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Aloe vera toxic effects: expression of inducible nitric oxide synthase (iNOS) in testis of Wistar rat

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ABSTRACT

Objective(s): Nitric oxide (NO), a product of inducible nitric oxide synthase (iNOS), contributes in germ cell apoptosis. This study was aimed to evaluate the effects of *Aloe vera* gel (AVG) on male Wistar rat reproductive organ, serum NO level, and expression of iNOS gene in leydig cells.

Materials and Methods: Adult male Wistar rats (n=36) were used for experiments in three groups. The experimental groups were orally administered with the AVG extract solution once-daily as follow: 150 $mg.kg^{-1}$; group A, 300 $mg.kg^{-1}$; group B, and only normal saline; group C (control group). They were mated with untreated females and the reproductive and chemical parameters were assessed for each group, including semen quality, serum testosterone, sperm fertility, gonad and body weight, serum NO concentration (by the Griess method), and iNOS gene expression (using RT-PCR).

Results: The testes weight, serum testosterone, as well as sperm count and fertility of the AVG treated groups were significantly reduced when compared to the control (P<0.001). Concentration of serum NO was significantly increased (37.1±4.63 µM) in the administrated group with higher AVG concentration, compared to the control group (P<0.001; 10.19±0.87 µM); however, iNOS mRNA expression was increased in the treated animals (P<0.001).

Conclusion: iNOS may play a functional role in spermatogenesis via apoptosis, reducing sperm count, but further studies are needed to illustrate the mechanisms by which AVG exerts its negative effects on spermatogenesis and sperm quality.

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Introduction

Nowadays, many plants are increasingly being used as medical plants (1-4). *Aloe vera* shows significant recuperative activities, such as repairing radiation skin damages and wounds, cancer therapeutic effects, as well as improving decubitus ulcers (2, 5-9). *A. vera* gel (AVG) contains high levels of carbohydrates (composed of long-chain polydispersed mucopoly-saccharides and a mannose monomer/acetyl), calcium malate, and protein as its predominant compositions (10-12). AVG enhances the release of several cytokines, including interleukins (IL-1, 2, and 6), interferon (IFN), granulocyte/monocyte-colony stimulating factor (GM-CSF) and tumor necrosis factor (TNF), as well as nitric oxide (NO) (11). Acemannan (ACM) is an important polydispersed mucopolysaccharide in AVG, known to have many pharmacological properties, including immune-stimulant, antiviral, antineoplastic, and gastrointestinal activities. Through stimulating the release of cytokines (e.g., IL-1, IL-6, TNF-α), AVG is able to activate macrophages and monocytes and produce NO (11, 13, 14). Recently, NO has shown several intra and inter cellular functions as a messenger and a basic role in regulation of male reproductive system, especially in human and rats; it has displayed autocrine and paracrine control over steroidogenesis of leydig cells (14-19).

In testis, macrophages are the most important sources of NO and control testicular NO signaling; indeed, during different phases of the reproductive cycle, a direct relationship exists between testicular macrophages and leydig cells (20, 21). Increasing of testicular macrophages at the beginning of testis recrudescence is related to the enhancement of leydig cells proliferation, reaching maximum

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number during breeding phase. However, throughout the reproductive cycle, the ratio of these reproductive cells to macrophages remains stable (1:3) (22). Low and high concentrations of NO stimulate leydig cells steroidogenesis, through the reproductive cycle soluble guanylatecyclase (GUCY1), and suppress their function, respectively (23).

To the author's knowledge, in spite of these early observations, the molecular mechanism of *A. vera* in male reproductive system has remained unclear. Since *A. vera* has an important role on NO signaling pathway in leydig cells, this paper evaluates the quantitative expression of nitric oxide synthase (NOS) in these cells. Moreover, it focuses on the possible side effects of AVG on rat male reproductive organ, sperm count and fertility, concentration of serum testosterone, gene expression of iNOS mRNA in testis, and serum NO concentration. The relation between variations in gene expression of inducible nitric oxide synthase (iNOS) mRNA in the testis with the relative weight of reproductive organ, sperm count and fertility, and concentration of serum testosterone has been also evaluated in this study.

Materials and Methods

Preparation of AVG extract

AVG was prepared from the fresh leaves (70-90 cm) as follow: the leaves were washed with clean water and cut transversely into slices and then the thick epidermis was selectively removed. The achieved gel was homogenized and lyophilized and thereafter was extracted using 95% ethanol. Its ethanol was evaporated in a rotary, under low pressure and dry condition, and the achieved extract was stored at 4 ˚C (24).

DPPH radical scavenging assay

The antioxidant activity of the prepared AVG was evaluated based on the radical scavenging ability of 2, 2-diphenyl-1-picrylhydrazyl (DPPH). The AVG stock solution was diluted with methanol at different concentrations: 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 µg/ml. The prepared DPPH methanol solution was added to these AVG solutions and allowed to react at room temperature. Finally, after 15 min, the absorbance values were measured at 517 nm and the activity was calculated by the following equation (25):

Ic_{50} (%) = 100 × ($A_{control}$-A_{sample})/$A_{control}$

Ic50 represents the sample concentration at which 50% of the DPPH radical was scavenged.

Total phenolic compounds of AVG

Total phenolic compounds were determined using a modified version of the Folin–Ciocalteu method. A low volume (0.1 ml) of the extract was added to 0.5 ml of Folin–Ciocalteu phenol reagent. The mixture was then allowed to stand for 5 min and 0.4 ml sodium carbonate was added to the mixture. The final mixture became blue and its absorbance was measured at 680 nm. The standard calibration curve was plotted with 12.5, 25, 50, 62.5, 100, and 125 $mg.l^{-1}$ of gallic acid in methanol and water (60:40, v/v). The amount of phenols was expressed based on $mg.g^{-1}$ in gallic acid equivalent (26).

Total flavonoid of AVG

Total flavonoid content of the methanolic extract was determined according to a colorimetric method. Briefly, a solution (1.5, 1, and 6 ml of 60% methanol, 2% aluminum, and 5% potassium acetate, respectively) was added to 1 ml of AVG extract, and kept at room temperature for 40 min. The absorbance of the final mixture was measured at 415 nm and the total flavonoids were expressed as the mg equivalent of Rutin per g of extracts (27).

Animals and treatment

Male Wistar rats (n=36; 8-week old) were purchased from Razi Institute, (Tehran, Iran) and randomly divided into 3 groups. The experimental groups were orally administered with the AVG extract solution as follow: 150 $mg.kg^{-1}$; group A, 300 $mg.kg^{-1}$; group B, and only normal saline; group C (control group) (28, 29). The rats were housed in a standard air-conditioned animal room; constant room temperature: 25±3 ˚C, humidity level: 10%, and a 12 hr light: 12 hr dark cycle, as well as food available *ad libitum*.

Tissue preparation

At the end of the experiment, the rats were weighted and deeply anesthetized with ketamine (110 $mg.kg^{-1}$) and xylazine (5 $mg.kg^{-1}$). Their testes were dissected out, weighted, and immediately frozen (-70 °C) until extracting their RNA content.

Sperm counting

Sperm counting was performed after dissecting out the epididymides. Briefly, the ducts were incised and their sperm content and epididymal fluid were mixed with saline solution (1 ml; pH=7.2) (30). The suspension was filtered through a nylon mesh and the number of sperms was evaluated using a counting chamber Neubauer (Deep 0.1 mm, LABART, Germany) (30). Five small squares per sample were counted in triplicate; the total sperm count was determined and expressed as million per milliliter.

Fertility and pregnancy test

At the end of experiment, each of the treated male was mated with three fertile females. Detection of sperm in the vaginal smear, as an excellent predictor of pregnancy and the presence of a vaginal plug, were the indicators of a successful mating. During

Table 1. Primers and probes used for RT-PCR of specific gene expression

Name	Primer and probe sequence
GAPDH	F: 5'-CCGAGGGCCCACTAAAGG-3' R:5'-GCTGTTGAAGTCACAGGAGACAA-3' 5'-(FAM) CATCCTGGGCTACACTGAGGACCA-3' (TAMRA)
iNOS	F: 5'-TGG TCC AAC CTG CAG GTC TT-3' R : 5'-CAG TAA TGG CCG ACC TGA TGT-3' 5'-(FAM) TGCCCGGAGCTGTAGCACTGCAT-3' (TAMRA)

pregnancy, changes in the body weight of female possessed vaginal plug were checked.

Radioimmunoassay of serum testosterone

The serum was separated from the blood specimens, which had been collected from the abdominal aorta of the treated rats, and stored at -20 ˚C. Testosterone kit (Demeditec Diagnostics GmbH. Lise-Meitner-Straße 2. D-24145Kiel, Germany), with an accuracy of 0.066 ng.dl, was used to measure the serum testosterone.

RNA extraction

According to the protocol of the manufacturer, total RNA was isolated from the rat testes using the Biozol total RNA extraction reagent (Bioflux, Japan).

Quantitative analysis of iNOS mRNA in the rat testis using real-time PCR (RT-PCR)

An aliquot containing 0.2 µg of total RNA was used for the reverse transcription reaction, which was conducted using the SuperScript first-strand cDNA synthesis system (Fermentas, Finland). The sequences of oligonucleotide primer and probe are shown in Table 1. The quantification of iNOS mRNA levels was analyzed using a Rotor-Gene 3000 (Corbett). RT-PCR reactions were performed in a total reaction volume of 10 µl, containing 1 µl of synthesized cDNA, 5 µl of Rotor-Gene Probe PCR Master Mix (Qiagen, Germany), 0.4 µl of each primer, and 0.2 µl of the TaqMan probe. Amplification program included a pre-warming step (5 min at 94 ˚C), denaturation step (94 ˚C for 15 sec), and an annealing/extension step (60 ˚C for 1 min). The mRNA expression levels of the other genes were normalized via quantification of GAPDH (glyceraldehyde 3-phosphate dehydrogenase), as a reference gene. The relative quantification of gene expression in each sample was analyzed using the 2ΔΔCt protocol and expressed as the ratio of related gene to GAPDH mRNA.

NO assays

Serum NO metabolites were assessed according to the Griess reaction supplemented by the enzymatic reaction of nitrite and nitrate with copper-plated cadmium (31). Briefly, Griess reagent was prepared from two solutions, including sulfanilamide and N-(1-naphthyl) ethylenediamine dihydrochloride, and stored at 4 ˚C. Nitrite and Griess reagent were mixed to form a purple azo dye in a reaction coil; its absorbance was measured at 540 nm by a flow-through spectrophotometer after 10 min. Finally, NO concentrations were determined relative to a standard graph derived from different concentrations of $NaNO_3$.

Statistical analysis

For the statistical analysis of the data, one-way analysis of variance (ANOVA) was used to compare the differences between the experimental groups and the control group (P<0.05 and 0.001), and the results were expressed as the mean±standard error (SEM).

Results

Standardization of A. vera extract

Total amount of phenolic compounds in *A. vera* extract was 17.2 mg galic acid equivalent per one g dried extract. Total amount of flavonoid compounds were 12.26 mg per one g of dry matter.

Table 2. Antioxidant activity of *A. vera* extract and Butylated Hydroxy Toluene (BHT), as a positive control)

Sample	Concentration (µg/ml)	Radical scavenging activity DPPH (µg/ml) (IC_{50}%)
Aloe vera extract	300	15.32
	400	12.98
	500	11.68
	600	12.20
	700	10.12
	800	9.87
	900	6.49
	1000	1.29
Butylated Hydroxy Toluene	10	22
	20	40.09
	25.41	50 (IC_{50})
	30	55.5
	40	78.3
	50	90.8

Table 3. Reproductive organ weight of male rats in different concentrations of *Aloe vera* gel (AVG) extract

Groups	Body weight (g)
Control	167.37±5.42
AVG 150 mg.kg^{-1}	165.45±3.44
AVG 300 mg.kg^{-1}	165.78±2.24

* Values are mean±SEM. Significant differences are indicated by *** (P<0.001)

Antioxidant activity of A. vera extract against DPPH

Different concentrations of *A. vera* extract showed low levels of radical scavenging activity against DPPH; calculation of IC_{50} was impossible in the higher concentrations (Table 2).

Relative body and reproductive organ weights in male adult rats

At the end of experiment, weight of testes in the AVG treated rats was significantly reduced compared to the control group (P<0.001), although body weight and seminal vesicle weight showed no significance difference between all of the groups (Table 3).

Spermatogenesis and sperm fertility

Spermatozoa concentration in the caudal epidi-dymis of the treated rats was significantly decreased compared to the control group (P<0.05; Figure 1). In addition, according to the percentages of the pregnant rats with vaginal plug, sperm fertility in the treated groups was significantly decreased (even more than 50 percent in 300 mg.kg^{-1} AVG concentration) when compared to the control group (P<0.05; Table 4).

Serum testosterone and NO levels

After 8 weeks of AVG administration, concentration of serum testosterone in the treated groups was statistically reduced in a dose-dependent manner, compared to the control group (Figure 2).

Figure 4 shows that higher concentration of AVG (300 mg.kg^{-1}) resulted in a dramatic increase in serum NO level, about four times higher than the control group (P<0.001).

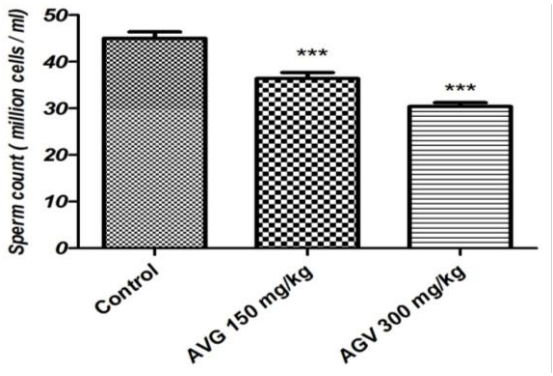

Figure 1. Sperm count in epididymis of the *Aloe vera* gel (AVG) treated rats and control group
*Values are mean±SEM. Significant differences are indicated by *** (P<0.001)

Table 4. Effects of oral administration of *Aloe vera* gel (AVG) on fertility in adult rats

Treatment groups	Pregnant female rats (%[a])
Control	100%
AVG 150 mg/kg	66.6%
AVG 300 mg/kg	45.5%

Quantitative RT-PCR analysis of the iNOS mRNA expression

Expression of iNOS mRNA in ledyig cells of the AVG treated rats was significantly increased compared to the control group (P<0.001). Expression of GAPDH served as a control to normalize the expressed mRNA of ledyig cells in all experimental groups.

Discussion

Nitric oxide plays several important functions in organisms and is synthesized from l-argenine through an enzymatic reaction of NOS. NOS possess three isoforms, including inducible (iNOS), neural (nNOS), and endothelial (eNOS) (32). The iNOS isoform, involved in immune response, releases high levels of NO, and its progressive generation leads to cytotoxic and neurotoxic effects. NO directly leads to DNA damage and thereby induces apoptosis in various cells (e.g., thymocytes, macrophages, B cells, neurocytes, and germ cells) (33-36). DNA damage is associated with increasing expression of the p^{53} tumor suppressor protein, known as the first indicator of NO induced apoptosis (37, 38).

Literature suggests that some compounds in *A. vera*, such as polysaccharides, stimulate the activity of testicular macrophages to produce iNOS. However, leydig cells steroidogenesis appears to be highly sensitive to the paracrine NO (39-41); NOS suppresses the conversion of cholesterol into pregnenolone through inhibition of the heme containing stero-idogenic enzyme (CYP17A1), thereby inhibits testosterone production. Furthermore, *in vitro* studies show that NO suppresses leydig cell functions through

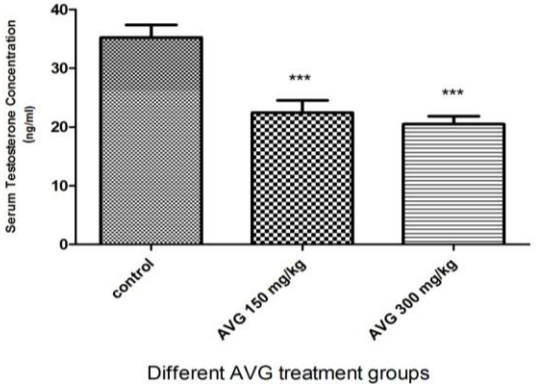

Figure 2. Effects of *Aloe vera* gel (AVG) on serum testosterone concentrations in the experimental groups
* Values are mean±SEM. Significant differences are indicated by *** (P<0.001)

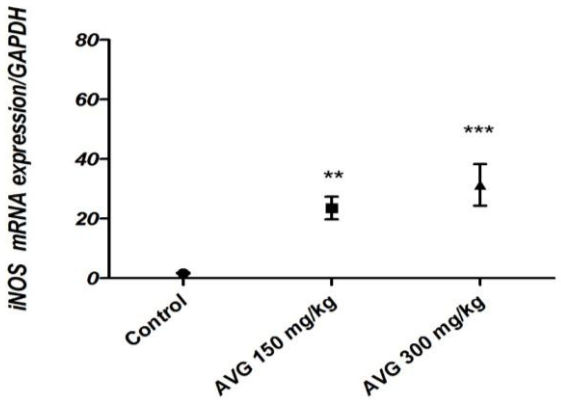

Figure 3. Quantitative expression of iNOS in leydig cells of the experimental groups

inhibiting the expression of CYP11A1, known as a side-chain cleavage enzyme of cytochrome p^{450} (42, 43). Therefore, the suppression of testosterone production in the AVG treated rats could be as a result of high iNOS level in these cells.

Mitochondria are the most important regulators in cell apoptosis. The key regulatory proteins of apoptosis are Bcl_2 and Bcl_x that promote cell survival by inhibiting the mitochondrial release of proapoptotic factors including Bax and Bak (44). NO is involved in mitochondrial dependent intrinsic signaling pathway, a key apoptotic pathway for male germ cell apoptosis (45, 46); iNOS induces release of cytochrome C and consequently the activation of the inhibitor caspase 9, the executioner caspases (3, 6, 7), as well as poly (ADP-ribose) polymerase (PARP) cleavage (33, 47-49). Therefore, our results showed a significant reduction in the relative weight of reproductive organ of the AVG treated groups. Moreover, epididymal sperm count decreased and iNOS expression increased in these groups. Therefore, it could be suggested that the probable induced apoptosis by AVG was the main cause of the

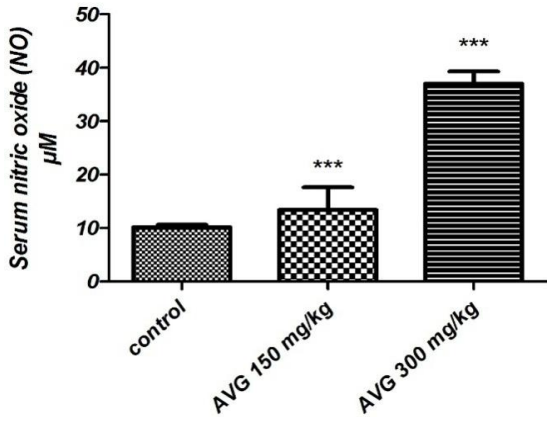

Figure 4. Serum NO concentrations in the experimental groups after 8-week administration of AVG
* Values are mean±SEM. Significant differences are indicated by *** (P<0.001)

reduction in sperm count, which in turn reduced the reproductive organ weight. These results are inconsistent with previous study showed that due to deficiency of iNOS in mice, a remarkable increase in testis weight and sperm output occurred after a testicular warming treatment (43 ℃ for 15 min) (36, 50).

Conclusion

The data confirm sever deleterious effects of AVG on fertility of male rat through overexpression of iNOs gene signaling pathway. Moreover, iNOS may play a functional role in rat spermatogenesis via apoptosis, and reduce the sperm count, but further studies are needed to illustrate the mechanisms and signaling pathway by which AVG exerts its negative effects of spermatogenesis and sperm quality.

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