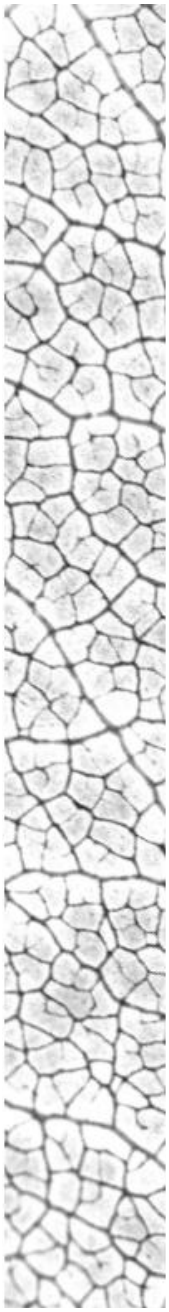

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Molecular and morphological study of the UV radiation on mice sperm formulation

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Abstract

After exposure of mice to ultra violet (UV) radiation for 30, 60 and 90 minutes for 8 days and after sacrificing the mice, sperm isolation and examine. The percentage of live and normal sperm decrease and the percentage of dead and abnormal sperm increase with the increase of exposure time as compared with the control, this is before fertilization. Also examine the sperm after fertilization to induce the new sperm formation. The results revealed that higher percentage of normal and live sperm was decreased and higher percentage of dead and abnormal sperm increased as compared with the percentage of them before fertilization and in compare with control. Also after each exposure, serum was examined to determine the concentration of reproductive hormones (FSH, LH and Testosterone). Before fertilization, the level of reproductive hormones increases with the increase of exposure time as compared with the control while after fertilization the level of reproductive hormones increases more than before fertilization.

There is a protein in humans called Zinc Finger Y-chromosomal protein which is encoded by the ZFY gene on the Y chromosome, there are two paralogous copies expressed in mice, Zfy1 and Zfy2, wrongful expression of one of them during spermatogenesis, results in apoptosis at the mid-pachytene checkpoint. Zfy genes in mice are useful for meiotic sex chromosome inactivation (MSCI) and may work as a transcription factor. In Zfy knockout spermatocytes, sex chromosomes are wrongly coding. Therefore, the Zfy has some functions at the mid-pachytene checkpoint: (1) promote MSCI, (2) monitor MSCI progress, and (3) execute cells, by program cell death, that fail to undergo MSCI.

Our results show that zfy1 increases its expression in group (1) 4.06 fold after exposed to UV ray in 30 min and increases its expression in group (2,3) 11.80 and 20.00 folds after exposed to UV ray in 60 and 90 min respectively, that means increases in meiosis and higher incidence of meiotic errors lead to lower sperm counts and abnormalities sperms.

Keywords: sperm, gene expression, reproductive hormones, Zfy

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INTRODUCTION

There are clear effects of UV radiation on organisms, it causes several biological reactions, formed by various photochemical processes. By way of the production of the free radicals and the "ROS" reactive Oxygen species. The free radicals are the cause of a series of events Contains changes in DNA (including breaks of the double-strand (dsB) or single strand (ssB)), peroxidation of lipid and denaturation of proteins. or cell damage (Akram et al., 2013).

UV irradiation can impair cellular functions by directly damaging DNA to induce apoptosis (Wäster & Ollinger, 2009). Sometimes the chromosomes in the sperm exposed to changes that lead to changes in the genetic information which also lead to abnormal shape, size and appearance (Armstrong & Kricker 2001). Genetic, environmental (more common), or both of them are the causes of sperm abnormalities. Fail to conception and male infertility causes have been associated with these factors (Gruijl et al., 2001). Classification of Sperm abnormalities are into two groups, primary (All defects which occur during spermatogenesis) and secondary (those developing subsequent to spermiation) abnormalities depending to their presumptive origin (Sun & Martin 2006). Head, middle piece or tail are classified as sperm abnormalities (defects in the sperm) (Saacke 2001). The phenotypic traits transfer through gene from parents to filial. Most biological properties resulted from the effect of many different genes as well as epigenetic interactions.

The ZFY gene in humans is coding for Zinc finger Y-chromosomal protein, whereas the expression be in the

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mice into the two paralogous copies: (Zfy1, Zfy2). A transcription factor is the function of this gene. This gene may be elect gene for the testis-determining factor (TDF).

Through spermatogenesis, mutant expression of Zfy1 or Zfy2 causes apoptosis, at the mid-pachytene checkpoint. https://en.wikipedia.org/wiki/ZFY - cite_note-Vernet_2016-5 ZFY in humans is largely expresses in the testis and prostate. Also ZFY express in twenty different tissues, such as bone marrow "urinary bladder" gall bladder and appendix. ZFY gene has correlation with other disease include campomelic dysplasia, cystadenofibroma, and Frasier syndrome (Nagamine et al., 1990).

Pituitary gland is a part of endocrine system which plays a main role in controlling many processes in the body as metabolism, growth, sexual maturation, blood pressure, reproduction and other processes (De Bellis et al., 2011a). It regulates their functions by secreting many different hormones into the bloodstream. The secreted hormones are controlled by two different ways either hormonal or nervous signals from the hypothalamus (Gore, 2010).

Complex interaction between gonads which include ovaries and testes, pituitary gland and hypothalamus, all of them have control on reproductive systems in both of genders (males and females). Hypothalamus secrets Gonadotropin releasing hormone (GnRH) which is a trophic peptide hormone. The anterior pituitary gland stimulated by GnRH to release gonadotropins which are follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Albanese et al., 1996 & Bliss et al., 2010). FSH and LH play main roles in controlling reproduction in both males and females. In males, after the hypothalamus elicits the pituitary gland to release both of FSH and LH. Then each of FSH and LH enters the testes. FSH plays an important role to stimulate spermatogenesis after it binds with receptor of Sertoli cells. LH is required to produce testosterone by Leydig cells. Testosterone is a hormone that is responsible on the secondary sexual characteristics (Anderson et al., 1997).

The aims of this study are to show the effect of UV light exposure on sperm parameters, gene expression and reproductive hormones FSH, LH and testosterone before and after fertilization.

MATERIALS AND METHODS

Animals

A mice of Swiss albino strain were obtained from animal house of Biotechnology division/ Technology University. 40 mice male were used in this study, at the beginning of experiment their ages range between 3.5 - 4 months old and the weight range was between 25-30 grams.

Table 1. The Primers used

Primers	Sequence: 5' →3' direction
Zfy1	
Forword	TGCCTGAACATGTCTTGATGAG
Reverse	CCCACTGGAATCTAAGACTGC
Eef2	
Forword	ATTCTGATGATGGGCCGCTA
Reverse	GCTGACGCTGAACTTCATCA

Exposure to UV

Males were randomly divided into 4 groups, each composed of 10 mice. The first group was exposed to UV for 30 minutes; the second group was exposed to UV for 60 minutes and the third group was exposed to UV for 90 minutes daily for 8 days and the fourth group didn't expose to UV as a control group. The exposure distance was 50 cm (Akram et al., 2013).

Sperm isolation and microscopic examination

Sperm were collected by flushing (Fakhrildin et al., 2001). According to world health organization Laboratory manual the sperms were assessed for percentage of dead/live sperm, motility, and abnormalities (WHO 1999). The sperm were examined before and after fertilization.

Gene expression of mRNAs

mRNAs expression was determined by (RT-qPCR) method. mRNA was extracted from testes using TransZol Up Plus RNA Kit (Transgen BioTec., China). Modified TRIzol method (TRIzol, 2012)

The isolated mRNA from samples was reversely transcribed to cDNA using EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (Transgen BioTec.). Quantitation for mRNA, total RNA was poly (A)-tailed, before reverse transcription, use an A-Plus Poly (A) Polymerase Tailing Kit (Transgen, Bio.Tec.). According to the instructions of the manufacturer the reaction was done in 20 µl volume. The reverse transcription quantitative polymerase chain reaction (RT-qPCR) was carried out using the TransStart® Green qPCR SuperMix Kit (TransGen Bio tech.) and cDNA as a template.

Design primers

Primers were designed according to bioinformatics programs: Forward and reverse oligonucleotide primers of target mRNA gene and for the housekeeping gene Eef2, were showed in **Table 1**.

25 µl is the final volume of the reaction mix according to the manufacturer which contains: 12.5µl GoTaq_ qPCR Master Mix (1X); for 2-Step RT-qPCR, 1µl of each primer (10 mM), 3 µl cDNA and 7.5 µL nuclease-free water. Then transfer the mix to a real-time thermocycler (Cephied, smartcycler Real-time PCR System, USA), that programmed for the optimized cycles: initial denaturation for 30 sec. at 4C° for one cycle only, then denaturation for forty cycles for 5 Sec at 94C°, annealing (15 second at 60C°) and extension (10 second at 72C°),

Table 2. Effect on UV radiation on mice sperm before fertilization

Groups	Live sperm % (mean ± SE)	Dead sperm % (mean ± SE)	Normal sperm % (mean ± SE)	Abnormal sperm % (mean ± SE)
Control	40.285 ± 0.11	2.85 ± 0.11	19.24 ± 13.47	2.33 ± 1.05
30 (min.)	6.038 ± 2.79	10.31 ± 0.11	15.078 ± 4.99	2.136 ± 1.136
60 (min.)	4.969 ± 0.033	10.72 ± 1.15	13.045 ± 0.045	4.929 ± 3.78
90 (min.)	4.20 ± 3.25	10.86 ± 7.95	5.746 ± 2.16	5.36 ± 2.20

Table 3. Effect on UV radiation on mice sperm after fertilization

Groups	Live sperm % (mean + SE)	Dead sperm % (mean + SE)	Normal sperm % (mean + SE)	Abnormal sperm % (mean + SE)
Control	40.285 + 0.11	2.85 + 0.11	19.24 + 13.47	2.33 + 1.05
30 (min.)	1.3 + 0.17	19.2 + 0.115	7.927 + 1.124	4.53 + 1.44
60 (min.)	0.932 + 0.011	28.914 + 4.225	6.538 + 0.577	6.73 + 5.96
90 (min.)	0.8 + 0.4	+ 2.95	3.786 + 0.059	9.26 + 4.95

and the end cycle of melt curve (Dissociation Stage) at 65–90 C°.

The expression was valued as "2-ΔΔCt", which demonstrates changes in the relative fold. So the results were expressed as a fold change in the level of expression a study gene that was normalized to housekeeping gene and relative to a calibrator, which is the study gene in control samples (Stephenson and Paul, 2016).

Quantitative Real Time PCR (qRT–PCR)

Quantitative Real Time PCR (qRT–PCR) The fold change and levels of gene expression were quantified by adjust the threshold cycle (Ct) using the TransStart® Green qPCR Super Mix Kit components.

Real Time qRT-PCR analysis of zfy1 gene expression (Livak and Schmittgen, 2001)

ΔCT: The expression ratio was calculated without a calibrator sample 2-ΔCt according to the following equation: (Livak and Schmittgen, 2008).

ΔCT (test) = CT gene of interest (target, test) – CT internal control

Finally, the expression ratio was calculated according to the formula

2-ΔCt = Normalized expression ratio

Hormonal analysis

The samples of blood were separated by centrifuge at (3000 rpm for 3 minutes). Then the samples of serum were analyzed to determine the concentration of reproductive hormones (FSH, LH and Testosterone).

FSH & LH hormonal assay

The concentrations of FSH and LH were determined by using Diagnostic automation, inc. for FSH and LH (Microwell ELISA Follicular –Stimulating Hormone (FSH) enzyme immunoassay test kit and Microwell ELISA Luteinizing Hormone (LH) enzyme immunoassay test kit) for human (Knobil, 1980).

Testosterone hormonal assay

The concentration of Testosterone was determining according to Bio merieux Italia S.P. a vidia campligliano, 58 50015-point A EMA (F1) Italia miniVIDAS (Wasnaa, 2014).

Statistics

At least three independent replicates were performed in all experiments. For statistical analysis, student t-test was used for two group comparisons and ANOVA, followed by Turkey's test for multiple group comparisons. $P < 0.05$ was considered significance.

RESULTS AND DISCUSSION

Effect of exposure to UV on mice sperm before and after fertilization

The mice were exposed to ultra violet (UV) radiation for 30, 60 and 90 minutes for 8 days and after sacrificing the mice, sperm isolation and examine the sperm under the microscope and count the number of live, dead, normal and abnormal sperm the results showed that when increase the exposure time the percentage of live and normal sperm will decrease on other hand the percentage of dead and abnormal sperm increase in compare with control as shown in **Table 2**.

Our results agree with (Amel, 2014) that the effect of radiation on sperm head abnormalities shows the number of normal cells in the control group decrease while the no head and no head and tail increase.

Also study the sperm (normal, abnormal, live and dead) after fertilization to induce the new sperm formation. The results after fertilization revealed that the percentage of normal and live sperm was decreased more than before fertilization and the percentage of dead and abnormal sperm increased as compared with the percentage of them before fertilization and in compare with control as shown in **Table 3**.

It can be concluded that, some degree of sperm head abnormalities related to UVC irradiation as a toxic effect. four types of sperm abnormalities found in this research: headless, abnormal tail, curved head & no hook and balloon head **Fig. 1**.

Extraction of total RNA

RNA was successfully isolated from all samples, concentration of total RNA ranged from 53 to 196 ng/ µl. The purity of total RNA samples ranged from 1.52 to 1.99 ng/ µl in all tissue sample.

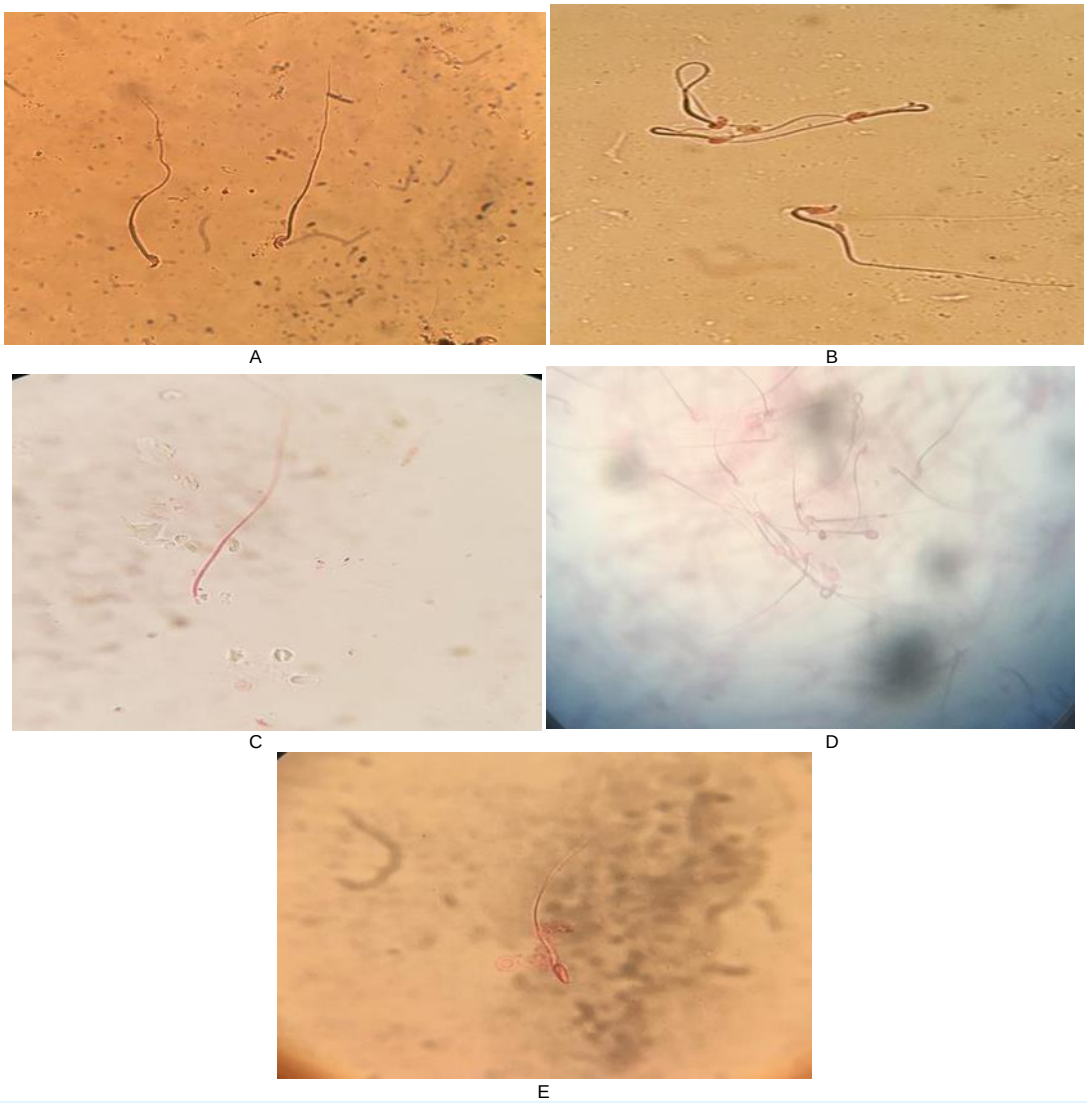

Fig. 1. (A) Normal mice sperm, (B) Abnormal mice sperm tail, (C) no head (D) balloon head and (E) Abnormal mice sperm (curved head & no hook head)

cDNA reverse transcription

cDNA reverse transcription was conducted to the extracted RNA. A common primer reaction was applied since it was needed to have cDNA for both the target gene in study and housekeeping gene. The efficiency of cDNA concentration was assessed through the efficiency of qPCR conducted later on. All steps were associated with perfect yield reflecting efficient reverse transcription.

Results of quantitative Real Time PCR

Real time PCR quantification utilized the SYBR green, a fluorescent dye which recognizes any double stranded DNA including cDNA. The amplification was registered as a Ct value "cycle threshold**"**. The lower value of Ct refers to the existence of higher copies of the study gene and vice versa. In phrase of gene expression, the high Ct values indicate low gene expression and vice versa (Royo et al., 2010).

Real time PCR quantification of Eef2 Expression

The Ct value of Eef2, the housekeeping gene shown in **Table 4**.

In molecular studies the inherent assumption in the use of housekeeping genes is that under investigation in the cells or tissue their expression remains constant. They found that using of *Eef2* is quite a reliable strategy for the normalization in qRT-PCR when applied in clinical studies.

Real time PCR quantification of zfy1Expression

The Ct value of *zfy1* cDNA amplification are shown in **Table 5**.

Each quantificative PCR reactions was run in triplicate for each sample, in each run, samples from mice group, radiation staff group and control group were run in addition to non-template and non- primer controls.

Table 4. Comparison of Eef2 Fold expression between groups in this study

Group	Means Ct of *Eef2*	2^{-Ct}	experimental group/ Control group	Fold of gene expression
Group 1 (control)	19.84	106 E6	106 E6/105 E6	1.009
Group 2 (30 min.)	19.81	108 E6	108 E6/105 E6	1.02
Group 3 (60 min.)	19.83	107 E6	107 E6/105 E6	1.01
Group 4 (90 min.)	19.86	105 E6	105 E6/ 105 E6	1.00

Table 5. Comparison between different groups in Ct, ΔCt and 2-ΔCt value (*zfy1*)

Group	Mean of Ct zfy1	Mean of Ct Eef2	Mean ΔCt = CT gene of zfy1 – CT Eef2	Mean 2-ΔCt
Group 1 (control)	23.78	19.84	3.94	0.065
Group 2 (30 min.)	22.17	19.81	2.36	0.19
Group 3 (60 min.)	21.43	19.83	1.6	0.32
Group 4 (90 min.)	25.81	19.86	5.95	0.016

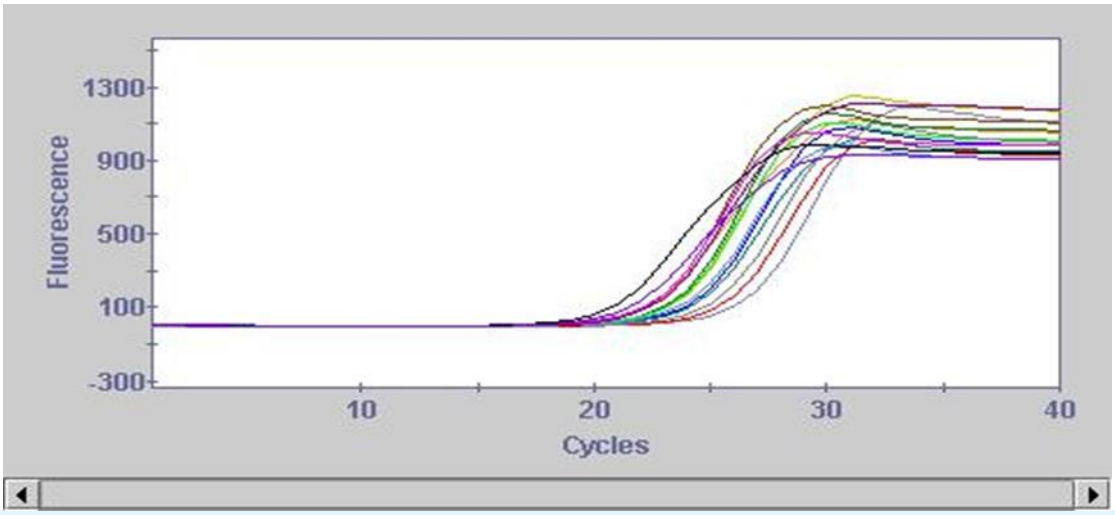

Fig. 2. *Eef2* and *Zfy-1* amplification plots by qPCR Samples included all study groups. Ct values ranged from 18.23-21.65. The image taken directly from cephied (smart cycler) qPCR machine

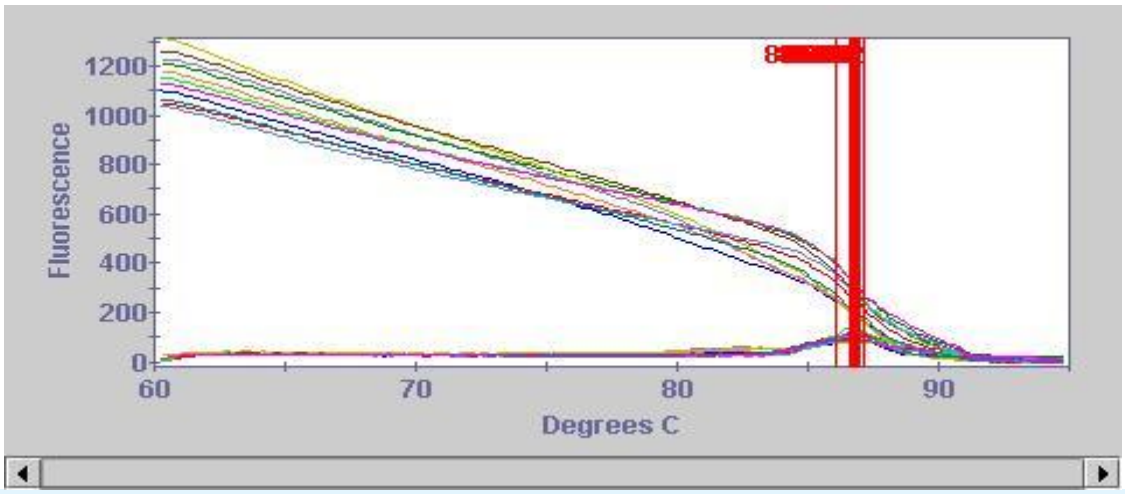

Fig. 3. *Eef2* and *Zfy-1* dissociation curves by qPCR Samples included all study groups. Melting temperature ranged from 86°C to 88°C, No primer dimer could be seen. The image taken directly from cephied (smart cycler) qPCR machine

This was important to make the statistical calculation of each group and in order to specify the calibrator. Plots of each run were recorded including the amplification plots and dissociation curves. **Figs. 2** and **3** show the amplification plots and dissociation curves for *Eef2* and *zfy1*.

Normalization of Ct (cycle threshold) Values: The RT-PCR examined the expression of mRNA of zfy1 and compared its expression between apparently healthy control group (not exposed to UV), and the other groups. The mathematical determination of gene expression fold change was made using relative quantification **Table 6** (Livak & Schmittgen, 2001).

ZFY genes of Mammals are existed on the Y chromosome; and code supposed transcription factors with (12-13) zinc fingers preceded by a large acidic

Table 6. Fold of *zfy1* expression Depending on $2^{-\Delta Ct}$Method

Groups	Means Ct of *Zfy1*	Means Ct of *Eef2*	ΔCt(Means Ct of *zfy1* - Means Ct of *Eef2*	$2^{-\Delta Ct}$	experimental group/ Control group	Fold of gene expression
Group 1 (control)	24.81	19.86	5.95	0.016	0.016/0.016	1.00
Group 2 (30 min.)	23.78	19.84	3.94	0.065	0.065/0.016	4.06
Group 3 (60 min.)	22.17	19.81	2.36	0.19	0.19/0.016	11.80
Group 4 (90 min.)	21.43	19.83	1.6	0.32	0.32/0.016	20.00

Table 7. Thermal profile of zfy1 expression

Step	Temperature	Duration	Cycles
Enzyme activation	95ºC	3 min	Hold
Denature	95ºC	5sec	40
Anneal/extend	60ºC	20 sec	
Dissociation	1min /95 ºC-30 sec /55 ºC-30sec/95 ºC		

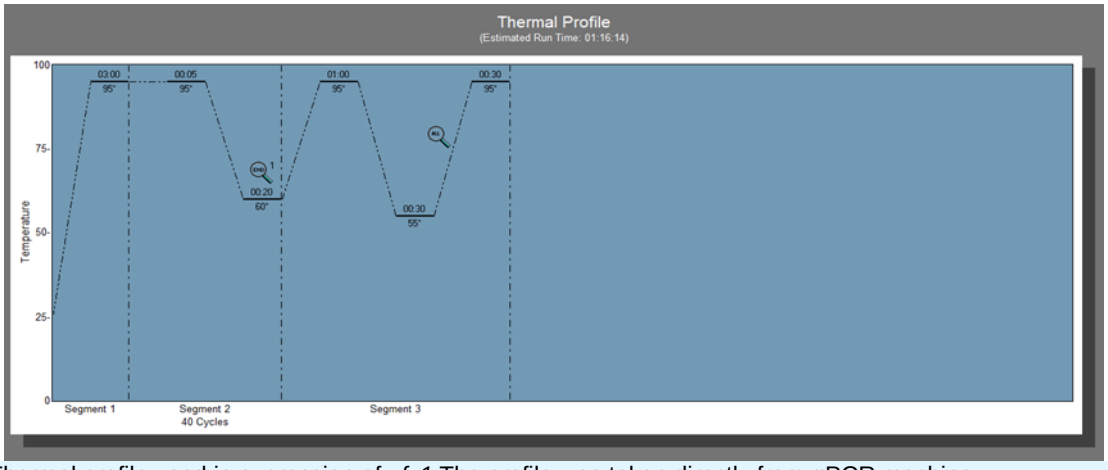

Fig. 4. Thermal profile used in expression of *zfy1*.The profile was taken directly from qPCR machine

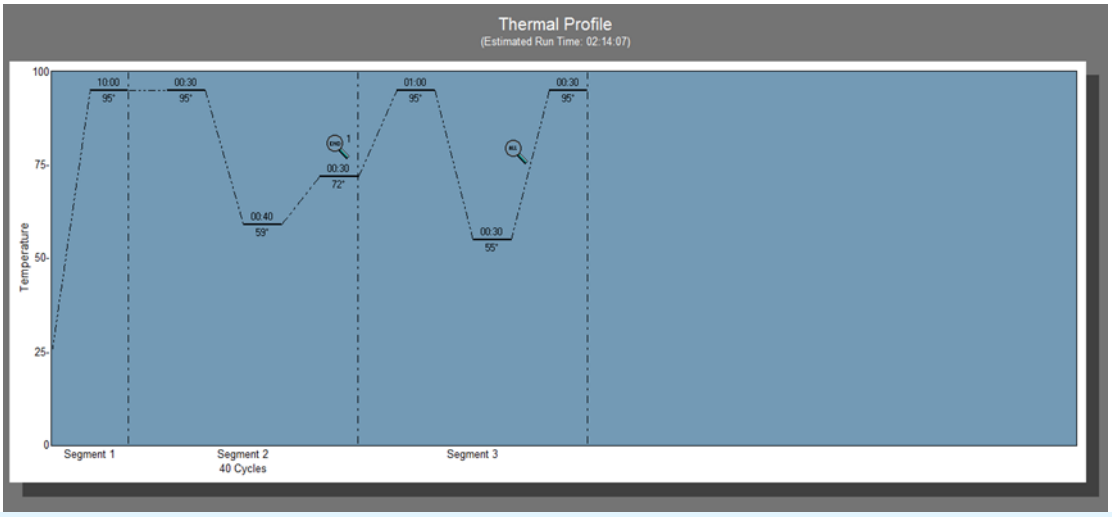

Fig. 5. Thermal profile used in *Eef2* Amplification. The image was taken directly from qPCR machine

(activating) domain. There are two genes in mice, (*Zfy1* and *Zfy2*), that are expressed mainly in the testis. As they enter meiosis their transcription raises in germ cells, during pachytene the two genes are silenced by meiotic sex chromosome inactivation (MSCI). Our results show that *zfy1* increases its expression when exposed to UV ray that means increases in meiosis and higher incidence of meiotic errors lead to lower sperm counts and abnormalities sperms (Amel, 2014 and Royo et al., 2010).

The qPCR Reaction run

According to the thermal profile mentioned in the **Table 7 Fig. 4** the cycling protocol was programmed:

Housekeeping Gene Amplification

The housekeeping gene (Eef2) used as an internal control to calculate the ΔCT value. A reaction of qPCR amplification of Eef2 was done with the Thermal profile shown in **Fig. 5.**

Table 8. Effect on UV radiation on mice hormones before fertilization

Groups	Testosterone (ng/ml) (mean + SE)	FSH (IU/ml) (mean + SE)	LH (IU/ml) (mean + SE)
Group 1 (control)	2.17 ± 0.18	0.22 ± 0.02	0.27 ± 0.07
Group 2 (30 min.)	2.78 ± 0.455	0.55 ± 0.002	0.5 ±0.023
Group 3 (60 min.)	3.66 ± 0.057	0.59 ± 0.011	0.53 ± 0.115
Group 4 (90 min.)	3.94 ± 0.023	0.61 ± 0.017	0.56 ± 0.001

Table 9. Effect on UV radiation on mice hormones after fertilization

Groups	Testosterone (ng/ml) (mean + SE)	FSH (IU/ml) (mean + SE)	LH (IU/ml) (mean + SE)
Group 1 (control)	2.17 ± 0.18	0.22 ± 0.02	0.27 ± 0.07
Group 2 (30 min.)	2.5 ± 0.28	0.7 ± 0.011	0.51 ± 0.011
Group 3 (60 min.)	3.55 ± 0.25	0.77 ± 0.003	0.64 ± 0.002
Group 4 (90 min.)	4.75 ± 0.25	1.3 ± 0.01	2.35 ± 0.215

Effect of exposure to UV on reproductive hormones FSH, LH and testosterone before and after fertilization

The mice were exposed to Ultra Violet (UV) radiation for 30, 60 and 90 minutes for 8 days and after sacrificing the mice, serum were analyzed to determine the concentration of reproductive hormones (FSH, LH and Testosterone). The results demonstrated when the exposure time increase, the level of reproductive hormones will increase also compared to control as shown in **Table 8**.

After fertilization, the results showed that the level of reproductive hormones (FSH, LH and Testosterone) was increased more than before fertilization as shown in **Table 9**.

The results that were obtained in the above experiments established that UV light exposure effects on pituitary function that will influence on the levels of reproductive hormones (FSH, LH and Testosterone). These results are not generally consistent with the work of (Falkenbach et al., 1997).

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