Attenuation of Senescence-Induced Oxidative Exacerbations in Aged Rat Testis by Ferula Elaeochytris Root Extract

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ABSTRACT
Objective: Age brings about changes to the oxidant and antioxidant balance of male testis that give rise to loss of fertility. The Ferula elaeochytris root extract (FE), contents antioxidant and anti-inflammatory component, have been used to treat infertility by local people for centuries. The main objectives of this study were to determine whether FE was effective on sperm quality, spermatogenesis, apoptosis and oxidative stress in aged rat.

Methods: Four groups were formed with 40 rats; young Control (YC), Aged Control (AC), Ferula elaeochytris administrated aged rat (A+FE) and vitamin E administrated aged rat (A+VE). Vitamin E and FE was administered orally for 8 weeks.

Results: The administration of FE significantly increased serum TAS, testosterone levels and decreased testicular malondialdehyde (MDA) activity, that these changes were accompanied by the reduced serum TNF-α, and TOS levels. Also, the apoptosis germ cell, the tubular diameter, the germinal epithelium height and Johnson’s score a have been regulated after administration of FE (p < 0.05). Meanwhile, in the present context, in aged group the sperm count, motility, testicular weight declined significantly. FE showed showed significantly increased effect on the motility and sperm count.

Conclusive: These findings support that aging induces stress oxidative and inflammation, and FE could protect the testis against these damaging effects via its anti-oxidative, anti-inflammatory action and modulate spermatogenesis.

Keywords: Ferula elaeochytris, Aging, Antioxidant system, Spermatogenesis.

INTRODUCTION
In parallel with the increasing world population, the world’s elderly population has promoted considerably in the last 20 years and it is obvious that it will gradually increase in the coming years. It is well known that aging is a long process that causes changes in most organ structures in the body. Although environmental and genetic factors are many factors that accelerate the aging process, it is widely accepted that aging is the most important cause of oxidative damage, which is caused by reactive oxygen species (ROS)1. The expression of enzymatic and non-enzymatic antioxidants (tocopherol, glutathione, etc.) systems that protect cells from ROS decreases with aging, and therefore the mechanism of protection from oxidative stress slows down1,2.

Despite the structural changes in all tissue structures due to chronic oxidative stress with aging process, the testicles are more sensitive because they produce steroids and have a weak antioxidant system3. Therefore, in addition to chronic diseases associated with aging, prevention of reproductive aging has become important recently. In order to reduce chronic oxidative stress in the aging process, more importance has been given to natural herbal resources that will reduce the accumulation of oxidative stress, as well...
as suggestions such as exercise, sports and diet that change life habits. Ferula species, grows naturally from the Mediterranean region to central Asia, is well known to contain substances that beneficial effect such as antimicrobial, antitumor, anticoagulant, antihyperlipidemic, antioxidant, anti-inflammatory, aphrodisiac, cytotoxic, antidiabetic, antispasmodic, anti-uloscerate and hepato-protective effects.

Ferula elaechochrysis, a species of Ferula genus, has been consumed for centuries by the local community as an aphrodisiac as well as for stimulating the mating of goats and sheep. Previous studies have found strong evidence that FE has some therapeutic and vitamin E, and C, type of vitamin E, and C. Large amounts of flavonoid compounds, as well as tocopherol, a main type of vitamin E and C. Ferula species have been reported to find large amounts of flavonoid compounds, as well as tocopherol, a type of vitamin E, and C. Therefore, the study aimed to investigate whether FE can prevent some testicular damage due to aging, by comparing it with vitamin E, which has a protective effect on the testicle.

METHODS
Preparation of Plant Extract
Extract was extracted using the Soxhlet method and stored in the refrigerator after extracting.

Experimental Animals
Twenty young male Sprague-Dawley rats (4 months; body weight 360 to 375 g), aged rat (24 months; body weight 390-420) were used in the study.

Experimental Procedure
A total of 40 rats were used. There were 10 rats in each group and the study was divided into 4 groups. The dose of F. elaechochrysis and vitamin E were administered (40 mg/kg, 50 IU/kg/day p.o. for 8 weeks 50U/kg, respectively) as described in previous studies.

Young Control: Adult (4 months-aged) rats, Control.
Aged Control: old (24 months-aged) rats, Control.
Aged+ Ferulo Elaeochytris: old (24 months-aged) rats administered F. elaechochrysis
Aged+ Vitamin E: old (24 months-aged) rats administered vitamin E

Main Points:
- Ferula extract boosted the spermatogenesis via modulation of oxidative stress, increased the apoptosis and proinflammatory cytokines such as TNF-α.
- Ferula extract is a versatile compound and can be used in advanced stages in the treatment of many diseases associated with hormonal disorders and oxidative stress.
- Examination of the effects of Ferula extract, which is used in the treatment of many diseases today.

Serum and Tissue Preparation
When the experimental part of the study was completed, no animal deaths were observed. At the end of the experiment, blood samples were centrifuged and their serums were collected. Some of the testicles were fixed in Bouin's solution for use in histopathological studies.

Sperm Quality
The evaluation of spermatozoa was performed as in previous studies. Sperm analysis was used with the microscope to evaluate spermatozoa parameters. Motility parameters from at least 10 areas were analyzed and 1000 sperms per sample were evaluated. Meanwhile, spermatozoa was treated with 1% Eosin to evaluate for sperm Vitality. While living spermatozoa that absorb the dye appear red, inanimate ones are colorless.

Histological Study
Twenty seminiferous tubules were taken into account for each rat under the light microscope, and the evaluations were evaluated after 5 Research Spermatogenesis Johnson scores. The Johnson score applies a score of 1 to 10 for each seminiferous tubule in each seminiferous tubule cross-section. In briefly, the scoring was as follows: score 10: Germinal epithelium is multi-row, there are many spermatozoa, score 9: Germinal epithelium is disorganized and agglomerated towards the lumen, there is spermatozoa, 8: Germinal epithelium is multi-rowed, but there are less than 10 spermatozoa in the lumen, 7: No spermatozoa, There are many spermatids, 6: No spermatids, less than 10 sperm- matids, 5: No spermatozoa, no spermatids, there are spermatocytes, 4: No spermatozoa, no spermatids, less than 5 spermato- cytes, 3: Only spermatogonia as germ cells, 2: No germ cells, only Sertoli cells, 1: No cells in the seminiferous tubulus.

DNA fragmentation-associated apoptosis was determined by the TUNEL assay. Apoptotic germ cell ApopTag Plus Peroxidase was designed using the InSitu Apoptosis Detection Kit (Chemicon, catno: S7101, USA) in accordance with the kit's procedures, and cells showing brown nuclear staining were considered positive in the evaluation of TUNEL staining. All prepared preparations were examined, photographed, and examined under a research microscope (Olympus, BX51, Japan). Staining index was determined by counting nuclear staining in at least 500 cells in 10 randomly selected microscopic fields.

Biochemical Analysis
Serum catalase (CAT), glutathione (GSH), and malondialdehyde (MDA) levels were determined by the methods described by Uchiyama and Mihara, respectively. Also, the activity of total antioxidant status (TAS) and Total oxidant status (TOS) (Product Code: RL0017 and Product Code: RL0024, Rel Assay Diagnostics® Mega Tip Ltd., Gaziantep, Turkey), as well as the levels of Testosterone (DRC testosterone ELISA, catalog num. E1A-1559) and Tumor necrosis factor-alfa (TNF-α) (cat. ab46070, UK) in serum were determined according to the ELISA kit providers instructions.

Statistical Analysis
Data were analyzed using the SPSS program version 24.0 (SPSS Inc., Chicago, IL, USA). The data were expressed as mean ± SD.
One-way ANOVA analysis of variance was performed, followed by post hoc Tukey tests to find changes between individual groups. Significant value was determined as $P < 0.05$.

**RESULT**

**Effect of FE on Body and Testis Weights**

While in YC and AC groups rat the body weight of rat increased by the end of the 8-weeks period, the testis mean weight decreased (Table 1). Moreover, there was described to statistical difference in body weight between the AC and A+FE ($p<0.05$). The testis weight increased in the A+FE group compared to AC, which was statistically significant ($p<0.05$) (Table 1). Result indicated the AG+FE exhibited significant decreased in food intake the ($p<0.05$), as compared to the AG. However, the water intake was increased in A+FE compared the AG (Table 1).

| Table 1. The effect of FE on food, Water intake and body, testicular weight |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Groups   | Initial body weight (gr) | Final body weight (gr) | Absolute testis weight (mg) | Relative testis weight (as% body weight) | Food intake (g/day) | Water intake (ml/day) |
| YC       | 289.1±10.6         | 318.3±10.3        | 1.62±0.04       | 0.49±0.002       | 19.78±6.65       | 28.5±4.54         |
| AC       | 372.6±11.5a        | 394.3±11.1a       | 1.48±0.031a     | 0.37±0.007a      | 28.58±7.05a      | 29.8±3.83         |
| A+FE     | 373.4±10.9         | 348.9±9.3b        | 1.55±0.07b      | 0.44±0.006b      | 25.18±4.34b      | 41.8±6.36b        |
| A+VE     | 371.5±12.1b        | 373.2±12.1b       | 1.61±0.03b      | 0.43±0.006b      | 33.78±4.78       | 42.4±5.72b        |

Mean ± SE (8 values).

*aSignificant difference compared with the young controls.

*bSignificant difference compared with the aged rat

**Effect of FE on the Germinal Epithelium, the Tubule Diameter and Spermatozoa Values**

In tests samples, the height of the germinal epithelium and the tubule diameter were evaluated under the microscope shown in (Fig 1/A). The height of the germinal epithelium was not significantly reduced in the AC compared to that in the YC ($p>0.05$). However, in AC group, the tubule diameter was showed a significant decrease compared to the YC ($p<0.05$). Meanwhile, the tubule diameter and the germinal epithelium height were observed to increase A+FE and A+VE as compared to the AC (Table 2) (Fig.1/A/B). The motility and number of sperm were observed to reduce in aged rat ($p>0.05$). However, There was no difference between sperm viability of young and old rats ($p>0.05$). The FE and Vitamin E significant improved sperm motility and count in aged rat compared the AC (Table 2).

| Table 2. Data of investigated tubular diameter, germinal epithelium height, sperm number, sperm motility, sperm viability in rat groups |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Groups   | Tubular diameter (µm) | Germinal epithelium height(µm) | Sperm Count(10⁶) | Sperm Motility(%) | Sperm viability(%) |
| YC       | 274.2 ± 48.1       | 84.5 ± 12.16     | 123 ± 22.4      | 73.2± 4.2       | 68.6± 3.3       |
| AC       | 248.5 ± 35.3a      | 77.4± 8.25       | 79 ± 19.3a      | 51.3± 6.8a      | 65.9± 4.4       |
| A+FE     | 263.8 ± 19.6b      | 82.1± 9.46       | 96 ± 13.8b      | 62.5± 5.2b      | 67.5± 7.2       |
| A+VE     | 265.4 ± 22.9b      | 81.9± 10.96      | 100 ± 15.2b     | 65,7± 5.5b      | 66.3± 5.8       |

Mean ± SE (7 values).

*aSignificant difference compared with the young controls.

*bSignificant difference compared with the aged rat

**Effect of FE on the Spermatogenesis**

Histopathological evaluation at week 8 revealed that spermatogenic activity in the AC group was dramatically decreased compared to YC. On the other hand, administration of FE to aged rats restored spermatogenic activity ($P>0.05$; Figure 1/C/D). To confirm the role of FE and also to compare it with vitamin E in germ cell apoptosis in aged rats, apoptosis of spermatogenic cells was also evaluated with TUNEL ($P>0.05$; Figure 1/E). TUNEL results clearly showed increased germ cell apoptosis in AC tests (Fig. 2/A). Compared to the AC group in the A+FE group (Figure 2/B), the number of apoptotic cells decreased and apoptosis was most common in spermatogonia (Figure 2/C) and spermatocytes (Figure 2/D). Statistically significant difference was found between AC and A+FE group ($p<0.05$) and also between AC and A+VE group ($p<0.05$; Fig. 2/E).
Figure 1A. Testicular tissue H&E staining of control young rats
Normal spermatogenetic activity and many spermatozoa were present in the lumen of most seminiferous tubules, as well uniform seminiferous tubules and regular germ cells: a: spermatogonia, b: spermatocyte, c: spermatid, d: spermatid, e: sertoli cell, X1,2: tubular diameter, Y: germinal epithelium height.

Figure 1B. Testicular tissue H&E staining of aged rats
Few spermatogenetic activity with few spermatozoa and degenerative changes in the seminiferous tubules.

Figure 1C. Testicular tissue H&E staining of aged of FE-treated aged group
Normal spermatogenetic activity with many spermatozoa in the lumen of most seminiferous tubules. Spermatogenetic activity in FE-treated aged rats was increased compared to aged rats.

Figure 1D. Testicular tissue tunnel staining of VE-treated aged group
Normal spermatogenetic activity with many spermatozoa in the lumen of most seminiferous tubules. Spermatogenetic activity in VE-treated aged rats was increased compared to aged rats.

Figure 1E. Comparison of johnson score in all groups
a Significant difference compared with the young controls.
b Significant difference compared with the aged rat was seen.

Figure 2A. Testicular tissue tunnel staining (arrow head) of control young rats

Figure 2B. Testicular tissue tunnel staining (arrow head) of aged rats

Figure 2C. Testicular tissue tunnel staining (arrow head) of FE-treated aged group

Figure 2D. Testicular tissue tunnel staining (arrow head) of VE-treated aged group

Figure 2E. Effect of FE and VE on apoptotic index of spermatogenetic cells in aged
a Significant difference compared with the young controls.
b Significant difference compared with the aged rat.
Effect of FE on Serum Testosterone and TNF-α and Oxidant/ Antioxidant Markers

Although the serum TAS level decreased in AC compared to the YC, there was no statistically difference between two groups (P>0.05; Fig. 3/A). Meanwhile, as a sign of more oxidative damage to tissue, serum TOS levels was showed significantly higher in AC, compared to YC rats. However, as a sign of restoration of tissue damage due to oxidative stress, both A+FE and A+VE rats showed significantly higher serum TAS concentrations compared to AC rats (P<0.001; Fig. 3/B).

The results illustrated that serum level of testosterone was importantly decreased in the AC rats compared with the YC rats (P<0.001). Meanwhile when compared in YC, TNF-α levels was seen considerably to increase (P< 0.05) in AC (Fig. 3/C). At the end of 8 weeks, it was seen that administration of FE and VE significantly increased the testosterone serum level and also the serum TNF-α level was significantly regulated (P< 0.05) (Fig. 3/D).

When the evaluated in testis tissue, although the CAT level of the AC was seen to be lower than YC, there was no statistical difference between the two groups (P>0.05). However, testicular GSH level, which had decreased in AC the testicular, it was observed to increase a significant degree in the A+FE and A+VE (P<0.001), compared to AC (Fig. 4). The AC group showed significantly higher tissue MDA levels compared to the YC group, representing greater oxidative damage to lipids (P<0.05).

DISCUSSION

The aging process and protection or elimination of problems caused by this process have become an important field of research. It have been a well-known fact that natural antioxidant production of an organism decreases with aging. Therefore, this study was designed to investigate whether FE has a protective effect on testicles during aging.
In many studies, it has been reported that we, as well as others, have reported body weight increases during aging. Recently, many studies have been conducted to prevent the body weight gain such as genetic, metabolic, hormonal, behavioural, social, and cultural aspects. It is well known that there are many studies reporting the anti- obesity effects of the plant extract such as green tea, Vitaceae, Melanthiaceae and also Ferula species on humans and animals. The study was indicated that FE consumption regulated to the gain body weight. In addition, FE was found to decrease food intake while increasing the water intake in aged rat.

In this study was seen that spermatogenesis activity increased in FE administered group rat compared to the control group. Although the mechanism of action of Ferula and its compounds on the testis needs to be explained, FE is known to have rich antioxidants and anti-inflammatory compounds such as Khusino, alf pinen, beto ionone. Meanwhile, numerous studies are stated that sperm cells are particularly susceptible to reactive oxygen species during spermatogenesis. Aging with accumulating radical oxygen species causes apoptosis in testis as well whole body. For the reasons, the apoptosis marker increased with aging causes to change in the testis germ cell that can lead to decrease spermatogenesis, and also sperms quality. The data of the current study showed that the FE improved the number, motility, of sperms and also may played a protective role against aged-induced apoptosis in germ cell in aged rat. The results obtained from this study were consistent with the results of researchers about ferula species previous reports.

Furthermore, it was well also known that the tubule diameter and the thickness of the germinal epithelium layer is an indicator for the status of spermatogenesis and also in aging and some chronic diseases, tubule diameter is reduced and the thickness of the germinal epithelium layer decreases. Our results also indicate that, unlike spermatogenesis activity and the tubule diameter, when the height of the germinal epithelium was analysed, there was only a slight difference between the aged rats and the young control rat as described by researchers. However, FE treatment led to recovered in the tubule diameters and germinal layers in aged rat.

One of the most important findings of the study was that serum TNF and T levels were regulated after FE administration. As known, aging is associated with increased TNF activity and low testosterone level in the blood. The deficiency of testosterone or TNF elevation is also known to increase in age-related diseases for example hipogonadizm, metabolic syndrome, diabetes, Alzheimer disease, cardiovascular disease and also erectile dysfunction (ED). Meanwhile, there is strong evidence that testosterone modulates TNF alpha, an important cytokine responsible for the immune system. We observed that age-related decreased the serum TNF and testosterone level as reported by the researchers. Additionally, serum testosterone level was seen to boost in the FE administered group as compared to the aged rat and the vitamin E administered aged rat. Researchers reported that after extract of some Ferula genus was administered, boosted the serum testosterone level and improved sexual functioning in young rat and mice, and also reduced TNF and IL 6. Thus, FE may be an alternative option for use in the testosterone replacement therapy in the future.

Lipid peroxidation is an oxidative stress indicator resulting from MDA. Some researchers stated that testis MDA level changed with age, while some claimed in contrast. In our study, MDA levels of aged rat testis tissue were found to be significantly higher than the aged control group (p<0.001). Meanwhile, FE-supplemented aged rats showed significantly lower testicular MDA level, as reported for ferula in rat testicular damage. Glutathione-S-transferase (GST) is an antioxidant enzyme that provides to conjugation the electrophilic and hydrophobic compounds with glutathione, which is generally easier to remove and convert to less toxic metabolites. Studies also show that GST is the enzyme most affected by the aging-related antioxidant changes occurring in the testis and that it decreases greatly in the testicular tissue during aging. It is known that most of the flavonoids have the ability to activate glutathione-S-transferase (GST). This mechanism is accomplished by Glutathione reductase (GR) which is a flavoprotein. GR uses β-nicotinamide dinucleotide phosphate (NADPH) as a hydrogen donor and catalyzes oxidized glutathione (GSGG) to reduced glutathione (GSH). In this study, GSH was observed to decrease with age in the testicular tissue. However, it has been seen that FE restored the decrease in GSH level in the aged rat as reported by authors. This compounds may have been increased the GSH levels in the FE administered group. Moreover, as in previous studies, it was found in our study that vitamin E increased the GSH levels statistically in aged group. However, when the rat fed with FE and the rat given VE were compared statistically, it was determined that there was no difference between the two groups.

The current study had several limitations. The results obtained are the study conducted on only one species of the experimental animal. Reliability coefficients can be further increased by increasing different animal species and numbers in this study.

CONCLUSION

The present results indicated that FE clearly inhibited histopathological damage in testicles caused by senescence and preserved spermetegenosis and improved serum testosterone levels. Meanwhile FE reduces inflammation and oxidative stress in the aged rat, it slows down apoptosis in testis. The positive findings that FE can reduce testicular dysfunctions due to aging are promising for future studies.

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