Original Article

In Vivo, Molecular Evaluation for Improvement of Sperms Mitochondrial Functions by Fenugreek, Extract and Nanoparticles

Mohammed T. Jaafar[1], Hayder A. N. Al-Zamely[2], Adnan Mansour Jasim[3]

[1,3]Department of Physiology, Biochemistry and Pharmacology, College of Veterinary Medicine, Al-Qasim Green University, Al-Qasim, Babil, Iraq

[2]Department of Physiology, Biochemistry and Pharmacology, College of Veterinary Medicine, University of Al-Qadisiyah, Al-Diwaniyah, Iraq

Email: dr.mohammed.t.g@gmail.com

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Abstract

This study was carried out to investigate the possibility of using the fenugreek extract and fenugreek NPs to improve sperms mitochondrial functions in rats through estimation the level of testicular protein using the Real-Time polymerase chain reaction (RT-PCR). Totally, 60 Wister rats were selected, prepared and equally divided into three groups; negative control that remains without any treatment and given only distilled water daily; T1 that orally treated with a daily dose (100) mg/kg of extract fenugreek, and T2 that orally treated with a daily dose (100) mg/kg of fenugreek NPs. After an experimental period continued for 60 days, all study animals were euthanized with chloroform and collection of testis samples that subjected for RT-PCR examination through targeting SDHA, CS, and GAPDH genes. The findings revealed that the Ct of the calculated genes were increased in SDHA-fenugreek and CS-fenugreek when compared to GAPDH-fenugreek; as well as in values of SDHA-NPs and CS-NPs in comparison with GAPDH-NPs. Additionally, values of SDHA-control and CS-control were significantly higher than those observed in GAPDH-control. Regarding the fold change of genes, significant elevation (P□0.05) was reported in values of fenugreek NP in both SDHA and CS genes against the control as well as the fenugreek extract. In conclusion, Analysis of mitochondrial activity can give important additional information on the quality of a given spermatozoa sample, and highlights the importance of genetic analysis in confirming the expression of SDHA and CS genes. Additionally, the administration of both fenugreek extract and fenugreek NPs was revealed in a significant increasing in protein expression with a more significant effectiveness of fenugreek NPs rather than fenugreek extract. However, we recommended that the augmentation of fenugreek NPs and the proper regulation of steroidogenesis and mitochondrial biogenesis related genes is notably need to be furthermore studied. The toxicity of chitosan NPs needs to be evaluated, in particular in embryo development aspects, because extensive research into their biomedical applications was based largely on the biodegradable and biocompatible profile of chitosan.

Keywords: Testis, SDHA gene, Chitosan, NP, PCR, Iraq

INTRODUCTION

Many mitochondrial functions are derived from ancient mitochondrial precursors also established in eukaryotic cells (Amaroli et al., 2022). Eukaryotic cells depend on mitochondrial function and thus require continuous regulation of mitochondrial production to meet energy demands and cellular stress. Eukaryotes also provide metabolic cooperation between different cells through the involvement of mitochondria (Walker and Moraes, 2022). However, endosymbiotic relationships determine the presence of various mitochondrial injuries, including a combination of proteotoxic stress, oxidative damage, immune activation of free mitochondrial contents, and transformation (Perdigoto, 2021). Sperms are composed of important component of mitochondrial parts that act as a link between sperm mitochondrial defects and decreased sperm motility (Anifandis et al., 2017). The molecular level has revealed that deletions and other changes to mitochondrial DNA (mtDNA) can result in decreased sperm functionality and male infertility. Comparing sperm from asthenozoospermic samples to control samples revealed that sperm from the first samples contain abnormally high levels of specific mitochondrial RNAs (mtDNA), as well as, transcripts encoding mitochondrial proteins that are encoded by the nucleus (Nowicka-Bauer et al., 2018; Ferramosca et al., 2021). Another significant discovery has been the relationship between the activity of sperm mitochondrial enzymes including the mitochondrial electron transport chain (ETC) complexes in addition to a variety of sperm parameters such as concentration, vitality and motility among other characteristics (Zhu et al., 2019).

Recently, the negative effects of chemicals with shortcomings of certain ailments have made traditional medical practices increasingly visible. Fenugreek (Trigonella foenum gracum) is one of the most globally medicinal plants which demonstrated to having many amazing medicinal properties (Bahmani et al., 2016; Srinivasa and Naidu, 2021). Many authors believe that fenugreek originates from the Mediterranean region, but at present, this plant is distributed in United States, Middle East and Southeast (Basu et al. 2019). Fenugreek contains various compounds including steroidal sapogenins present in fenugreek seed oil such as components of diosgenin, in addition to alkaloids such as coumarin, niacin, trimethylcoumarin and trigonelline. Nanoparticles (NPs) are an active area of research and a highly technological economic field with many applications in particular biological applications, cellular imaging, and photothermal therapy carried out on the basis of physical characteristics (Azlan, 2021). NP carriers have a major challenge with proper surface chemistry, preparation, immunoassays, thermolysis, and drug differentiation (Xie et al., 2019), and this because drugs can be effectively delivered to target sites thereby increasing therapeutic efficacy while reducing side effects (Yang et al., 2017). Chitosan is a biodegradable, biocompatible polymer regarded as safe for human dietary use and approved for wound dressing applications. Chitosan has been used as a carrier in polymeric nanoparticles for drug delivery through various routes of administration. Chitosan has chemical functional groups that can be modified to achieve specific goals, making it a polymer with a tremendous range of potential applications (Mohammed et al., 2017). Nanoparticles (NP) prepared with chitosan and chitosan derivatives typically possess a positive surface charge and mucoadhesive properties such that it can adhere to mucus membranes and release the drug payload in a sustained release manner (Seyam et al., 2020).

Male sperm activity must be maintained at normal level in order to ensure the normal male fertility and to avoid a male infertility. Therefore, this study was carried out to investigate the possibility of using the fenugreek extract and fenugreek NPs to improve sperms mitochondrial functions in rats through estimation the level of testicular protein using the molecular assay, Real-Time polymerase chain reaction (RT-PCR).

Materials and methods

Ethical approval

The current study was performed under the license of the Department of Physiology, Biochemistry and Pharmacology, College of Veterinary Medicine, Al-Qasim Green University (Al-Qasim, Babil, Iraq).

Preparation of fenugreek extract and NPs

Initially, a total 300 g of fenugreek seeds were purchased from the local market of herbal medicine in AL-Qasim city, grinded using a blender max. The powder of fenugreek seeds was dissolved then into a totally 1000 ml of alcohol solution, hydroalcoholic acid (70%), with mechanical shaking by magnetic starrier at 55°C for 6 hours. The mixture was filtered, dried under the vacuum for 20 hours, incubated at 37ºC for 36 hours, and stored in deep freezes at -20ºC (Banso and Adeyemo, 2006). Chitosan was used to prepare of the NPs by the ionic gelation method with some modifications (Agarwal et al., 2018; Shafiei et al., 2019).

Study design and sample collection

Sixty Wister rats were randomly selected and subjected for preparatory period; during which, they kept at 23 ± 2ºC, fed on basal laboratory diet and tap water, and exposed to 12/12 light and dark conditions. After one week, the study animals were equally divided into three groups as following:

1. First group (Negative control group): Rats of this group still without any treatment and given only distilled water daily.

2. Second group (T1): Rats of this group treated with a daily dose (100) mg/kg of extract fenugreek that given orally by stomach tube.

3. Third group (T2): Rats of this group treated with a daily dose (100) mg/kg of fenugreek NPs that given orally by stomach tube (Al-Zamely and Kshash, 2021). After an experimental period continued for 60 days, all study animals were euthanized with chloroform and subjected for sampling of testis.

Molecular examination

According to manufacturer instructions of RNA Extraction Kit (Intrron, Korea), a part of testicular tissues for animals of each study group were subjected for extraction of RNAs. Three sets of primers were designated for detection of SDHA gene [(F: 5'-AACACTGGAGGAAGCA CACC-3') and (R: 5'-GCAACTCGAGTCCCTCACAT-3')] (Piantadosi and Suliman, 2008), CS gene [(F: 5'-CCGTGCTCATGGACTTGGGCCTT-3') and (R: 5'- CCCCTGGCCCAACGTAGA TGCTC-3')] (Siu et al., 2003), and GAPDH gene [(F: 5'- ATGACTCTACCCACGGCAAG-3') and (R: 5'- CTGGAAGATGGTGATGGGTT-3')] (Kunst et al., 2013) at a product size of 302 bp, 176 bp and 89 bp, respectively. Then, the Mastermix tubes were prepared using the GoTaqTM qPCR Mastermix kit (Intrron, Korea) from the primers of each gene at a final volume of 25µl, and then subjected to the conditions of the thermal Cycler GoTaq® 1-Step RT-qPCR System as following: 1 cycle Reverse transcription (42ºC for 15 min), 1 cycle RT inactivation / Hot-start activation (95ºC for 10 min), 40 cycles for denaturation (95ºC for 10 sec), annealing / data collection (60ºC for 30 sec), extension (72ºC for 30 sec) and 1 cycle dissociation.

The obtained experimental data results from the qRT-PCR for both target and housekeeping gene were statistically analyzed using the fold change test based on the relative quantification of gene expression levels. The fold change method is originally described by Livak and Schmittgen (2001).

Statistical analysis

All collected data were analyzed by ANOVA at P□0.05 in GraphPad Prism Software (version 6.0.1), (Al-Gharban and Al-Taee, 2016; Gharban et al., 2019).

Results

The findings of molecular examination were revealed a significant variation in values of amplification plot of SDHA, CS as targets as well as GAPDH as house-keeping gene (Figure 1).

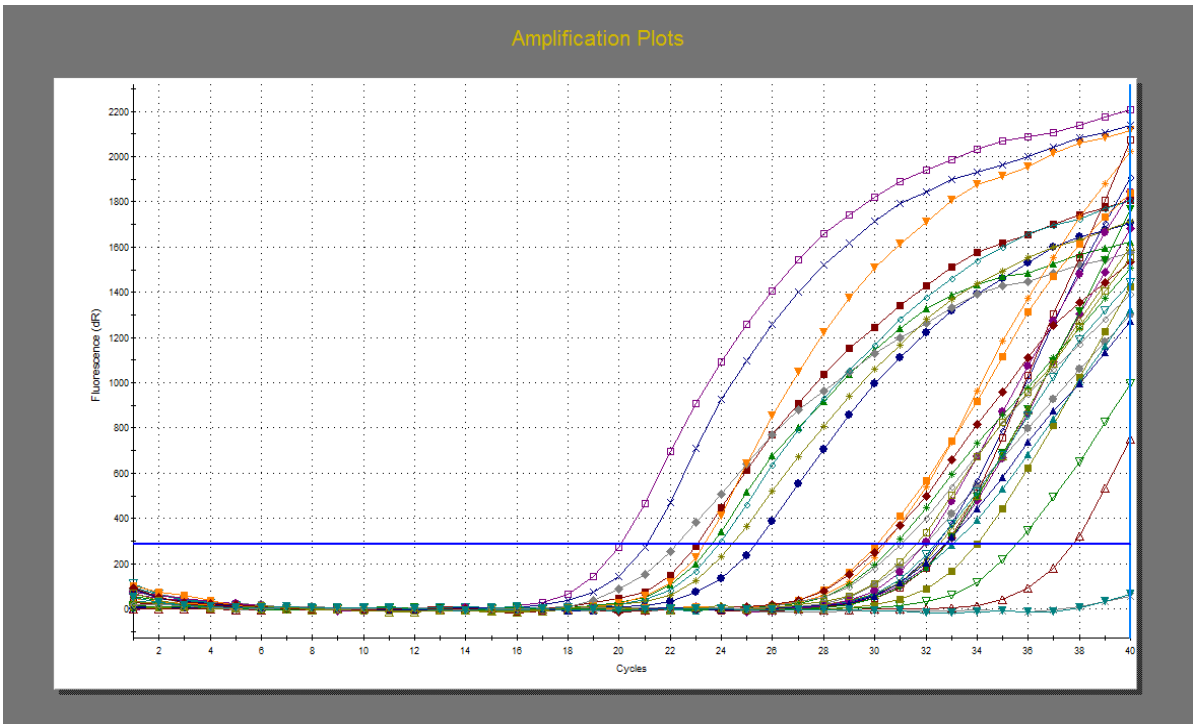

Figure (1): Amplification plot of SDHA, CS as targets; and GAPDH as a house-keeping gene by the Mx3005P Stratagene system

The Ct of the calculated genes were increased in SDHA-fenugreek (33.54 □ 0.29) and CS-fenugreek (32.3 □ 0.18) when

compared to GAPDH-fenugreek (23.003 □ 0.41); as well as in values of SDHA-NPs (32.41 □ 0.24) and CS-NPs (31.74 □ 0.23) in comparison with GAPDH-NPs (23.82 □ 0.4). Additionally, values of SDHA-control (32.7 □ 0.11) and CS-control (31.17 □ 0.25) were significantly higher than those observed in GAPDH-control (21.7 □ 1.15), (Table 1).

Table (1): Cycle threshold (CT) values of the calculated genes

Group	Value	Group	Value	Group	Value
GAPDH-Fenugreek	23.07	SDHA- Fenugreek	33.6	CS-Fenugreek	32.57
GAPDH- Fenugreek	23.67	SDHA- Fenugreek	34	CS-Fenugreek	32.39
GAPDH- Fenugreek	22.27	SDHA- Fenugreek	33.02	CS-Fenugreek	31.95
M ± SE	23.003 ± 0.41	**M ± SE**	33.54 ± 0.29 *	**M ± SE**	32.3 ± 0.18 *
GAPDH-NP	24.47	SDHA-NP	32.8	CS-NP	32.11
GAPDH-NP	23.92	SDHA-NP	32.46	CS-NP	31.77
GAPDH-NP	23.08	SDHA-NP	31.97	CS-NP	31.33
M ± SE	23.82 ± 0.4	**M ± SE**	32.41 ± 0.24 *	**M ± SE**	31.74 ± 0.23 *
GAPDH-C	20.09	SDHA-C	32.47	CS-C	30.83
GAPDH-C	23.92	SDHA-C	32.79	CS-C	31.02
GAPDH C	21.1	SDHA-C	32.83	CS-C	31.66
M ± SE	21.7 ± 1.15	**M ± SE**	32.7 ± 0.11 *	**M ± SE**	31.17 ± 0.25 *

Significance * (P□0.05), NS: Non-significance (P□0.05)

Regarding the fold change of genes, significant elevation (P□0.05) was reported in values of fenugreek NP in both SDHA and CS genes against the control as well as the fenugreek extract (Tables 2-4).

Table (2): Results of fold change of the study genes

Gene/ Sample	Fold change	Gene/ Sample	Fold change
SDHA- Fenugreek	1.56193383	CS- Fenugreek	1.10700878
SDHA- Fenugreek	1.79419082	CS- Fenugreek	1.90087896
SDHA- Fenugreek	1.3410224	CS- Fenugreek	0.97715997
M ± SE	1.566 ± 0.131	**M ± SE**	1.328 ± 0.289
SDHA- NP	7.17676327	CS- NP	4.0185267
SDHA- NP	6.20457905	CS- NP	3.47416595
SDHA- NP	4.86801405	CS- NP	2.63292544
M ± SE	6.083 ± 0.669 **	**M ± SE**	3.375 ± 0.403 *

Significance * (P□0.05), ** (P□0.01), NS: Non-significance (P□0.05)

Table (3): Statistical results of the fold change of SDHA gene

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significance	Summary	*p-value*
C vs. Fenugreek extract	-0.5657	-2.274 - 1.143	No	NS	0.5945
C vs. Fenugreek NP	-5.083	-6.791 to -3.375	Yes	***	0.0002
Fenugreek extract vs. Fenugreek NP	-4.517	-6.226 to -2.809	Yes	***	0.0005

Significance * (P□0.05), NS: Non-significance (P□0.05)

Table (4): Statistical results of the fold change of CS gene

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold	Summary	*p-value*
C vs. Fenugreek extract	-1.3283	-1.570 for 0.9137	No	NS	1.7104
C vs. Fenugreek NP	-3.375	-3.617 for -1.133	Yes	**	1.0026
Fenugreek extract vs. Fenugreek NP	-3.047	-3.289 for -0.8048	Yes	**	1.0056

Significance * (P□0.05), NS: Non-significance (P□0.05)

Discussion

Previous reports revealed the role of mitochondria role to regulation cell death. Under certain conditions, such as stress, profound changes occur in mitochondria, including mitochondrial energy expenditure, energy release, inflammation, and internal damage (Orrenius et al., 2015; Bock and Tait, 2020). Worldwide, the number of preventable male infertility cases is increasing, suggesting that the underlying pathophysiology of testicular dysfunction is unknown (Djari et al., 2021; Wu et al., 2021). Assisted reproductive technology has improved the treatment of male infertility, but the technology has raised concerns about potential risks such as genetic defects, structural defects, and potential mechanical and biological damage (Hindryckx et al., 2010; Glaser, 2015; Ribas-Maynou and Yeste, 2020). This fact suggests that further studies are needed to provide detailed information on the role of mitochondrial proteins in spermatogenesis in vivo by assessing potential stress.

Mitochondrial dysfunction, the mitochondrial respiratory chain, and the discovery of the related protein SDHA and CS, the first enzyme to catalyze the formation of oxaloacetate and citrate from acetyl-CoA in the tricarboxylic acid cycle, were the aim and outcome of this study shown an important increase expression of this protein. Our results suggested that increased expression of these proteins may play an important role in testicular and non-testicular impotence. Lee et al. (2017) showed that SDHA is a good choice for pig breeding, allowing constant expression in real-time quantitative PCR and allowing normalization of genes of interest.

Previous studies have found statistical differences in the expected uptake of her SDHA genotype between infertile patients and controls, suggesting that SDHA could control and regulate spermatogenic events. However, changes in the activity of SDHA and CS enzymes associated with the efficiency of spermatogenesis are not the result of genetic or SNP changes, so the activity of these enzymes should be assessed at the time of implantation and/or level of translation (Ruiz-Pesini et al., 2000; Bonache et al., 2007). A recent report indicates that the SDH subunit is the only mitochondrial resting-state complex that is fully encoded at the nuclear level. The efficiency of the spermatogenic process (measured by sperm concentration within the plant) depends on the activity of mitochondrial enzymes produced in the nucleus (Alston et al., 2017; Rasheed and Tarjan, 2018; Barros and McStay, 2020). Other studies have shown that many SDHA mutations may open future medical targets (Dahia (2014) and is an oncogene that prevents uncontrolled cell growth and division (Jakoube et al., 2021). Using a mouse model, the National Reproductive Science Research Group found that CS in semen acts as a sperm factor that activates the fertilized egg. Furthermore, their study showed that CS can cause hypersensitivity, senescence, and an inability to synthesize citrate, which plays an important role in sperm aging, suggesting possible infertility due to susceptibility (Kang et al., 2020; Rahimizzadeh et al., 2020).

Various recent studies have shown that TCA dysfunction (tricarboxylic acids are the main source of cellular energy and are involved in many metabolic processes within cells) causes human diseases such as neuro-metabolic disorders and tumors is shown. These studies therefore demonstrate that CS can use this modified SDHA to control the reductive and oxidative orientation of the TCA cycle (Liu et al., 2021). In addition, metabolomics analysis of aged sperm indicated that loss of CS increased her TCA turnover in mitochondria with age, which could lead to a decrease in extra-mitochondrial citrate. Data from other studies show that CS is age-related in male fertility, suggesting that male fertility declines with age (Nunoura et al., 2018; Kang et al., 2020, 2021).

Conclusion

Analysis of mitochondrial activity can give important additional information on the quality of a given spermatozoa sample, and highlights the importance of genetic analysis in confirming the expression of SDHA and CS genes. Additionally, the administration of both fenugreek extract and fenugreek NPs was revealed in a significant increasing in protein expression with a more significant effectiveness of fenugreek NPs rather than fenugreek extract. However, we recommended that the augmentation of fenugreek NPs and the proper regulation of steroidogenesis and mitochondrial biogenesis related genes is notably need to be furthermore studied. The toxicity of chitosan NPs needs to be evaluated, in particular in embryo development aspects, because extensive research into their biomedical applications was based largely on the biodegradable and biocompatible profile of chitosan.

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