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Original Article

The GGC Medium Reduces the DNA Fragmentation of Human Spermatozoa via *in vitro* Activation

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Abstract

In vitro sperm stimulation by the addition of certain chemicals has become one of the most significant techniques for dealing with sperm DNA fragmentation, which is one of the leading causes of male infertility. The GGC medium is an invented triple antioxidant-containing medium (10 mM/ml green tea extract, 10 mM/ml glutathione, 60 mM/ml vitamin C, sodium pyruvate 0.01g/L, and 10% human serum albumin applied to 1L Ringer solution) for *in vitro* human sperm activation. This study aimed to evaluate the quality of the human sperm DNA after *in vitro* activation using a GGC medium. For this purpose, 200 semen samples were employed in this study. Before activation by swim-up technique, the samples were split into three groups, consisting of a control group (G1) with no activation medium, and G2 and G3 groups activated with Ferticult flushing medium and GGC medium, respectively. Afterward, the sperm DNA fragmentation index (DFI) was assessed pre- and post-swim-up activation. Based on the comparison of pre- and post-activation stages, the findings of DNA fragmentation revealed a significant increase at the pre-activation stage. Furthermore, relative to the other treatment groups, there was a strong significant (P<0.05) reduction of DFI in samples activated with GGC medium. The G2 and G3 showed a substantial reduction of DFI at the post-activation stage, compared to the pre-activation stage (P<0.05). According to the findings, both mediums can reduce DNA fragmentation; however, the GGC medium showed the most significant outcomes, compared to the Ferticult medium used for the *in vitro* activation of spermatozoa.

Keywords: DNA fragmentation, Ferticult medium, GGC medium, Spermatozo

1. Introduction

Male chromosomal abnormalities, characterized by degraded deoxyribonucleic acid (DNA), suggest male infertility regardless of typical sperm features (1). Several studies have found that sperm destruction concentrations can predict pregnancy outcomes and the likelihood of multiple pregnancy losses when utilizing assisted reproductive technology (2-4). Therefore, the existence of DNA damage would not be a reliable predictor of reproductive potential (5). Despite the fact that DNA damage is not detectable in ordinary sperm concentration, these tests are an important part of the evaluation of an infertile man (5, 6).

There are several methods for the assessment of sperm DNA damage (7), including disinfection with acridine orange (AO), terminal deoxynucleotidyl transferase-mediated deoxy-uridine triphosphate-nick-end labeling assay, single cell gel electrophoresis (COMET) assay, the sperm chromatin structure assay (SCSA), flow cytometer, and the DNA fragmentation index to determine the degree of DNA fragmentation index (DFI) (8, 9). Sperm nucleus abnormalities include DNA strand breaks, statistical and physical

genetic disorders, Y chromosome microdeletions, and alterations in the epigenetic regulation of the paternal genome, all of which affect reproductive success. Sperm DNA breakage studies are increasingly used as a sperm quality measure (10).

Single and double DNA strand breaking, metabolic alteration of a base, such as oxidation or alkylation, inter- or intra-strand cross-linkage, and DNA-protein covalent interactions are the most prevalent kinds of sperm DNA damage discovered (11). Damage to the DNA of a single sperm that fertilizes a female egg can significantly impact the growth of the embryo (12). The chromatin state of mammalian sperm is crucial for proper embryo development, and the integrity of mammalian sperm DNA is crucial for the paternal genetic contribution to a normal progeny (13).

By comparison of DFI (percentage of cells showing denatured DNA) and HDS (cells with defective chromatin condensation) in spermatozoa with threshold values, the SCSA enables the fast identification of DFI and HDS percentages. The findings of SCSA can be used to predict pregnancy success (13, 14). Several ideas have been postulated about the molecular mechanism of sperm DNA damage. Apoptosis, oxidative stress, and aberrant chromatin packing are the most critical (15).

Tejada, Mitchell (16) were the first researchers to discuss this issue (1984). The AO technique quantifies the metachromatic shift of AO fluorescence from green (native or double-stranded DNA) to red (acid-induced denaturation) *in situ* to determine the susceptibility of sperm nuclear DNA to acid-induced denaturation (denatured or single-stranded DNA). The AO test is affordable and accessible; however, the color fades quickly which necessitates rapid assessment (17).

According to Zwamel and Kadhim (18), GGC medium contains antioxidants (10 mM/ml green tea extract, 10 mM/ml glutathione, 60 mM/ml vitamin C, sodium pyruvate 0.01g/L, and 10% human serum albumin applied to 1L Ringer's solution). It decreases sperm stickiness and lipid peroxidation, allowing the sperm to flow more freely (19). Green tea has a high polyphenol content, and polyphenols have antioxidant properties that include scavenging and removing free radicals. Due to its high cell division and metabolic rates, low oxygen pressure, impaired arteries, and higher levels of unsaturated fatty acids, testicular tissue is well-prepared to fight the impacts of free radicals and oxidative stress.

Furthermore, anti-inflammatory effects have been found in green tea. In addition, glutathione protects the sperm membrane from oxidative damage, while sulfhydryl groups are important in sperm motility and metabolism. Sulfhydryl-containing compounds, such as cysteine and glutathione, are found in normal sperm (20). In addition, vitamin C is considered to combat free radicals and minimize oxidative damage due to its complete chain-breaking antioxidant potential, making it a possible treatment for unexplained male infertility (16). In this regard, the present study aimed to evaluate the quality of the human sperm DNA after *in vitro* activation using a GGC medium.

2. Materials and Methods

2.1. Sample Collection and Semen Analysis

The present study was performed on 200 semen samples. The semen samples were collected from individuals within the age range of 18-45 years old after 3-5 days of sexual abstinence. The samples were held at 37 ℃ for up to 40 min to allow for liquefaction, and then the seminal fluid analysis was performed according to the standard criteria of WHO (2010). The semen samples were divided into three groups before activation by swim-up technique. The control group (G1) received no GGC medium, while G2 and G3 were activated with Ferticult flushing medium and GGC medium, respectively. Afterward, the sperm DFI was assessed at the pre- and post-swim-up activation.

2.2. Preparation of the Acridine Orange

The AO stain was prepared according to the instructions provided by Tejada, Mitchell (16). As a stock solution, 1 g of AO was soaked in 1,000 mL of distilled water and kept at 4 ℃ in the dark. Moreover, 40 mL of 0.1 M citric acid, 2.5 mL of 0.3 M

$Na_2HPO_4.7H_2O$, and 10 mL of stock suspension were pipetted. It should be mentioned that during dyeing, the pH was adjusted to 2.5. Except for the AO, which was stored in the refrigerator, the other stock solutions were stored at ambient temperature.

2.3. Preparation of Tyrode's Solution

For the preparation of Tyrode's solution, a small portion of warmed distilled water (0.24 g) was applied to $MgCl_2$, which was followed by all of the NaCl components (7.054 mg). The KCl is a salt of potassium chloride (0.439 mg), $CaCl_2.2H_2O$ is a salt of calcium chloride (0.24 gm), and $Na_2HPO_4.2H_2O$ is a salt with the formula $Na_2HPO_4.2H_2O$ (0.187 mg). Moreover, $NaHCO_3$ is a salt of sodium chloride (1.302 mg). When the volume reached 1 L, it was dialyzed to 7.3 using the method provided by Tejada, Mitchell (16).

2.4. Preparation of Fixative Solution (Carnoy's Solution)

This mixture was produced using three main parts methanol and one part glacial acetic acid, as reported by Tejada, Mitchell (16).

2.5. Preparation of GGC Medium

The GGC medium was prepared by adding 10 mM/ml green tea extract, 10 mM/ml glutathione, 60 mM/ml Vitamin C, sodium pyruvate 0.01 g/L, and 10% human serum albumin to 1 L Ringer's solution (18).

2.6. Assessment of DNA Fragmentation Index

The AO technique was used to evaluate the chromatin stability of sperm. A smear was collected from each specimen and placed on a transparent microscope slide on the same day of the inspection. Afterward, it was mixed with a freshly prepared Carnoy's solution. The next day, the slides were inserted into the AO solution for 5 min. Afterward, they were thoroughly washed with tap water before being stored in a cool and dark place until the evaluation was finished. The fraction of spermatozoa with normal DNA was determined by measuring at least 200 spermatozoa under a fluorescence microscope at a magnification of 400. Spermatozoa with normal, undamaged, and double-stranded DNA fluoresced green at a wavelength of 450-490 nm, while those with denatured DNA fluoresced red or orange. Genomic instability was measured using the DFI, which measures the amount of transient transfection as a percentage of total DNA (27).

3. Results

Based on the comparison of pre- and post-activation stages, the findings of genomic instability indicated a significant reduction at the post-activation stage. Figure 1 shows that the samples activated with GGC medium (G3) had the lowest DFI post-activation, compared to other groups. Despite the fact that the level of DFI was lower in G3, compared to that in G2, the difference between them was non-significant. Normal sperm DNA and sperm DNA fragmented in the AO test are represented in figures 2 and 3.

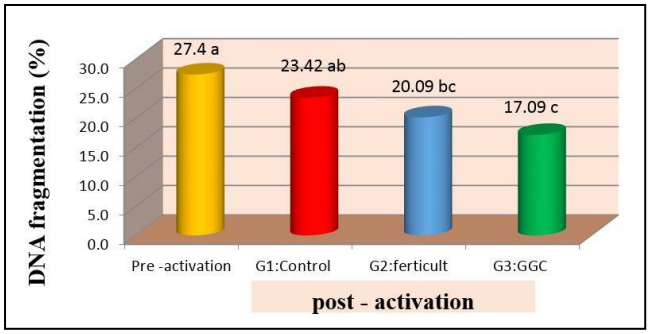

Figure 1. DNA fragmentation of pre- and post-*in vitro* sperm activation using GGC for subjects.
Means with different superscripts within each column are significantly different (P<0.05).
Means with similar superscripts within each column are non-significantly different (P>0.05).

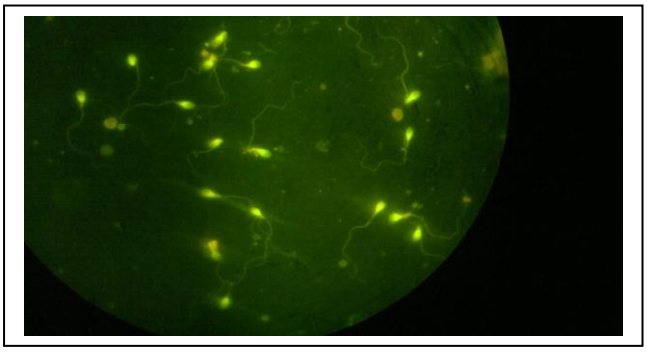

Figure 2. Sperm head under magnification power of (1000) of oil immersion displaying green fluorescence as sperms with intact DNA

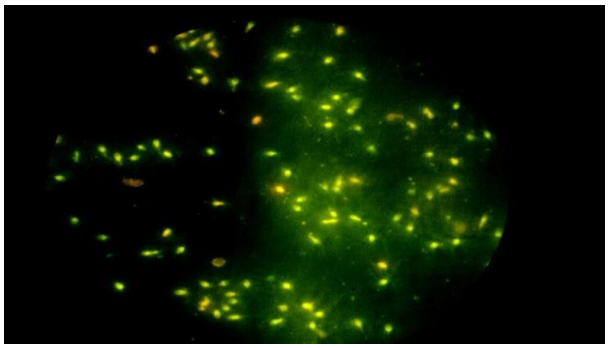

Figure 3. Sperms head under (X40) HPF displaying intact and abnormal fragmentation DNA of human sperm. The green fluorescence refers to sperms with intact DNA, while the orange and yellow fluorescence considers sperms with fragmented DNA

4. Discussion

Sperm DNA fragmentation was a helpful technique for the evaluation of male fertility and infertility (21). Peroxidation is the most frequent source of DNA fragmentation in spermatozoa (22). The high content of unsaturated fatty acids in the plasma membrane of human sperm renders them more susceptible to free radicals. These unsaturated fatty acids enhance extracellular matrix protein processes (e.g., acrosome activation and sperm-oocyte interaction) and sperm motility. Due to their unsaturated nature, many components are also vulnerable to free radical assault and lipid peroxidation of the sperm plasma membrane (23). Lipid peroxide in human spermatozoa has been associated with a significant decrease in motility (24). In the present study, the increase in sperm motility might be related to the GGC medium stopping oxidative damage pathways in human spermatozoa when effective free radical hunters play a protective role.

Sperm DNA integrity was more desirable at the post-activation stage, compared to that at the pre-activation stage. However, the GGC medium exhibited the highest recovery rate in sperm DNA, compared to the control group, which is in concordance with the method of Harrison (25). Despite a reasonable balance of oxygen reduction reactions, glutathione has a positive impact, minimizing oxidative stress and decreasing DNA strand breaking (26). It may be deduced from the data that sperm motility and DNA damage have a strong connection.

Some defensive effects of green tea used in *in vitro* culture medium include low water solubility, limited absorption, and decreased accessibility of polyphenols, like quercetin, which possess high *in vitro* antioxidant properties (27). The GGC medium also contains glutathione, which protects sperm-oocyte fusion potential by inhibiting ferrous ion-catalyzed oxidative damage *in vitro*. Superoxide dismutase and glutathione peroxidase/reductase strands are always considered to have two superoxides (O_2-) and H_2O_2 protective factors (28). It was also discovered that the direct addition of vitamin C to the sperm has a more therapeutic effect, as inadequate seminal vesicle activity causes low ascorbate levels in these people. A wide range of reactive oxygen species (ROS) is trapped by vitamin C, which explains its capacity to prevent free radical damage, either mediated gene damage or ROS generation (29).

In conclusion, the novel GGC medium was found to be effective in improving sperm motility and reducing sperm DNA fragmentation in asthenozoospermic individuals by *in vitro* sperm stimulation utilizing the straight swim-up approach.

Authors' Contribution

Study concept and design: A. H. Z. and N. K. K.
Acquisition of data: N. K. K.
Analysis and interpretation of data: A. H. Z.
Drafting of the manuscript:
Critical revision of the manuscript for important intellectual content: A. H. Z.
Statistical analysis: A. H. Z.
Administrative, technical, and material support: N. K. K.

Ethics

Informed consent was obtained from all the participants before the collection of semen samples. Besides, the local medical Ethics Committee approved this study.

Conflict of Interest

The authors declare that they have no conflict of interest.

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