frozen in liquid nitrogen, where they were kept at -70 °C until processing.

### **Treatments**

Systemic treatment was performed for one month before surgery, with NAC dose 600 mg/day. In these doses, systemic treatments were used in respiratory diseases with the task of increasing the level of GSH in tissues [16]. Topical treatment: was performed for one month before surgery with 10% solution of NAC 1 drop four times a day. A comparison of

both treatments was used for practical purposes and as a scientific instrument to evaluate possible interaction between NO and GSH in pterygium pathogenesis.

## Samples processing

Samples were processed as they accumulated in groups of approximately 20, with average time two-three weeks after collection. Samples of homogenates were obtained by placing the tissue in a 30 mmol cold phosphate buffer solution (pH 7.2) and adding 0.1% of Triton 100 (1 mg of tissue per 10 µl buffer). Tissues were homogenized and centrifuged at 10 000 rpm for 1 min at 4 °C, and the supernatants were stored at -70 °C to be processed within two weeks. In the tissue homogenates, the Cayman chemistry (USA, MI) chemical kits was employed for measurement of total proteins (TP, No.704002), NO (nitrate/nitrite colorimetric assay kit, No.780001), and total reduced GSH y oxidized form of GSH (GSSG) (glutathione assay kit, No. 703002), catalase (CAT, No. 707002). The level of 3-nitrotyrosine (3NT, 3-nitrotyrosine Elise kit, Abcam, No. ab116691, UK) was established in homogenates by the enzyme-linked immunosorbent assay. The values of NO, GSH, GSSG, and 3NT, are expressed as nmol/mg of TP/ml that correspond to µmol of concentration. GSH oxidation grade was calculated according to  $GSSG\% = \frac{GSSG}{2GSH} \times 100$ .

## Western blot assay

100 µg of protein from the different groups of pterygium were subjected to 10% SDS-PAGE under non reducing conditions and transferred to polyvinylidene fluoride membranes (Immobilon PVDF, 0.45 µm; Millipore, USA). Subsequently, the membranes were blocked with 5% albumin seric bovine in TBS-0.1% Tween 20 (TBS-T, pH 7.4) for two hours. After that, were washed three times with TBS-T and incubated with primary antibody eNOS (Cat. SC-5302) at 4°C for 18 h in continuous agitation. After adequately washing with TBS-T, the membranes were incubated with secondary antibody (Cat. SC-516102) for 2h in constant agitation. Detection was then carried out by the enhanced chemiluminescence method (Western Blotting Luminol Reagent, Santa Cruz Cat. 2048). Membranes were photographed and the image digitalized to carry out densitometric analysis using software Imagen Studio Lite (LI-COR Biosciences). The relative presence of each protein was normalized with β-actin as a housekeeping protein. Before implementation of western blot the study group were

separated in subgroups with 6 samples inside. Consequently final level of groups C, R, and Ps was average of 3x6 determinations, group P of 6x6 and group Pt – 4x6 determinations.

## Statistical analysis

GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analysis. The means values and standard deviations obtained for each group. The count data were analyzed by ANOVA, followed by Tukey post hoc test, considering significance at  $P<0.05$ . Bivariate Pearson correlation was examined concerning the parameters in all experimental groups studied.

## Results

### All patients

Figure 1 presented data on levels of total reduced GSH (a), GSSG (b), CAT (b), GSSG% (d), NO (e), and 3NT (f) in all patients regardless of gender.

Surprisingly in all patients the level of GSH shows substantial increase in recurrent pterygium group, even more significant increase in the primary pterygium group with systemic pretreatment with NAC, while topical pretreatment shows no effect on GSH in group P, comparing with groups C or P. GSSG and CAT activity showed GSH-like behavior. GSSG% shows decreases in all groups, compared to control group. The level of GSSG% is very high in investigated ocular tissues: in group C, it is about 35% and experimental groups it is about 25%, while in other tissues of human organism, this value was reported between 1-2%. The behavior of NO and 3NT in study groups is similar to that of the GSSG%, only that 3NT was significantly lower in all pathological groups, compared to control group, while NO only in primary pterygium group.

Figure 2 presents the behavior by groups of all patients regardless of the sex of the activity of eNOS.

Comparing with group C, a decrease in eNOS activity was observed in primary pterygium, slighter reduction of recurrent pterygium, absence of change in systemic treatment, and decreased level in topical treatment, similar to the group of recurrent pterygium.

### Gender differences

In Figure 3, we can see the analysis of gender differences by study groups.

Regarding GSH, women showed a higher level of GSH in the P group and a lower level in the Ps group, compared to men. GSSG or GSSG% showed no gender differences. CAT in group R was higher in women compared to men, which coincides with higher NO levels in women in the same group.

Results of behavioral pattern analysis of parameters measured by study groups (bivariate Pearson correlation) regardless of gender and separately men and women were presented in Table 2.

Table 2. Bivariate Pearson correlation coefficients, starting from 0.8. Empty cells – with no significance.

	ALL	MEN	WOMEN
GSH-CAT	0.949*	0.962**	0.940*
GSH-GSSG	0.966**	0.983**	0.921*
NO-GSSG%	0.992**	0.905*	
3NT-GSSG%	0.824		0.948*
ENOS-NO	0.889*		
ENOS-3NT	0.802		

Empty cells – without significance. \* -  $P < 0.05$ ,

\*\* -  $P < 0.01$  statistical significance of the

correlation coefficient.

In all patients regardless of sex, the correlations are positive, both for GSH with CAT or GSSG, as for NO and 3NT with GSSG% and eNOS with NO and 3NT. If positive associations in men and women are similar for GSH, correlation with NO was observed for GSSG% in men and with 3NT in women.

## Discussion

In general, the importance of GSH in eye tissue protection is established [8]. Metabolism of GSH and its transport through cell membranes or between different tissues are essential for the maintenance of redox homeostasis in eye tissues. GSH efflux outside eye tissues through Na<sup>+</sup>-independent efflux system is established for conjunctive towards “tear film” [19, 20, 21], which is considered as an essential mechanism of H<sub>2</sub>O<sub>2</sub> neutralization in “tear film” [22]. Also, GSH efflux was shown in the liver to have much higher

concentrations of GSH than all other tissues [23] and can maintain redox homeostasis in the multitude of tissues by releasing GSH into the blood flow.

GSH cannot pass into direct cells and must be metabolized to its constituent amino acids, which pass through the membrane and can be substrates for intracellular GSH re-synthesis. Usually, a limiting factor for GSH synthesis is the availability of cysteine [24], which justifies the use of NAC as a cysteine precursor in the treatment of different pathologies, including ocular. L-cysteine or L-glutamate transport across cell membrane was shown in several eye cell types including human retinal pigment epithelium (RPE), and it was found, that it does increased L-cystine uptake (oxidized form of cysteine), raising intracellular GSH levels. GSH is assumed to be involved in intracellular repair of protein damaged by oxidative stress [13]. Also, nitrosative stress increased uptake of L-cystine and enzyme activity  $\alpha$ -glutamylcysteine synthetase (GCS) responsible for GSH synthesis in conjunctival epithelial cell layers [12].

Positive effects of treatment with GSH or its prodrugs were primarily seen in pathologic eye tissues with decreased GSH levels [8, 12, 25]. In this study, we have surprisingly observed the elevated levels of GSH in patients with recurrent pterygium compared with the control group or primary pterygium, while systemic pretreatment of pterygium with NAC further increased the level of GSH and topical treatment did not affect it compared with group C or P. Last, at least, means that cysteine does not enter directly into pterygium tissue.

An increase in the level of GSH in recurrent pterygium can be in three main ways: decrease of oxidative stress, stimulation of its synthesis, or by efflux (mobilization) of GSH from the nearby tissue with higher concentration, which could be cornea. The latter could recover this loss of GSH from blood flow proceeding from the liver. In this study, we have observed a decrease in eNOS, NO, and 3NT in pterygium tissue, while in the previous research, no significant change in lipoperoxidation (TBARS) was found in pathological tissues [6]. Then it is likely that nitrosative stress slope decreases the inactivation rate of GSH and CAT in recurrent pterygium resulting in their increase. Also a positive correlation of the CAT level with GSH in study groups may mean a reduction in the inactivation rate of this enzyme due to a rise in the level of GSH in pathological tissues.

A high positive correlation between NO and 3NT levels with eNOS activity in our study groups confirms that this enzyme regulates NO production in pterygium tissue, decreasing its level without affecting significantly GSH level in primary pterygium. But the degree of GSH oxidation (GSSG%) decreases from 35% to 25% in this group and remains decreased in other pathological groups, which can be related to the decrease in nitrosative stress. The behavior of NO and especially of 3NT shows a high positive correlation with this parameter of the metabolism of GSH by the groups of the study, confirming a close relationship between these parameters.

As mentioned above, the decrease in the level of NO in tissue stimulates GSH synthesis, and its increase decreases during endotoxemia in the animal model [11]. In our case, reduced levels of NO and 3NT could stimulate GSH synthesis in primary pterygium tissue, which we have not observed. This discrepancy in the

stability of GSH with a reduced level of NO in group P makes it possible to assume that the increase in GSH in recurrent pterygium could be due to the mobilization of GSH from nearby tissue, which is cornea.

Comparing the effects of two pretreatments with NAC does not contradict this assumption. The positive impact of the use of NAC were observed in diabetic retinopathy [26], in protection against alloxan-induced diabetes in mice by increasing the synthesis of GSH in platelets [27] and in patients with dry eye syndrome, where treatment improved axis-related symptoms [18]. In literature, there are data on GSH efflux from the conjunctiva to tear film [21, 22]. We do not have data on the possible efflux of GSH from cornea to extracellular space despite its millimolar versus micromolar concentration in the conjunctiva. But its efflux from the liver to blood flow is real, and gradient of its level concerning all other tissues is even higher. We also assume that in an organism, there is a precise balance between efflux of GSH of the liver and its concentration in different tissues, and pre-treatment with NAC could stimulate the synthesis of GSH in the liver with consequent redistribution to a multitude of various tissues, including oculars. Topical pre-treatment showed no effect on intracellular levels of GSH in pterygium, which may be by blocking the direct transport of cysteine into pathological tissue.

NO is a radical and has a short life but high capacity to penetrate cells. It is supposed that the reaction between NO and proteins (S-nitrosylation) allows lengthening its lifetime. Under the term S-nitrosylation, we determine the modification of protein thiols by NO [14]. GSH has a high affinity to NO, and S-nitrosoglutathione (GSNO) is the main intermediate in the production of other metabolic regulating nitrosothiols [14, 15]. In this case, nitrosothiols are NO stabilizers and their possible carriers (carrier molecule) instead of their action [15]. Possible NO release reaction of S-nitrosoglutathione is as follows: GSNO = GSSG + 2NO [28]. If it is true, the NO release product of this reaction is GSSG and there was observed the correlation between NO and grade of GSH oxidation.

Evaluating gender differences, we found that women showed a higher level of GSH and CAT in primary pterygium group, lower level of GSH and a higher level of NO in recurrent pterygium. Women showed a positive correlation in research groups between GSSG% and 3NT, while men with NO. As a NO release reaction of S-nitrosoglutathione has as a product GSSG, women likely show more significant role of S-nitrosation compared with men. The nitrosothiols have a different half-life in water: S-nitrosoglutathione (hours), S-nitrosocysteine and S-nitroso-N-acetylcysteine (minutes) and can affect the synthesis of intracellular GSH or its efflux of tissues with a high level of GSH as the liver for all tissues or cornea for eye tissues. In our study high positive correlation between NO and 3NT with percentage of oxidized GSH in total GSH (GSSG%) in pathological tissues indirectly shows the possible effect of S-nitrosation on GSH metabolism and/or its efflux (release) of tissues.

One of the possible sequences of events in pathogenesis in primary pterygium: decreased activity of eNOS, bioaccessibility of NO and S-nitrosation of GSH or other nitrosothiols, possible release of NO resulting in modulation of the intracellular level of GSH through synthesis and/or mobilization of different tissues. If we are right, treatment with prodrugs-NO substances should be investigated regarding pterygium.

In this study, only systemic pre-treatment with NAC significantly increased the level of GSH in pterygium, which we interpreted as a positive effect, but the number of patients is not sufficient to evaluate the frequency of recurrence of the disease.

## List Of Abbreviations

NO = nitric oxide

eNOS = endothelial nitric oxide synthase

3NT = 3-nitrotyrosine

GSH = total reduced glutathione

GSSG = oxidized GSH

GSSG% = grade of GSH oxidation

CAT = catalase

NAC = N-acetylcysteine

GSNO = S-nitrosoglutathione

## Declarations

### Ethics approval

All patients signed informed consent, and approval obtained from the Hospital “Nuestra Señora de la Luz” Ethics Committee (N° 3-2015 FHNSL-CE).

### Patent consent for publication

Not applicable

### Availability of data and materials

The database used and analyzed during study are available from corresponding author on reasonable request.

### Competing interests

We have not competing interests regarding the products or procedures used in the present study

## Funding

Not applicable

## Authors' contributions

All authors participated in the performance of the experiment and analysis of results. FP, AK, GG and MC designed the research. FP obtaining tissues samples during surgery and participated in its proceedings. AK main contributor in writing the manuscript and performed measurement procedures. GG and MC performed literature analysis. AF and MT analyzed and interpreted the western blot procedure. AJ and EL analyzed the patient's data regarding clinical history and statistical analysis. All authors read and approved the final manuscript.

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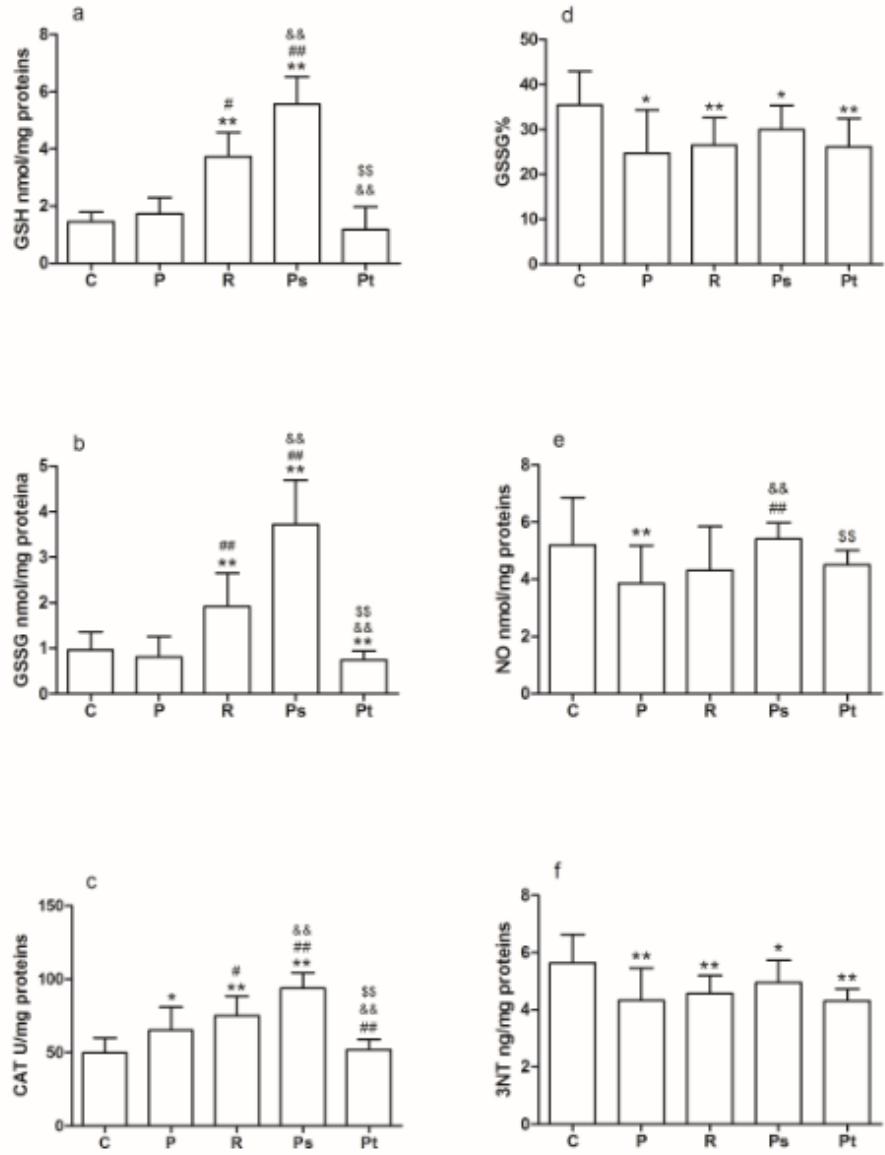
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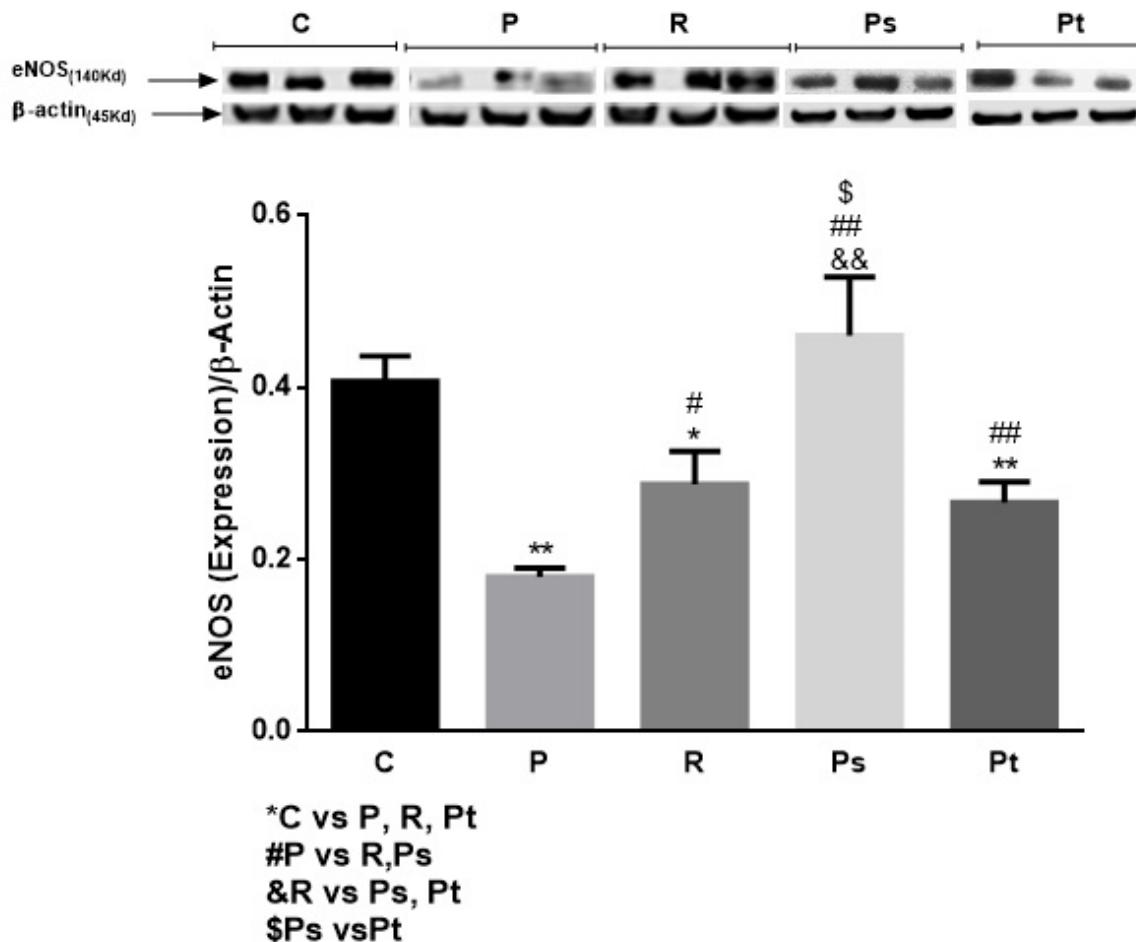
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## Figures



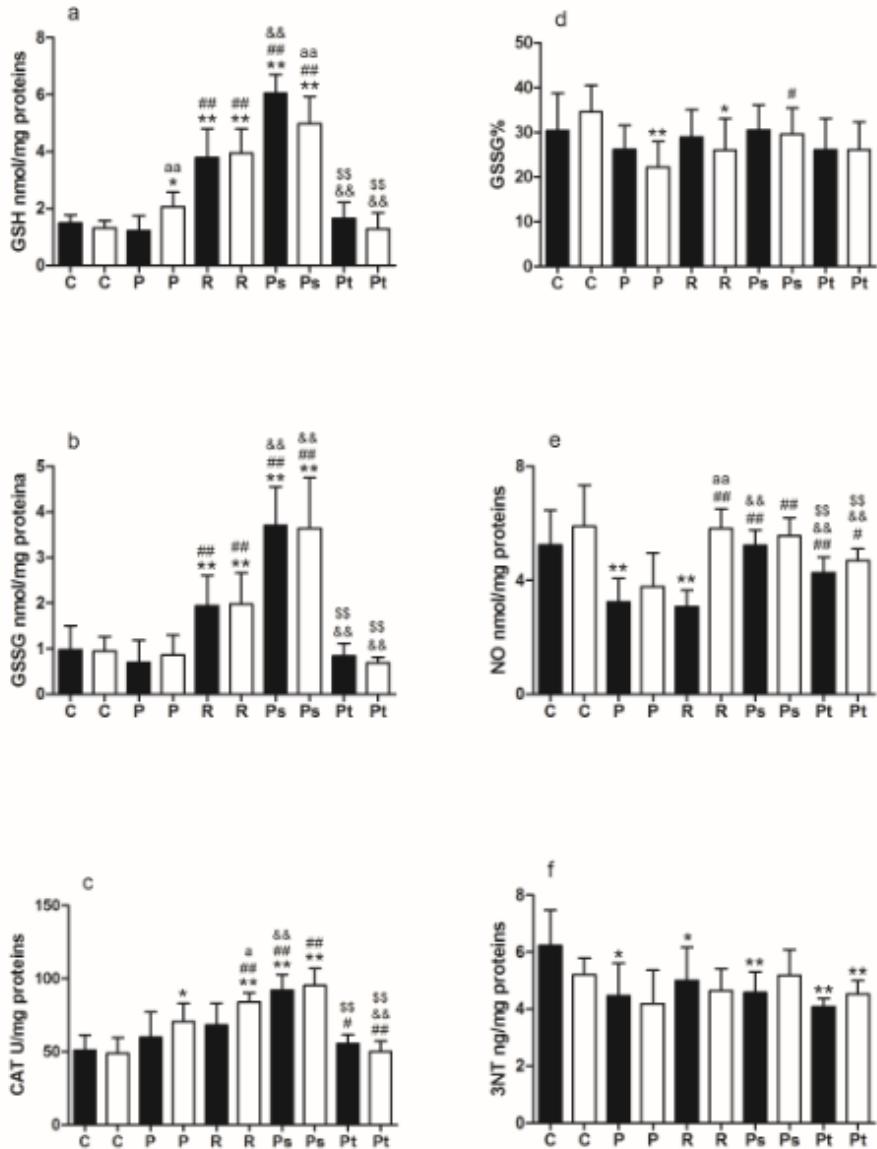
**Figure 1**

Response in all patients regardless of gender. Levels of reduced and oxidized GSH, CAT, degree of GSH oxidation, NO and 3-nitrotyrosine in control group (C), primary pterygium (P), recurrent pterygium (R), primary pterygium with systemic (Ps) or topical (Pt) pretreatment in all patients regardless of gender. \* -  $P<0.05$ , \*\* -  $P<0.01$  compared to group C. # -  $P<0.05$ , ## -  $p<0.01$  compared with primary pterygium group. & -  $P<0.05$ , && -  $p<0.01$  comparing with group R. \$\$- P<0.01 compared groups with treatments.



**Figure 2**

Response of eNOS in all patients regardless of gender. Levels of eNOS activity in control group (C), primary pterygium (P), recurrent pterygium (R), primary pterygium with systemic (Ps) or topical (Pt) pretreatment in all patients regardless of gender. \* -  $P<0.05$ , \*\* -  $P<0.01$  compared to group C. # -  $P<0.05$ , ## -  $P<0.01$  compared with primary pterygium group. & -  $P<0.05$ , && -  $p<0.01$  comparing with group R. \$ -  $P<0.05$  comparing groups with treatments.



**Figure 3**

Gender differences in response. Levels of total GSH reduced (a), oxidized (GSSG, b), CAT (c), degree of GSH oxidation (GSSG%, d), NO (e) and 3-nitrotyrosine (f) in control group (C), primary pterygium (P), recurrent pterygium (R), primary pterygium with systemic (Ps) or topical (Pt) treatment in men (black columns) and women (white columns). a -  $P<0.05$ , aa -  $P<0.01$  gender differences within groups. \*  $P<0.05$ , \*\* -  $P<0.01$  compared to group C. # -  $P<0.05$ , ## -  $p<0.01$  compared to primary pterygium group. & -  $P<0.05$ , && -  $p<0.01$  comparing with group R. \$ -  $P<0.05$ , \$ -  $P<0.01$  comparing groups with treatments.