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# Infliximab prevents dysfunction of the vas deferens by suppressing inflammation and oxidative stress in rats with chronic stress

Tuğçe Demirtaş Şahin[a,*], Semil Selcen Gocmez[a], Gökhan Duruksu[b], Yusufhan Yazir[b,c], Tijen Utkan[a]

[a] Department of Pharmacology, Medical Faculty, Kocaeli University, Kocaeli, Turkey
[b] Center for Stem Cell and Gene Therapies Research and Practice, Kocaeli University, Kocaeli, Turkey
[c] Department of Histology and Embryology, Medical Faculty, Kocaeli University, Kocaeli, Turkey

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ABSTRACT

*Aims:* Chronic stress leads to the development of male sexual problems such as ejaculatory dysfunctions. The rhythmic contractions of vas deferens (VD) play an important role on the ejaculatory process. In the current study, we investigated whether infliximab (IFX) treatment has any beneficial effects on possible alterations in contractility of VD obtained from rats exposed to unpredictable chronic mild stress (UCMS).

*Materials and methods:* The rats were randomly divided into four groups: control, control + IFX, UCMS and UCMS + IFX. IFX (5 mg/kg/week, i.p.) was administrated for 5 weeks during UCMS period. Depressive like-behaviors were evaluated using locomotor activity, forced swimming and sucrose consumption and preference tests. The blood was collected for serum biochemical determinations. VD tissues were harvested for functional studies and, measurements of oxidative stress, inflammatory and apoptotic biomarkers.

*Key findings:* We observed increased serum concentration of corticosterone and depressive-like behaviors in rats exposed to UCMS. In VD tissues of UCMS-exposed rats, noradrenaline- and adenosine triphosphate (ATP)-induced contractile responses significantly enhanced and electrical field stimulation (EFS)-induced contractile responses markedly decreased. UCMS exposure induced inflammation, oxidative stress and apoptosis in VD. However, IFX treatment significantly improved all the aforementioned parameters.

*Significance:* The results of the present study revealed that chronic stress-induced depression caused VD dysfunction by promoting inflammation and oxidative stress in VD. IFX protected against VD dysfunction through its anti-inflammatory and antioxidant effects.

## 1. Introduction

Ejaculation, a complex physiological process regulated by various neuronal, neurochemical and hormonal mechanisms, mainly occurs due to rhythmic contractions of the vas deferens (VD). These series of contractions that propel the spermatozoa from the epididymis toward the urethra, are initiated and commanded by the release of noradrenaline from the adrenergic nerve endings [1]. There is also evidence that the adrenergic neurons innervating the VD utilize adenosine triphosphate (ATP) as a co-transmitter with noradrenaline [1]. In this context, the impairment of the contractile activity of the VD might result in ejaculatory dysfunction and the mechanisms regulating the motor function of the VD may therefore be of interest as pharmacological targets for the treatment of ejaculatory disorders.

Chronic physiological stress elicits diverse alterations within the nervous, endocrine and immune systems, leading to impairment of emotional and sexual behaviors [2]. Unpredictable chronic mild stress (UCMS) has been shown to trigger despair- and anhedonic-like behaviors in animals that resembles some of the symptoms of human depression [3]. Also, chronic stress has been indicated to result in a variety of male reproductive and sexual problems such as low sperm quality, reduced sex drive and erectile/ejaculatory dysfunctions [4]. A recent animal study reported testicular dysfunction induced by chronic stress [5]. In our previous study, we have suggested the link between chronic stress-induced depression and erectile dysfunction in rats [6]. However, no evidence of functional changes in the VD tissue has been shown in a preclinical study, which focused on the association between chronic stress and ejaculatory dysfunction.

Inflammation and oxidative stress are important mechanisms contributing to the development of depressive-like behaviors induced by

* Corresponding author at: Kocaeli University, Medical Faculty, Department of Pharmacology, Umuttepe Campus, İzmit, Kocaeli 41380, Turkey.
*E-mail address:* tugce.demirtas@kocaeli.edu.tr (T.D. Şahin).

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chronic stress [7]. They have also been suggested to play a crucial role in the chronic stress-induced disruption of male sexual and reproductive functions such as erectile and testicular dysfunction [8,9]. Emerging evidence from the chronically stressed animals demonstrated increased production of proinflammatory cytokines in penile and testicular tissues [6,8]. On the other hand, inflammation and oxidative stress have been linked to impaired function of the VD in lipopolysaccharide (LPS)-treated rats [10] and diabetic rats [11]. Infliximab (IFX), an anti-tumor necrosis factor-α (TNF-α) monoclonal antibody, is used safely in many inflammatory diseases [12]. It has also been reported to reduce oxidative stress by enhancing antioxidant activities, and decreasing lipid peroxidation and oxidant status in rats [13]. We have previously revealed that IFX diminished depressive-like behaviors in rats exposed to chronic mild stress [14]. However, in the literature, there is no evidence showing the protective effects of IFX on VD function in any animal study. In this background, we hypothesized that IFX might protect against the possible impairment of VD contractility in rats exposed to UCMS. Thus, the aim of the present study was to determine whether IFX treatment may prevent UCMS-induced impairment of VD contractility by its anti-inflammatory and/or antioxidant effects.

## 2. Materials and methods

### 2.1. Animals

All study and animal care procedures were approved by the Animal Research Ethics Committee of Kocaeli University, Turkey (Approval Number: KOU HADYEK 2/9–2017) and followed the Guide for the Care and Use of Laboratory Animals (National Institutes of Health (NIH) Publications No. 8023, revised 1978). Thirty two male Wistar albino rats (4 months old, weighing 250–300 g) were obtained from Experimental Medical Research and Application Center, Kocaeli University, Kocaeli, Turkey. The rats were kept at 22 ± 2 °C temperature, 45% humidity with 12-h light/12-h dark cycle under environmentally controlled conditions in cages of up to 5–6 rats. Tap water and standard chow were available ad libitum.

### 2.2. Drugs and chemicals

Noradrenaline, ATP, prazosin and suramin were obtained from Sigma-Aldrich, (St. Louis, MO, USA). IFX (Remicade®) was purchased from Merck Sharp Dohme (Brinny, Ireland). All drugs were prepared immediately before use and all other chemicals used in the current study were of analytical grade and commercially available.

### 2.3. Experimental design

After one week of acclimatization to laboratory conditions, the rats were randomly allocated to one of the following four groups, with eight rats in each group: control, IFX treatment with no exposure to UCMS (control + IFX), exposure to UCMS (UCMS) and exposure to UCMS with IFX treatment (UCMS + IFX). IFX (5 mg/kg/week, i.p) was dissolved in physiological saline and administered in a volume of 2 ml/kg body weight of the rats during 5-week-UCMS period. IFX dose was chosen based on its therapeutic relevance [15] and use in previous studies [14,16]. Body weight was measured at the beginning of the experiments and then weekly until the end of UCMS protocol. After 5 weeks of UCMS period, behavioral tests (sucrose consumption and preference test, forced swimming test and locomotor activity test) were performed. At the end of the behavioral experiments, rats were anesthetized by intraperitoneal ketamine (90 mg/kg) + xylazine (10 mg/kg) and blood samples were collected for the measurement of serum corticosterone concentration. Rats were sacrificed and VD tissues were removed and cleaned from adhering tissues for the functional studies and, determination of inflammatory and oxidative stress biomarkers.

### 2.4. Unpredictable chronic mild stress protocol

UCMS-induced depression rat model was determined compatible with the protocols described in our previous reports by subjecting animals to various mild stressors randomly during 5 weeks [6,17]. The stressors used were: tail clamping (1 min), hot water swimming (45 °C, 5 min), restraint (4 h), cold water swimming (4 °C, 5 min), wet bedding (24 h), level shaking (10 min), cage tilting (24 h), pairing with another stressed rat (48 h) and reversing light/dark cycle (24 h). One of the nine stressors was applied starting at 9:00 AM each day. In order to be stressors unpredictable for the rats, the same stressor was not applied for two consecutive days. Water and food were freely available in this protocol.

### 2.5. Determination of serum corticosterone

Corticosterone levels in the serum was analyzed with a commercial enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Sciences, Farmingdale, NY, USA) according to the manufacturer's instructions. The results were expressed as pg/ml.

### 2.6. Behavioral analysis

#### 2.6.1. Sucrose consumption and preference test

Sucrose consumption and preference test procedure were accomplished in line with Sarkisova et al [18]. All rats were allowed to consume 20% sucrose solution in an individual cage for 15 min every day over a period of 5 days and their sucrose consumption (g) was recorded. Following 5 days of training period, the rats were deprived of their water and food for 23 h. Then each rat was placed individually again and given both one bottle of 20% sucrose and one bottle of water for a period of 1 h. Water and sucrose consumption were measured by reweighing preweighed bottles of sucrose and water. The preference for sucrose was determined as: Sucrose preference (%) = sucrose consumption [g]/total fluid consumption [g] ×100.

#### 2.6.2. Forced swimming test

The forced swimming test was based on the method of Porsolt et al [19]. Briefly, the rats were placed in a glass cylinder (38 cm height and 47 cm diameter) filled with water at 22 ± 1 °C and forced to swim individually. Two consecutive sessions were performed: a pretest (15 min) and the test (5 min). The test session was conducted 24 h after the pretest session. The sessions were recorded and the duration of immobility behavior observed in the test session were calculated.

#### 2.6.3. Locomotor activity test

The rats were placed individually in a plexiglas chamber, which recorded their stereotypic, vertical and ambulatory activities by infrared vertical (mounted every 4.5 cm, upper) and horizontal (mounted every 2.5 cm, bottom) detectors attached to a data transfer software (Commat Ltd., Ankara, Turkey). Each rat was placed in the center of the chamber and observed for 5 min. After each trial, the chamber was cleaned with ethanol. The aforementioned activities of the rats were evaluated as total locomotor activity.

### 2.7. Functional studies in vas deferens strips

#### 2.7.1. Isolated organ bath preparation

VD was carefully isolated in a fresh Krebs solution of the following composition (mM): NaCl, 118.4; KCl, 4.7; $MgSO_4$, 1.2; $KH_2PO_4$, 1.2; $NaHCO_3$, 25; $CaCl_2$, 1.9 and glucose, 11. Prostatic portions of VD was dissected (length 1 cm) and each strip was suspended under 1 g tension in an organ bath containing Krebs solution at 37 °C (pH 7.4) and bubbled with a mixture of 95% $O_2$ and 5% $CO_2$. Changes of isometric tension were recorded using a force-displacement transducer (FDT 05, Commat Ltd) connected to a data acquisition system (MP30, Commat

Ltd.). Each strip was washed with Krebs solution at 15-min intervals and allowed to equilibrate for 1 h prior to initiating the experiments.

*2.7.2. Concentration–response curves for agonists*

After a stabilization period of 1 h, tissues were contracted with a high potassium chloride (KCl) solution (80 mM). The adrenergic or purinergic contractile responses were evaluated by cumulative concentration-response curves to noradrenaline ($10^{-8}$–$10^{-4}$ M) or ATP ($10^{-8}$–$10^{-4}$ M), respectively. The contractile responses induced by noradrenaline and ATP were expressed as percentage of 80 mM KCl-induced contraction responses for each VD strip.

*2.7.3. Neurogenic contractions*

To obtain nerve-evoked contractions, VD tissues were placed between two platinum wire electrodes connected to a stimulator (ST 95 PT, Commat Ltd.) inducing an electrical field stimulation (EFS). EFS was conducted at 50 V, 0.5 ms pulse width and trains of stimuli lasting 10 s at varying frequencies (2–32 Hz). Also, in a series of experiments, nerve-evoked contractions were obtained in the presence of prazosin (0.3 μM) or suramin (100 μM) in order to check the participation of purinergic or adrenergic neurotransmission, respectively. The responses induced by EFS were expressed as the percentage (%) of the contractile response to 80 mM KCl for each VD strip.

*2.7.4. Pharmacological parameters*

$E_{max}$ (the maximum contraction response induced by a full agonist) and $pD_2$ (potency, the negative logarithm of $EC_{50}$) values were calculated to assess the effects of noradrenalin and ATP on different experimental groups.

*2.8. Determination of inflammatory biomarkers*

The gene expressions of nuclear factor kappa B (NF-κB), TNF-α, interleukin-1 beta (IL-1β) and interleukin 6 (IL-6) in the VD tissues were assessed by real-time polymerase chain reaction (RT-PCR). Total RNA was isolated using GeneJet RNA Purification Kit (Thermo Scientific, Vilnius, Lithuania). Genomic DNA was removed by DNaseI (Thermo Scientific). The quality of RNA samples was determined by absorbance at 260 and 280 nm, and cDNA was synthesized using Thermo Scientific cDNA synthesis kit (Thermo Scientific, Rockford, IL, USA), according to the manufacturer's instructions. The cDNA products were subjected to PCR amplification with gene-specific primers. 50 ng of cDNA was amplified in each well using PowerUp SYBR Green Master Mix by LC480-II (Roche, Basel, Switzerland). Actin-β (ActB) was used as the internal control for normalization. The gene-specific primers used for inflammatory markers as well as the internal control ActB gene were as follow:

NF-κB: Forward primer; 5′-AGAGAAGCACAGATACCACTAAG-3′; Reverse Primer; 5′-CAGCCTCATAGAAGCCATCC-3′.
TNF-α: Forward primer; 5′-TGAACTTCGGGGTGATCG-3′; Reverse Primer; 5′-GGGCTTGTCACTCGAGTTTT-3′.
IL-1β: Forward primer; 5′-TGTGATGAAAGACGGCACAC-3′; Reverse Primer; 5′-CTTCTTCTTTGGGTATTGTTTGG-3′.
IL-6: Forward primer; 5′-CCTGGAGTATGTGAAGAACAACT-3′; Reverse Primer; 5′-GGAAGTTGGGGTAGGAAGGA-3′.
ActB: Forward primer; 5′-AGAGAAGCTGTGCTATGTTG-3′; Reverse Primer; 5′-GTACTCCTGCTTGCTGATCC-3′.

*2.9. Determination of oxidative stress biomarkers*

*2.9.1. GSH*

The reduced glutathione (GSH) levels were measured using QuantiChrom GSH Assay kit (DIGT-250, BioAssay Systems) based on the DTNB (5,5′-dithiobis-[2-nitrobenzoic acid]) method. After tissue homogenization in ice cold PBS, the samples were deproteinized and mixed with DTNB. After the incubation for 25 min at room temperature, the optical density at 412 nm was determined. Results were expressed as mmol/l.

*2.9.2. Superoxide dismutase (SOD)*

SOD activity was assessed by EnzyChrom SOD Assay Kit (ESOD-100, BioAssay Systems), according to the manufacturer's protocol. Briefly, 20 μl of each sample was mixed with 160 μl working solution containing xanthine and WST-1. Absorbance was measured immediately at 440 nm and the reagent was incubated at 25 °C for 60 min. Following the incubation, the absorbance was measured at 440 nm. The activity of SOD was estimated by subtracting the two consequent absorbance reading (before and after the incubation) and comparing this value with the SOD concentration standard curve. The activity was represented as U/ml.

*2.9.3. Catalase*

Catalase activity in tissues was determined by EnzyChrom Catalase Assay Kit (ECAT-100, BioAssay Systems) according to the manufacturer's instructions. Tissues were homogenized in 200 μl cold PBS buffer and centrifuged for 10 min at 14,000 rpm to remove any debris. Clear supernatant (10 μl) was used for the direct measurement of catalase degradation of $H_2O_2$. The change in color intensity was estimated at 570 nm. One unit was defined as the amount of catalase decomposing 1 μmol of $H_2O_2$ per min at pH 7 and room temperature. The activity was presented in U/l.

*2.9.4. Lipid peroxidation*

Malondialdehyde (MDA) content, an index of lipid peroxidation in tissues, was determined using QuantiChrome thiobarbituric acid reactive substances (TBARS) Assay Kit (DTBA-100, BioAssay Systems, Hayward, CA, USA) according to the manufacturer's protocol. Tissues were homogenized in cold PBS buffer and centrifuged for 10 min at 14,000 rpm. Ice cold 10% trichloroacetic acid was added into the supernatant to deproteinize. After the neutralization, the clear sample was mixed with thiobarbituric acid (TBA) solution and incubated at 100 °C for 60 min. The optical absorbance was determined at 535 nm using the VIS-Spectrophotometers (Molecular Devices). Results were expressed as nmol/ml.

*2.9.5. Protein concentration*

After the homogenization of tissues for enzyme activities or metabolites, all values were normalized with respect to the protein level by bicinchoninic acid (BCA) assay. Total protein concentration was analyzed by SMART BCA Protein Assay Kit (Intron Biotechnology, Gyeonggi-do, Korea) according to manufacturer's protocol.

*2.10. Enzyme-linked immunosorbent assay for NF-κB phosphorylated (p-) p65 subunit and active caspase 3*

The protein level of NF-κB p-p65 subunit was quantified from the homogenized VD tissue using NF-κB/p-p65 ELISA kit (Bioassay Technology Laboratory, Shanghai Korain Biotech, Shanghai, China) in accordance to manufacturer's instructions. The sensitivity of the NF-κB/p-p65 ELISA kit was 26 μg/ml. The active caspase 3 level in VD tissues was estimated using rat cleaved-caspase 3 ELISA Kit (Bioassay Technology Laboratory). The sensitivity of the cleaved-caspase 3 ELISA kit was 13.17 pg/ml. Shortly, each tissue sample was rinsed in PBS (pH 7.4) before homogenization in 1:20 (*w/v*) of tissue to T-PER Reagent (Thermo). The sample was centrifuged at 3000 rpm for 20 min at 4 °C. 40 μl of sample was mixed with 50 μl of streptavidin-HRP and 10 μl of either anti-NF-κB p-p65 antibody or anti-Casp-3 antibody supplied within the kit. After the incubation for 60 min at 37 °C, wells were washed 5 times and HRP substrate solution was added. After the incubation for 10 min at 37 °C, the reaction was stopped and the optical density was determined at 450 nm. The concentrations of both NF-κB/

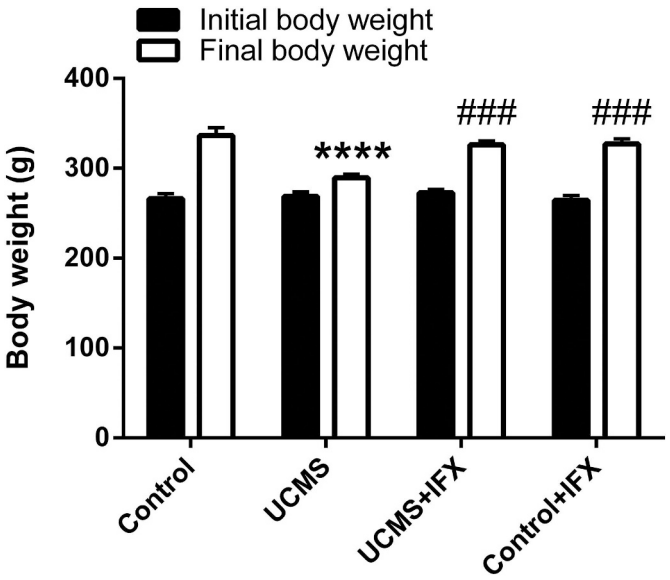



**Fig. 1.** Effect of UCMS and infliximab on body weight of rats. Values represent the mean ± SEM of 8 rats/group. $^{****}p < .0001$ vs. control group; $^{###}p < .001$ vs. UCMS group.

p-p65 and cleaved-caspase 3 in each sample were normalized to 1 μg/ml of total protein concentration in the sample after the homogenization.

### 2.11. Statistical analysis

Data was expressed as mean ± standard error of the mean (SEM). The GraphPad Prism software (San Diego, CA, USA) was used for statistical analysis. The statistical significance was analyzed by one-way analysis of variance (ANOVA) post hoc Bonferroni's test. $p < 0.05$ was considered to be statistically significant. Correlations between parameters were determined by Pearson's correlation using the combined results from all tested rats.

## 3. Results

### 3.1. Effect of infliximab on body weight

No significant difference in initial body weight of rats was observed between the experimental groups ($p > 0.05$; Fig. 1). At the end of UCMS period, chronic stress caused a significant weight reduction compared to control group ($p < 0.0001$; Fig. 1), while treatment with IFX markedly increased the body weight of rats exposed to UCMS ($p < 0.001$; Fig. 1). IFX treatment showed no effects on body weight of control rats ($p > 0.05$, Fig. 1).

### 3.2. Effect of infliximab on corticosterone levels

As illustrated in Fig. 2, chronic stress induced a significant increase in serum corticosterone levels compared to control group ($p < 0.0001$). This stress-induced elevation in the corticosterone concentrations significantly decreased in rats treated with IFX ($p < 0.01$). However, IFX treatment exhibited no significant effect on serum corticosterone levels of control rats ($p > 0.05$; Fig. 2).

### 3.3. Effect of infliximab on locomotor activity

As presented in Fig. 3, no significant difference between all experimental groups was found in terms of locomotor activity ($p > 0.05$).

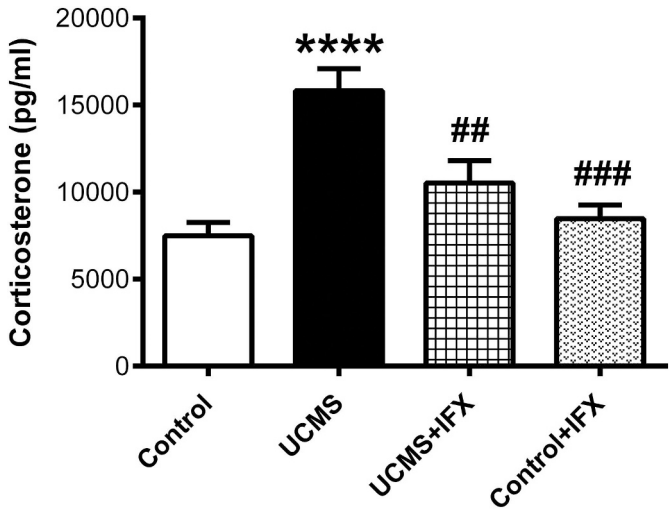



**Fig. 2.** Effect of UCMS and infliximab on serum corticosterone levels of rats. Values represent the mean ± SEM of 8 rats/group. $^{****}p < .0001$ vs. control group; $^{##}p < .01$ and $^{###}p < .001$ vs. UCMS group.

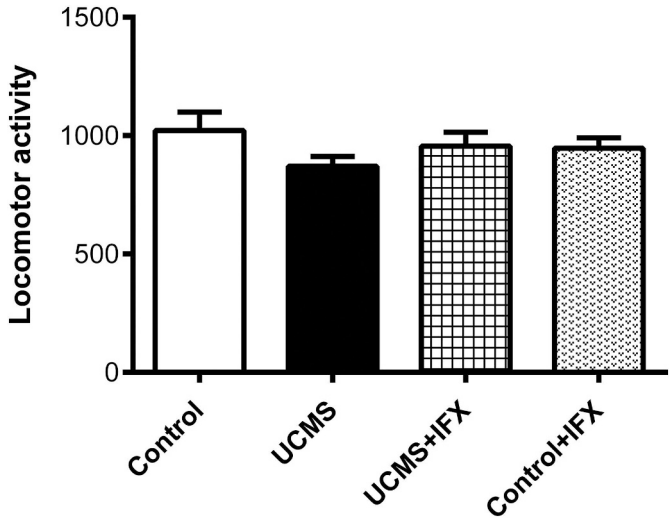



**Fig. 3.** Effect of UCMS and infliximab on locomotor activity of rats. Values represent the mean ± SEM of 8 rats/group.

### 3.4. Effect of infliximab on depressive-like behaviors

UCMS exposure significantly decreased the sucrose consumption compared to control rats ($p < 0.001$; Fig. 4A). IFX treatment of stressed rats induced a marked increase in sucrose consumption ($p < 0.05$; Fig. 4A). However, IFX showed no effects on the sucrose consumption in control rats ($p > 0.05$; Fig. 4A). Chronic stress significantly reduced the sucrose preference of rats compared with control rats ($p < 0.001$; Fig. 4B). IFX treatment of stressed rats resulted in a significant increase in the sucrose preference ($p < 0.01$; Fig. 4B), while treatment with IFX did not affect the sucrose preference in control rats ($p > 0.05$; Fig. 4B).

Chronically stressed rats displayed a significant increase in duration of immobility compared to control rats in the forced swimming test ($p < 0.0001$; Fig. 5). IFX treatment of stressed rats significantly alleviated the increase in duration of immobility ($p < 0.01$; Fig. 5). In control rats, chronic treatment with IFX did not change the duration of immobility in the test ($p > 0.05$; Fig. 5).

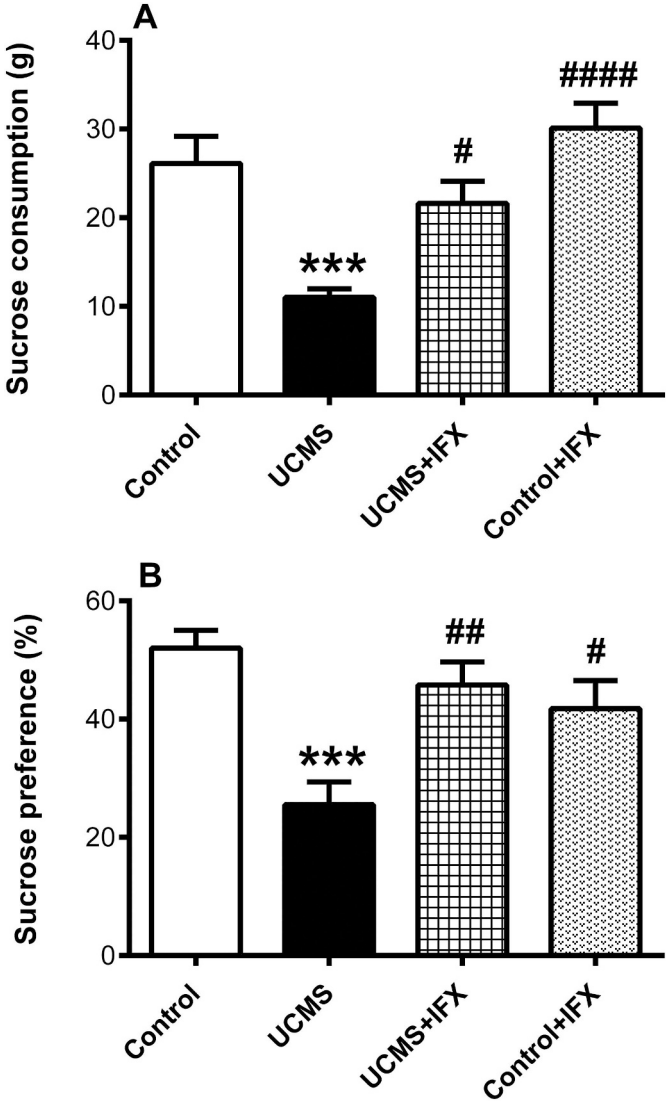



**Fig. 4.** Effect of UCMS and infliximab on sucrose consumption (**A**) and sucrose preference (**B**) of rats. Values represent the mean ± SEM of 8 rats/group. $^{***}p < .001$ vs. control group; $^{\#}p < .05$, $^{\#\#}p < .01$ and $^{\#\#\#\#}p < .0001$ vs. UCMS group.

### 3.5. Effect of infliximab on vas deferens contractility

Noradrenaline ($10^{-8}$ to $10^{-4}$ M) resulted in concentration-dependent contraction responses in the VD strips (Fig. 6A). The contractile responses of VD induced by noradrenaline were significantly higher ($p < 0.05$) in stressed rats ($E_{max}$ of 35.93 ± 1.68%) compared to control rats ($E_{max}$ = 23.08 ± 1.12%). Treatment with IFX significantly decreased the contractions in VD strips of stressed rats ($E_{max}$ = 28.60 ± 2.06%) ($p < .05$). IFX treatment did not alter the noradrenaline-induced contractions in control rats ($E_{max}$ = 22.98 ± 0.99%) ($p > .05$). The potency ($pD_2$) values for noradrenalin were not affected by UCMS or IFX treatment ($pD_2$ for control group = 5.06 ± 0.09, $pD_2$ for UCMS group = 5.10 ± 0.05, $pD_2$ for UCMS + IFX group = 5.05 ± 0.12 and $pD_2$ for control + IFX group = 5.11 ± 0.08).

The contractile responses of the VD tissues induced by ATP ($10^{-8}$ to $10^{-4}$ M) in all experimental groups were presented in Fig. 6B. Chronic stress caused a significant increase in ATP-induced contractions ($E_{max}$ = 20.45 ± 0.86%) compared to control group ($E_{max}$ = 14.54 ± 0.83%) ($p < 0.05$). The increased contractile responses were normalized by treatment with IFX in stressed rats

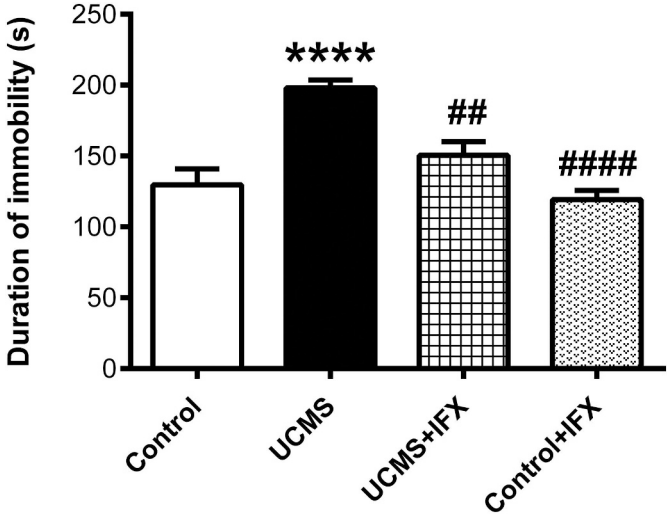



**Fig. 5.** Effect of UCMS and infliximab on duration of immobility of rats in the forced swimming test. Values represent the mean ± SEM of 8 rats/group. $^{****}p < .0001$ vs. control group; $^{\#\#}p < .01$ and $^{\#\#\#\#}p < .0001$ vs. UCMS group.

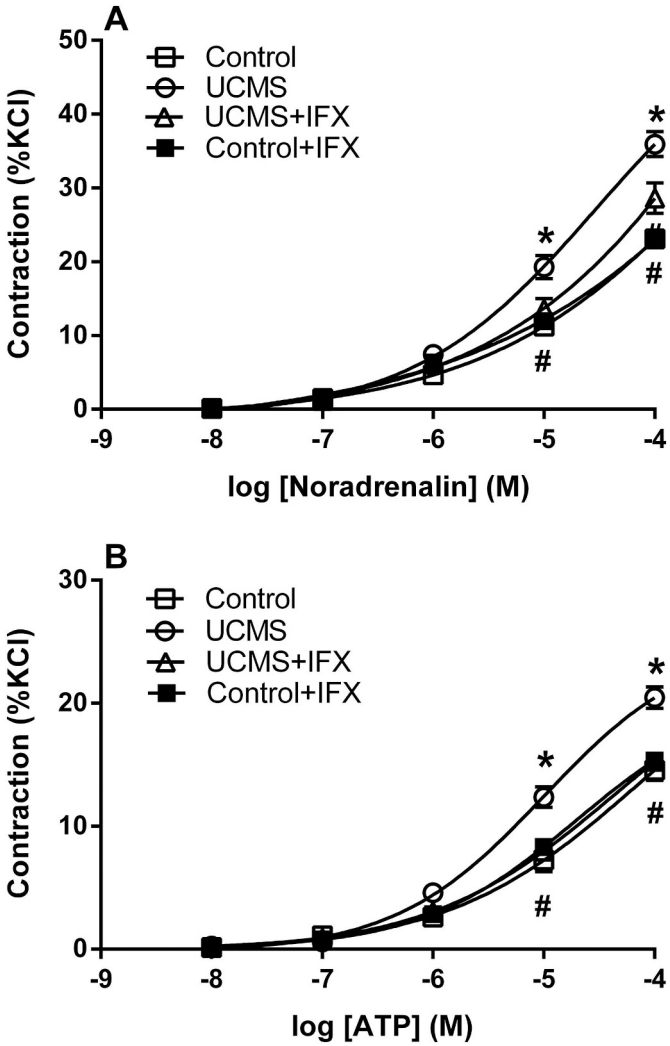



**Fig. 6.** Effect of UCMS and infliximab on rat vas deferens contractions induced by noradrenaline (**A**) and ATP (**B**). Values represent the mean ± SEM of 6 rats/group. $^{*}p < .05$ vs. control group; $^{\#}p < .05$ vs. UCMS group.

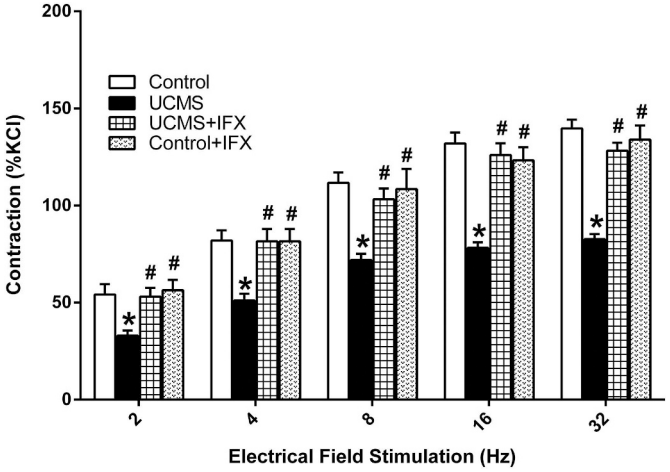



**Fig. 7.** Effect of UCMS and infliximab on rat vas deferens contractions induced by EFS. Values represent the mean ± SEM of 6 rats/group. *$p < .05$ vs. control group; #$p < .05$ vs. UCMS group.

($E_{max}$ = 15.26 ± 0.57%) ($p < 0.05$). IFX did not affect the contractions in response to ATP in control tissues ($E_{max}$ = 15.34 ± 0.55%) ($p > 0.05$). UCMS or IFX treatment did not change the potency values for ATP ($pD_2$ for control group = 5.04 ± 0.12, $pD_2$ for UCMS group = 5.32 ± 0.07, $pD_2$ for UCMS + IFX group = 5.09 ± 0.07 and $pD_2$ for control + IFX group = 5.10 ± 0.09).

EFS (2–32 Hz) evoked frequency-dependent contraction responses in VD strips (Fig. 7). Chronic stress significantly decreased EFS-induced contractile responses in VD strips when compared to those of controls ($p < 0.05$). Treatment with IFX significantly restored the decreased contractile responses in stressed rats ($p < 0.05$). The contractions induced by EFS in control rats did not alter by IFX treatment ($p > 0.05$). Chronic stress also caused a marked reduction in nerve-mediated contractile responses of VD in the presence of suramin or prazosin ($p < 0.05$; Table 1). Treatment with IFX significantly restored the impairment of EFS-induced contractile responses in the VD of stressed rats ($p < 0.05$; Table 1), while it did not change the contractions induced by EFS in control rats in the presence of suramin or prazosin ($p > 0.05$; Table 1). Also, the magnitude of nerve-mediated contractile responses was significantly lower in the presence of suramin or prazosin compared with those obtained in the absence of these agents. These nerve-mediated contractile responses were dramatically decreased in the presence of suramin + prazosin (not shown).

There was no significant difference between the groups for the

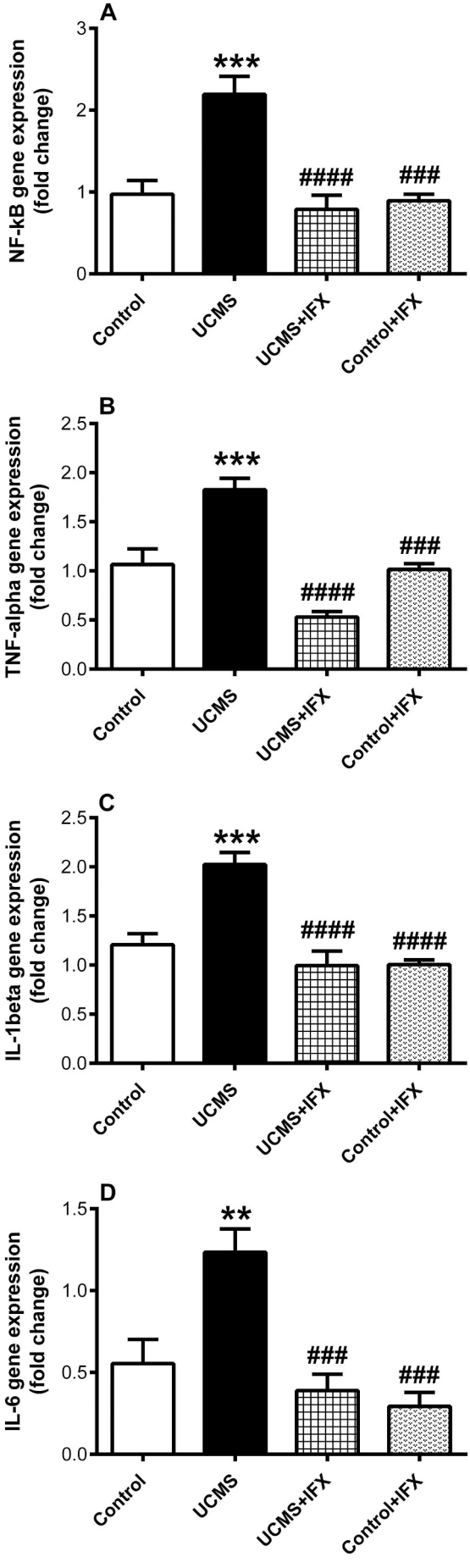
**Table 1**
Effect of UCMS and infliximab on rat vas deferens contractions induced by EFS in the presence of suramin or prazosin.

| | Control | UCMS | UCMS + IFX | Control + IFX |
|---|---|---|---|---|
| In the presence of suramin | | | | |
| 2 Hz | 13.77 ± 1.44 | 7.82 ± 1.15* | 14.26 ± 1.05# | 14.87 ± 1.58# |
| 4 Hz | 21.52 ± 1.04 | 14.61 ± 1.14* | 21.88 ± 1.47# | 20.73 ± 1.82# |
| 8 Hz | 29.87 ± 1.65 | 18.82 ± 0.99* | 27.05 ± 2.36# | 31.21 ± 2.49# |
| 16 Hz | 33.05 ± 1.85 | 21.68 ± 0.96* | 31.66 ± 2.62# | 34.54 ± 1.66# |
| 32 Hz | 34.64 ± 1.87 | 22.33 ± 1.49* | 31.48 ± 2.29# | 35.67 ± 1.85# |
| In the presence of prazosin | | | | |
| 2 Hz | 30.80 ± 5.12 | 15.80 ± 1.36* | 28.20 ± 2.08# | 24.80 ± 2.03# |
| 4 Hz | 46.20 ± 5.13 | 24.94 ± 1.78* | 38.71 ± 2.22# | 40.93 ± 3.35# |
| 8 Hz | 58.73 ± 4.44 | 37.05 ± 2.53* | 51.35 ± 2.88# | 53.69 ± 2.37# |
| 16 Hz | 66.05 ± 2.52 | 42.11 ± 2.65* | 63.04 ± 2.54# | 67.67 ± 4.28# |
| 32 Hz | 75.24 ± 2.31 | 45.71 ± 3.18* | 65.39 ± 3.44# | 71.82 ± 4.19# |

Values are expressed as percentage of 80 mmol/L KCl-induced contraction and represent the mean ± SEM. $n$ = 5–6 for all groups.

* $p < .05$ vs. control group.

# $p < .05$ vs. UCMS group.




*(caption on next page)*

**Fig. 8.** Effect of UCMS and infliximab on NF-κB **(A),** TNF-α **(B),** IL-1β **(C)** and IL-6 **(D)** gene expressions in rat vas deferens. Values represent the mean ± SEM of 8 rats/group. $^{**}p < .01$ and $^{***}p < .001$ vs. control group; $^{\#\#\#}p < .001$ and $^{\#\#\#\#}p < .0001$ vs. UCMS group.

contractile response induced by KCl (not shown) ($p > 0.05$).

### 3.6. Effect of infliximab on inflammatory biomarkers

As indicated in Fig. 8A, UCMS resulted in a marked increase in the gene expression of NF-κB in tissues ($p < 0.001$). IFX treatment significantly reversed this increase in stressed rats ($p < 0.0001$). However, treatment with IFX in control rats did not affect the gene expression of NF-κB in the VD tissues ($p > 0.05$).

The gene expression of TNF-α in VD tissues from experimental groups were presented in Fig. 8B. UCMS caused a significant increase in the gene expression of TNF-α compared to control group ($p < 0.001$), while IFX treatment markedly alleviated this elevation in stressed rats ($p < 0.0001$). The gene expression of TNF-α did not significantly change by IFX treatment in control rats ($p > 0.05$).

The gene expressions of IL-1β and IL-6 in VD tissues were significantly elevated in stressed rats compared with the control rats ($p < 0.001$, $p < 0.01$; Fig. 8C,D, respectively). However, in IFX treated stressed rats exhibited a significant reduction in these expressions ($p < 0.0001$, $p < 0.001$; Fig. 8C,D, respectively). The gene expressions of IL-1β and IL-6 did not significantly alter by IFX treatment in control rats ($p > 0.05$; Fig. 8C,D).

As presented in Fig. 9, chronic stress induced a significant increase in NF-κB p-p65 subunit level in VD tissues compared to control group ($p < 0.0001$), while IFX treatment significantly alleviated this elevation in chronically stressed rats ($p < 0.0001$). However, IFX treatment caused no significant effect on these protein levels of control rats ($p > 0.05$; Fig. 9).

### 3.7. Effect of infliximab on oxidative stress biomarkers

Effects of IFX treatment on reduced glutathione was depicted in Fig. 10A. GSH levels significantly diminished in the VD of stressed rats compared to control rats ($p < 0.001$). Treatment with IFX markedly inhibited the decrease of GSH levels in the VD of stressed rats ($p < 0.001$). However, IFX displayed no effects on GSH levels in the VD of control rats ($p > 0.05$).

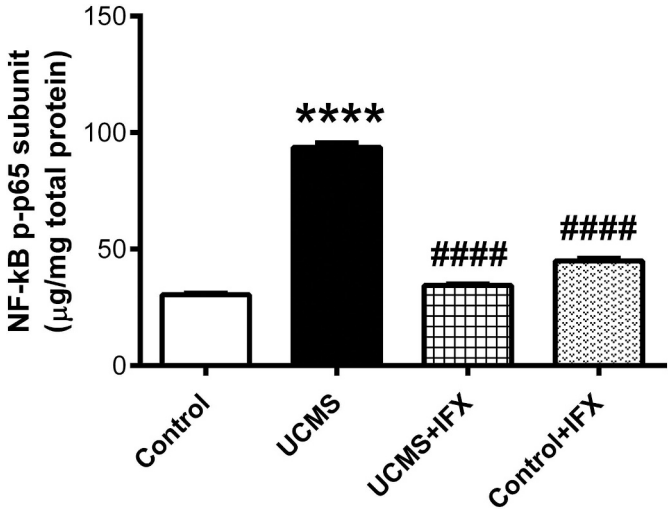
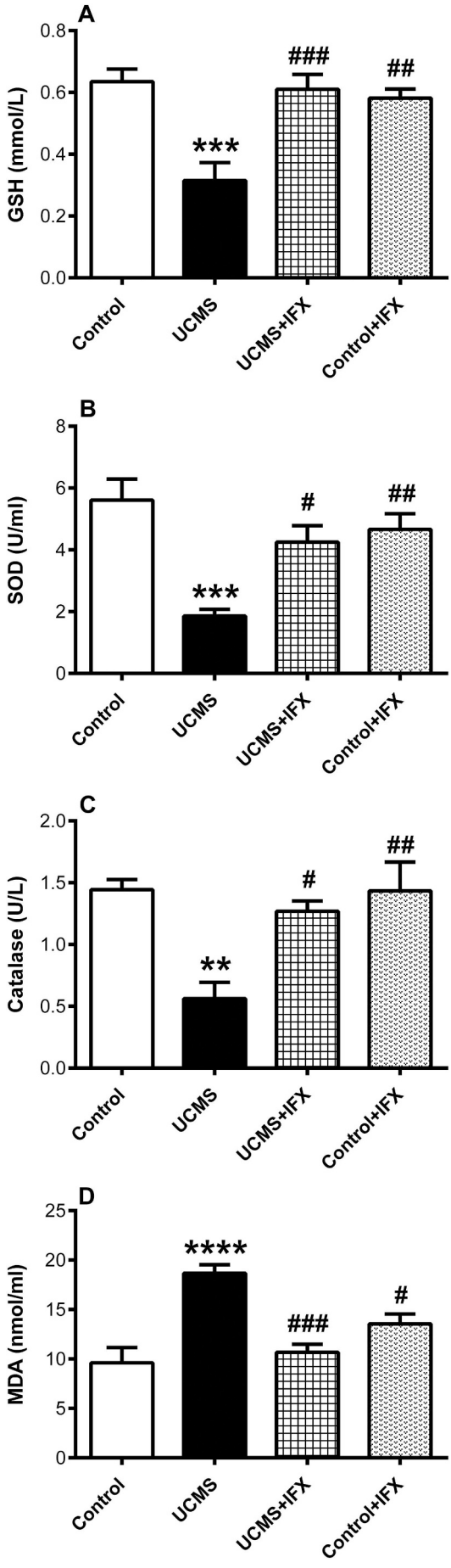



**Fig. 9.** Effect of UCMS and infliximab on NF-κB p-p65 subunit level in rat vas deferens. Values represent the mean ± SEM of 8 rats/group. $^{****}p < .0001$ vs. control group and $^{\#\#\#\#}p < .0001$ vs. UCMS group.




(caption on next page)

**Fig. 10.** Effect of UCMS and infliximab on GSH level (**A**), SOD activity (**B**), catalase activity (**C**) and MDA level (**D**) in rat vas deferens. Values represent the mean ± SEM of 8 rats/group. $^{**}p < .01$, $^{***}p < .001$ and $^{****}p < .0001$ vs. control group; $^{\#}p < .05$, $^{\#\#}p < .01$ and $^{\#\#\#}p < .001$ vs. UCMS group.

The alterations in SOD activity was presented in Fig. 10B. A marked reduction was observed in SOD activity in the VD of stressed rats in comparison to control rats ($p < 0.001$). This decrease was significantly improved by IFX treatment in the VD of stressed rats ($p < 0.05$). Treatment with IFX in control rats did not alter SOD activity ($p > 0.05$).

As shown in Fig. 10C, catalase activity significantly decreased in the VD of stressed rats compared to control rats ($p < 0.01$). Administration of IFX to stressed rats resulted in an increase in catalase activity ($p < 0.05$). IFX did not affect catalase activity in control rats ($p > 0.05$).

As indicated in Fig. 10D, MDA levels markedly elevated in the VD of stressed rats compared to control rats ($p < 0.0001$), whereas IFX treatment of stressed rats significantly reversed the increase in these levels ($p < 0.001$). Treatment with IFX did not alter MDA levels in the VD of control rats ($p > 0.05$).

### 3.8. Effect of infliximab on apoptosis

As indicated in Fig. 11, chronic stress resulted in a significant increase in active caspase 3 level in VD tissues ($p < 0.0001$). IFX treatment markedly reversed this increase in chronically stressed rats ($p < 0.0001$). Treatment with IFX did not change the active caspase 3 level in VD tissues of control rats ($p > 0.05$).

### 3.9. Correlation

Using the combined results from all tested rats, inflammatory biomarkers (NF-κB and TNF-α) were positively correlated with noradrenaline- and ATP-induced maximal contractions and, negatively correlated with EFS-induced maximal contractions in the VD tissue of rats ($p < 0.0001$; Table 2, Supplementary Fig. 1). SOD activity in the VD was found to be negatively correlated with noradrenaline- and ATP-induced maximal contractions and, positively correlated with EFS-induced maximal contractions in the VD tissue of rats ($p < 0.0001$; Table 2, Supplementary Fig. 2). However, MDA level was observed to be positively correlated with noradrenaline- and ATP-induced maximal contractions and, negatively correlated with EFS-induced maximal contractions in the VD tissue of rats ($p < 0.0001$; Table 2, Supplementary Fig. 2).

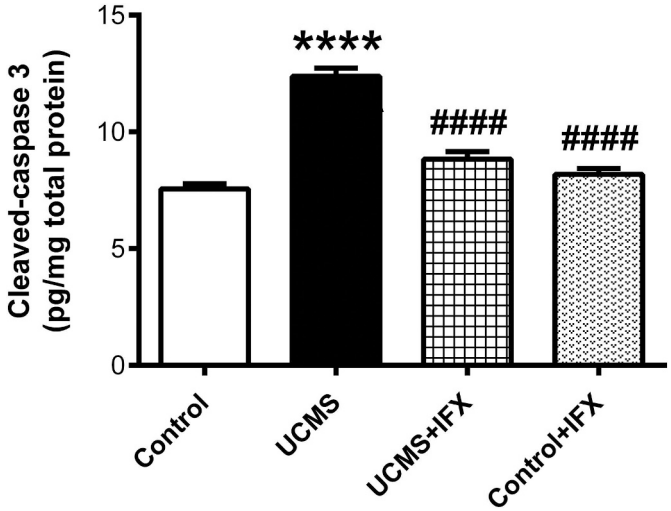



**Fig. 11.** Effect of UCMS and infliximab on cleaved-caspase 3 level in rat vas deferens. Values represent the mean ± SEM of 8 rats/group. $^{****}p < .0001$ vs. control group and $^{\#\#\#\#}p < .0001$ vs. UCMS group.

## 4. Discussion

In the current research, behavioral, functional, biochemical and molecular approaches were applied to explore the protective effects of IFX on UCMS-induced depression rat model. We found that rats exposed to UCMS exhibited typical anhedonia and despairing behavior after 5 weeks of UCMS, as evidenced by their reduced sucrose consumption/preference and enhanced duration of immobility in the behavioral tests, which demonstrated that the rat model of depression was successfully established. These results were similar to earlier reports [20]. Treatment with IFX in UCMS-exposed rats significantly inhibited the decrease of sucrose consumption/preference and reversed the increase of immobility duration, indicating an obvious protective effect of IFX on the depressive-like behavior, which was consistent with our previous study showing the antidepressant-like effect of IFX in rats exposed to 8-week-UCMS [14]. Moreover, no differences in locomotor activity were observed among the experimental groups, indicating that depressive-like behaviors observed in stressed animals were not dependent on any alterations in the locomotor activity. The effect of chronic stress on locomotor activity remains controversial with some studies showing impaired locomotor activity [21] while other studies, including ours, reported no alterations [6,22]. This discrepancy might be due to differences in stress schedule, animal strain and/or stimuli intensity.

Hyperactivity of the hypothalamo-pituitary-adrenal (HPA) axis is one of the most crucial factors that contribute to the development of depression [23]. It has been reported that chronic stress-induced depression is associated with the attenuation and disruption of the negative feedback action of corticosterone as indicated by elevated circulating levels of corticosterone [24]. Similarly, in the present study UCMS resulted in a significant increase in circulating concentrations of corticosterone, which validated our stress protocol. We also found for the first time that treatment with IFX decreased serum corticosterone level in UCMS-exposed rats, suggesting the regulatory effect of IFX on the chronic stress-induced HPA axis hyperactivity. The upregulation of GR expression and/or the restoration of GR function by reducing the GR resistance might be an explanation for the effects of IFX on normalizing corticosterone levels [20,25]. Besides, the antidepressant-like effect of IFX may be mediated by the modulation of hyperactivity of HPA axis.

It has been well established that the activation of the inflammatory response system promoted by chronic stress contributed to the development of depression [26]. However, chronic stress not only induces inflammation in the central nervous system, but also results in a low-grade inflammatory response in the periphery [27]. Earlier studies reported a significant elevation in the penile and testicular expression levels of NF-κB, TNF-α, IL-1β and IL-6 in UCMS-exposed rats [6,8]. Consistently, the results of the current study revealed a marked increase in the gene expressions of NF-κB, TNF-α, IL-1β and IL-6 in VD of rats exposed to UCMS. Also, we found elevated NF-κB p-p65 subunit level, indicating increased NF-κB activity in VD tissues of stressed rats. NF-κB is a major transcription factor that stimulates transcription of a number of different genes, including those coding for TNF-α, IL-Iβ and IL-6 [28]. Thus, the increased production of proinflammatory cytokines might be dependent on NF-κB activation. IFX treatment caused a significant reduction in the gene expression levels and activity of NF-κB and, decreased gene expression levels of aforementioned proinflammatory cytokines in tissues of stressed rats, suggesting the ability of IFX to inhibit chronic stress-induced inflammatory response in VD tissues. These effects are in agreement with previous studies which indicated that IFX can block the activation of NF-κB signaling pathway and therefore, suppress the tissue production of proinflammatory cytokines [29].

Oxidative stress has also been proposed to be involved in chronic

**Table 2**
Positive and negative correlations of EFS-, noradrenaline- and ATP-induced maximal contractions ($E_{max}$) between inflammatory (NF-κB and TNF-α) and oxidative stress biomarkers (SOD and MDA) in the VD tissues obtained from all tested rats.

| | $E_{max}$ for EFS (% KCl) | | $E_{max}$ for noradrenaline (% KCl) | | $E_{max}$ for ATP (% KCl) | |
|---|---|---|---|---|---|---|
| | r | *p* | r | *p* | r | *p* |
| NF-κB Gene expression | −0.8504 | < 0.0001* | 0.8705 | < 0.0001* | 0.9548 | < 0.0001* |
| TNF-α Gene expression | −0.8365 | < 0.0001* | 0.7342 | < 0.0001* | 0.8765 | < 0.0001* |
| SOD activity (U/ml) | 0.9287 | < 0.0001* | −0.8226 | < 0.0001* | −0.8912 | < 0.0001* |
| MDA level (nmol/ml) | −0.9327 | < 0.0001* | 0.8797 | < 0.0001* | 0.9302 | < 0.0001* |

r: Pearson's correlation coefficient.
$N = 24$ for all parameters.
* Statistically significant correlation.

stress-induced depression and related various pathologies [5,30,31]. In the present study, UCMS caused decreased GSH level as well as SOD and catalase activity and, elevated MDA content in the VD of rats. These results were in accordance with previous experimental reports showing oxidative stress induced by UCMS in a variety of peripheral tissues such as kidney, liver, pancreas and testis [5,32,33]. The interplay between oxidative stress and inflammation is well-known. TNF-α has been demonstrated to induce ROS production in various tissues leading to oxidative stress [34]. Increased oxidative stress leads to activation of NF-κB signaling pathway, which in turn enhances the production of proinflammatory cytokines and many mediators involved in inflammatory responses [8]. Thus, this increased oxidative stress in VD tissues of stressed rats may be associated with TNF-α-induced inflammation. In the current research, we also observed that IFX diminished oxidative stress through alleviation of MDA generation and improvement of the antioxidant status in VD tissues by enhancement of GSH, SOD and CAT levels. It has been proposed that IFX may suppress oxidative stress via the following pathways: (A) reducing lipid peroxidation and oxidant status [35], (B) improvement of the antioxidant status [36], (C) decreasing the production of ROS [37]. In line with these studies, our findings demonstrated the antioxidant effects of IFX in VD tissues of UCMS-exposed rats. These effects of IFX might be attributed to the suppression of inflammatory response via blockade of TNF-α, which is involved in ROS production [38].

It is well known that TNF-α is a major triggering factor for the extrinsic pathway of apoptosis. Hence, we evaluated the activation of caspase 3 (a good indicator of apoptosis) in VD tissues and found increased cleaved-caspase 3 level in UCMS-exposed rats. However, IFX treatment markedly inhibited the UCMS-induced activation of caspase 3 in VD tissues which may be due to its anti-inflammatory and antioxidant effects.

The contractile behavior of the VD is primarily regulated via endogenous noradrenaline and ATP released from sympathetic nerve endings which act at specific $\alpha_1$-adrenoceptors and P2X-purinoceptors on the smooth muscle of the VD, respectively [1]. It is well established that EFS of the sympathetic nerves in the rat VD lead to biphasic contractile responses, the first phase to ATP and the second phase to noradrenaline [1]. In our study, we demonstrated reduced contractile responses induced by EFS in the VD obtained from chronic stress exposed rats. We also assessed EFS-induced contractile responses of the VD tissues in the presence of prazosin and suramin to clarify how chronic stress influences purinergic or adrenergic components independently and we found that UCMS caused similar impairment on the responses to purinergic and adrenergic neurotransmission. This finding showed that these two separate systems were similarly affected by chronic stress. Moreover, chronic stress resulted in augmented ATP- and noradrenalin-induced contractility in VD of stressed rats. These increased purinergic and α-adrenergic responsiveness of VD might be attributed to upregulation of P2X-purinoceptors and α-adrenoceptors or enhancement of post-receptor events rather than an increase in the affinity of aforementioned receptors, since pD2 values for the contractile effects of ATP and noradrenaline were not significantly different. Additionally, KCl-induced contractile responses did not alter among the groups, suggesting that the changes observed in VD contractility of UCMS-exposed animals in the current study were not related with calcium movements.

Inflammation has been previously suggested to be linked to impaired function of the VD in LPS-treated rats [10]. In our study, we found strong correlations between the contractile responses of the VD and inflammatory biomarkers in VD tissues. These findings provide vivid evidence that inflammation is one of the mechanisms contributing the impaired and abnormal contractility of the VD in chronically stressed rats. Besides, we observed significant correlations between oxidative stress biomarkers and VD contractions, indicating that oxidative stress may be another reason for the impairment of the contractility of VD. Our findings were supported by an earlier study which reported a significant correlation between oxidative stress level and VD contractility [39]. Moreover, IFX restored the impaired contractile response induced by EFS in the absence or presence of suramin or prazosin, showing that IFX prevented chronic stress-induced oxidative stress in the peripheral adrenergic nerves in the VD. Also, IFX treatment markedly decreased the abnormal contractions in response to both noradrenalin and ATP in VD of UCMS-exposed rats. These protective effects of IFX on the neurogenic, noradrenaline- or ATP-induced contractile responses of the VD might be dependent on its ability to attenuate inflammation, to increase the production of intracellular antioxidants and scavenging capacity of free radicals as we reported here.

## 5. Conclusions

In conclusion, current study revealed that treatment with IFX abrogated chronic stress-induced VD dysfunction in rats by suppressing inflammation and oxidative stress, indicating that anti-inflammatory and antioxidant agents might be considered as a candidate for treatment of ejaculatory disorders associated with chronic stress.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2020.117545.

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*Declaration of competing interest*

The authors declare that they have no conflict of interest.

*Author contribution*

TDŞ conceived and designed the study. TDŞ, SSG and GD performed experiments. TDŞ, SSG, GD, YY and TU analyzed data. TDŞ wrote the manuscript. All authors read and approved the manuscript.

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