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Original Research Article

Intracellular Generation of Reactive Oxygen Species during Testicular Ischemia Reperfusion Injury and Effectiveness of Antioxidants in Rats

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ABSTRACT

The generation of Reactive Oxygen Species (ROS) occurs constantly during normal cell metabolism in all living cells (Yu, 1994). Free radical generation that exceeds the capacity of antioxidant defences results in oxidative stress, which possibly elicits irreversible, degenerative response including apoptosis or necrosis, in living cells (Buttke and Sandstrom, 1994). Higher amounts of ROS play a role in the ageing process as well as in a number of human disease states, including cancer, ischemia, and failures in immunity and endocrine functions. As a safeguard against the accumulation of ROS, several non-enzymatic and enzymatic antioxidant activities exist. The purpose of this study is to determine whether inducible nitric oxide synthase (iNOS) is involved in the pathogenesis of testicular ischemia-reperfusion (I/R) injury in association with germ cell death, through either necrosis or apoptosis. The objective of our study was to investigate involving excessive iNOS expression shows intracellular ROS (NO) generation, during ischemia reperfusion injury, is identified by Immunohistochemistry, on both sides of testis (ipsilateral and cotralateral) and antioxidant property of guava leaf extract may provide protection against testicular torsion ischemia reperfusion injury in rats.

Keywords: Ischemia Reperfusion, ROS Generation, Apoptosis, Antioxidants, Immunohistochemistry.

INTRODUCTION

The testis is sensitive to a variety of stressors, such as hyperthermia, inflammation, radiation, ischemia and exposure to agents that induce apoptosis of germ cells. Because oxidative stress in the testis is one of the major factors that induce germ cell apoptosis, this organ has fairly high concentrations of antioxidants, such as GSH, ascorbic acid and vitamin E. These antioxidants protect germ cells against oxidative DNA damage, and play important roles in spermatogenesis (Emiko Kasahara et al., 2002). Testicular torsion causes an enhanced formation of reactive oxygen species which contributes to the pathophysiology of ischemia-reperfusion injury in the testis (Esin Atik et al., 2006).

Reactive oxygen species (ROS) are involved in the cell growth, differentiation, progression, and death. Low concentrations of ROS may be beneficial or even indispensable in processes such as intracellular signaling and defense against

micro-organisms (Jose et al., 1999). On the other hand, low (physiological) levels of lipid peroxidation reflect the influence of reactive oxygen species (ROS) on sperm metabolism enhancing the ability of human spermatozoa to interact with zona pellucida. A reason for higher, pathological lipid peroxidation of sperm membranes can be unbalanced oxidative stress (Sanocka and Kurpisz, 2004). Lipid peroxidation is a complex process whereby polyunsaturated fatty acids (PUFAs) in the phospholipids of cellular membranes undergo reaction with oxygen to yield lipid hydroperoxides (LOOH). The reaction occurs through a free radical chain mechanism initiated by the abstraction of a hydrogen atom from a PUFA by a reactive free radical, followed by a complex sequence of propagative reactions (Palmieri and Sblendorio, 2007). Reactive Oxygen Species (ROS) comprise a class of radical and nonradical oxygen derivatives that play a significant role in reproductive biology. Because they have an unpaired electron in their outer orbit, ROS are highly reactive and interact with a variety of lipids, proteins, and nucleic acids in the body. Such reactions are not only harmful for reproductive potential, but they also generate more free radicals, thereby perpetuating a chain of reactions and creating high amounts of oxidative stress.

Reactive nitrogen species (RNS) is a subset of free oxygen radicals called reactive oxygen species (ROS). RNS is especially prominent in different areas of the male reproductive system and these sources can be categorized by structure and various cell types such as seminal ejaculate, accessory glands, epididymis, penis, testis, and ducts.

Nitric Oxide (NO) is produced from L-arginine via Nitric Oxide Synthase (NOS) (Sejal et al., 2012). Inducible nitric oxide synthase (iNOS) is one of the three known mammalian nitric oxide synthase (NOS) isoforms responsible for nitric oxide (NO) production (Jaffrey and Snyder, 1995). Inducible nitric oxide synthase (iNOS) is induced via the activation of nuclear factor kappa B (NF-κB) after I/R in rat (Gang Min Hur et al., 1999).

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a protein complex that controls transcription of DNA. NF-κB is found in almost all animal cell types and is involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, oxidized LDL, and bacterial or viral antigens (Gilmore, 2006). It is activated in response to a variety of stress- and injury-related stimuli including exposure to cytokines such as tumor necrosis factor-α (TNFα), and excitotoxic and oxidative insults (Mark et al., 1997).

ROS/RNS are usually too reactive and/or have a half-life too short (even much shorter than seconds) to allow direct measurements in cells/tissues or body fluids (Aitken and Roman, 2008). There are so many factors capable of inducing oxidative stress in the testis strongly suggests that this is a vulnerable tissue that is both highly dependent on oxygen to drive spermatogenesis and yet highly susceptible to the toxic effects of reactive oxygen metabolites; in this context, the testis is very like the brain. While the testis clearly do possess highly specialized antioxidant defence enzymes such as extracellular SOD, PHGPx etc, therefore clear benefits to be gained by treating susceptible individuals with exogenous antioxidants. Despite the evident clinical market for an antioxidant preparation specifically designed to support male reproductive health, it is remarkable how little effort has gone into the development of such a preparation and how poor most of the clinical trials in this area have been. In animal models an impressive range of antioxidant preparations has been examined and compounds identified that are

clearly capable of crossing the blood testes barrier and protecting the germinal epithelium and Leydig cells from oxidative stress (Aitken and Roman, 2008).

Current studies reported, The testicular injury score was lower in the Interleukin 10 (IL-10) treated group rats compared with the I-R/untreated group. IL-10 might play a protective role in reducing reperfusion injury (Hulya Ozturk et al., 2014). Treatment with Quercetin may have some benefits in controlling I/R-induced tissue injury through its anti-inflammatory, anti-apoptotic, and antioxidant effects (Çevik et al., 2013). Propofol as an anesthetic agent may attenuate germ cell-specific apoptosis and decrease NO biosynthases through downregulation of iNOS expression in an animal model of testicular torsion and detorsion (Yagmurdur et al., 2008). Numerous substances have been proposed as important in the prevention of post-ischaemia–reperfusion testicular injury. A range of chemicals and drugs has been successfully tested in animal models for the purpose of mitigating the dangerous effects of ischaemia - reperfusion in testis torsion (Ersagun Karaguzel et al., 2014).

Therefore we hypothesized that ethanol Guava leaves extract might elicit oxidative stress in the torsed testis, so our Immunohistochemistry study, describes the changes of oxidative stress in the testis and extent of germ cell apoptosis after administration of Guava leaves extract to the rat.

MATERIALS AND MEDHODS

The experimental protocol of the study was approved by the local ethics committee. Thirty pre-pubertal male westar –albino rats (8-9 weeks old, weighing 180-230g) were used. Rats were given guava leaves orally at a dosage of 10 µg/ml/kg body weight/day. Animals are divided into 5 groups (1) control group, (2) testicular torsion - 2 days, (3) testicular torsion - 2 days + Guava leaves treated, (4) testicular torsion - 15 days, (5) testicular torsion – 15 days + Guava leaves treated.

All surgical procedures were performed under xylazine/ketamine anaesthesia (10/90mg/kg,i.p.). The incision was made through the scrotum and tunica vaginalis was opened and the gubernaculum was divided. The right testis was rotated 720 degrees in a clockwise direction and fixed within the hemiscrotum for 1 hour. The torsed testis was then detorsed. The incision was then closed using a 4-0 silk suture (Turner et al., 1997).

General health and behaviour:

At the end of experimental period all the animals showed normal feeding habit, no sign of distress and showed normal behaviour, all animals appeared throughout the period of study. Rats were weighed and sacrificed by cervical dislocation. Orchiectomy was performed, removed testis, cleared of the adhering tissues and weighed.

Immunohistochemistry:

Paraffin-embedded sections (5microns) which were mounted on gelatine coated slides were used for the study. The sections were brought up to distilled water after which treated with 10 mm sodium citrate buffer for antigen unmasking. Later incubated with 1% H_2O_2 to quench endogenous peroxidase activity and followed by 10% normal goat serum for 20 minutes to block non specific binding antibody. Then incubated with primary antibody (anti iNOS monoclonal) for 1 hour and secondary antibody TRITC conjugated (Tetramethyl Rhodamine Iso-Thiocyanate) for 45 minutes. Finally counter stained with DAP1 (4',6-Diamidino-2-Phenylindole). After mounted with glycerol, observed under fluorescence microscope. iNOS expressed areas were stained red and nuclei

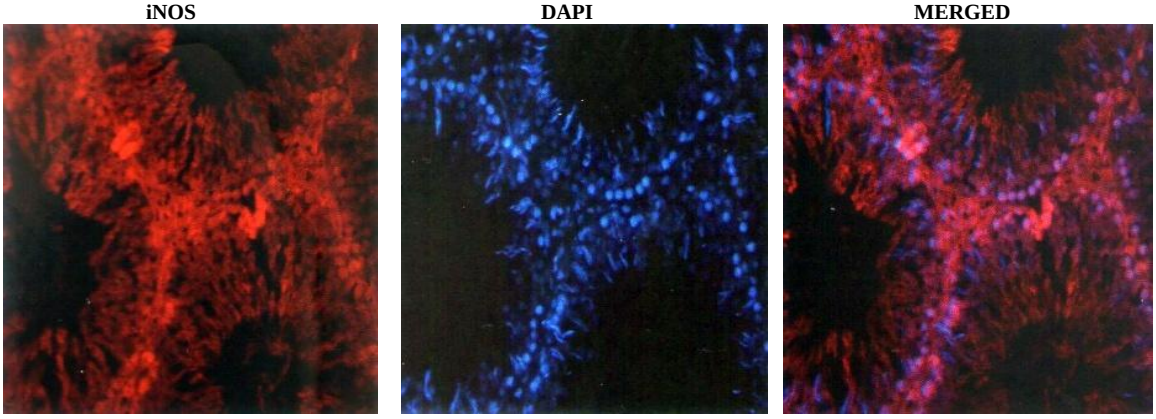
with blue colour (Giorgio Cattoretti et al., 1993).

RESULTS

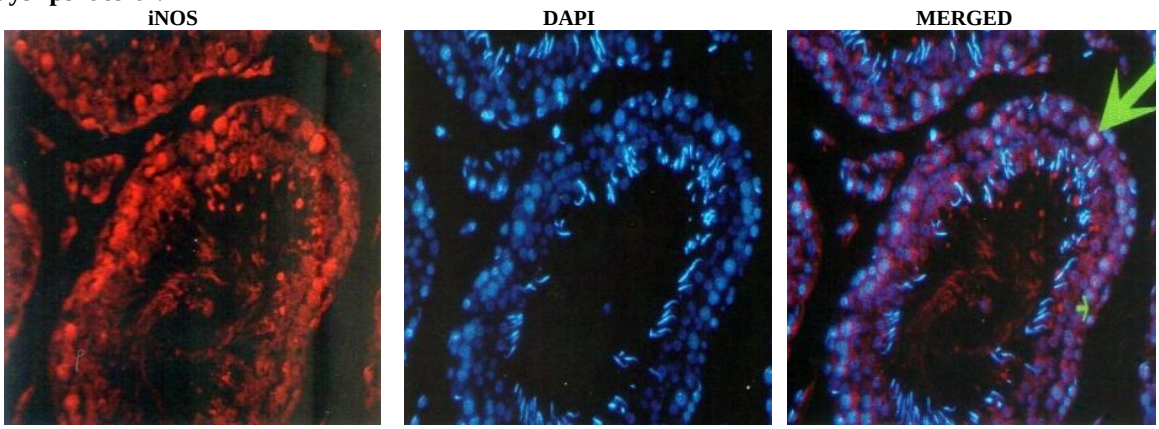
Immunohistochemistry:

iNOS expression shows up-regulation in group (4) and in group (2) , whereas down regulation in group (4) and group (3). Control group shows no expression.

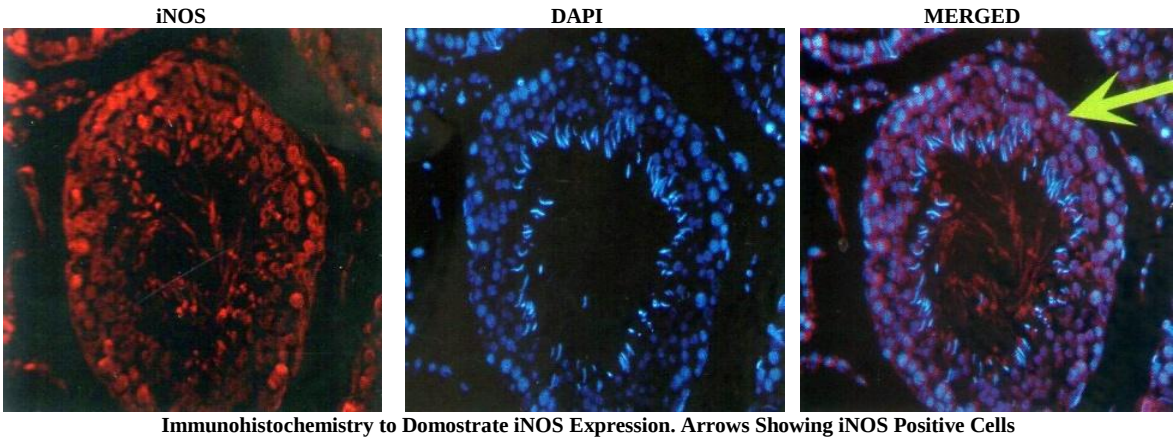
Control group:



2 Days Ipsilateral:

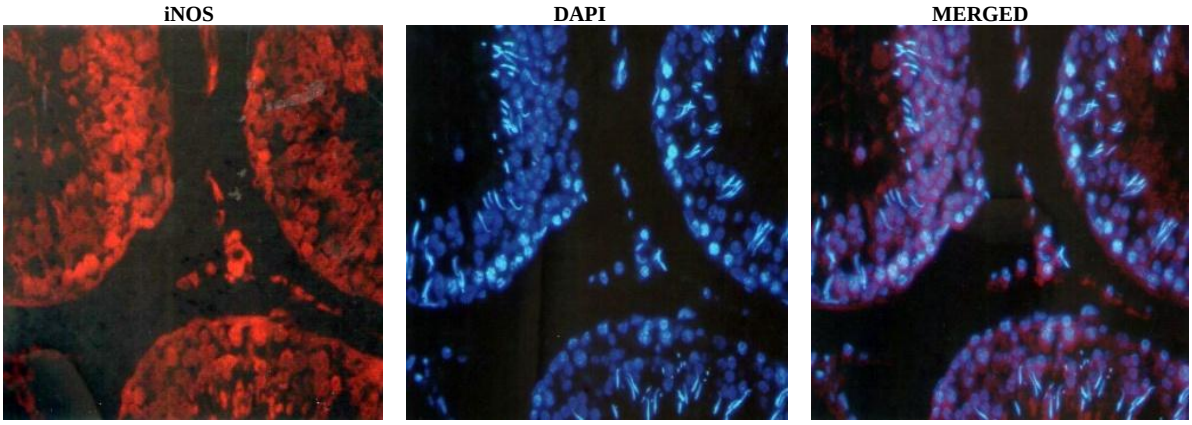


2 Days Drug Treated Ipsilateral:

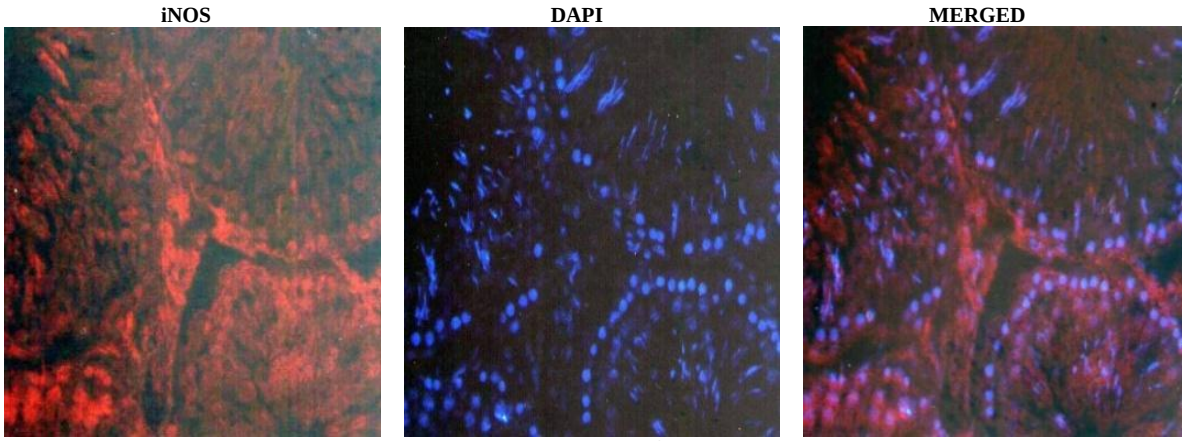


Immunohistochemistry to Domostrate iNOS Expression. Arrows Showing iNOS Positive Cells

2 Days –Contralateral

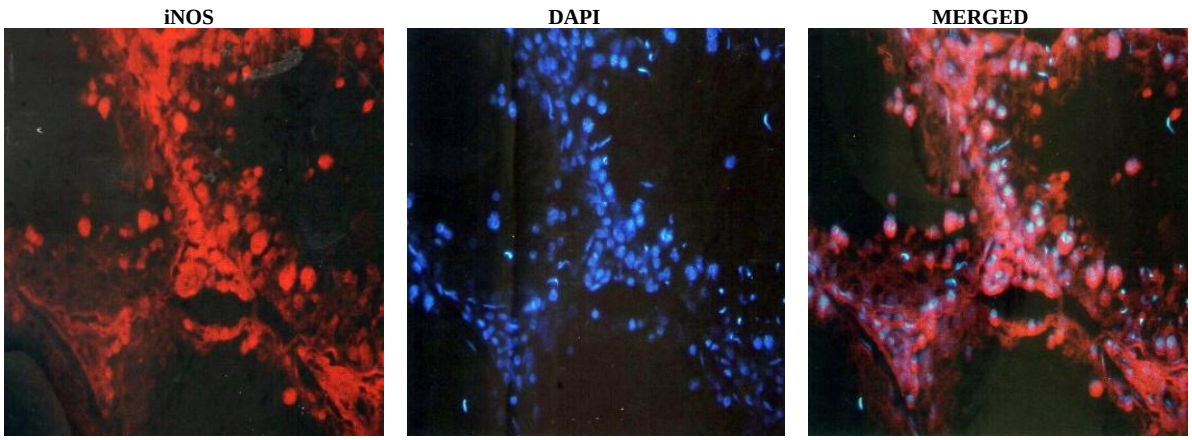


2 Days - Drug Treated Contralateral:

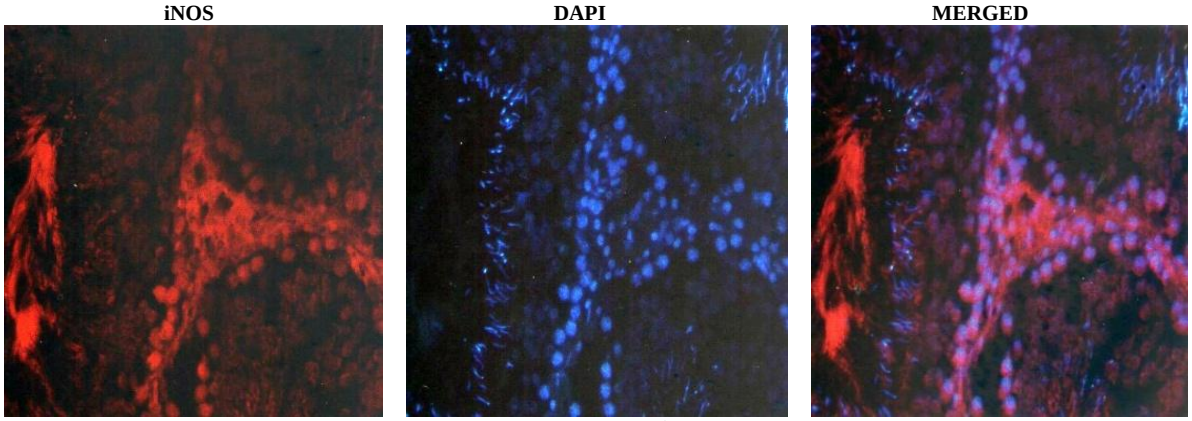


Immunohistochemistry of iNOS -20x

15 Days - Ipsilateral :

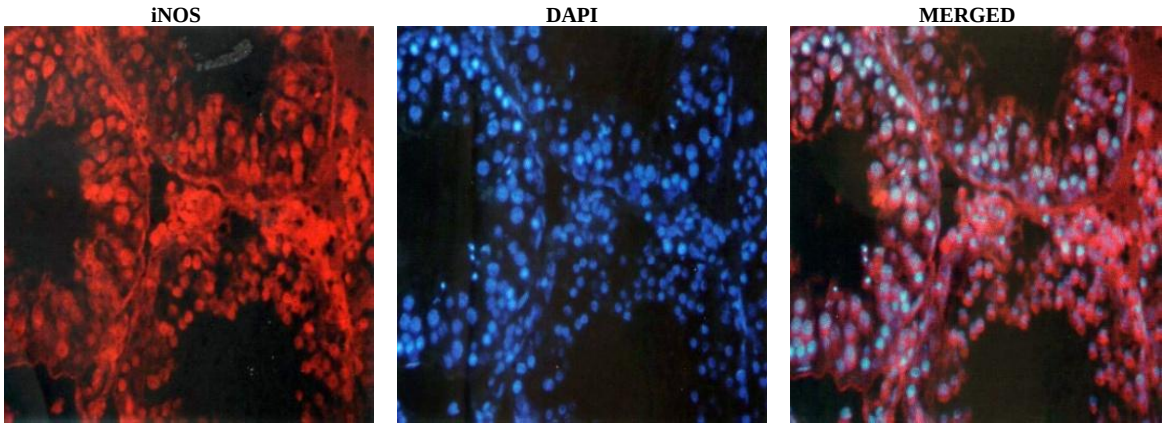


15 Days - Durg Treated Ipsilateral:

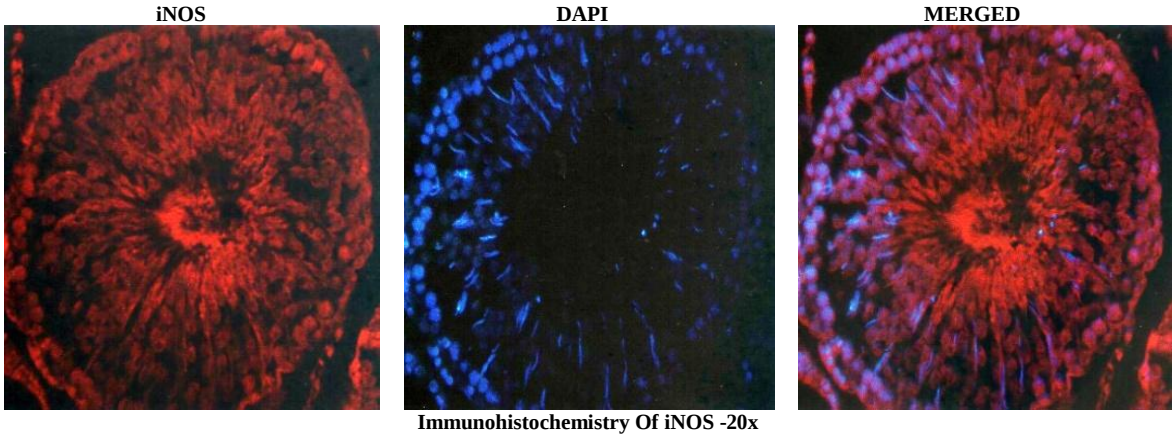


Immunohistochemistry Of iNOS -20x

15 Days - Controlateral:



15 Days - Drug Treated Controlateral:



Immunohistochemistry Of iNOS -20x

DISCUSSION

Nitric oxide is a highly reactive free radical with a multitude of organ septic regulatory function. NO plays a major role in many organ systems, and deranged NO synthesis causes a number of path-physiological states (Ferguson et al., 2000). NO is a free radical produced from L-

arginine by the enzyme NO synthase, which has three forms: endogeneous NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) (Knowles and Moncada, 1994). Of these, eNOS and nNOS are constitutively expressed, while iNOS is produced in response to cytokines and NF-B. eNOS has a protective effect on microcirculation and always produces NO in small amounts, which predominate in the circulation , producing a productive effect on the microcirculation.

Evidence suggests release of ROS, cytokines, and NO into the venous effluent following ischemia reperfusion activates NF-B. These signalling pathways induce iNOS (Edward Eelly et al., 1995) in the target organ. Inducible nitric oxide synthase (iNOS) through its product, nitric oxide (NO), contribute to the induction of germ cell apoptosis. The expression of iNOS in ipsilateral groups of group (2) and group (4) shows there is a release of cytokines in the inflammatory process of testicular torsion.

Oxidative stress is a major factor in the etiology of male infertility. At the level of the isolated spermatozoon, ROS attack can induce lipid peroxidation and DNA fragmentation disrupting both the motility of these cells and their ability to support normal embryonic development. At the level of the testis, oxidative stress is capable of disrupting the steroidogenic capacity of Leydig cells as well as the capacity of the germinal epithelium to differentiate normal spermatozoa. A large number of independent clinical studies have demonstrated a correlative relationship between male infertility and evidence of oxidative stress in the ejaculate (Sikkaet al., 2001), (Agarwal et al., 2006).

The present study shows that reperfusion for 24 h or more following 1 h of ischemia strikingly induces iNOS that elevates NO production in the model of testicular torsion of the rat. By contrast, the huge amount of NO after 24 h or more of reperfusion is thought to be derived from the iNOS induced after reperfusion for 24 h or more, peaking at 48–96 h of reperfusion. The inflammatory (mononuclear and polynuclear) cells in the interstitial tissue expressed iNOS (Koji Shiraishi et al., 2000).

CONCLUSION

I/R of the testis induces iNOS, which promotes germ cell injury, possibly through necrotic cell death induced by NO and cytokines in the delayed phase of reperfusion. The treatment of ethanol guava leaves extract to testicular torsed rats showed marked improvement, in group (3) and (5) shows it might be the active substances present in the extract like flavanoids, alkaloids and alkylamines, which play an important role in increasing the anti oxidant levels in rat testis.

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