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RESEARCH ARTICLE

EVALUATION OF ANTI OXIDANT EFFECT OF GREEN TEA EXTRACT IN CRYO PRESERVED HUMAN SEMEN SAMPLES

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ABSTRACT

Anti oxidant effect of green tea extract on 47 cryo preserved human semen samples were studied. Each sample was divided into two- one with green tea extract and the second without green tea extract. Percentage of motility, viability and DNA fragmentation were examined. While comparing the study results of control vs. green tea extract samples, the mean value of percentage of motility in control group was 51.2 with standard deviation of 10.18 vs. 53.03±10.40 in green tea extract group. The percentage of viability and standard deviation in control yielded 59.63±10.13 and it was 61.38±10.29 in green tea extract group. The mean and standard deviation in percentage of DNA fragmentation was 73.49±5.24 in control and it was 67.04±5.02 in green tea extract group. The students paired t-test results showed statistically significant increase in percentage of motility and viability and reduction of DNA fragmentation in green tea extract group compared to control with p-value less than 0.05. Green tea is an effective anti oxidant and it reduces the DNA fragmentation and it increases the motility and viability of sperms, thus it can be use as an effective anti oxidant in cryo preservation of human sperms.

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INTRODUCTION

Recent studies show that Oxidative stress is a major cause of male factor infertility. The definition of oxidative stress (OS) states that it is an imbalance between the systemic manifestation of reactive oxygen species (ROS) and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides (Suresh *et al.,* 1995) and free radicals that damage all components of the cell, including proteins, lipids, and DNA. Production of reactive oxygen species is a particularly destructive aspect of oxidative stress. ROS production in the sperm is a part of normal physiological process. Proper amount of ROS are needed in the sperm for capacitation, signaling process for fertilization, acrosome reaction etc. But when the level of ROS in the body is more, it causes adverse effect in the normal physiological function. It causes damage to proteins, phospho lipids, amino acids etc. In sperm it can cause damage to sperm morphology, loss of motility and viability, DNA damage and finally causes male infertility (Eve *et al.,* 1997; Aitken *et al.,* 1994; Ford, 2004).

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The balancing in the level of ROS in the body is by the antioxidants which are present in the body. If there is any imbalance between the level of anti oxidants and ROS, it leads to a state called oxidative stress. Many studies were published about the role of anti oxidants in male infertility (Agarwal *et al.,* 2004; Kartikeya *et al.,* 2009; Raghuveer Choudhary *et al.,* 2010). All these studies are specifying the oral supplementation of the anti oxidants. Developments in the infertility treatment, andrology and embryology leads to preserve the normal sperms for a long time especially for the case of patients with reduced number of sperms, couple who wants to have children in future through invitro fertilization methods, cancer patients who will undergo for chemotherapy or radiotherapy etc. One disadvantage of sperm cryo preservation is that, it causes the formation or production of high amount of ROS and DNA fragmentation thus reducing the quality of sperm (Wang *et al.,* 1997; Watson, 2000; Marlea *et al.,* 2012; Ribas *et al.,* 2014; Florence *et al.,* 2012). Many studies were published about the anti oxidant supplementation before and after cryo preservation (Taylor *et al.,* 2009; Angel *et al.,* 2010; Malo *et al.,* 2010). Among the anti oxidants, vitamin C, Vitamin D, Vitamin E, and pentoxifyllene are commonly used anti oxidants during cryo preservation (Anel-López *et al.,* 2012; Wittayarat *et al.,* 2012; Ashok *et al.,* 2010; Francesco *et al.,* 2011).

Many studies have shown that green tea is a good anti oxidant and daily green tea consumption is good for health. Scientific studies indicate that green tea may be a helpful antioxidant for many health conditions (Jian *et al.,* 2004; Christian *et al.,* 2008; Hsu *et al.,* 2011; Cooper *et al.,* 2005; Inoue *et al.,* 2001). Till now no studies were published regarding the effect of green tea in cryo preservation of human sperm. This study aimed to assess the anti oxidant effect of natural green tea extract on cryo preserved human semen sample.

MATERIALS AND METHODS

Study design

This study was conducted in 47 male patients who came with infertility problem at an infertility hospital, Bahrain. The study period was from July 2014 to December 2014. Each sample was divided into two- one with green tea extract and the second without green tea extract as control group.

Routine cryo preservation of sperm

After semen collection, the sample was allowed to liquefy at room temperature for 30 minutes. Count and motility was checked by using Makler Counting Chamber. The entire volume of semen sample was taken into a 15ml Falcon tube. Equal volume of sperm freezing media was taken into a 6ml tube and allowed for equilibration at room temperature for 20-30 minutes. Sperm freeze media was taken into a sterile glass pipette and added drop wise to the sample and mixed it continuously for 30sec.The sample was allowed to rest for 3minutes for equilibration. The cryo vials were labeled with patients ID and transferred the entire volume of sample to the cryo vials. The cryo vials were placed in water bath and kept for 10minutes at room temperature. This was transferred to a refrigerator at 4^0 C for 30-90 minutes. Then the vials were fixed on an aluminum cane and placed it in liquid nitrogen vapor for 30minutes. Vials should be kept above 10-20cm in the liquid nitrogen. Then the vials were quickly transferred for final storage in liquid nitrogen in the corresponding liquid nitrogen tank. All details regarding total count, motility, volume, tank number, canister number, cane number and position of vial were recorded in sperm freeze register (Amann, 1999).

Cryo preservation with anti oxidant supplement

(i) Preparation of Green Tea Extract

10gm natural green tea leaves were taken and dissolved in 100ml dissolved water. Then heat at 30-40^{0}C for 45minutes with a magnetic stirrer and then filtered with whattman filter paper. The solution was extracted to a sterile culture flask (Haghparast *et al.,* 2011; Ibrahim *et al.,* 2014). This extract kept for storage at 25^{0}C for future use. For cryopreservation with green tea, the semen sample was allowed to liquefy at room temperature for 30minutes after semen collection and then checked for count, motility and volume in Makler Counting Chamber. Then the sample was spun with equal volume of sperm wash medium for 10minutes at 1200RCF. In the centrifuged pellet add 0.25% of green tea extract and kept for 10minutes. Then equilibrated sperm freeze media was added drop wise to the sample and cryopreserved the sample.

Analysis of the sperm parameters

(i) Motility

To determine the motility, Makler counting chamber (Sefi Medical Instruments, Israel) was used. After liquefaction 10 micro liter of the semen sample was loaded into the counting chamber. Then the sample was allowed to settle for 1 minute. Objective lens magnification was at 10x. Sperm count was then carried out in 10 squares. The entire grid was counted only if the sperm concentration was less than ten sperm per row. Semen concentration was expressed in millions/milli liter. Then the total count was calculated as per the following formula.

Total count = motile sperm + immotile sperm in 100squares of Makler counting chamber divided by 10.

The sperm motility was assessed in at least 100 sperm and expressed as percent of motile sperm (sum of rapid progression plus slow progression sperm). The following formula was used.

% Motility = (motile sperm/Total count) x 100

(ii) Viability

The traditional method for assessing whether the sperm membrane is intact or disrupted involves examining a percentage of viable sperm by a stain exclusion assay (Agnieszka *et al.,* 2012). In this study the viability was measured using eosin-nigrosin assay by assessing the plasma membrane integrity (World Health Belgium, 2010). One gram of eosin B (Merk, Belgium) was added to 1 gm of sperm and 3 gm of nigrosin. In eosin-nigrosin stain under the microscope, live spermatozoa appeared white and unstained against the purple back ground of nigrosin. Dead damaged spermatozoa which have a permeable plasma membrane appeared in pink colour. The percentage of viable and non viable spermatozoa was calculated and tabulated.

(iii) DNA fragmentation test

For DNA fragmentation study, the Halo sperm kit (Halotech Dna, SL) was used. The test procedure was based on the sperm chromatin dispersion (SCD) test (Fernández *et al.,* 2003 and 2005). As an initial step, the lysis solution was kept at room temperature of 22^{o}C. The semen sample was diluted in culture medium to a concentration of 5-10 million per milliliter. The agarose gel was then dissolved by melted in a microwave or in water for 5minutes at 90-100^{o}C. The agarose eppendorf was transferred to a water bath where temperature was maintained at 37^{o}C and left it for 5minute until temperature became even.

25 micro liter of semen sample was added to agarose eppendorf and mixed well.14-20 micro liter of cell suspension was taken from agarose eppendorf on to the treated slides and covered with glass cover slip.

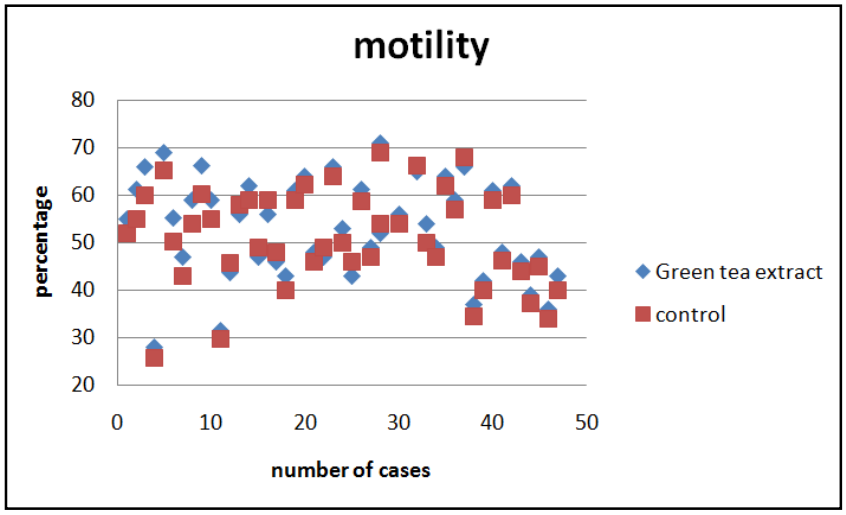

Figure 1. percentage of motility vs. number of cases in controll and green tea extract group

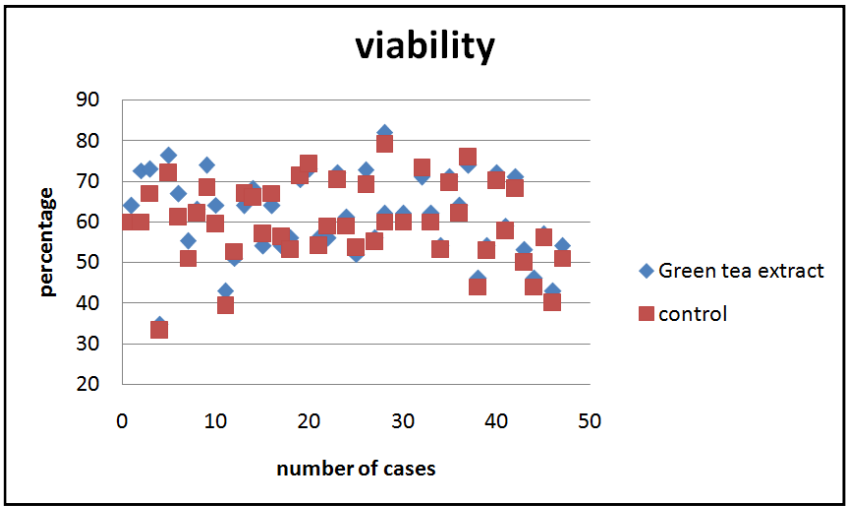

Figure 2. percentage of viability vs. number of cases in controll and green tea extract group

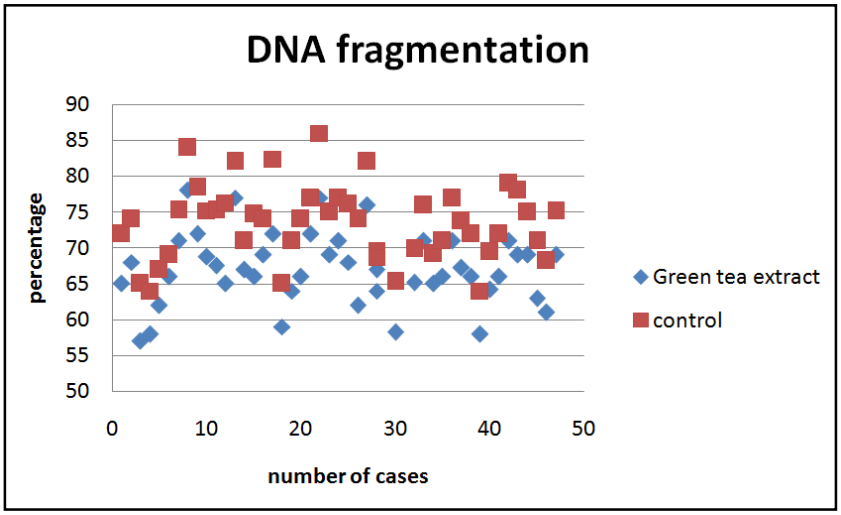

Figure 3. percentage of DNA fragmentation vs. number of cases in controll and green tea extract group

Table 1. mean and standard deviation of percentage of motility, viability and DNA fragmentation - students paired t-test results

Parameter	Mean +/- SD		P-Value (Students paired t-test)
	control	with anti oxidant green tea extract	
Motility %	51.2±10.18	53.03±10.40	0.00090
Viability %	59.63±10.13	61.38±10.29	0.00042
DNA fragmentation %	73.49±5.24	67.04±5.02	0.00000

Care was taken to avoid air bubbles. The slides were kept in horizontal position throughout the process. Then the slides were placed on the cold surface in refrigerator at 4^{o}C and left the sample 5minute to become gel form. Then acid denaturation (AD) solution was prepared. For this, 80microliter of AD was added to 10milli liter of distilled water and the resultant mixture was placed in the incubation tray. The slides cover was then removed by sliding it of gently with the help of land set. After this, immediately the slides were immersed into AD solution and left to incubate for 7minutes. Afterwards, placed this in another incubation tray containing 10ml of lysis solution and kept it for incubation for 25minutes. Then slides were kept horizontally into a tray containing abundant distilled water in order to wash lysis solution. This was kept for incubation of 5minutes. The slides were then placed horizontally into a tray with 70% ethanol (2min) followed by 90% ethanol (2min) and finally 100% ethanol (2min). This slides were kept for air dry at room temperature followed by diff quick staining. After staining, slides were visualized to analyze the DNA fragmentation.

(E) Statistical analysis

For the data analysis, statistical tests were performed. Basic descriptive statistics (mean ± standard deviation) were calculated for different parameters such as total motility, viability and DNA fragmentation. Student's paired t-test was performed to find out the significance in above parameters while comparing sample with green tea vs. control A P-value < 0.05 was considered statistically significant.

RESULTS

While comparing the study results of control vs. green tea extract samples in terms of motility, viability and DNA fragmentation (table.1), the mean value of percentage of motility in control group was 51.2 with standard deviation of 10.18 vs. 53.03±10.40 in green tea extract group. The percentage of viability and standard deviation in control yielded 59.63±10.13 and it was 61.38±10.29 in green tea extract group. The mean and standard deviation in percentage of DNA fragmentation was 73.49±5.24 in control and it was 67.04±5.02 in green tea extract group. The students paired t-test results showed statistically significant increase in percentage of motility and viability and reduction in percentage of DNA fragmentation in green tea extract group compared to control group with p-value less than 0.05.

DISCUSSION

The green tea extract contain nutrients and substances which includes catechin, caffeine, the amino acid called anine, butyric acid, Vitamins A, B1, B2, B3, C, E, F, P, & U, chlorophyll, minerals, pectin and saccharides saponin. Because of its multi vitamin effect and powerful anti oxidant property, the effect is supposed to be better compared to the anti oxidant effects of commonly available or routinely using anti oxidants like vitamin C, vitamin E, L carnithene, glutathione and pentoxifyllene. More over studies were published regarding the green tea extract supplementation in cryo preservation of animal sperms (Ibrahim *et al.,* 2014). In this study results using green tea as an anti oxidant supplement in cryo preservation, it was obvious that green tea extract made statistically significant increase in motility and viability. More over the DNA fragmentation percentage also reduced compared to the control. Since the results are favoring the efficiency of green tea, it indicates that the green tea extract is an efficient anti oxidant and it can be used in cryo preservation of human sperms. More comparison studies are needed to make a conclusion that whether it can replace the other routinely using anti oxidants in cryo preservation like vitamin C, vitamin E. pentoxifyllene etc. In this study the green tea extract is prepared in a particular way by following a previous study. Here the question is that which type of green tea gives better antioxidant effect and whether the nature of these ingredients of green tea will change or not, based on how green tea is processed. The effects of these variables are not included in this study.

Conclusion

Green tea is an effective anti oxidant and it reduces the DNA fragmentation and it increases the motility and viability of sperms, thus it can be use as an effective anti oxidant in cryo preservation of human sperms.

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