Original Article

Redox Imbalance and Reproductive Side Effects of Long and Short Term Nitroglycerin Treatment in Rat Uterus

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Abstract

Background: Bioactivation of nitroglycerin (NTG) leads to the production of reactive oxygen species and reactive nitrogen species. The aim of this study was to investigate the effects of NTG treatment on the redox homeostasis in rat uterus around the time of implantation and the number of pups.

Materials and Methods: The rats in long-term test groups were treated subcutaneously with NTG (15mg/kg BW) and normal saline (1ml/kg B) in control groups for 4 weeks. Afterwards, they were mated and divided into four groups. Two groups were treated until 5 days after mating. Thereafter, they were sacrificed and the activities of glutathione peroxidase (GPx), catalase (CAT), and glutathione reductase as well as the levels of reduced glutathione (GSH) and malondialdehyde (MDA) in the uterus homogenates were measured. In other two groups, treatments were continued until their pups were counted. In the short-term groups, treatments were started after mating, and all above parameters were measured as similar as long-term groups.

Results: Long-term NTG treatment significantly increased MDA level and decreased the GPx activity and the pups number compared to the controls ($p<0.05$), whereas no marked alteration in the activities of GR and CAT and the levels GSH were observed. However, short-term NTG treated groups showed no significant changes in all the parameters mentioned above as compared with the controls.

Conclusion: Long-term NTG treatment, unlike short-term treatment, may cause impaired implantation and infertility, but there is also room for further improvement.

Keywords: Redox imbalance- Implantation- Infertility- Pups- Uterus homogenates

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Introduction

Infertility is one of the most critical problems in public health, affecting 15%–20% of couples of reproductive age[1]. Forty to fifty percent of infertility cases are related to a gynecological problem[2]. It is clearly well known that oxidative stress has an important role in the pathophysiology of gynecological diseases, which can lead to the infertility [3-7]. High levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in uterine tissue may results in oxidative stress-induced infertility. According to the

reports, 0.2-4% of all pregnancies in the western world are influenced by cardiovascular diseases (CVD). During pregnancy, the most frequent cardiovascular events are related to hypertension (6–8%). On the other hand, the most common manifestations of CVD during pregnancy is congenital heart disease-CHD (75%), and most of the patients depend on nitroglycerin (NTG) to relieve their chest pain and hypertension[8]. Bioactivation of NTG produces high levels of ROS and RNS, such as hydroxyl (OH^-), super oxide ($O2^{\cdot-}$), and nitric oxide (NO) radicals, and these species trigger oxidative stress-induced damage to key cellular targets[9, 10]. Oxidative stress degrade polyunsaturated lipids, forming malondialdehyde, as a marker of oxidative stress[11-13]. Gori et al, showed that NTG triggers ROS production by direct uncoupling of the mitochondrial respiratory chain[14]. One study of 9 fertile women with regular cycles and 30 infertile women with endometriosis revealed that high endometrial levels of NO, as observed in patients with endometriosis, may produce an unfavorable environment for implantation[15]. In another study, Barroso RP et al, showed that higher concentrations of NO inhibit both embryo development in vitro and implantation in vivo in mouse models[16]. The human body has several mechanisms to counteract oxidative stress. One of these mechanisms is antioxidant enzymes, such as glutathione peroxidase (GPx), glutathione reductase (GR)[17, 18], and catalase (CAT) as well as reduced glutathione (GSH), which have pivotal roles in ameliorating oxidative stress[19-22]. Based on the observations described above, we aimed to assess the long- and short-term effects of NTG treatment on oxidative features of rat uterine tissue around the time of implantation.

Methods

Animals: Eighty female sprague-dawley rats (175±15g) were provided by the animal breeding center of Shiraz University of Medical Sciences (Shiraz, Iran). All rats were maintained on a 12-h light/dark schedule and fed ad libitum. All experiments were performed according to the approved protocol by ethics committee of shiraz university of medical sciences. Eight groups of rats, each containing 10 animals, were used in the present study.

Experimental protocol and groups: To study the long-term effects of NTG, 40 female rats were divided into four groups of 10 animals (test and control). We used a commercial formulation of NTG (Caspian, Rasht, Iran) in the form of 5 mg/mL ampules and 450 µL of NTG was administered each morning at a specific time (10 a.m.) to each rat. NTG [15 mg/kg, subcutaneously (s.c.)] was administered to test group (a) and normal saline vehicle (1 ml/kg, s.c.) was administered to control group (b) daily for 4 weeks[23]. After 4 weeks, the rats were mated and mating was confirmed by the presence of spermatozoa in the vaginal smear in the next morning (day 1 of pregnancy)[24]. The rats were sacrificed on day 5 of pregnancy (implantation time) and the uteri were removed, washed in saline to remove blood, and kept at −70 °C until further analysis. Another test and control groups (c and d) were treated continuously until their pups were born and counted. To study the short-term effects of NTG, 40 female rats were mated, and after confirmation of mating, they were divided into four groups of 10 animals [24]. NTG (15 mg/kg, s.c.) was administered to test groups (e and f) and controls (g and h) were treated with normal saline vehicle (1 ml/kg, s.c.) daily until implantation time (five days after mating)[23]. Finally, all rats were sacrificed and the uteri were removed, washed in saline, and kept at −70 °C until further analysis. As described in the case of long term treatments, short term groups f and h were also treated with NTG until their pups were born and counted.

Homogenization of uterine tissues: The frozen uterine tissue samples were cut into small pieces and homogenized in ice-cold saline to produce 10% (w/v) homogenates which were centrifuged at 10 000 × g for 20 min at 4 °C. The supernatants were used for the measurement of GPx, GR, CAT activities as well as MDA and GSH levels. The protein in the uterine supernatants was measured by the Bradford method, using bovine serum albumin as a standard[25].

Measurement of MDA concentration: Uterine malondialdehyde (MDA) was assayed by a colorimetric method as described by Mostafavi-Pour et al. The MDA concentration was calculated using 1, 1, 3, 3-tetraethoxy propane (TEP) as a standard. The results were expressed as nmol/mg protein of the

uterine supernatant[26].

Measurement of GSH concentration: The assay of GSH with DTNB [5, 5′-dithiobis-(2-nitrobenzoate)] dye was performed with some modifications of Ellman's method[27]. Standard curve was made by serial dilutions of the 1mM GSH solution. Clear uterus supernatant was analyzed for GSH level. 2.3 ml of potassium phosphate buffer (0.2 M, pH 7.6) was added to 0.2 ml of supernatant and then 0.5 ml DTNB (0.001 M) was added to this solution. Concentrations of GSH were determined by the measurement at 412 nm and were expressed as µmol/mg protein.

Determination of GPx activity: Activity of glutathione peroxidase (GPx) in the uterus supernatant was measured by the method of Fecondo and Augusteyn, by monitoring the regeneration of reduced glutathione (GSH) from oxidized glutathione (GSSG) upon the action of GR (Sigma Chemical Company, USA) and NADPH (Fluka Chemical Company, Switzerland) [28]. The enzyme activity in the uterus supernatants was expressed as mU/mg of the protein using a molar extinction coefficient of 6.22×10^6/M/cm for NADPH.

Determination of CAT activity: CAT activity was estimated by monitoring H_2O_2 decomposition using the procedure of Aebi [29]. Activity of this enzyme was expressed as the mmol of H_2O_2 consumed per min/mg of the uterus supernatant protein content using a molar extinction coefficient of 43.6/ M/cm for H_2O_2.

Determination of GR activity: GR activity was determined by the method of Carlberg and Mannervik, as previously described[30]. This enzyme catalyzes the reduction of GSSG to GSH, using NADPH as a reductant. Accordingly, the measurement of NADPH consumption will result in the determination of GR activity. Results were based on a molar extinction coefficient for NADPH of $6.22\times10^6 M^{-1}cm^{-1}$. One unit of GR is defined as mU/mg cell protein.

Statistical analysis: The data were analyzed using SPSS version 19 software (SPSS, Chicago, IL, USA). All data were analyzed by Mann-Whitney tests for group comparison and are expressed as mean ± standard error of the mean. The 0.05 level was used for statistical significance.

Results

Long and short term effects of NTG on MDA and GSH levels: Figure. 1a shows the uterus MDA level in long and short-term NTG treated test and control groups. The MDA levels in the long-term test group were increased significantly by 51.3% compared to the control group ($P<0.05$). According to our results, there was no difference between MDA levels of short-term NTG treated test and control groups (Figure 1a). As depicted in Figure. 1b, there was no remarkable difference between the long term test and control groups in GSH levels. Short-term NTG treated test group had a mildly elevated GSH levels compared with the control group, but the difference is not significant.

Long- and short- term effects of NTG on GPx, GR and CAT activity: According to the Figure. 2a, long-term NTG treatment of the test group resulted in a significant decrease in GPx activity by %48.7 as compared to the controls ($P<0.05$). Compared with the control group , the Long-term administration of NTG had no significant effect both on the GR and CAT activity. However, no significant differences were found in the activities of these three enzymes in short-term NTG treated group compared with the controls.

Long and short term effects of NTG treatment on the number of pups: As shown in Figure.3, long-term NTG treatment significantly decreased the number of pups by 49.9% compared to control group ($P<0.05$). However, in the short-term NTG treated groups ,there were no significant differences in the number of pups between the test and the control groups.

Discussion

Our observations indicated that GPx activity in the long-term NTG treated group was significantly decreased, when compared with the controls ($p<0.05$). These data are consistent with results of previous study, which showed that the activity of the antioxidant enzymes, catalase and GPx in red blood cells were significantly decreased after intravenous NTG treatment[31]. In addition, some investigations have shown that NTG treatment and RNS formation could lead to the inactivation of glutathione S-transferase (GST), a glutathione-mediated enzyme, by nitration or oxidation of some amino acid residues in

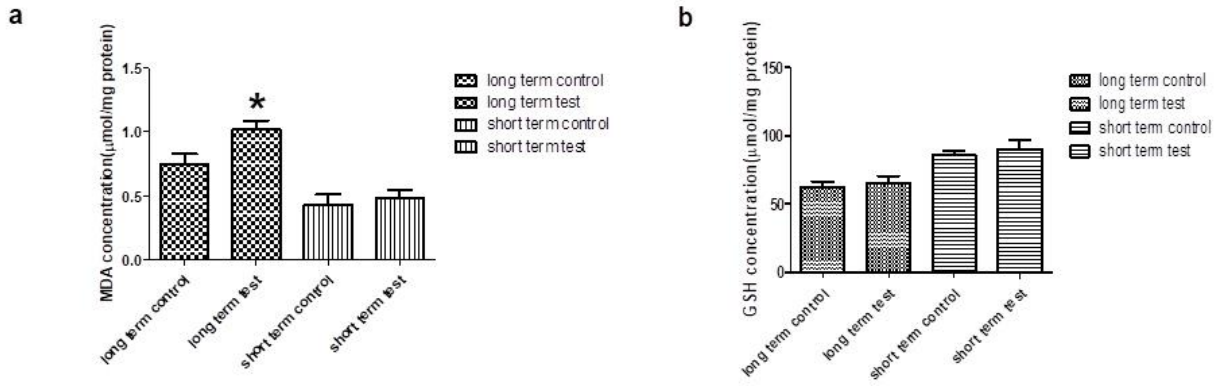

Figure 1. Effects of long and short-term NTG treatment on the concentration of (a) malondialdehyde (MDA) and (b) reduced glutathione (GSH) (mean±SEM) in rat uterine supernatant as compared with the control groups (normal saline). * P< 0.05.

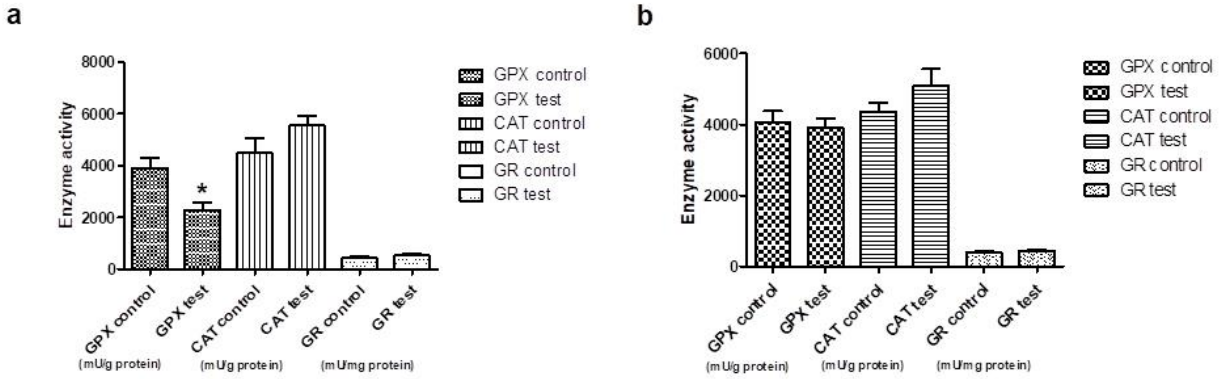

Figure 2. Effects of (a) long and (b) short-term NTG treatment on the activities of glutathione peroxidase (GPx), catalase (CAT), glutathione reductase (GR) (mean±SEM) in rat uterine supernatant compared to the control groups (normal saline). * P< 0.05.

the enzyme structure[32, 33]. Accordingly, it appears that the mechanism of the reduction in GPx activity in our study was similar to that of GST inactivation.

Glutathione peroxidase is one of the first lines of defense against free-radical damage to tissues, thus inactivation of this enzyme in the uterus at implantation window may be detrimental to this process. In addition, the levels of malondialdehyde (MDA), as a marker of lipid peroxidation, were significantly increased in the long-term NTG treated group compared to the controls ($p<0.05$). This observation is consistent with other studies that have investigated the effects of NTG treatment on MDA levels. Dudek et al., and Knorr et al., showed that NTG treated rats have significantly increased MDA levels as compared with the corresponding values in the control group[34,35]. In our study, long-term administration of NTG showed no significant differences in the levels of GSH as well as in the

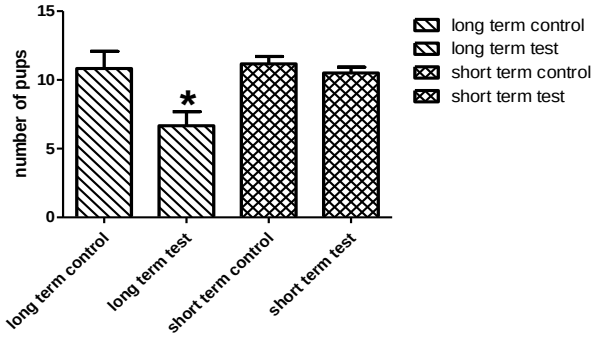

Figure 3. Effects of long and short-term NTG treatment on the number of pups (mean±SEM) compared to the control groups

activities of GR and CAT as compared with the control groups. GPx and GR are involved in oxidizing and reducing GSH respectively. According to our results, GPx activity decreased, but GR activity and GSH levels remained unchanged. A possible

explanation for these data by considering the mechanism of reaction is that the inactivated GPx and unchanged GR activity could result in the intact GSH. This indicates that there was an imbalance in the redox homeostasis of uterine tissue. These findings are consistent with previous observations.

Husain showed that long-term administration of NTG had no significant effects on activity of some antioxidant enzymes and GSH levels in the rat heart tissue[36]. However, one study reported that NTG treatment increased formation of superoxide and peroxynitrite leading to the significant decrease in plasma levels of lipophilic antioxidants , alpha and beta carotene, and superoxide dismutase activity[37]. As shown in Figure. 3, long-term NTG treatment of test group significantly decreased the number of pups compared with the control group ($p<0.05$). Although, some studies have found that nitric oxide may be needed in the process of implantation and improving pregnancy rate[38,39], nevertheless, nitric oxide-overwhelmed environment in the boy tissues may exert an inhibitory effect on the expression of adhesion molecules and could lead to impaired implantation[40-42]. Accordingly, it appears that decreased number of pups in long term NTG treatment in our study may be due to the implantation failure. However, as shown in Figures.1a, 1b, 2b, and 3, no significant differences were observed in the activities of GPx, CAT, and GR or in the levels of MDA and GSH and number of pups in the short-term treated NTG groups as compared with the controls.

Conclusion

According to our data, long-term NTG treatment may result in redox imbalance, impaired implantation and can lead to the infertility. However, it appears that the short-term NTG treatment may not have reproductive side effects.

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