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Excessive ROS generation and oxidative stress induction trigger chromatin dispersion and apoptosis in sperms of parents with recurrent implantation failure

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Abstract

There is insufficient evidence on correlation between DNA fragmentation and recurrent implantation failure (RIF) to introduce sperm DNA testing as a part of the clinical practice. Thus, this study investigated the influence of sperm DNA fragmentation on the incidence of RIF. Partners of 110 women with RIF following intracytoplasmic sperm injection (ICSI), and 50 recent fathers (control) were examined. Sperm DNA fragmentation was examined by the sperm chromatin dispersion (SCD) test, Aniline blue test (AB), Acridine orange (AO) test and DNA diffusion assay. The study also examined different aspects causing DNA fragmentation such as oxidative stress by biochemical measuring of reduced glutathione (GSH) and malondialdehyde (MDA) levels, and the reactive oxygen species (ROS) level was also studied using dichlorofluorescin diacetate. There were no obvious differences in sperm DNA fragmentation index measured among different used tests. However, AB was found to provide clear and stable clinical cut-off levels, and considered as the most easy and inexpensive assay. Therefore, AB can be recommended for a clinical sperm DNA damage evaluation as a routine test. Moreover, impaired chromatin packaging, apoptosis and ROS result in significant DNA damage in human spermatozoa of RIF group compared to control and positive pregnancy groups.These results support the hypothesis that sperm DNA fragmentation is an important cause of RIF, and that sperm DNA integrity testing has value as a diagnostic tool in investigation and treatment of infertility.

Keywords: Recurrent implantation failure; DNA fragmentation; Oxidative stress; Apoptosis and ROS generation

Introduction

For patients undergoing assisted reproductive technology (ART), recurrent implantation failure (RIF) is relevant only. While no official definition for recurrent implantation failure is appropriate, some studies describe RIF as failure to achieve pregnancy after 2–6 cycles of in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI), in which at least 10 good quality embryos were transferred into the uterus **(Shufaro and Schenker 2011)**. It was suggested that the embryo and the endometrium can play a good role in RIF **(Simon and Laufer 2012)**.

Embryonic factors are the most common causes of RIF, because the good quality embryo needs to have the correct number of cells corresponding to the day of its development. Implantation failure can result from arresting embryo to develop in uterus, and also possible that low-quality sperm may result in the formation of low-quality embryos **(Cutting et al. 2008; Avendaño et al. 2010)**.

The examination of the sperm quality depends on the evaluation of a semen sample in a basic andrology laboratory **(Organization, 2010)**, which are poor predictors of reproductive outcome. So, sperm DNA integrity testing has been suggested to be a test with promising potential to compliment the standard semen analysis **(Aitken and De Iuliis, 2007)**.

Fragmentation of sperm DNA (SDF) may increase the risk of failure to conceive. **(Evenson and Wixon 2008)**, Bad result following stimulated intrauterine insemination **(Bungum et al., 2007)**, longer times to pregnancy **(Spanò et al., 2000)**, deficient embryonic development **(Morris et al., 2002)**, high abortion rate **(Robinson et al., 2012)** and an increased risk of loss of pregnancy after both *in vitro* fertilization (IVF) and intracytoplasmic sperm injection **(Zini et al., 2008)**. So, damage of sperm DNA may have long-distance ramifications for reproductive outcome.

The etiology of SDF is involving a number of factors. Some of cellular events participate to reduced fertility and damage of sperm DNA such as abnormal chromatin packaging or remodeling during spermatogenesis **(Sakkas et al., 2002, Shamsi et al., 2008)**, increased reactive oxygen species (ROS) production **(Moustafa et al. 2004; Venkatesh et al., 2009)**, reduced seminal antioxidants **(Shamsi et al., 2010)**, and the apoptosis within the epididymis during sperm maturation **(Gosálvez et al., 2015)**. Also was suggested that exposition to the toxins and pollutants in the environment, chemoradiation, drugs, febrile illness, smoking, varicocele and raised age is a factors that can increase SDF **(Rubes et al. 2005, Sharmam et al., 2013, Sharma et al., 2016)**.

Some studies from the American Society for Reproductive Medicine **(Medicine 2008)**, the European Society for Human Reproduction and Embryology **(Barratt et al., 2010)**, and the British Fertility Society **(Tomlinson et al., 2013)** all have reported that nowadays, there is a few evidence for sperm DNA testing to be inserted as aspect of clinical application with the need to identify more research.. However, the three studies showed that the most powerful evidence is actually for a sperm DNA examination role in patients of recurrent miscarriage (RM) and implantation failure.

The current study was therefore conducted to investigate the prevalence of sperm DNA fragmentation in couples with idiopathic recurrent implantation failure, by evaluating the various aspects that cause DNA fragmentation including chromatin condensation, oxidative stress markers, ROS and apoptosis, to determine the main reason of DNA fragmentation, and the possible correlation between DNA fragmentation and RIF.

Subjects and methods

Research ethics committee approval:

The plan and all experiments of this study have been approved by the ethical committee in the General Organization for Teaching Hospitals and Institutes, Egypt with the approval number "HAM00092.

Selection of subjects:

The current study included 160 semen samples, 110 samples of sperm from couples with RIF, including those who had a positive and negative pregnancy test after 2 to 6 cycles of ICSI, and 50 samples of sperm from men who had become recent fathers as a control group**.**

Preparation of semen samples

Semen samples (one per subject) were obtained by masturbation 3–4 days after sexual abstinence. Samples were collected in sterile plastic containers (Sarstedt, Leicester, UK), and allowed to liquefy at 37°C for 30 min prior to semen analysis according to WHO methods. After semen analysis, sperms were isolated from seminal plasma using an 80-40% density centrifugation gradient (Cook UK Ltd., Hitchin, UK), and centrifuged at 500 × *g* for 20 min before being washed in 3 ml fertilization medium, and then finally re-suspended in a further 0.3 ml of fertilization medium.

Measuring the integrity of sperm DNA

The integrity of sperm DNA was assessed in all semen samples using Sperm chromatin dispersion, Acridine Orange, Aniline Blue tests and DNA Diffusion assay.

Sperm chromatin dispersion (SCD) test

The Sperm chromatin dispersion (SCD) test was done using the Halosperm G2 kit (Halotech DNA; Madrid, Spain), based on the principle that sperms with fragmented DNA can't produce the characteristic halo of dispersed DNA loops, which are observed on

the sperms with non-fragmented DNA. Sperms were mixed with aqueous agarose, following acid denaturation, and removal of chromatin nuclear proteins, and finally, 500 spermatozoa were examined **(Fernandez et al., 2003)**.

Sperm chromatin structure assay using

The Acridine Orange (AO) test was performed to assess single and double-stranded DNA breaks in all samples, based on denaturation of sperm DNA by acid which makes metachromatic shift of AO fluorescence from green (non-denatured DNA) to red (denatured DNA), thus, the monomeric AO bound to native DNA fluoresce green, while the aggregated AO on denatured DNA fluoresces red **(Tejada et al., 1984; Hoshi et al. 1996)**. Briefly: semen samples were spread on clean slides, air-dried and fixed overnight in methanol-glacial acetic acid (3:1) at room temperature. Slides were then removed from the fixative, stained with AO (0.19 mg/mL, pH 2.5) for 7 minutes at room temperature, rinsed gently in deionized water and sealed under a coverslip with nail polish. Slides were read on the same day with a fluorescent microscope using 490 nm excitation filter, and 530nm barrier filter. The heads of sperm cell with good DNA integrity had a greenish fluorescence, while those with diminished DNA integrity had orange-red color.

Aniline Blue (AB) test

To assess the chromatin packing of sperm DNA, the Aniline Blue (AB) test was conducted by spreading 10 µL of the prepared spermatozoa onto clean glass slides, leaving them to dry, then fixing them in 4% formalin for 5 minutes at room temperature, and rinsing them in deionized water. Finally, stained slides with 5% AB in 4% glacial acetic acid (pH 3.5) solution for 5 minutes then rinsed them with deionized water, and left to dry. Under light microscope, 200 sperms were counted using 1000x magnification. Immature sperms were stained dark blue. The percentage of abnormal sperm chromatin condensation was calculated as the ratio of the number of dark-blue sperm to the total number of sperm examined.

DNA diffusion assay

A small aliquot of the semen sample was analyzed for apoptosis using a DNA diffusion assay **(Singh, 2000)**. Micro-gel electrophoresis slides were prepared by coating with 50 µL of 0.7% agarose, and the first layer of the microgel was made by pipetting 50 µL of agarose on top of the frosted portion of the slide, and smearing the agarose in one motion, holding it horizontally and then air drying. The second layer of the microgel was made by mixing approximately 10000 sperm cells in 5 mL of PBS with 50 µL of 0.6% agarose 3:1 for each slide, and 50 µl of this mixture was layered onto the pre-coated slides with the first layer of microgel, and the slides were left for air drying. 200 mL of 2% superfine resolution (SFR) agarose solution was layered as before to make a third layer of microgel, the slides were then immersed and maintained for 10 minutes in a lysis solution.

For neutralization and DNA precipitation, slides were immersed twice in freshly prepared 20 mM Tris for 10 minutes, then in ethanol 2 min, and then air dried. One slide at a time was stained with ethidium and examined immediately under a fluorescent microscope. Sperm exhibiting diffuse halos in the DNA diffusion assay were considered apoptotic. The percentage of apoptotic cells with diffuse DNA and a hazy outline was calculated from a total of 1000 cells counted.

Estimation of oxidative stress

Induction of oxidative stress was studied by estimating the reactive oxygen species (ROS) generation, and measuring the level of malondialdehyde (MDA) and reduced glutathione (GSH) in all semen samples.

Measuring the MDA level

The level of MDA was determined by measuring the absorbance of the pink product resulting from the reaction of thiobarbituric acid with MDA in an acidic medium, at temperature of 95°C, at wavelength of 534 nm **(Ohkawa et al., 1979)**.

Measuring the GSH level

The level of GSH was measured based on the reduction ability of Ellman's reagent [5, 5' dithio bis-(2- nitrbenzoic acid)] with GSH to form 1 mole of 2-nitro-5 mercaptobenzoic acid (yellow compound) per mole of GSH. Thus, the reduced chromogen is directly proportional to the GSH concentration, and its absorbance is determined by spectrophotometer at 405 nm **(Beutler, 1963)**.

Estimation of ROS using Dichlorofluorescin diacetate stain

Intracellular ROS production was measured using 2,7 dichlorofluorescin diacetate (DCFH-DA) dye, that passively introduces sperms, and interacts with ROS forming a highly fluorescent compound dichlorofluorescein (DCF) **(Siddiqui et al., 2010)**. Sperm cells were washed twice with PBS, then 50 µL of the semen sample was incubated for 30 min in dark with DCFH-DA (20mM), and 20 µL of this mixture was layered onto the slides. The cells were visualized and photographed using a fluorescence microscope.

Statistical analysis

All results are represented as mean ± standard error of the mean, and were analyzed using Statistical Package for Social Science (SPSS) software version 22. One Way Analysis of Variance ANOVA) followed by Duncan's test was applied to show the similarities among the studied groups.

Results

Integrity of sperm DNA

SCD assay

In the SCD test processed sperm nuclei, four dispersion patterns were observed including: large halo sperm cells, medium halos, very small halo cells and halo-free sperm cells., as well as, sperm nuclei scoring showed that in the semen samples of couples with RIF who had a negative pregnancy test, the mean percentage of Sperm DNA

fragmentation (SDF) was significantly higher in comparison to the control group stage. On contrast, the SDF was unchanged and still in the control level in couples with RIF who had a positive pregnancy test as shown in Fig. 1& 2.

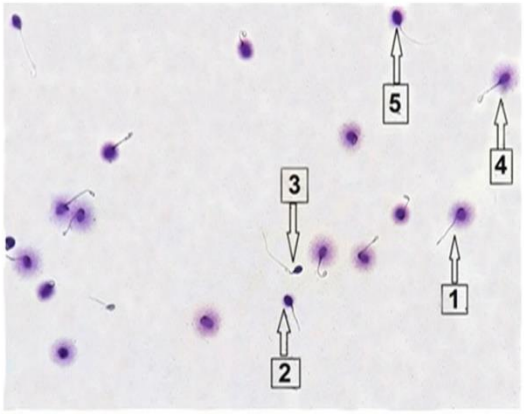

Fig. 1: Integrity of DNA assessed by the sperm chromatin dispersion test. Sperm nuclei 1 and 4: Large halo (un-fragmented DNA); nuclei 5 with medium-sized halo; sperm nuclei 2 and 3 with small halo-fragmented DNA. Magnification: 400x.

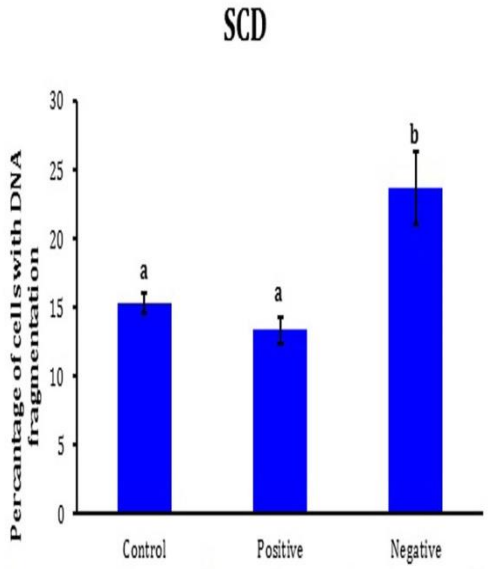

Fig. 2: The percentage of sperms with DNA fragmentation using sperm chromatin dispersion (SCD). Different letters indicated statistical significant difference at $p<0.05$

AO assay

In the AO test, red-orange fluorescence was emitted by sperm DNA with single or / and double stranded breaks stained with AO fluorescence dye, while intact sperm DNA emitted green fluorescence, as shown in Fig. 3.The percentage of DNA fragmentation was significantly higher in the RIF couples with negative pregnancy, as indicated by the significant higher number of sperm nuclei scored with red fluorescence, compared to those scored in RIF couples with positive pregnancy test and control couples (Fig 4).

AB assay

In AB assay the protamine-rich nuclei of mature spermatozoa with abundance of arginine and cysteine negatively respond and remain unstained, while increased AB sperm staining indicates loose packaging of chromatin. The sperm cell heads with a good structure of chromatin were thus stained light blue, whereas those with an abnormal structure of chromatin were stained dark blue, as shown in Fig 5. Sperms scoring indicated that abnormal sperm chromatin condensation was statistically increased in the semen samples of RIF couples with negative pregnancy test compared to control couples. On contrast, the incidence of DNA damage was unchanged and still in the control level in semen samples of RIF couples with positive pregnancy test (Fig. 6).

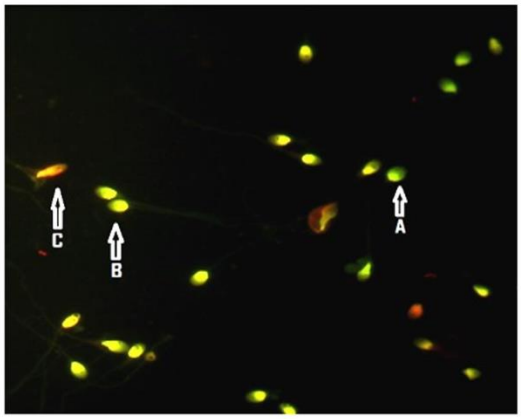

Fig. 3: Human spermatozoa stained with Acridine Orange showing A: nc denatured DNA emitted green color; and B & C: denatured DNA w DNA breaks emitted red and yellow color. Magnification 400x

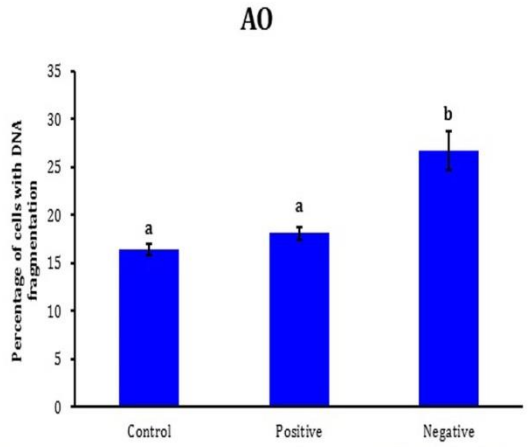

Fig. 4: The percentage of DNA damage (double and single breaks) in sperms stained using Acridine Orange dye. Different letters indicated statistical significant difference at $p<0.05$

DNA diffusion assay

Results of DNA diffusion assay showed that the percentage of sperms with DNA damage was statistically higher in the semen samples of RIF couples with negative and positive pregnancy test, compared to their level in the control couples as shown in Fig.7& 8.

GSH level

Reduced glutathione (GSH), is an important endogenous antioxidant in human which suppress the formation of ROS. The level of GSH was statistically decreased in the semen samples of RIF couples with negative and positive pregnancy test, compared to its level in the semen samples of control couples.

MDA level

MDA level is an important marker of oxidative stress, and it is an end product of lipid peroxidation. Elevated level of MDA indicates a high rate of peroxidation of lipids. The MDA level was statistically increased in the semen samples of RIF couples with negative pregnancy test, compared to the RIF couples with positive pregnancy test and control couples as shown in Fig. 10. There was a strong correlation coefficient between high level of MDA and low levels of GSH in semen samples of RIF couples with negative pregnancy test, in comparison with the control couples and RIF couples with positive pregnancy test (Fig.11).

Correlation between different DNA integrity tests and oxidative stress

By comparing the correlation coefficient of the four techniques used to assess DNA damage in semen samples, the data showed positive correlations between the level of MDA and DNA damage characterizing all four techniques used as sensitive enough to estimate sperm DNA integrity in situ (Fig. 12-13).

ROS generation

Staining of semen samples with 2,7-DCFH-DA stain revealed higher intracellular ROS production in the sperms of the RIF

couples with negative and positive pregnancy test compared to that generated in the sperms of control couples by the observed high emission of fluorescent light as shown in Fig. 14.

Discussion

At present, scientists have become increasingly interested in studying the effect of fragmentation of sperm DNA on the incidence of RIF. Therefore, the current study was conducted to estimate the possible relationship between the sperm DNA fragmentation in the semen of couples with RIF after ICSI, and the sperms of control couples whose men had become recent fathers. Moreover, the oxidative stress markers were evaluated to shed more light on the possible reasons that may cause DNA damage

In this study, the results of SCD, AO, AB and DNA diffusion assays demonstrated that the level of sperm DNA fragmentation (SDF) was statistically higher in the semen samples of the RIF couples with a negative pregnancy test compared to RIF couples with a positive pregnancy test and control couples in harmony with previous studies **(Evenson et al., 1980; Tejada et al., 1984; DE YI and BAKER, 1992; Evenson et al., 1993; Perreault et al., 2003)**, which suggested that sperm from infertile men showed an increase in the red fluorescence light and increased DFI may be one of the possible causes of RIF. On contrast, a negative correlation between the semen quality and abnormal DNA integrity test has been reported recently in 187 men **(mainly infertile) by Erenpreiss et al., (2006)**.

In addition to measuring sperm DNA fragmentation and its correlation with RIF, the study also examined different aspects that cause DNA fragmentation such as markers of oxidative stress and ROS generation to determine the possible reasons of DNA fragmentation.

ROS are chemically reactive free radicals having the capability to interact and damage a number of biomolecules, including proteins, lipids and DNA **(Epe et al. 1993; Von Sonntag, 1994)**. Damage to sperm DNA caused by ROS can cause pre- and post-implantation failure and early miscarriage **(Dada, 2010)**. Our detection of high fluorescent light emission from the sperms of RIF couples with positive or negative pregnancy test confirmed a higher ROS generation in infertile men of RIF couples compared to the control couples, and supported previous studies of the US population showing a significantly higher level of ROS in infertile semen compared to fertile controls **(Pasqualotto et al. 2000; Agarwal et al., 2006; Pasqualotto et al. 2008)**.

Consequently, the higher level of MDA and the lower GSH level detected in the semen of the RIF couples can be attributed to the generation of a significantly higher ROS compared to the control couples. Likewise, it was reported that the seminal GSH level was

significantly lower in infertile males compared to normozoospermics, **(Raijmakers et al., 2003)**. Conversely, no difference in GSH concentration between fertile and infertile men could be observed in another study (Ebisch et al., 2007), and this may be due to the contribution of sperm ROS leading to the greater control of thiol synthesis to protect sperm from oxidative damage. The present findings have therefore shown that GSH levels in seminal plasma may play a role in protecting against oxidative damage by reducing sperm membrane lipid peroxidation. It can therefore be proposed that the concentration of GSH can be used as a chemical parameter for male fertility estimation. GSH's beneficial role in reducing sperm oxidative damage might make it an effective candidate for clinical use in the treatment of male infertility.

The DNA diffusion assay is a very responsive method of detecting apoptosis, as the specificity of the assay is more than 98% **(Singh, 2000)**. The induction of apoptosis demonstrated in the semen of RIF couples can be attributed to the observed higher ROS generation as ROS can interact with DNA causing single- and double-strand DNA breaks and impair chromatin packaging as manifested by AO and AB assays in our study.

Conclusion

Based on previous results, we concluded that fragmentation of sperm DNA is an important cause of RIF through higher ROS generations that can interact with DNA causing DNA breaks, impeding chromatin mobilization, and induction of apoptosis. Therefore, the sperm DNA integrity test is of value in such patients.

Acknowledgment

Great thanks and appreciation for Zoology Department; Faculty of Science Cairo University for providing us with chemicals and equipment required for experiments of this study.

Consent for publication

Not applicable

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Consent for publication

Not applicable

Consent for participation

Not applicable

Authors' contribution

H El-S and H.R.H.M: designing the study, conducting the molecular and Biochemical experiments, reviewing and editing manuscript and performing statistical analysis; A.M.M.A: conducting the experiments and writing manuscript.

Funding

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Declaration of interest

Authors declared that they have no conflict of interest.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

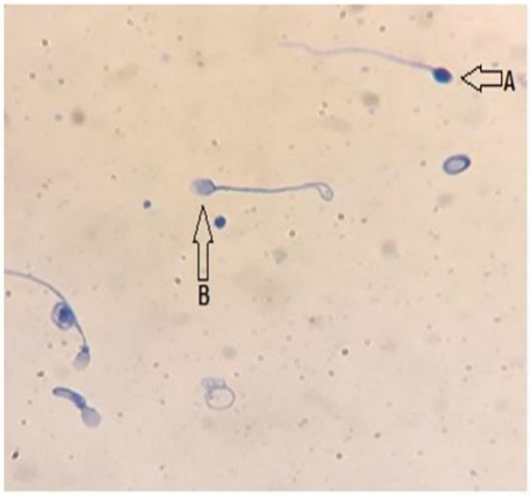

Fig. 5: Sperm chromatin condensation measured by Aniline Blue showing A: abnormal chromatin structure stained dark blue; B: S cell heads stained light blue, with a strong chromatin struc Magnification 400x

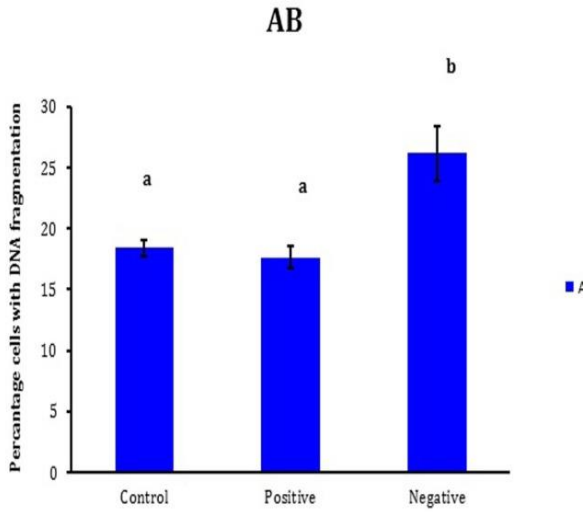

Fig. 6: The percentage of sperm with DNA damage in sperms using Aniline Blue (AB) assay. Different letters indicated statistical significant difference at $p<0.05$

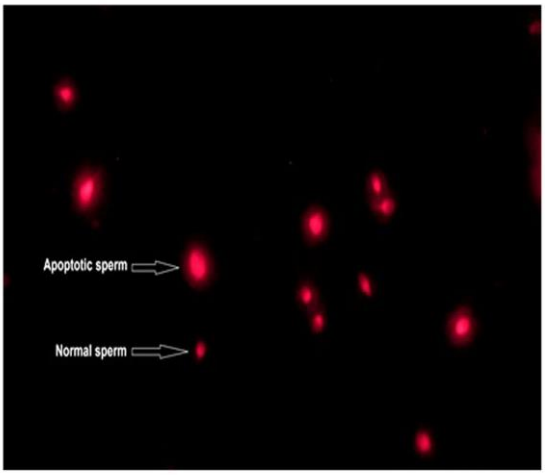

Fig. 7: Representative photograph of the DNA diffusion assay examined for apoptotic sperm and normal sperm. Magnification 400x

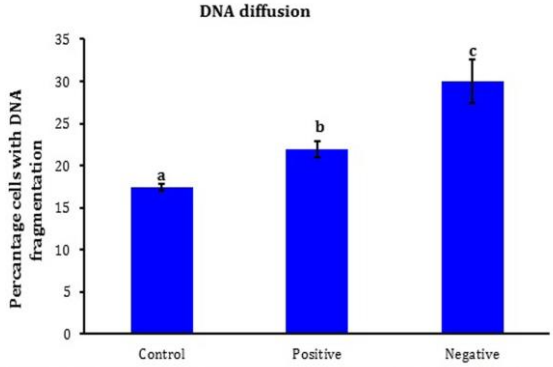

Fig. 8: The percentage of sperms with DNA fragmentation using DNA diffusion technique. Different letters indicated statistical significant difference at $p<0.05$

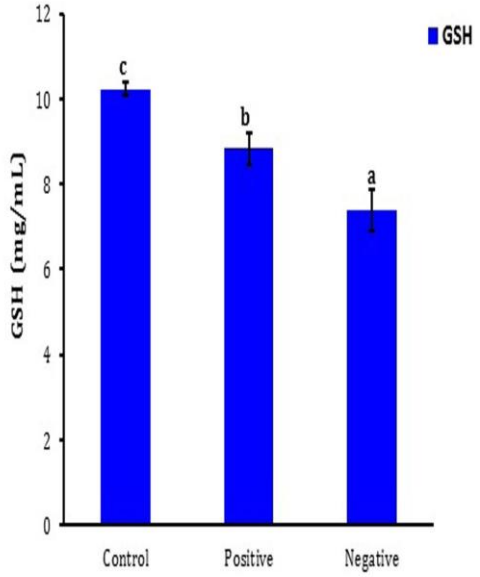

Fig. 9: The level of GSH in semen samples of RIF couples with positive and negative pregnancy test and control couples using colorimetric groups.
Different letters indicated statistical sgnificant difference at p<0.05

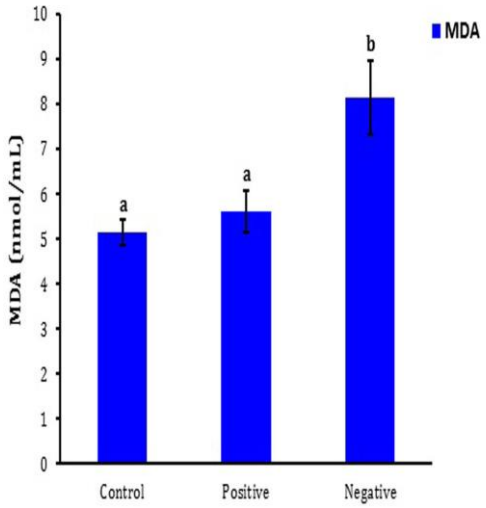

Fig. 10: The level of MDA in semen samples of RIF couples with positive and negative pregnancy test and control couples using colorimetric. Different letters indicated statistical difference at p<0.05.

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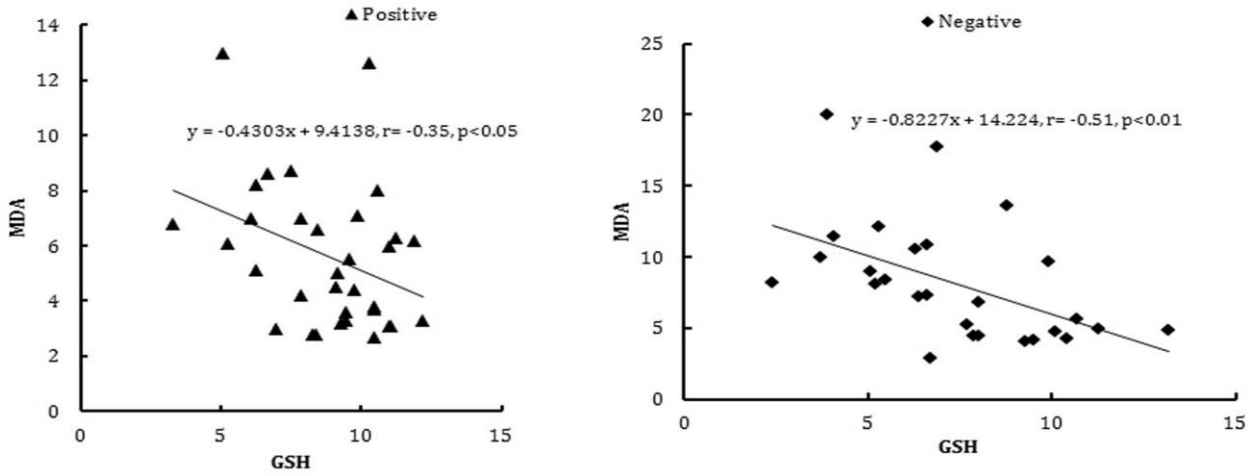


Fig. 11: Regression analysis and correlation coefficient (r) depicting the relationship between the level of GSH and MDA in the RIF couples with positive and negative pregnancy test.

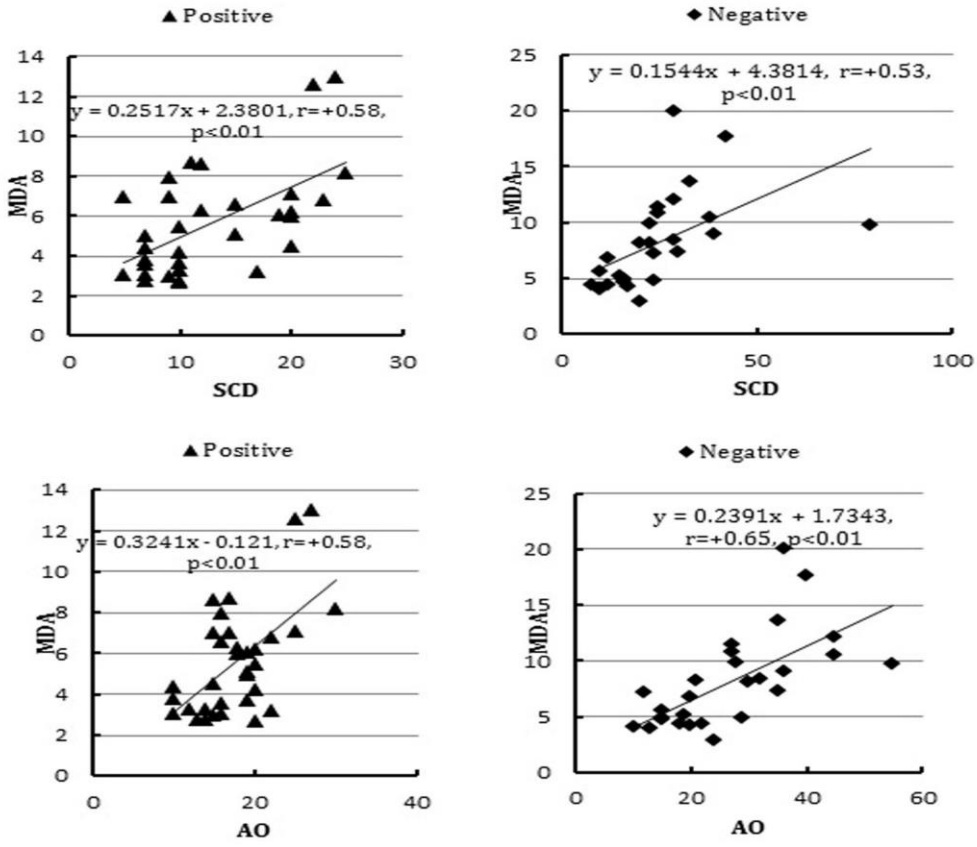


Fig. 12: Regression analysis and correlation coefficient (r) depicting the relationship between MDA level and DNA fragmentation assessed by SCD and AO techniques in the positive and negative groups.

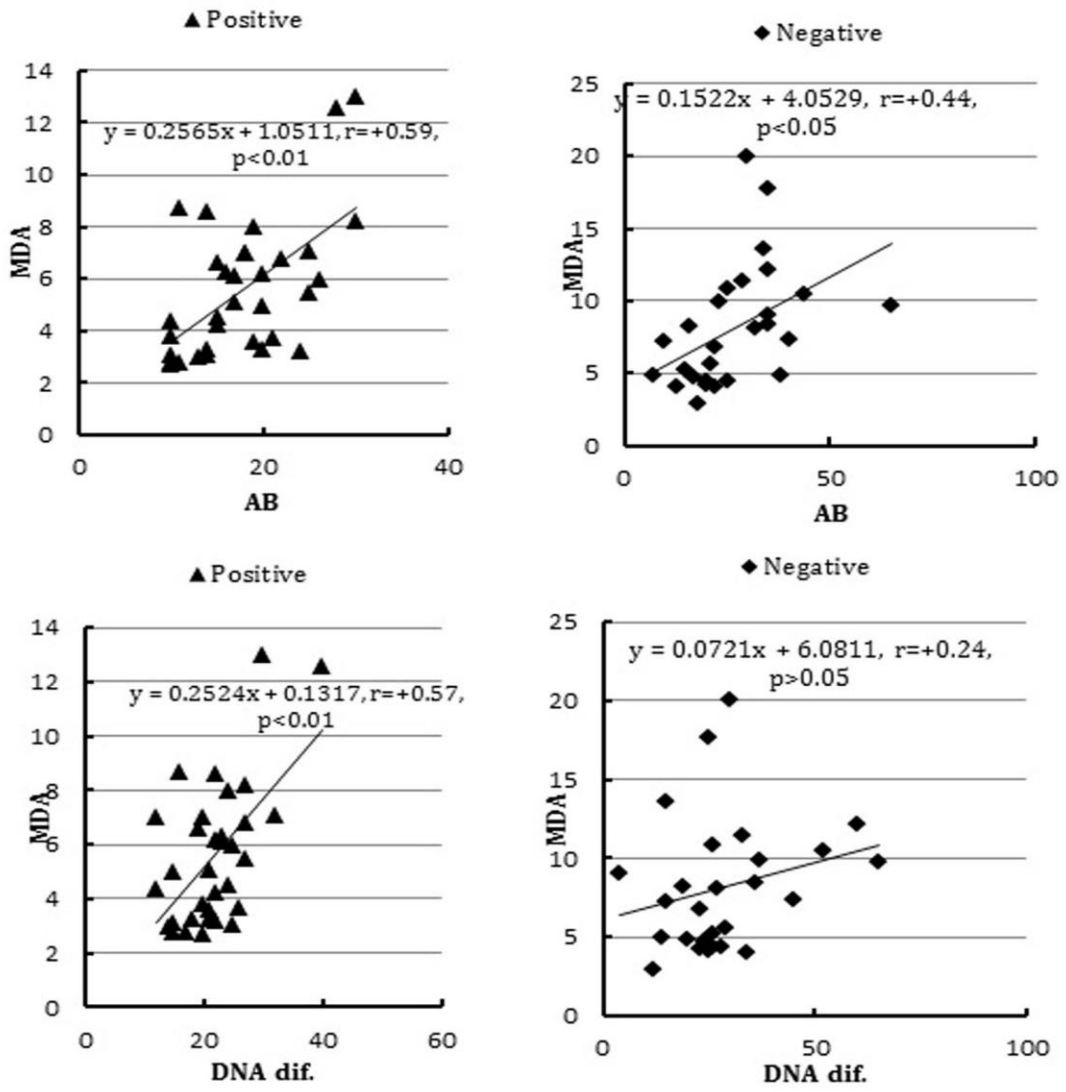


Fig. 13: Regression analysis and correlation coefficient (r) depicting the relationship between the MDA level and DNA fragmentation assessed by SCD, AO, AB and DNA diffusion techniques in the positive and negative groups.

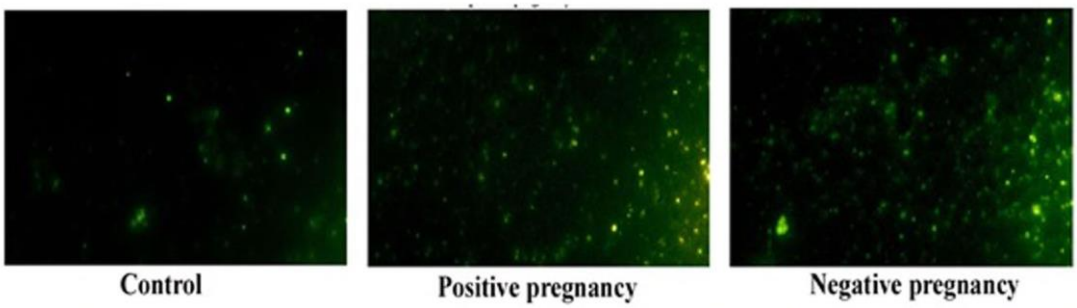
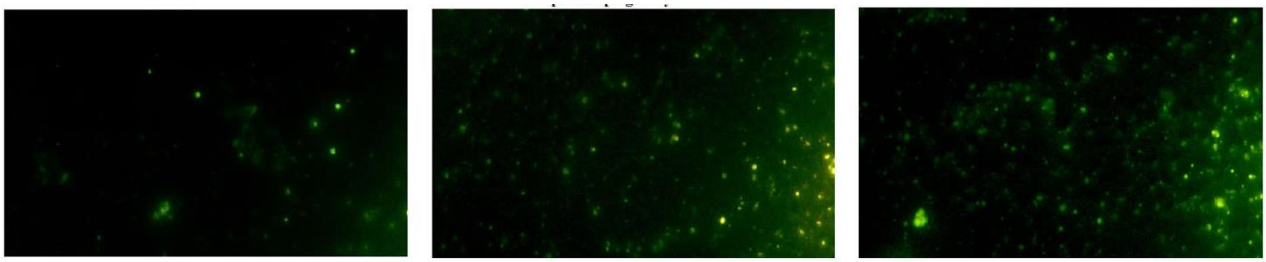


Fig. 14: Representative photos for the observed ROS in the semen samples of the RIF couples with positive and negative pregnancy test and control couples



Control Positive pregnancy Negative pregnancy

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