The sensitivity of male rat reproductive organs to sodium benzoate

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Abstract:

In order to investigate the effect of sodium benzoate dosage on reproductive efficiency in mature male rats , The current study was conducted at the college of Science ,AL-Qdisiyah University for the period from 15\10\2016 to 15\4\2017. mRNA expression level of *E1f1ay, Ddx3y, Cyp17al and Cyp19* genes have been evaluated in testes and epididymis tissues as well.

Sixty adult male rats (aged 56 days and weighted 138±8.8 g) have been used in the present study .The rats have been divided randomly in to three equal groups (20 rats to each group). The first group included the control (C) group and was injected with physiological saline solution daily .The second group was injected with sodium benzoate at a concentration of(50 mg\kg) .The third group was injected with sodium benzoate at a concentration of(100 mg\kg). Each group was divided into two sub groups , each containing 10 rats, depending on the duration of the dosage , 2-3 weeks .

After 24 hours on the last day of the experiment , Samples from testes ,epididymis tissues in all groups have been quickly removed, dipped in liquid nitrogen for RNA extraction and molecular study.

The result showed a significant decrease (P>0.05) in sperm concentration , sperm ratio, live sperm ratio and normal sperm ratio in the two groups (T1,T2) during the experimental period compared to control group .

Quantification analysis results of gene expression, performed by real-time RT-PCR, revealed that treatment with sodium benzoate caused significant decrease of mRNA expression levels of *E1f1AY, Ddx3y, Cyp17al and Cyp19* genes during the studied period.

The result of the histological study on the testes showed a significant decrease in the composition of the sperm in the two groups (T1,T2) where the spermatozoa showed a small number of primary and secondary sperm cells as well as a decrease in the number of sperm and leydig cells. This decrease increases with increasing concentration and duration compared to control group .

It can be concluded that treated with 50 , 100 mg/ kg, bw, of sodium benzoate effect in the reproductive efficiency of male rats. As well as its negative role in lower the expression level of *E1f1AY, Ddx3y, Cyp17al and Cyp19* genes, when used at the given dose and for 21 days.

Key words : Gene expression , Sodium benzoate , Rat

Introduction

One of the great advances in human history was the ability to conserve food ,And with the beginning of the twentieth century and the qualitative and evolutionary leaps at all levels, One of the areas in which the development of technology has been the production and manufacture of food, which led to the abundance of food and increase circulation and transport not only within the product country ,but exceeded the geographical area and across the border, which in turn led to the impossibility of preparing and packing and storage and transport of many food without the addition of materials maintained by of damage.[1]

Many methods were used to conserve food. Initially, food preservation techniques started from freezing and drying , and then developed into preservatives ,which were subsequently increased food species . As a result, the food needed to consume a lot of time instead of moving from place to place. To get fresh food, there must be a way to save it until it is used.

In the past, food was produced and consumed locally, but at present it is produced in a place and treated elsewhere and then distributed elsewhere. This means that the period between production and consumption is longer and it becomes necessary to conserve food to prevent damage and prevent undesirable changes in taste and color. [2] With the increase in food and fast food production, the use of preservatives has become important in food technologies.The availability and consumption of healthy food that equips the body with its basic needs is necessary for humans , Food and its active ingredients have a significant effect on human health and food safety is not a new concept in the modern world, It is deeply rooted in the history of human civilization ,Food damage has been a common problem

throughout history, For the activity of organisms or enzymatic activity during food preservation. [3]About one-third of the population in developed countries suffering from food-borne diseases and more than 250 microbiological, chemical or physical agents responsible for these diseases were estimated in 2011.The center for diseases control in America estimated about 128,000 people hospitalized for food borne diseases and three Thousands die every year. [4]The use of chemicals to prevent or delay food damage is partly due to the great success of these substances in the treatment of diseases in humans, animals and plants. Although a large number of chemical compounds are effective food preservatives but due to strict food safety laws that have been approved by the FDA Minimally scheduled in foreign studies All compounds showed antimicrobial effects and the addition of these compounds to some food products has no effect and only a few of them are allowed in food products. [3,4]In our day the food factory forms about 75% of the diet of Western societies. [5] Sodium benzoate is one of the industrial additives which is widely used in the food industry and is known as a safe substance(GRAS),It is widely used as a preservative in various products such as pickles, vinegar, jams, juices and other materials such as shampoo and medicine.[6]It is also used to disturb the cycle of urea and prevents inflammation .Its use is permitted in oral medications and cosmetics. [7]

Sodium benzoate has an antimicrobial effect such as fungi and some bacteria and its use as an ideal preservative in products that are naturally acidic, especially food and beverages with PH<4.5. [8]

Although sodium benzoate is classified as a safe preservative, damage preservatives industrial was noted to him in the research prior .[9]

Some research has shown that small amounts of benzene can be composed of benzoic acid with vitamin C and gasoline is a carcinogen that breaks down DNA-deficient RNA in the mitochondria, Sodium benzoate is also linked to cancer, as mixing it with vitamin C in soft drinks leads to the formation of benzene.[10]Short –term exposure to benzoates can cause irritation in the skin, eyes and bronchi, but prolonged and continuous contact may cause large skin sensitization. [11]

Materials and Methods

Experimental animals Sixty five days old adult male Wistar rats (average weight : 138+-8.8g), were breed at the animal house of the college of Veterinary medicine ,Al - Qadisiyah University**.** For the period between 15/10/2016 and 15/4/2017.

Experimental protocol Sixty adult male rats aged 56 days with a mean weight of 138±8.8 g were randomly divided into three groups. Each group included20 rats .The first group represented the control group and was treated with saline physiological solution .The second group represented the treatment group T1, which was injected with sodium benzoate at a concentration of 50 mg/kg. The third group T2 which was injected with sodium benzoate at a concentration of 100 mg/kg. Each group was then divided into three equal secondary groups according to the duration of the dosage: two weeks and three weeks. Each secondary group included10 rats. After the last administration of (sodium benzoate) , the animals were sacrificed after general anesthesia by combination of Xylazine and Ketamine (10mg and 90mg/kg , i.p. respectively).After scarification testes tissue will be removed for histological examination.

Study of semen parameters:

Semen Analysis: The total number of spermatozoa was counted using the new improved Neuber's counting chamber (haemocytometer), expressed as number of sperm cells in millions/ml. The fluid from the caudal epididymis was diluted with Tris buffer solution to 0.5 ml, in order to determine sperm motility, which was expressed in percentage (%). Abnormal features of sperm morphology were observed and categorized as tail defects, neck and middle piece defects, and head defects; and the findings were expressed as percentage (%) of morphologically abnormal sperm. [12,13,14,15,16]

Molecular analysis:

RNA isolation from rat pancreatic tissues:

RNA was isolated from rat testes and epididymis according to the protocol described by the TRIzol® reagent manufacturer with some modification. [17]

Statistical analysis

All statistical analysis were carried out using the GraphPad Prism (SAS Institute, Inc., USA).

Result

Semen analysis:

The sperm concentration of all the groups show in (Table 1). There was decreased sperm motility in all the treatment groups, with Group T2 given 100 mg/kg MSG having the least % motility. This same group had the highest number of dead sperm cells, as revealed by the lowest Life Death ratio .All the treatment groups however had increased in cell death compared to the Control, but not as much as Group T2 animals treated with 100 mg/kg MSG.

Table (1) Shows the effect of treatment with sodium levels on sperm parameters in male white rats

Groups / parameters	C control		Treatment (T1) (50 mg/kg)		Treatment (T2) (100 mg/kg)	
	2week	3week	2week	3week	2week	3week
Count*(10^6 /ml)	56.60 ±0.45 A a	58.60 ± 0.43 A a	48.8± 0.8 B a	44.60± 1.28 C b	42.40± 1.45 C a	36.60 ± 0.45 D b
Motility	85.6± 0.2 A a	85.8± 0.1 A a	76.6± 0.6 B a	65.6 ± 0.2 C b	57.9 ± 2.06 C a	47.6 ± 2.14 D b
Viability	85.4 ±0.4 A a	86.5 ±0.2 A a	74 ±1.3 B a	66.8 ± 2.5 C b	60 ±2.3 C a	51.8 ± 0.74 D b
Morphology	76± 0.3 A a	75.1± 0.2 A b	66.8± 0.8 B a	56± 0.3 C b	53.8± 1.21 C a	43.6 ± 1.3 D b

- The results represent the mean ± standard error
- The different small letters indicate significant differences ($P < 0.05$) between the periods for each group
- The large different characters indicate significant differences ($P < 0.05$) among the three groups for each period
- C : control group
- T1: Treatment group with 50 mg / kg body weight in sodium benzoate
- T2: Treatment group with 100 mg / kg body weight in sodium benzoate

Molecular analysis:

The concentrations and purity of total RNA

Total RNA concentrations (ng/µl) and purity were estimated using Nanodrop spectrophotometer in absorbance readings (260/280 nm). All tissue samples that used in the present study gave high concentrations of total RNA and appeared quantitatively enough to proceed in quantitative reverse

transcriptase real-time PCR as shown in tables (1).treated male rats recorded significant lower concentrations of RNA throughout the experiment as show in figure (1).The present results have been shown that the ratio between optical density at 260 nm and 280 nm was within normal range (more than 1.8 and less than 2.1). The purity of total RNA samples (also assessed using agarose gel electrophoresis) of tissues that obtained from experimental adult male rats recorded different band thickness.

Quantitative Reverse Transcriptase Real-Time PCR:

Data analysis of SYBR® green based reverse transcriptase real-time PCR assay were divided into primer efficiency estimation and relative quantification of (*E1f1ay, Ddx3y, Cyp17a1, Cyp19a1*) genes expression levels normalized by housekeeping gene expression (*GapdH*).

Primer efficiency estimation:

The data result, threshold cycle numbers (Ct) were calculated from amplification plot of real-time PCR detection system, during exponential phase of fluorescent signals of SYBR® green primer of different genes that react with complementary DNA (cDNA) of rat pancreas mRNA, where, the amount of PCR product (DNA copy numbers) in master mix reaction is approximately doubles in each PCR cycle. First prepared series dilution of testes cDNA of control group, this concentrations was used with the primer of different genes (*E1f1ay, Ddx3y, Cyp17a1, Cyp19a1*and *GapdH*) separately to formation the amplification plot of each gene and then from this amplification plot threshold cycle (Ct) was used to calculate a linear regression based on the data points, and inferring the efficiency of each primer from the slope of the line. (Fig. 2)

4.5.2.2. Relative quantification of target genes expression:

To calculate the relative expression of target genes in pancreas, the 2^-ΔΔCt

livak and Schmittgen method has been used by normalize gene expression of target gene with expression of housekeeping gene (*GapdH*) as reference gene. The gene expression in control was expressed as (calibrator) or control in both target genes and reference gene (*GapdH*), at first, the threshold cycle number of target gene normalized to that of reference gene in all treatment groups and calibrator. Second, the ΔCt of treatment groups normalized to the ΔCt of calibrator, and finally the expression ratio (fold change) was calculated.

Relative quantification of *E1f1ay* gene expression:

Reverse transcriptase real-time PCR results shown in figure (2a) revealed that ***E1f1ay*** mRNA expression level was decreased in T2-3W compared to the control group.

Relative quantification of *Ddx3y* gene expression:

Results clarify in figure (2a), showed that ***Ddx3y*** gene mRNA expression level was significantly decreased in testes tissue in the T2-3W group compared to the control group while the decrease in half was in the groups T1-2W and T1-3W (2.673 and 2.736) respectively In comparison to the control group, but there is no significant difference between them. The T2-2W group has a moral decline of less than half as it reached (1.898) compared to control group (2a).

Relative quantification of *Cyp17a1* gene expression :

Results of ***Cyp17a1*** gene expression levels quantification, clarified in figure(2a), showed that ***Cyp17a1*** gene expression levels was decreased significantly ($P<0.05$) in testicular tissue obtained from T1 and T2 groups compared to control group.

Relative quantification of *Cyp19a1* gene expression :

Results of ***Cyp19a1*** gene expression levels quantification, illustrated in figure (2a) revealed that ***Cyp19a1*** gene expression levels was decreased

significantly (P<0.05) in testecular tissue obtained from T1 and T2 groups compared with control.

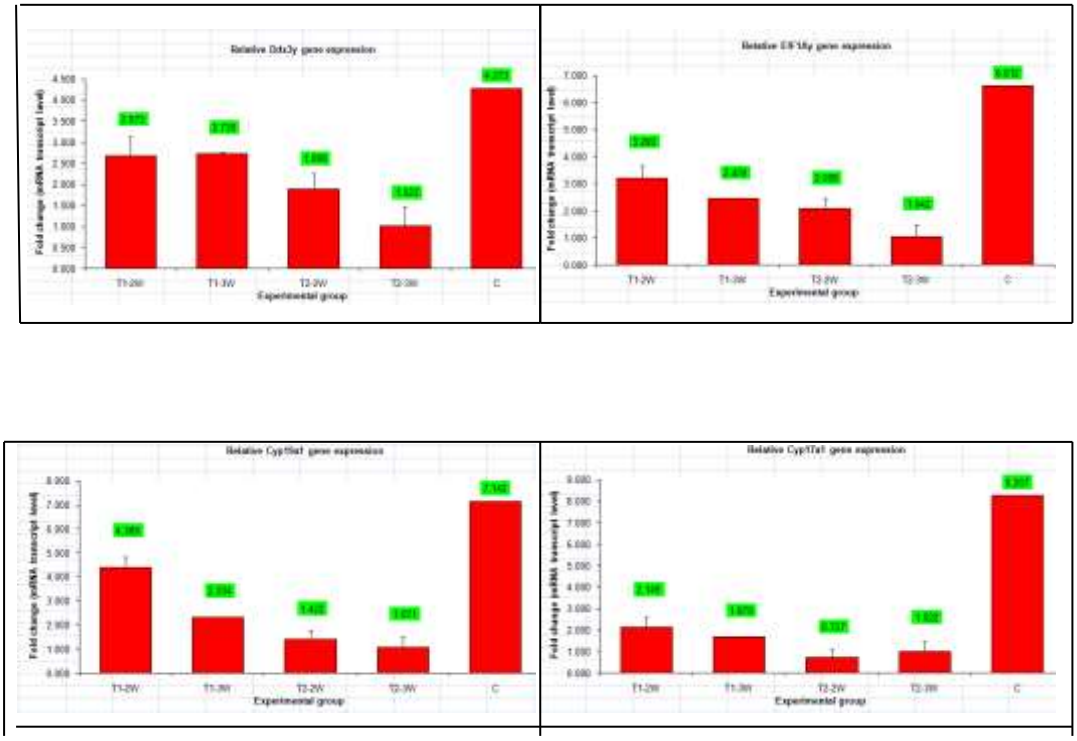

Figure (2-a) Genetic variation of *E1f1ay*, *Ddx3y*, *Cyp17a1 and Cyp19a1* in testicular tissue

T1-2W: rats drenched with sodium benzoate at a concentration of 50 mg / kg for two weeks

T1-3W: rats drenched with sodium benzoate at a concentration of 50 mg / kg for three weeks

T2-2W: rats drenched with sodium benzoate at a concentration of 100 mg / kg for two weeks

T2-3W: rats drenched with sodium benzoate at a concentration of 100 mg / kg for three weeks

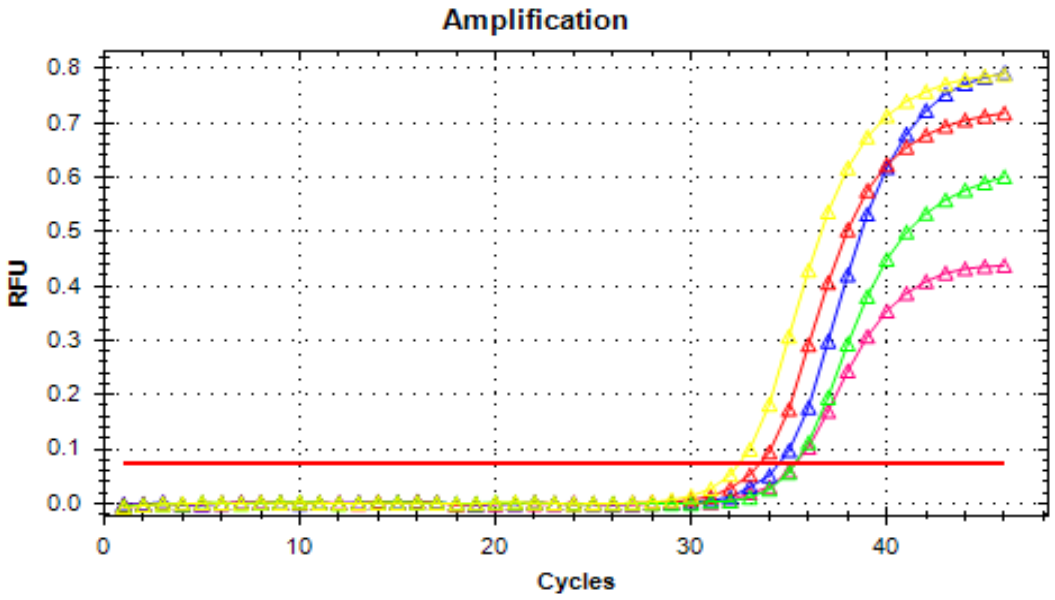

Figure show effect of sodium benzoate on *E1f1ay* gene expression (fold change) in the testes showing the difference in the Ct value between the control group and the other groups

Red Line (2W-T1): animals drenched with benzoate at a concentration of 50 mg / kg for two weeks

Blue line (T1-3): animals drenched with benzoate at a concentration of 50 mg / kg for three weeks

Green Line (T2-2): animals drenched with benzoate at a concentration of 100 mg / kg for two weeks

Pink line (T2-3): animals drenched with benzoate at a concentration of 100 mg / kg for three weeks

Yellow Line (C): Control Group

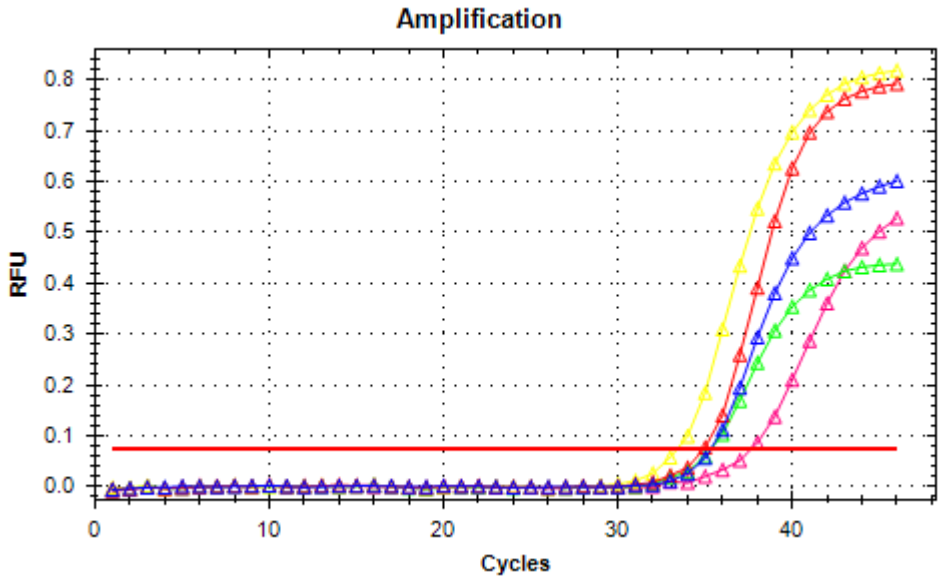

Figure show effect of sodium benzoate on *Ddx3y* gene expression (fold change) in the testes showing the difference in the number of the ct value between the control group and the other groups

Red line (2-T1) :animals drenched with benzoate at a concentration of 50 mg / kg for two weeks

Blue line (T1-3): animals drenched with benzoate at a concentration of 50 mg / kg for three weeks

Blue line (T2-2): animals drenched with benzoate at a concentration of 100 mg / kg for a week

Pink line (T2-3): animals drenched with benzoate at a concentration of 100 mg / kg for three weeks

Yellow Line (C): Control Group

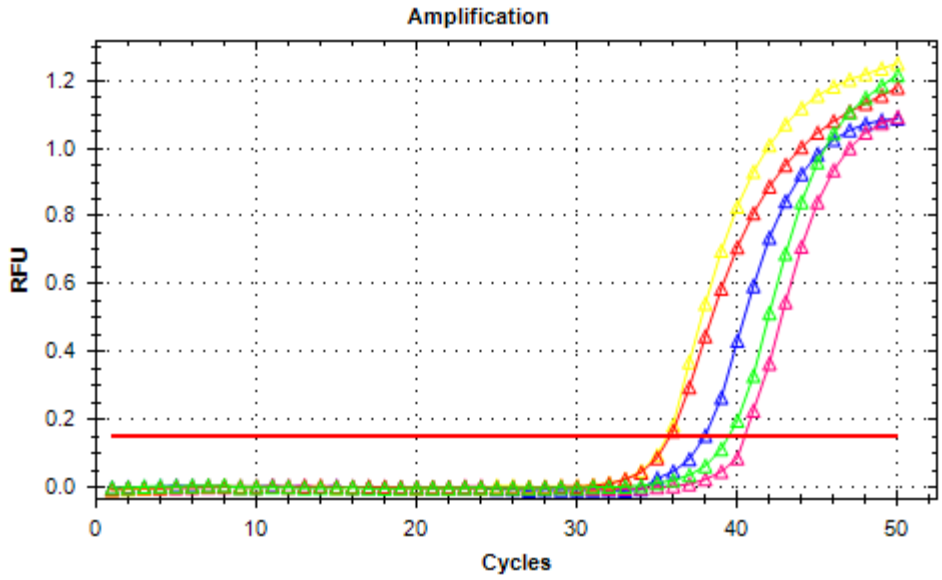

Figure show effect of sodium benzoate on *Cyp19a1* gene expression (fold change) in the testes showing the difference in the number of the ct value between the control group and the other groups

Red line (2-T1): animals drenched with benzoate with benzoate at a concentration of 50 mg / kg for two weeks

Blue line (T1-3): animals drenched with benzoate with benzoate at a concentration of 50 mg / kg for three weeks

Green Line (T2-2): animals drenched with benzoate with benzoate at a concentration of 100 mg / kg for two weeks

Pink line (T2-3): animals drenched with benzoate with benzoate at a concentration of 100 mg / kg for three weeks

Yellow Line (C): Control Group

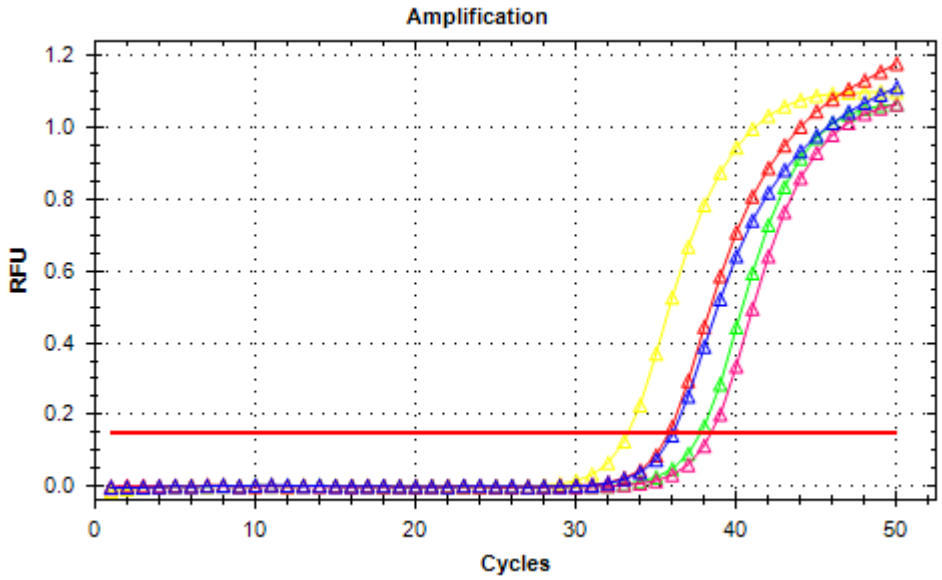

Figure show effect of sodium benzoate on *Cyp17a1* gene expression (fold change) in the testes showing the difference in the number of the ct value between the control group and the other groups

Red line (2-T1): animals drenched with benzoate at a concentration of 50 mg / kg for two weeks

Blue line (T1-3): animals drenched with benzoate with benzoate at a concentration of 50 mg / kg for three weeks

Green Line (T2-2): animals drenched with benzoate at a concentration of 100 mg / kg for two weeks

Pink line (T2-3): animals drenched with benzoate at a concentration of 100 mg / kg for three weeks

Yellow Line (C): Control Group

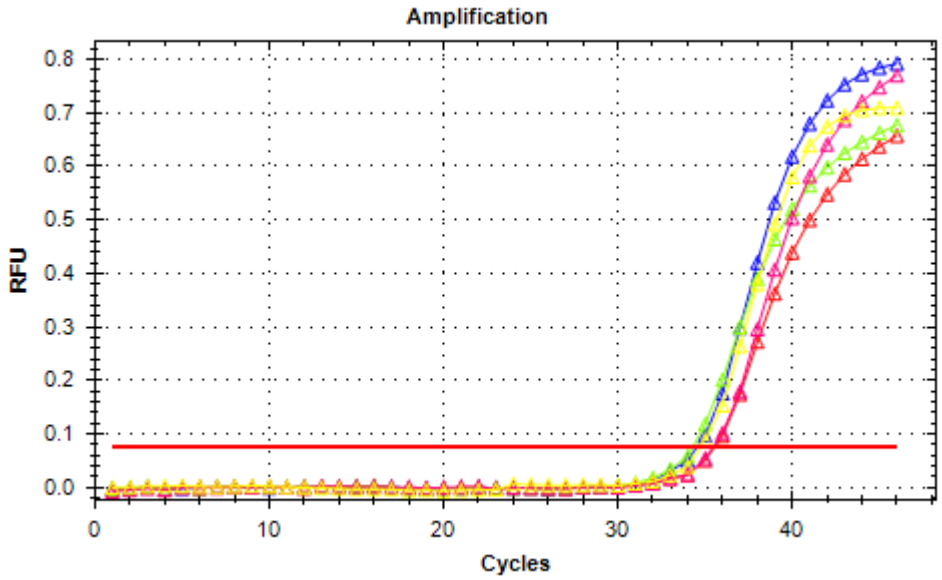

Figure show effect of sodium benzoate on *GAPDH* gene expression (fold change) in the testes showing the difference in the number of the ct value between the control group and the other groups

Red line (2-T1): animals drenched with benzoate at a concentration of 50 mg / kg for two weeks

Blue line (T1-3): animals drenched with benzoate at a concentration of 50 mg / kg for three weeks

Green Line (T2-2): animals drenched with benzoate at a concentration of 100 mg / kg for two weeks

Pink line (T2-3): animals drenched with benzoate at a concentration of 100 mg / kg for three weeks

Yellow Line (C): Control Group

2-B relative quantity of *E1f1ay* gene expression, *Ddx3y*, in epidermal tissues

The results of the qPCR interaction showed a significant decrease of P> 0.05 in the gene expression level of *Ddx3y* gene in the adult male rat tissue. This decrease was gradually increased by increasing the concentration and age to reach the lowest level in the T2-3W group where the expression levels of *Ddx3y* (2.134, 1.776, (T1-2W, T1-3W, T2-2W, T2-3W) respectively compared to the control group (Fig. 2b).As for the levels of ***E1f1ay*** gene expression in epidermal tissue, the results of qPCR showed a significant decrease of P> 0.05 in the T1-2W group with a level of (2.873). This decrease continues gradually in totals T1-3W, T2-2W, T2-3W, Levels of gene expression (2.055, 1.444, and 1.125), respectively, compared to control group (Fig. 2b)

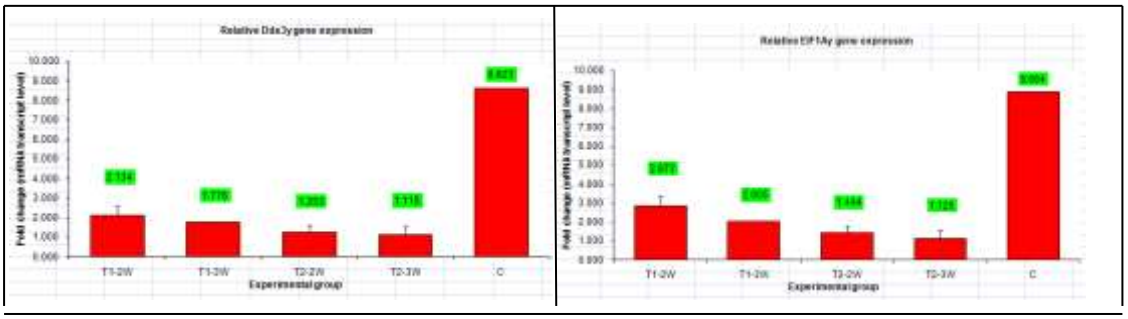

Figure (2-b) Genetic variation of *E1f1ay* and *Ddx3y* in epidermal tissues

T1-2W: rats drenched with sodium benzoate at a concentration of 50 mg / kg for two weeks

T1-3W: rats drenched with sodium benzoate at a concentration of 50 mg / kg for three weeks

T2-2W: rats drenched with sodium benzoate at a concentration of 100 mg / kg for two weeks

T2-3W: rats drenched with sodium benzoate at a concentration of 100 mg / kg for three weeks

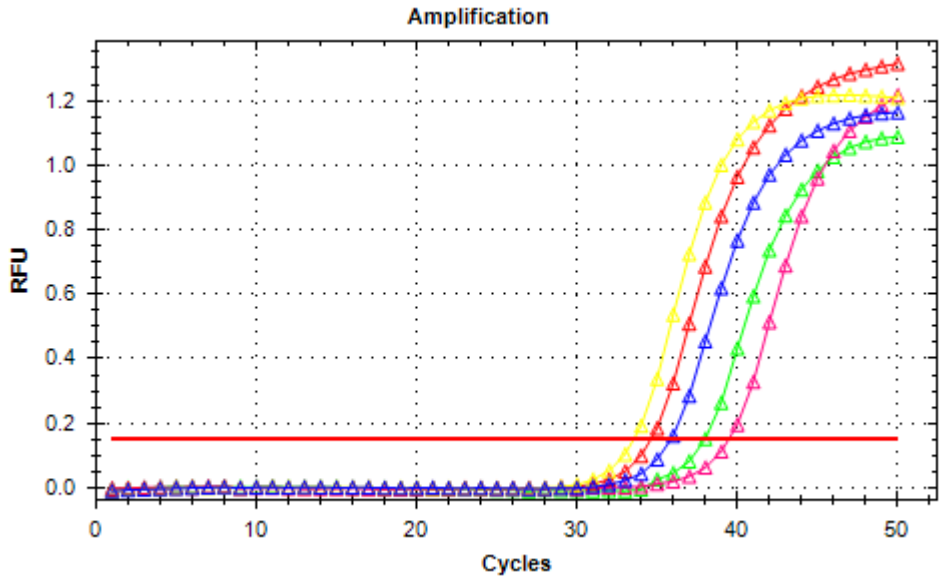

Figure show effect of sodium benzoate on *E1f1ay* gene expression (fold change) in the epididymis showing the difference in the number of the ct value between the control group and the other groups

Red line (2-T1): animals drenched with benzoate at a concentration of 50 mg / kg for two weeks

Blue line (T1-3): animals drenched with benzoate at a concentration of 50 mg / kg for three weeks

Green Line (T2-2): animals drenched with benzoate at a concentration of 100 mg / kg for two weeks

Pink line (T2-3): animals drenched with benzoate at a concentration of 100 mg / kg for three weeks

Yellow Line (C): Control Group

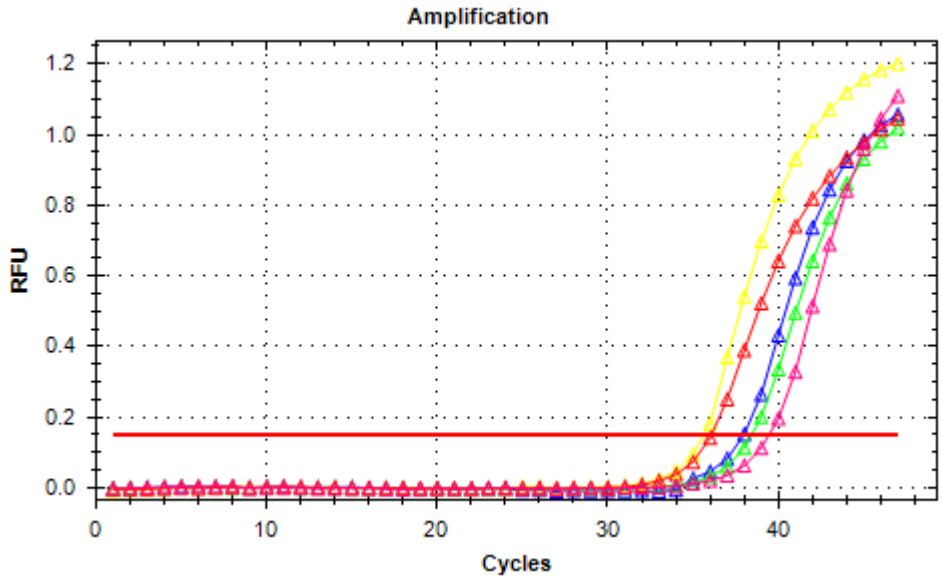

Figure show effect of sodium benzoate on *Ddx3y* gene expression (fold change) in the epididymis showing the difference in the Ct value between the control group and the other groups

Red line (2-T1): animals drenched with benzoate at a concentration of 50 mg / kg for two weeks

T1-3: animals drenched with benzoate at a concentration of 50 mg / kg for three weeks

Green Line (T2-2): animals drenched with benzoate at a concentration of 100 mg / kg for two weeks

Pink line (T2-3): animals drenched with benzoate at a concentration of 100 mg / kg for three weeks

Yellow Line (C): Control Group

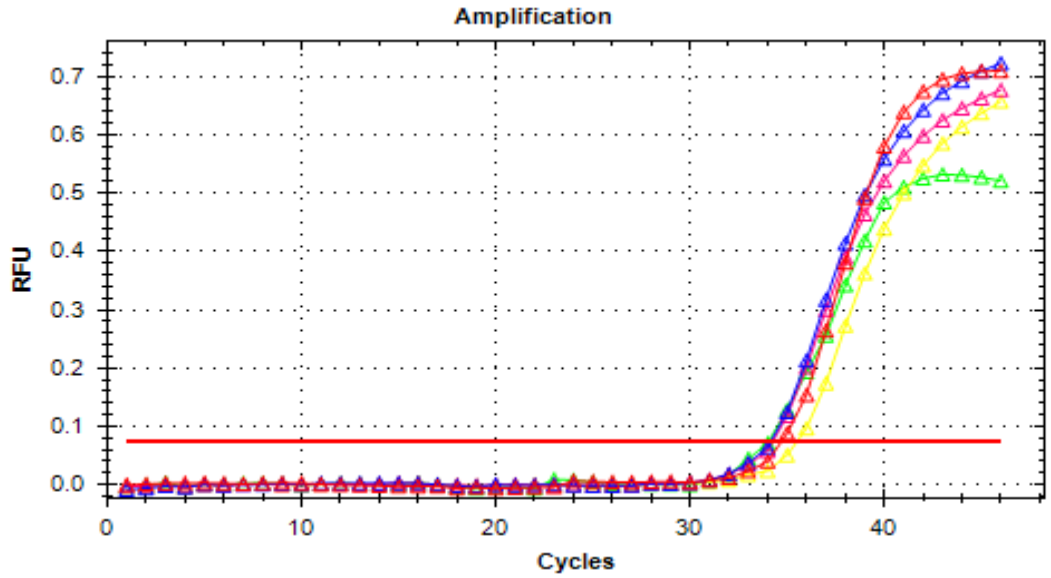

Figure show effect of sodium benzoate on *GAPDH* gene in the epididymis showing the difference in the number of the ct value between the control group and the other groups

Red line (2-T1): animals drenched with benzoate at a concentration of 50 mg / kg for two weeks

Blue line (T1-3): animals drenched with benzoate at a concentration of 50 mg / kg for three weeks

Green Line (T2-2): animals drenched with benzoate at a concentration of 100 mg / kg for two weeks

Pink line (T2-3): animals drenched with benzoate at a concentration of 100 mg / kg for three weeks

Yellow Line (C): Control Group

Discussion

Effect of Sodium on testicular changes in some parameters of the sperm

Sperm count is one of the most sensitive tests for spermatogenesis, since it gives the cumulative result of all stages in sperm production, and it is highly correlated with fertility. Our results show that sodium benzoate is cytotoxic to the sperm since it decreases the sperm count significantly in the experiment period . The duration of spermatogenic cycle in rats is 52 to 60 days and our findings point out that the germ cells affected are approximately the spermatids, spermatocytes, and spermatogonia. The count was reduced even at the end of the 30 days. This in all probability signifies its effect on the stem cells. The decline in sperm count was highest at the T2 group, which possibly indicates that the spermatogonia are more vulnerable to the toxic effects of sodium benzoate. It is also possible that the Sertoli cells might have been affected and the other possibility might be due its effect on the epididymal function.

Exposure to preservatives, such as paraffin, benzoic acid (a preservative of food derived from benzoic acid), adversely affects the function of the male reproductive system and affects the secretion of adipose hormone [18]. And its maturity requires a combination of hormones together, such as prolactin hormones and growth hormone, as well as hormones that secrete from the frontal lobe of the pituitary gland, LH, FSH[19] . This is explained by the moral decline in the concentration of sperm in both The two groups during the duration of the dosage

Effect of Sodium on Relative Quantities of Gene Expression in Testicular and The epididymis Tissue

The results of the study showed a significant decrease in the levels of *Cyp19a1, Cyp17a1, E1f1ay, Ddx3y* in the tissues of the testes during the studied period may be due to the effect of sodium benzoate on the hormone lipid , lutein hormone and their effect on the formation of steroids as studies have shown that the treatment of male rats with concentrations Different sodium benzoate leads to decreased production of testicular lipid hormone and lutein. [20]

The decrease in the production of lutein hormone contributes to the reduction of building lipid hormone testosterone by inhibiting the genetic expression of the protein StAR, which is one of the most important proteins that are responsible for the transfer of cholesterol to the internal membrane in the mitochondria, which uses enzymes that contribute to the formation of steroids .[19] The decrease in lutein secretion also inhibits the enzymes that contribute to the formation of steroids .[21]

The stimulation of the hypothalamic-adrenal axis (the primary epithelial system activated in the lobes) by the effect of stress strains leads to inhibition of the hypothalamic-pituitary-hypothalamic axis by inhibiting the secretion of Gonadotropin releasing hormone (GnRH) .[22]where male reproductive functions start hormonal control from the hypothalamus region where GnRH is released. When this protein reaches the frontal lobe of the pituitary gland, it stimulates the secretion of two hormones, LH and follicle stimulating hormone (FSH) These hormones, in addition to other hormones, have to be present the emergence of spermatozoa process .[23]

Song and his group (2002) confirmed that the LH is essential in the synthesis of the lipid hormone produced in Leydig cells. The follicle

stimulating hormone (FSH) stimulates sertoli cells, a key component of the spermatozoa and the estrogens, and play an important role in the conservation of sperm as well as the process of its formation .[24]

Cyp17a1 is an intermediate substance in cortisol and sexual steroids .[25]Some studies have suggested that sodium benzoate can cause chemical stress, which can cause an increase in the levels of glycated corticosteroids such as cortisol .[26]

The results of the study indicate that increased cortisol levels lead to a decrease in the secretion of LH and GnRH hormones, as well as a reduction in the toxic function of the test. The increase in cortisol and the reduction of testicular fat are stress indicators.[27]

The results showed a significant decrease in the genetic expression of the gene *Cyp19a1*. This gene is expressed initially in the brain ,where the expression is in the area of the hypothalamus and is higher before birth and less mature, the lack of building estradiol (which turns into steroids by the enzyme Aromatase Which encodes this gene) leads to hormonal changes and leads to a lack of aromatase, which has been directly linked to changes in the genetic behavior of animals and humans .

The construction of estradiol is a key to nerve growth, gonads and sexual behavior .[28]

And found that estradiol and adipose lipid hormone regulated mRNA aromatase in the hypothalamus as well as enzymatic activity .[29].The results showed a significant decrease in the expression of genes (*E1f1ay*, *Ddx3y*) in the testes and bacterium. This can be attributed to the effect of sodium benzoate on the chromosome and DNA. In a study conducted by Ishidate and his group (1984)[30], sodium benzoate was found to cause chromosomal anomalies in the hamster's fibroblasts In another study, sodium benzoate was found to inhibit DNA synthesis at 100 mg

concentration .There was also a decrease in mitochondrial and chromosome breakdown when treated with sodium biodegradation and increased by increasing concentration and duration .[31]

Since these genes are found in a region known as male specific chromosome (msky), the Y chromosome is a repository of genes necessary to determine male sexual characteristics and sperm composition . These genes are due to the factors responsible for undernutrition (AZF). The lack of expression of the *E1f1ay* gene contributes to Azoospermia .[32] The decreased function of the *Ddx3y* gene leads to infertility .[33] Which explains the occurrence of abnormalities in the sperm and their number due to the effect of benzoate on the chromosome in addition to the effects on hormones. This explains the moral decline in the level Wyatt expression of the genes studied and increased this decline increased focus and duration.

References

1- Saad, B. ;Bari, M.F. ; Saleh,M.I. ;Ahmad,K.and Talib, M.K.(2005). Simultaneous determination of Preservatives(benzoic acid, sorbic acid,methylparaben and propylparaben) in foodstuffs using high performance liquid chromatography.J. Chromatogr.A. 1073(1-2):393-397.

2-Wroblewska ,B.(2009) .Influence of Food Additives and Contaminants(Nickel and Chromium) on Hypersensitivity and other adverse health reactions-A Review.pol.J.Food Nutr.Sci.59(4):287-294.

3-Jay, JM.; Loessner, MJ.and Golden, DA. (2005). *Modern food microbiology.* New York: Springer Science.

4-Makwana, S.; Choudhary, R.; Dogra, N.; Kohli, P.and Haddock, J.(2014). Nanoencapsulation and immobilization of cinnamaldehyde for developing antimicrobial food packaging material. *LWT-Food Sci Technol.* 57(2):470-6

5-Zengin, N.; Yuzbasioglu, D.; Unal, F.; Yilmaz ,S.and Aksoy, H.(2011). The evaluation of the genotoxicity of two food preservatives: sodium benzoate and potassium benzoate. *Food Chem Toxicol.* 49(4):763-9

6-Yazar, K.; Johnsson, S.; Lind, ML.; Boman, A.; Lidén, C.(2011). Preservatives and fragrances in selected consumer-available cosmetics and detergents. Contact Dermatitis , 64: 265-272.

7- Batshaw,M.L.;MacArthur,R.B. and Tuchman,M.(2001). Alternative pathway therapy for urea cycle disorders:twenty years later.J.Pediatr.138(1):46-54.

8-Stanojevic, D.; Comic, L.; Stefanovic, O. and Solujic-Sukdolak, S.(2009). Antimicrobial effects of sodium benzoate, sodium nitrite and potassium sorbate and their synergistic action in vitro. *Bulg J Agric Sci J.*15(4):307-11.

9-Mamur, S.; Yuzbasioglu, D.; Unal ,F. and Yilmaz, S.(2010). Does potassium sorbate induce genotoxic or mutagenic effects in lymphocytes? *Toxicol In Vitro.*24(3):790-4.

10- Nyman, P.J. ; Wamer, W.G. ;Begley, T.H. ;Diachenko, G.W. and Perfetti,G.A. (2010). Evaluation of accelerated UV andthermal testing for benzene formation in beverages containing benzoate and ascorbic acid .J.Food Sci.75(3):263-267.

11- do Nascimento Filho, I.; Schossler, P.; Santos Freitas,L.; Melecchi, MI.; Rodrigues Vale, MG. and Bastos Caramao, E.(2004). Selective extraction of benzoic acid from landfill leachate by solid-phase extraction and ion-exchange chromatography. *J Chromatogr A.*1027(1-2):167-70.

12- Cheng, D. .; Zheng, Xin.; Li, S.; Yang, Z.; Hu ,L.(2006). Effects of epidermal growth factor on sperm content and motility of rats with surgically induced varicoceles. *Asian J Androl; 8 (6): 713-717.*

13-Levin, R.J. ;Brown, M.H. ; Bell,M. ; Shue, F.;Greenberg,G. N. ;and Brodson, B. L.(1992). Air-conditioned Environments do not prevent

deterioration of human semen quality during the summer .*Fertil.Steril*.57:1065-1083.

14-Bambe, K. (1998). Evolution of acrosomal integrity of boar spermatozoa by bright field microscopy using an Eosin-Nigrosin stain. Theriogenology,29:1245-1251.

15-Lucio ,R.A.; Tlachi, J.L.; Lopez, A.A.; Zempoalteca, R.; Velázquez-Moctezuma, J.(2009). Analysis of the parameters of the ejaculate in the laboratory Wistar rat: technical description. Vet Méx;40:405–15.

16-Axiner, E. ; Malqvist, M. ; Linda-Forsberyg, C .and Rodringuez-Mertias, H. (1999). Regginal histology of the duct epididymis in the domestic cats .J.Report.Develop.45:151-160.

17- Wang, G. and Hardy, M.P.(2004).Development of leydig cells in the insulin-like growth factor-I(igf-I)knockout mouse: effects of igf-I replacement and gonadotropic stimulation.Biol.Repord., 70:632-639.

18-do Nascimento Filho, I.; Schossler, P.; Santos Freitas,L.; Melecchi, MI.; Rodrigues Vale, MG. and Bastos Caramao, E.(2004). Selective extraction of benzoic acid from landfill leachate by solid-phase extraction and ion-exchange chromatography. *J Chromatogr A.*1027(1-2):167-70.

19- Cheng, D. .; Zheng, Xin.; Li, S.; Yang, Z.; Hu ,L.(*2006*). Effects of epidermal growth factor on sperm content and motility of rats with surgically induced varicoceles. *Asian J Androl; 8 (6): 713-717*

20-Filaire, E. ;Bernain,X. and Sagnol, L. G.(2001).Preliminary results on mood state, salivary testosterone: cortisol ratio and team performance in a professional soccer team.Eur J.Appl.Physiol.86(2):179-184.

21- Anuka, E. ;Yivgi-Ohana, N.;Eimerl, S. ;Garfinkel, B. ;Melamed-Book, N. ; Chepurkol, E. ; Aravot, D. ; Zinman, T. ; Shainberg, A. ; Hochhauser, E. and Orly, J.(2013).Infarct-Induced Steroidogenic Acute Regulatory Protein :A Survival Role in Cardiac Fibroblast .Mol. Endrocrinol.27(9):1502-1517.

22-Arukwe, A. (2005). Modulation of brain steroidogenesis by affecting transcriptional changes of steroidogenic acute regulatory (StAR) protein and cholesterol side chain cleavage (P450Scc) in juvenile Atlantic salmon (Salmo

salar) is a novel aspect of nonylphenol toxicity. Environ. Sci. Technol.39(24):9791-9798.

23- Breen, K.M. and Karsch, F. J. (2004).Does cortisol inhibit pulsatile luteinizing hormone secretion at the hypothalamic or pituitary level . Endocrinology,145(3):692-698.

24- Weltzien,F.A.; Norberg,B. and Swanson ,P. (2003). Isolation and characterization of FSH and LH from Pituitary glands of Atlantic halibut (Hippoglossus hippoglossus L.).General and Comparative Endocrinology .131:97-105.

25- Xiong,X. ; Wang,A.;Liu, G.; Liu,H.; Wang,C.; Xia,T. ; Chen, X.and Yang,K.(2006). Effect of p,p`-dichlorodiphenyl dichloroethylene on the expressions of transferrin and androgen-binding protein in rat Sertoli cells. *Environ.Res.*101(3):334-339.

26-Hakki, T.; Bernhardt, R.(2006). CYP17- and CYP11B-Dependent Steroid Hydroxylases as Drug Development Targets. Pharmacol. Ther. 111, 27–52

27- Sorg,B.A.;Bailie T. M. ; Tachergi, M. L. ;Li, N. and Wu, W. R. (2001). Exposure to repeated low-level formaldehyde in rats increases basal corticosterone level and enhances the corticosterone response to subsequent formaldehyde .Brain Res.898(2):314-320.

28- Debus,N.; Breen, K. M. ;Barell, G. K. ;Billings, H. J. ;Brown, M. and Young, E. A. (2002). Does cortisol mediate endotoxin-induced inhibition of pulsatile luteinizing hormone and gonadotropin-releasing hormone secretion ? Endocrinology ,143(10):3748-3758.

29-Bulun, SE.; Lin, Z.; Imir, G.; Amin, S.; Demura, M.; Yilmaz, B.; Martin, R.; Utsunomiya, H.; Thung, S.; Gurates, B.; Tamura, M.; Langoi, D.; Deb, S. (2005). Regulation of aromatase expression in estrogenresponsive breast and uterine disease: from bench to treatment. Pharmacol Rev. 57:359–83.

30- Livonen, S.; Heikkinen, T.; Puolivali, J.; Helisalmi, S.; Hiltunen, M.; Soininen, H.; Tanila, H. (2006).Effects of estradiol on spatial learning,

hippocampal cytochrome. P450 19, and estrogen alpha and beta mRNA levels in ovariectomized female mice. Neuroscience.137: 1143–1152.

31- Ishidate, M. Jr.; Sofuni, T.; Yoshikawa, D.; Hayashi, M.; Nohmi, T.; Sawada ,M. and Marsouka, A.(1984). Primary mutagenicity screening of food additives currently used in Japan. Food and Chemical Toxicology, 22, 623-633.

32- Turkoglu, S.(2007). Genotoxicity of five food preservatives tested on root tips of Allium cepa L. *Mutat Res.* 626(1-2):4-14.

33-Kleiman, S. E.; Yogev, L.; Hauser, R.; Botchan, A.; Maymon, B. B. S.; Paz, G. and Yavetz, H. (2007). Expression profile of AZF genes in testicular biopsies of azoospermic men. Hum. Reprod. 22, 151–158.

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