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Research Article

Effect of Naringin nanocomposite in improving reproductive system efficiency in white male rats exposed to cyclosporine-induced oxidative stress

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Abstract

Naringin is a natural plant compound, found in the juice, flower, and peel of grapefruit and many other citrus fruits. Cyclosporine can affect the male reproductive system in a complex way and may cause male and female infertility when used for a long time. This work aimed to study the role of the nano-naringin compound in reducing the effect of cyclosporine on the efficiency of the reproductive system of male rats. The study conducted by 32 adult rats for 60 days that were divided randomly into four groups. Control group (C) was administrated by distilled water only, (T1) received CsA at the dose of 15mg/kg/day, (T2) CsA administered for 30 days, then nano-naringin at a dose 40mg/kg/day for 30 days, (T3) cyclosporine and nano-naringin have been administered simultaneously for 60 days. No significant differences were observed in the weight of the testis of rats treated with CsA, in contrast to the epididymis. The results showed a significant decrease in the level of gene expression of DDX3Y and LHr genes in testicular tissues of rats treated with CsA. CsA administration to rats led to a significant decrease in the level of Testosterone hormone and an increases in the level of luteinizing hormone. CsA treatment caused a significant increase in MDA level and significant decreases in GSH level and CAT activities when compared with the control group. The above parameters were improved to a near-normal level by the combined administration of nano-naringin. The results indicate a curative effect of nanonaringin as an antioxidant that attenuates the side effects of CsA on testicular injury.

Keywords: Cestode, Echinococcosis, Taenia, ELISA.

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Introduction

Nanoparticles (NPs) are small materials ranging from 1 to 100 nanometers that possess unique physical and chemical properties due to their increased surface area and nano-sized size, making them useful for many industrial and medical applications. Nanotechnology allows the introduction of a range of biomedical applications, such as tissue engineering i.e. tissue repair within the body, drug delivery to target cells, and biosensors through early disease detection (Nasrollahzadeh et al. 2019). Naringin (4´, 5, 7- trihydroxy flavonone -7- rhamnoglucoside) is a natural phytochemical pigment found in the peel of citrus fruits, which belongs to the group of bioflavonoids (Singh et al. 2019). Naringin regulates signaling pathways and interacts with signaling molecules giving it a variety of biological activities and pharmacological effects. These include anti-inflammatory, anti-viral, anti-carcinogenic, anti-mutagenic, free-radical scavenging, antioxidant, and immune-boosting activities (Chen et al. 2016). Its role in combating inflammation and oxidative

damage makes it a potential candidate for treating various disorders related to oxidative stress (Arafah et al. 2020). Accordingly, naringin has a broad pharmacological activity that may reduce CsA-induced testicular toxicity.

Cyclosporine (CsA) is one of the most important and widely used immune suppressants. It is used after organ transplants to reduce the chance of the body rejecting the transplanted organ (Kaminski 2008). It is an antibiotic produced by fungi as a lipophilic and hydrophobic cyclic polypeptide complex consisting of 11 amino acids (Duttagupta et al. 2016). CsA inhibits the production of interleukin-2, which leads to inhibition of T-lymphocyte differentiation and proliferation. It selectively inhibits immune responses mediated by T-helper lymphocytes (Amor et al. 2010). The major adverse effects of CsA are neurotoxicity, nephrotoxicity, hypertension, hyperglycemia, and gastrointestinal disturbances (Barbarino et al. 2013). CsA can affect the male reproductive system in a complex way and cause male and female infertility when used for a long time (Kant et al. 2009). This work aimed to study the role of the nano-naringin compound in reducing the effect of cyclosporine on the efficiency of the reproductive system of male rats.

Materials and Methods

In this experiment, 32 adult male Wister rats of 16-14 weeks' age and weighed between 200-225g were obtained from an animal house in the college of veterinary medicine at the University of AL-Qadisiyah. The animals were housed in ventilated wire-plastic cages (40×60cm) and reared under 12h light and 12h dark conditions at 22°C. The animals were allowed to acclimatize for 14 days before the experiment.

Cyclosporine A was obtained from medicine stores in the form of soft gelatin capsules under the traditional name of Sandimmun Neoral (Oral Solution; 100mg/ml; Novartis Pharma AG, Switzerland) and has been administered at a dose of 15mg/kg per day dissolved in 1ml of distilled water depending on the average body weight of the animal. The dose used in this study was proportional to the therapeutic doses of CsA, according to Freitas et al. (2013).

Naringin (98% purity) we obtained from drug stores in powder form, and the dose was prepared at a concentration of 40mg/kg of body weight, according to Rajadurai & Prince (2006). The method described by Guzman-Villanueva et al. (2013) was modified to prepare the nanocomposite. For this purpose, 2g of naringin was dissolved by adding 50ml of deionized distilled water and 50ml of absolute ethyl alcohol in a glass beaker. Then 2g of Chitosan (NP) carrier (which had a volume of 40nm) was weighed to obtain a 1:1 loading ratio, dissolved in 50ml of acetic acid, and its volume with deionized distilled water reached 100ml and stirred well by a hot plate magnetic stirrer for 30min at 900rpm. Then it was placed in a Probe Sonicator for 4min at a power of 600W with the addition of a drop and drop of the alcoholic naringin solution to mix and homogenize the solutions. After that, the solution is filtered with filter paper to eliminate the unconnected particles. The solution is placed in the electric oven at a temperature of 60°C for 24 hours for drying.

A total of 32 adult male Wistar rats were divided randomly into five groups i.e. eight animals for each group and treated for 60 consecutive days as follows: Control group (C) given 1ml distilled water orally, (T1) given CsA orally in a dose of 15mg/kg B.W dissolved in 1ml distilled water for 60 days, (T2) given CsA for 30 days then given nano-naringin orally at dose 40 mg/kg b.w/day for 30 days, and (T3) both CsA and nano-naringin have been administered simultaneously till the end of the experiment.

Twenty-four hours after the end of the experiment, rats were anesthetized by injecting 0.3ml ketamine+0.1ml xylocaine intraperitoneally and left three minutes for the anesthesia. The blood was taken from the heart directly using syringes, and been kept in plastic tubes without heparin, then the tubes were centrifuged for 15min at 3000rpm for obtaining the serum that kept at a temperature of -20°C until

Table 1. Show Primers of the Ddx3y, LHr and GAPDH regulator genes.

Gene	Primer	Sequence	Amplicon size (bp)
Ddx3y	Forward	ACTCCGGTACAAAAGCATGC	91
	Reverse	AAGCTGCCGTTTTTCCAGAC	
LHr	Forward	CTGAAAACACTGCCCTCCAAAG	95
	Reverse	TTCGGCAAATTCCTGAAGGC	
GAPDH	Forward	TGATGGGTGTGAACCACGAG	132
	Reverse	TCATGAGCCCTTCCACGATG	

NCBI- Reference Sequence: Testosterone subunit gene Ddx3y (NM_001108858.1), LHr NM_012978.2) and GAPDH (NM_017008.4).

laboratory tests are performed. The rats were dissected by opening the abdominal cavity to excise the right testis and the surrounding fatty and connective tissues were removed, weighed using a digital scale. A piece of the left testis fragment in Eppendorf containing Trizol has been kept at -20°C to assess the mRNA expression levels of the Ddx3y and LHr genes using Syber Green dye-based qRT-PCR technology.

Body Weight (g) gain was measured using the formula of Weight gain rate = final body weight - initial body weight and the weight of teste was calculated according to the following equation of Percentage of member's weight = Member weight (mg) / Final body weight (g) × 100.

Quantitative reverse transcription real-time PCR (RT-qPCR): Measuring the quantitative levels of mRNA was done to indicate the amount of gene expression of the Ddx3y and LHr genes. In addition, the GAPDH gene as a standard regulator gene has been used to calculate the gene expression, using a reagent kit (TRIzol® reagent) supplied by the Korean Bioneer Company and performed according to the company's instructions.

Measurements of hormones: The concentrations of hormones of testosterone and luteinizing hormone (LH) in the serum have been measured using the analysis kits for each of the aforementioned hormones produced by the Chinese company BT Lab.

Measurement of the level of oxidants and antioxidants in the serum

Assessment of MDA concentration: It was done using the modified method of Jo & Ahn (1998). This method depends on the interaction between lipid peroxides, mainly MDA, and Diethyl-2-thiobarbituric acid (DETBA) to give a pink color that is read at 532nm.

Determination of glutathione (GSH) concentration: The method is based on using Ellman's reagent detector as it reacts rapidly with GSH and is reduced by the sulfate group (SH group) of the clotathione, forming a color product whose absorption is read at 412nm (Sedlak & Lindsay 1968).

Catalase activity estimation: Evaluation of the activity of the enzyme catalase has been done according to Hadwan & Kadhum (2018). Its principle is based on the reactions of ammonium meta-vanadate with hydrogen peroxide H_2O_2 resulting in a red-orange peroxovanadium compound at wavelength 452nm as in the following reaction: $H_2O_2 + NH_4VO_3 + H2SO_4 \rightarrow NH_4 [VO(O_2)SO_4] + 2H_2O$.

Statistical analysis: SPSS (Version 27) was used to analyze data that expressed as means ± standard errors (SE). Differences between group means were estimated using a one-way analysis of variance (ANOVA) with the calculation of Least Significant difference (LSD) at the 5% probability level (Schiefer 1980).

Results

The results showed a significant decrease ($P<0.05$) in the mean weight gain of male rats treated with CsA (T1), and no significant difference ($P>0.05$) in the testes weights of the control group (C) compared to the other groups (Table 2). The results also showed the level of gene expression of DDX3Y and LHr in

Table 2. Effect of nano-naringin compound on body weight and testes weight, and on the level of gene expression of (Ddx3y, LHr) genes in male rats treated with cyclosporine for 60 days.

Groups / Parameters	C	T1	T2	T3
body weight (gm)	79.6±8.61[a]	29.2±9.62[d]	50.6±17.[2c]	66±4[b]
testes weight %	512.5±7.07[a]	507.5±35.93[a]	530±78.04[a]	532±41.12[a]
Ddx3y	1.424±0.90[b]	0.238±0.1[c]	1.440±1.07[b]	1.919±1.68[a]
LHr	1.397±0.09[c]	0.196±0.07[d]	1.870±0.52[b]	2.177±1.05[a]

Numbers = Mean±Standard Error (S.E). Different litters = Significant Differences (P<0.05). (C): The negative control group: has been given distilled water only. (T1): The group treated with CsA at dose of 15mg/kg of body weight. (T2): The group treated with CsA at dose of 15mg/kg, then has been given nano naringin at dose of 40mg/kg. (T3): The group treated with CsA at dose of 15 mg/kg and nano naringin at dose of 40mg/kg simultaneously.

testicular tissues of rats treated with CsA (T1) had a significant decrease (P<0.05) compared to other groups (Table 2).

Cyclosporine treatment (T1) results in a significant (P<0.05) decrease in serum testosterone levels and LH levels were also significantly increased (P<005) in the same group. However, concurrent administration nano-naringin for CsA-treated animals has almost naturally improved the levels of the above-mentioned hormones (Table 3). CsA treatment has been caused a significant (P<0.05) increase in the MDA and a significant decrease (P<0.05) in the GSH and CAT activities. In contrast, administration of nano-naringin to CsA-treated rats resulted in a decrease in MDA level and a significant improvement in GSH level and CAT activity compared to the CsA group (Table 3).

Discussion

Nano-drug delivery systems protect drugs from degradation, maintain their efficacy and increase their therapeutic efficiency by increasing the solubility of naringin, improving its biodistribution, and impeding its biotransformation, and thus can improve the performance of naringin in the treatment of various diseases and enhance its clinical applications (Budel et al. 2020). Despite its therapeutic importance, the oral bioavailability and biodistribution of naringin are low, and its limited permeability, low water solubility, and instability at gastric pH reduced its effectiveness (Zeng et al. 2020). Nallamuthu et al. (2021) showed that the nano-naringin compared to the free form, has a higher efficiency in scavenging free radicals; the nanoparticles can remove 62.5% of the DPPH radical compared to 55.2% of the free form and the high function of naringin in the nano form. This may be due to the enhancement of cellular uptake by Endocytosis.

The body weight index is an important parameter associated with risks to public health, and weight loss can indicate a deterioration in the health status of rats (Yousef et al. 2008). There was a significant decrease in the weight gain of CsA-treated rats, and these results agreed with the findings of Monteiro et al. (2008) and Lin et al. (2019). These rats are treated with the same CsA dose for the same period. However, some studies did not agree with these findings due to significant differences in the sensitivity of animals to CsA, which could be related to the dose and duration of treatment (Freitas et al. 2012). CsA decreases the appetite of rats to eat the food and impairs the intestinal absorption of glucose, cholesterol and some fatty acids, so the decrease in weight gain could be explained by changes in insulin secretion or impaired intestinal absorption of nutrients (Sigalet et al. 1992). Changes in drug-induced oxidative parameters result in significant oxidation of the amino acid pool and significantly affect lipids and proteins in parallel with cytotoxicity, causing a lower rate of weight gain (Alozhy et al. 2019). Naringin has a regulating effect on body weight and food consumption (Wang et al. 2021). Weight gain is linked to the availability and

Table 3. Effect of nano-naringin compound on the concentrations of (testosterone and LH) and on the level of (Malondialdehyde-glutathione-catalase) in male rats treated with cyclosporine for 60 days.

Groups / Parameters	C	T1	T2	T3
Testosterone (ng/L)	322.34±11.43^{a}	193.38±13.09^{d}	289.34±22.4^{c}	306.32±30.65^{b}
LH (mIU/ml)	3.08 ±0.25^{c}	4.42 ±0.31^{a}	3.47±0.37^{b}	3.17±0.18b^{c}
MDA (µmol/L)	1.34±0.06^{d}	3.52±0.21^{a}	1.92±0.15^{b}	1.56±0.09^{c}
GSH (µmol/L)	64.44±4.52^{a}	30.93±3.08^{d}	51.81±2.68^{c}	59.17±2.23^{b}
CAT (katal/ml)	3.20±0.18^{a}	0.91±0.21^{c}	2.07±0.25^{b}	2.90±0.71^{a}

Numbers = mean ± Standard Error (S.E). Different litters = Significant Differences (P<0.05). (C, T1, T2 and T3): the same as the previous indications.

absorption of nutrients. Naringin, when administered orally, increases the absorption of nutrients in the body by blocking some digestive enzyme actions so that no nutrients are broken down and absorbed directly into the bloodstream (Singh et al. 2019). Naringin also improves various digestive disorders caused by CsA by activating the ghrelin receptor, a receptor that promotes intestinal motility when activated (Jang et al. 2013).

Giving rats CsA drug according to this dose did not affect testis weight, and it was determined that the dose of CsA that decreases in the weights of male rats' genitals is 20mg/kg or higher (Seethalakshmi et al. 1990). The results show a significant decrease in the gene expression level of mRNA Ddx3y and LHr in testicular tissues of male rats treated with CsA. One of the main reasons for the decreased gene expression is DNA damage, which happens due to the formation of free radicals and reduced protein synthesis (Mahdi 2018). Wang et al. (2013) confirmed that the decreased Ddx3y gene expression results from hyperoxidation in the nuclear microenvironment. Nucleic acids and regulatory proteins in microenvironments within the nucleus are fragmented by free radicals because biological control of gene expression requires the regulation of genes in these environments within the cell nucleus (Zaidi et al. 2007). Decreased Ddx3y in testicular tissue is usually associated with severe failure of spermatogenesis and is closely related to male reproductive toxicity (Luddi et al. 2009). Ravula & Yenugu (2021) also reported that oxidative stress caused reproductive dysfunction in male rats and disrupted the expression of genes involved in germ cell production.

CsA significantly reduced the expression of LHr in Leydig cells, which affects the biosynthesis of testosterone and thus, CsA impairs natural steroid synthesis (Cavallini et al. 1989). It is known that CsA interferes with protein synthesis; there is a clear possibility that the drug interferes with receptor protein formation, resulting in a qualitative and quantitative reduction of LH receptors (Krueger et al. 1991). While in the groups treated with nano-naringin, a clear increase in the level of gene expression is observed, this improvement may be due to the role of nano-naringin in protecting testicular tissues because of its radical scavenging activity. Naringin showed antioxidant activity and the ability to protect DNA from free radical damage in rats exposed to oxidative stress (Akondi et al. 2011). In addition to supporting the enzymatic antioxidants, naringin has shown anti-lipid peroxidation activity and ROS generation. This results in the cell membrane's stability and integrity, thus reducing the leakage of enzymes into the bloodstream (Ali et al. 2020). Based on Maatouk et al. (2018), naringin at doses of 20 and 40 mg/kg body weight significantly reduced DNA damage suggesting its protective role against genotoxicity. Naringin maintains DNA biosynthesis by reducing DNA damage and increasing its repair capacity (Bacanli et al. 2015). All this leads to an increase in the gene expression of the studied genes. The presence of a significant decrease in testosterone concentration and a significant increase in the level of LH for male rats

treated with CsA has two indications: The first is that CsA within the used dose range not have an inhibitory effect on pituitary gonadotropin secretion. Elevated levels of LH combined with the low levels of circulating testosterone in the circulatory system in rats are indicative of the integrity of the hypothalamic-pituitary-testicular axis (Blanco-Rodríguez & Martinez-Garcia 1997). The second is that CsA affects the synthesis and secretion of testosterone in the testicle and reduces its levels in the serum. CsA has an inhibitory effect on the synthesis of testosterone in Leydig cells through a decrease in mitochondria and smooth endoplasmic reticulum, which are organelles that contain biosynthesis enzymes for testosterone (Cavallini et al. 1989). Rajfer et al. (1987) reported that oral CsA administration in doses equal to or greater than 15mg/kg per day for one month leads to a decrease in serum and intra-testicular testosterone levels in rats after using three doses of CsA of 7.5, 15, 30mg/kg of body weight. The deficiency in LHr receptors also underlies the failure of the testes to respond to elevated serum LH levels in rats when treated with CsA (Krueger et al. 1991).

There is an improvement and a rise in testosterone level compared to the drug group (T1) by treating with nano-naringin. The treatment with naringin at a dose 40 and 80mg/kg of body weight has increased the levels of low testosterone in rats treated with Bisphenol A (BPA) and this improvement may be due to the direct or indirect effect of naringin on preventing oxidative damage to cells (Alboghobeish et al. 2019). Also, the nano-naringin gradually improves the elevated LH. This improvement happens through an increase in the number of LHr receptors in the groups treated with nano-naringin due to the improvement in the testicular tissues. This increases the binding of LH with Leydig cells and stimulates increased biosynthesis of testosterone.

Increased MDA in serum and tissues were observed with CsA administration. There has been an increase in hydrogen peroxide production in living cells, leading to an increased formation of hydroxyl radicals (Khan et al. 2006). The presence of polyunsaturated fatty acids in the membrane of testicular tissue cells makes them more sensitive against oxidative stress (Aksu et al. 2016). The Raising ROS is directly related to the oxidative metabolism of CsA during oxidation by cytochrome P450 enzymes in the liver so that ROS leakage can occur (Zhong 1998). This was along with a decline in the antioxidant enzymes GSH and CAT's effectiveness because they are antioxidants contributing to preventing oxidation induced by the drug directly removing free radicals. These enzymes are depleted because they represent a line of defense against ROS toxicity and thus are reduced due to the increased demolition or lack of manufacture (Hudson 1999).

When CsA was treated with nano-naringin, a decrease in the MDA, an improvement, and an increase in the antioxidants GSH and CAT were observed. Aksu et al. (2018) demonstrated that the administration of naringin at different doses (50 and 100 mg/kg body weight) reduced oxidative stress in testicular tissues by increasing the intracellular enzymatic and non-enzymatic antioxidants inside cells to eliminate free radicals. The treatment with naringin has reversed the decrease in the level of GSH, and the antioxidant effects of naringin have been shown to be similar to those of GSH (Papasani et al. 2014). Thus, the treatment of naringin at a concentration of 40mg/kg with cyclosporine reduces the levels of lipid peroxides and hydroxyl radicals and re-regulates the levels of enzymatic and non-enzymatic antioxidants (Chandramohan & Parameswari 2013). Naringin has an important role in regulating antioxidant capacity by upregulating mRNA expression of SOD, CAT, and GSH-Px by inhibiting the activity of ROS-forming enzymes (Kanno et al. 2003).

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