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Full Length Research Paper

Anti-oxidative effects of citro flavonoids on spermatogenesis in rat

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Citrus fruits have long been recognized as containing valuable sources of important nutrients which are biologically active in humans. Citrus fruits, such as oranges, contain compounds called phytochemicals that can be included into three major groups: the flavonoids, limonoids and carotenoids. The flavonoids are a group of benzopyran derivatives which occur widely in plants. The flavonoids typically consist of a benzene ring fused with the heterocyclic six-membered ring containing an oxygen atom. As citrus has an antioxidant's potential, we want to evaluating its useful effect on spermatogenesis and sperm parameters. Wistar male rat (n=30) were allocated into three groups, control (n=10) and test groups (n=20), that subdivided into groups of 2 that received citrus extract powder (400 and 600 mg/rat) for 30 consequence day. Animals were kept in standard conditions. In twentieth day the testes tissue of rats in whole groups were removed and sperm was collected from epididymis and prepared for analysis. TAC, SOD levels and percentage of sperm viability and motility in both test groups significantly increased ($p<0.05$) in comparison to control group, whereas, sperm concentration, morphology and testes weights in both experimental and control group were similar. The level of MDA in both extract groups were significantly decreased ($p<0.05$). Results revealed that administration of 600 mg/kg/day citrus extract significantly increased the TAC, SOD levels and sperm percentage, viability, motility and decreased MDA levels. This suggested that citrus may be promising in enhancing sperm healthy parameters.

Key words: Anti-oxidative, citrus, spermatogenesis, super oxide dismutase, testis.

INTRODUCTION

The use of herbal medicines (medicinal plants or phytotherapy) has recently gained popularity in Europe and the United States. Citrus fruits, such as oranges, contain compounds called phytochemicals that can be included into three major groups: the flavonoids, limonoids and carotenoids. The flavonoids are a group of benzopyran derivatives which occur widely in plants. The flavonoids typically consist of a benzene ring fused with the heterocyclic six-membered ring containing an oxygen atom. Many flavonoids may also exist as glycosides. The flavonoids in citrus also include the flavone polymethoxylated flavone. This compound is represented by flavones substituted by methoxy groups and is unique to citrus. The polymethoxylated flavones have shown cholesterol and lipid lowering potential in animals and possibly humans, and the potential for treating diabetes and inflammation. Hesperidin is a flavanone glycoside (flavonoid) (found abundantly in citrus fruits. Its aglycone form is called hesperetin. It's name is derived from the Hesperides nymphs of Greek mythology. Hesperidin is believed to play a role in plant defense. It acts as an antioxidant according to *in vitro* studies (Hirata et al., 2005; Monforte et al., 1995). In human nutrition, it contributes to the integrity of the blood vessels. Various preliminary studies reveal novel pharmaceutical properties. Flavonoids are products of plant metabolism and have different phenolic structures (Guzmán and

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Navarrete, 2009; Ohtusuki et al., 2003). They are effective antioxidants because of their free radical scavenging properties and because they are chelators of metal ions (Trivedi et al., 2001); thus, they may protect tissues against free oxygen radicals and lipid peroxidation. Flavonoids may also be activated by mechanisms that apparently are not directly dependent on their antioxidative properties. Under certain conditions they may also behave as preoxidants (Garg et al., 2001). A wide range of different biological activities, including antibacterial, antithrombotic, vasodilatory, antiinflammatory, and anticarcinogenic effects mediated by different mechanisms, are associated with flavonoid compounds (Middleton et al., 2000). Hesperidin reduced cholesterol and blood pressure (Ohtsuki et al., 2003) in rats. In a mouse study, large doses of the glucoside hesperidin decreased bone density loss (Chiba et al., 2003). Hesperidin has anti-inflammatory effects (Galati et al., 1994; Kawaguchi et al., 2004). Hesperidin is also a potential sedative, possibly acting through opioid or adenosine receptors (Loscalzo et al., 2008). Hesperidin also showed the ability to penetrate the blood-brain barrier in an *in vitro* model. Infertility is one of the major health problems in life, and approximately 30% of infertilities are due to a male factor (Isidori et al., 2006; Carlsen et al., 1992).

Several conditions can interfere with spermatogenesis and reduce sperm quality and production. More factors such as drug treatment, chemotherapy, toxins, air pollutions and insufficient vitamins intake have harmful effects on spermatogenesis and sperm normal production (Mosher et al., 1991). Several studies have reported that antioxidants and vitamin A, B, C, and E in diet can protect sperm DNA from free radicals and increase blood testis barrier stability (Jedlinska-krakowska et al., 2006). Evidence suggests that certain phytochemicals found in citrus sources, such as flavonoids and limonoids, play a major role in treating or retarding chronic diseases, including anti-oxidative, anti-carcinogenic, cardiovascular protective, neuro-protective, bone health promotion and anti-inflammatory diseases. Antioxidants protect DNA and other important molecules from oxidation and damage, and can improve sperm quality and consequently increase fertility rate in men (Yang et al., 2006). Therefore, the role of nutritional and biochemical factors in reproduction and sub- fertility treatment is very important. The present study was planned to assess the ability of citrus to promote sperm parameters and modulate MDA concentration, spermatogenesis and oxidative stress. The results obtained will provide further insights into appropriate treatment of male patients by improving spermatogenesis and sperm parameters.

MATERIALS AND METHODS

Experimental animals

Adult Wistar albino male rats (n=30) were included in the present study. The rats were 8 weeks old and weighing 250±10 g each. They were obtained from animal facility of Pasture Institute of Iran. Male rats were housed in temperature controlled rooms (25°C) with constant humidity (40 to 70%) and 12 h/12 h light/ dark cycle prior to experimental protocols. All animals were treated in accordance to the Principles of Laboratory Animal Care [NIH]. All rats were fed a standard diet and water. The daily intake of animal water was monitored at least one week prior to start of treatments in order to determine the amount of water needed per experimental animal. Thereafter, the rats were randomly divided into control (n=10) and experimental (n=20) groups. The control group just received 4CC distilled water daily. However, the experimental groups split into two groups each included ten rates (Ct.1) received 400 mg/kg/rat and (Ct.2) received 600 mg/kg/day citrus extract for 30 consequence days. Body weight daily intake of food and water were determined several times per week throughout the study (Feng et al., 2001).

Phytochemical screening

Hesperidin as a major flavonoid in orange peel identified in Hyroalcoholic Extract by thin layer chromatography (TLC) on silica gel 60 F254 sheet (Merck, Darmstadt, Germany) with EtOAc:MeOH:H2O (80:15:1) as the mobile phase. After development, the plate were dried and sprayed with AlCl3 5% reagent to visualize the hesperidin spot at Rf=0.28.

Preparation of extract

Citrus sinesis fruit were collected from north of Iran. Peels were removed and dried in room temperature. 300 g powdered peels were extracted using maceration with ethanol (80%,v/v) for 24 h. The solvent was then evaporated under reduced pressure. This hydroalcoholic extract was kept in refrigerator for all experiments.

Surgical procedure

In thirtieth day, the Pentobarbital sodium (40 mg/kg) was administered intra peritoneal for anesthesia, and the peritoneal cavity was opened through a lower transverse abdominal incision. Thereafter testis in control and experimental groups were immediately removed. The weights of testis in each group were registered. The animals were decapitated between 9:00 and 11:00 AM, and blood samples were obtained. Blood samples were centrifuged at 4°C for 10 min at 250 × g and the serum obtained was stored at –20°C until assayed.

Epididymis sperm count, viability and motility

Sperms from the cauda epididymis were released by cutting into 2 ml of medium (Hams F10) containing 0.5% bovine serum albumin (Feng et al., 2001). After 5 min incubation at 37°C (with 5% CO_2), the cauda epididymis sperm reserves were determined using the standard hemocytometric method and sperm motility was analyzed with microscope (Olympus IX70) at 10 field and reported as mean of motile sperm according to WHO method (WHO, 1999).

Total antioxidant capacity (TAC), Malondialdehyde (MDA) and super oxide dismutase (SOD) concentration measurement in serum

A TAC detecting kit was obtained from Nanjing Jiancheng Bioengineering Institute-China. According to this method, the antioxidant defense system, which consists of enzymatic and

Table 1. The effect of the 400 and 600 mg/kg/rat citrus on Sperm parameters, TAC, SOD, MDA and testis weight of control and experimental groups in the rats.

	Control (n=10)	Ct. 1, (400 mg/kg-per day citrus) (n=10)	Ct.2 (600 mg/kg-per day citrus) (n=10)
Testis (gram)	1.38±0.33	1.48±0.55	1.49±0.55
Sperm concentration (total count) (No of sperm/rat ´10^6)	50.11±7.70	51.77±4.66	62.44±1.33*
Motility (%)	30.75±5.33	70±1. 44*	82±0.33*
Viability (%)	69.15±4.56	94.80±1.66*	96.80±0.11*
Total Antioxidant capacity (TAC),	0.61±0.55	0.92±0.016*	0.90±0.11*
Malondialdehyde (MDA), (mmol/ml)	3.90±0.55	1.55±0.12*	0.81±0.12*
Super oxide dismutase(SOD),(u/g Hb)	1000±0.55	1500±0.55*	1550±0.55*

Data are presented as mean ± SE.*Significant different at p< 0.05 level, (compared with the control group).

non-enzymatic antioxidants, is able to reduce Fe^{3+} to Fe^{2+}. TAC was measured by the reaction of phenanthroline and Fe^{2+} using a spectrophotometer at 520 nm. At 37°C, a TAC unit is defined as the amount of antioxidants required to make absorbance increase 0.01 in 1 ml of serum (Huang et al., 1995). Free radical damage was determined by specifically measuring MDA. MDA was formed as an end product of lipid peroxidation which was treated with thiobarbituric acid to generate a colored product that was measured at 532 nm (MDA detecting kit from Nanjing Jiancheng Bioengineering Institute-China) (Quintanilha et al., 1982). The activity of SOD was measured by following the method of Beyer and Fridovich (1987).

Statistical analysis

Statistical comparisons were made using the ANOVA test for comparison of data in the control group and the experimental groups. The results were expressed as mean ± standard error of means (S.E.M). Significant difference is written in parentheses.

RESULTS

Weight of individual male testis

The obtained results in this study illustrated in Table 1 are true. There was no significant difference in testes weights between the groups.

Results of sperm motility, viability and count

Administration of 400 and 600 mg/rat citrus extract for thirty consecutive days significantly increased sperm motility and viability in both experimental groups as compared with the control group. The motility and vitality were (73±4.35% and 95.80±1.68%) in Ct.1 and the corresponding values in Ct.2 were (81±5.33% and 98.80±80%). However, the motility and vitality in control group were significantly lower in comparison to the values in Ct.1 and Ct.2 (33.75±6.88% and 66.25±4.73%) (Table 1). In addition, sperm concentrations were similar in control and both experimental groups. The results were as follow: Control group, 48.68±7.70 mill/ml, Ct.1= 51.90±5.36 mill /ml and 61.60±2.34 mill/ml in Ct.2) (Table 1).

Results of total antioxidant capacity (TAC) and malondialdehyde (MDA) concentration measurement in serum

The mean concentration of MDA level was significantly ($p<0.05$) lower in Ct.1 (2.64±0.193) and Ct.2 (0.81±0.192) in comparison to control group (4.80±0.212). TAC was significantly higher ($p<0.05$) in Ct.1 (0.92±0.016) and Ct.2 (0.88±0.341) as compared with control group (0.53±0.77) (Table 1).

DISCUSSION

An herbal remedy is a type of alternative medicine that originates from plants and plant extracts. Used to heal illnesses and disease and to address psychological concerns, herbal remedies have been around for centuries, and were the precursor to modern medicine. Herbal remedies are obtained from a wide variety of natural resources including plant leaves, bark, berries, flowers, and roots. The citrus bioflavonoids include hesperidin (a glycoside of the flavanone hesperetin), quercitrin, rutin (two glycosides of the flavonol quercetin), and the flavone tangeritin. Flavonoids are phenolic compounds widely distributed in plants, which display a variety of biological activities, such as antioxidant, anti-inflammatory, blood lipid-lowing, and anticarcinogenic activities (Kipnis et al., 2001; Emim et al., 1994). In addition to possessing *in vitro* antioxidant activity and an ability to increase intracellular levels of vitamin C, rutin and hesperidin may have beneficial effects on capillary permeability and blood flow. They also exhibit anti-allergy and anti-inflammatory benefits of quercetin from *in vitro* studies. Quercetin can also inhibit reverse transcriptase,

part of the replication process of retroviruses (Khaki et al., 2010). The therapeutic relevance of this inhibition has not been established. Hydroxyethylrutosides (HER) have potential for use in the treatment of abnormal capillary permeability, bruising, hemorrhoids, and varicose veins (Emim et al., 1994). Hesperidin is a flavanone glycoside abundantly found in sweet orange and lemon and is an inexpensive by-product of citrus cultivation (Monforte et al., 1995). Hesperidin is effectively used as a supplemental agent in the treatment protocols of complementary settings. Its deficiency has been linked to abnormal capillary leakiness as well as pain in the extremities causing aches, weakness and night leg cramps. Supplemental hesperidin also helps in reducing oedema or excess swelling in the legs due to fluid accumulation.

A number of researchers have examined the antioxidant activity and radical scavenging properties of hesperidin using a variety of assay systems (Emim et al., 1994; Galati et al., 1994). Oxidants and antioxidants have attracted widespread interest in nutrition research, biology and medicine. It has become clear that constant generation of pro-oxidants, including oxygen free radicals, is an essential attribute of aerobic life (Sikka et al., 1996; Khaki et al., 2009). A disturbance in the prooxidant/antioxidant system has been defined as oxidative stress. Reactive oxygen species (ROS) are very reactive molecules ranked as free radicals owing to the presence of one unpaired electron such as a superoxide ion (O─2), nitrogen oxide (NO) and hydroxyl radical (HO─). Even though naturally present in the organism, they are mainly confined to cell compartments and counterbalanced by natural antioxidant molecules, such as glutathione, glutathione peroxidase, superoxide dismutase, vitamin E and vitamin C, acting as free radical scavengers (Sharma et al., 1996; Miller et al., 1993). Cellular damage in the semen is the result of an improper balance between ROS generation and scavenging activities. Excessive ROS production that exceeds critical levels can overwhelm all antioxidants defense strategies of spermatozoa and seminal plasma causing oxidative stress (Aitken et al., 1995; Sies, 1991).

Therefore, ROS production and TAC can be used as a marker of oxidative stress in seminal fluid and is correlated with male infertility. Infertile men with male factor or idiopathic diagnoses had significantly lower ROS-TAC scores than controls (Jedlinska et al., 2006). Besides, Said et al. (2005) suggested that abnormal sperm morphology combined with elevated ROS production may serve as a useful indicator of potential damage to sperm DNA. On the other hand, spermatozoa are highly susceptible to damage by excessive concentrations of ROS due to the high content of polyunsaturated fatty acids within their plasma membrane. The lipid peroxidation destroys the structure of lipid matrix in the membranes of spermatozoa, and it is associated with loss of motility and impairment of spermatogenesis (Said et al., 2005; Jedlinska et al., 2006). In the present study, administration of 400 and 600 mg/rat citrus extract for thirty consecutive days significantly increased sperm motility and viability in both experimental groups as compared with the control group, beside this research showed levels of TAC and SOD in both extracts groups were significantly increased and levels of MDA was decreased ($p<0.05$) and this results were consequential in 400 mg/kg extract (Table 1). These results are supported by the finding of Aitken et al. (1995), who reported that the conventional basic semen characteristics other than motility are not obviously influenced by the oxidative state of semen (Aitken et al., 1995; Feng et al., 2001).

This increase in sperm motility of experimental groups in comparison to control group could be due to the protective effect of citrus extract administration. Beside, these productive effects are reflected by the decrease of malonaldehyde level and increase in total anti-oxidants capacity (Table 1). In according with these results, Trivedi et al. (2011) showed Hesperetin protects testicular toxicity of doxorubicin in rat, prevention of oxidative stress, DNA damage and the cellular toxicity and protection against doxorubicin-induced germ cell toxicity was further evident from the sperm count and sperm head morphological evaluation. The role of nuclear factor-kappa B, p38 and caspase-3 on hesperetin-mediated protection against doxorubicin-induced testicular toxicity was confirmed (Trivedi et al., 2011). In conclusion, the present study has demonstrated that, citrus possess an antioxidant activity in doses 400 and 600 mg/rat citrus extract, and have useful effects on spermatogenesis and sperm parameters in rats.

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