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Impact of density gradient technique and *Glycyrrhiza glabra* medium mixed with autologous platelet rich plasma on certain sperm function parameters and sperm DNA fragmentation index of asthenoteratozoospermic men: A comparative cross-sectional study

Noor Kadhim Khudhur*[1], Saad S. Al-Dujaily[2], Laith Amer Al-Anbari[3]

[1]*Applied Embryology, Medical Laboratory Techniques Department, Al-Farahidi University, Baghdad, Iraq.*
[2]*Clinical Reproductive Physiology and ART, Higher Institute for Infertility Diagnosis and Assisted Reproductive Technology, Baghdad, Iraq.*
[3]*Surgery (Urology Surgery), College of Medicine, Al-Nahrain University, Baghdad, Iraq.*

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ABSTRACT

Background: Although there is evidence of a global decline in sperm motility and an increasing frequency of male infertility, there is minimal documentation regarding prospective treatments and techniques to activate and enhance certain sperm parameters to overwhelm male infertility.

Objectives: This study used Glycyrrhiza glabra (Gg) and Platelet Rich Plasma (PRP) media to establish a culture medium that aimed to improve sperm function parameters by preparing in vitro men's semen complaining of asthenoteratozoospermic factor.

Materials and methods: Semen samples from 80 participants were investigated in a comparative cross-sectional study. After semen fluid analysis 34 samples were revealed normozoospermia and 46 samples were with asthenoteratozoospermia. Certain sperm parameters and DNA fragmentation index were assessed. PRP and Gg were used as a sperm culture media. Certain sperm parameters and DNA fragmentation index were assessed using World Health Organization 2021 and 1999 guidelines.

Results: After activation using the density gradient method and Gg plus PRP, sperm function parameters improved significantly (p<0.001), including increased motility, morphologically normal sperm percentage, and decreased DNA fragmentation index.

Conclusion: It is concluded that the density gradient technique with any medium used in the present study revealed a significant improvement in certain sperm parameters and a reduction in DNA fragmentation index.

Introduction

Assisted reproductive technology treatments have improved in recent decades. However, intrauterine insemination, in vitro fertilization, and intracytoplasmic sperm injection have low pregnancy rates.[1] Gene polymorphism and insufficient semen treatment in vitro can affect the latter. For instance, oxidative stress, an imbalance between reactive oxygen species (ROS) and antioxidant defense, affects sperm. The imbalance can impede spermatozoa's ability to produce an

* **Corresponding contributor.**
Author's Address: *Applied Embryology, Medical Laboratory Techniques Department, Al-Farahidi University, Baghdad, Iraq.*
E-mail address: Noor.kadhim@uoalfarahidi.edu.iq
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embryo.[2] The sperm culture medium, incubation time, centrifugation, and sperm preparation method can all raise ROS levels. Density gradient centrifugation is an effective way to pick healthy spermatozoa and decrease ROS.[3]

The density gradient procedure is the most recommended for selecting a higher number of mobile sperm cells in cases of severe low sperm count, abnormal sperm morphology, or reduced sperm motility.[4-5]

On the other hand, Glycyrrhiza glabra (Licorice, Gg) is a tiny perennial widely used medicinal plant from the Fabaceae family, that is regularly used as feed and food.[6] Among its many beneficial components are glycyrrhizin, glycyrrhetinic acid, and others.[7-8] Since 2003, licorice has been introduced in vitro to gamete preparation and embryonic development culture mediums.[9-10] Some licorice elements may modulate steroidogenesis and influence testosterone and cortisol levels, according to research. These hormones are necessary for sperm production and motility.[11]

Platelet-Rich Plasma (PRP) is a form of plasma that is derived from the blood and contains a higher concentration of platelets compared to regular plasma.[12] Platelets release seven essential protein growth factors that activate all processes involved in tissue repair and medical disciplines e.g., dermatology, and reproductive medicine.[13] The supplementation of sperm culture media with PRP-derived compounds triggered a cascade of reactions including cell growth and protection against freezing, and improving motility.[14]

Thus, the current study aims to create a culture medium composed of Gg and PRP to improve certain sperm function parameters and reduce the DNA fragmentation index through in vitro activation of men's semen suffering from asthenoteratozoospermia factor.

Materials and methods

Participants

This comparative cross-sectional study (STROBE) examined 80 samples of semen including asthenoteratozoospermic (N=46) and normozoospermic (N=34) individuals. Prior to gathering each sample, all participants provided their consent and were informed of the purpose of the questionnaire as well as the goal of the research. The study was performed in the High Institute for Infertility Diagnosis and Assisted Reproductive Technologies, Al-Nahrain University and Al-Harithiya International Lab in Baghdad Iraq and extended from October 2023 to January 2024.

Inclusion criteria represented by fertile normozoospermic men and infertile asthenozoospermic men. On the other side exclusion criteria include azoospermic men, oligozoospermia/teratozoospermia, and men with history of undescended testes, hyperprolactinemia, genetic anomalies, and diabetes mellitus.

Sample size

The sample size was determined considering the given time frame for completing the research and the challenges associated with collecting samples during this timeframe, in line with the research's selection criteria. Hence, we formulated the study to include a sample size ranging from 80-100 samples. However, due to insufficient results and unforeseen errors, 13 samples were eventually removed.

Semen collection

Semen was obtained through masturbation and collected in a sterile wide-mouth container. The specimen was then placed in an incubator at a temperature of 37 °C and gently mixed. After the semen had liquefied, macroscopic and microscopic measurement was done as recommended by WHO.[15] Additionally, the evaluation of morphologically normal sperm was performed based on the instructions provided in the WHO manual because computer aided semen analysis (CASA) was not offered in the laboratories when the research done. [16]

Platelet rich plasma preparation

Blood from participants was processed to extract PRP.[17] Eight mL of venous blood yielded 2-3 mL of PRP [15] blood-drawing recommendations were followed for PRP procedures. Protocol specificity, equipment, and platelet concentration determined PRP centrifugation speed and time. This study used conventional criteria of 3200 RPM for 15 mins. Separated PRP were frozen at -8 °C.[18]

Preparation of Gg

To generate the working solution, 10 mg of Gg aqueous extract was added to 10 mL Phosphate Buffered Saline (PBS) in plastic plain tubes with broad- spectrum antibiotic (Ampicillin 0.004g) to prevent bacterial growth. The solution was filtered with a Millipore filter (0.22M) and adjusted to pH 7.2-7.4. Gg medium was then moved to a suitable storage container and stored in a cold, dark place away from sunlight and heat. [19]

Control media preparation

The ready-to-use medium FertiCult™ (FertiPro Company, Belgium) were also available. The medium was stored appropriately according to manufacturer instructions, typically in a refrigerator at the recommended temperature (4-8°C). For each sample, 4.5 mL of flushing medium was warmed in the incubator (37 °C) and used in the semen preparation and activation in vitro.

Density gradient centrifugation protocol

Sil-Select PlusTM gradients consist of silane-coated colloidal silica particles suspended in HEPES-buffered EBSS (Earle's balanced salt solution) supplemented

with 0.4-2.2 gm/L human serum albumin (medicinal substance derived from human blood plasma). Some product codes are supplemented with phenol red or 10mg/L gentamicin (medicinal substance). Our technique was done by adding 1 mL of 80% lower layer medium in a conical tube, followed by 1 mL of 40% upper layer medium (Sil-Select Plus™, FertiPro Company, Belgium) as a second layer, and then 1 mL of liquefied human semen. Four replicates of this initial step have been done with different superficial layers above the semen, as follows:

1. First tube: Density gradient media + liquefied semen + control medium (0.5 mL) Second tube: Density gradient media + liquefied semen + PRP (0.5 mL)
2. Third tube: Density gradient media + liquefied semen + Gg (0.5 mL)
3. Fourth tube: Density gradient media + liquefied semen + PRP and Gg (0.5+0.5 mL)

As soon as loading media and semen was finished, all tubes were moved to a centrifuge at 4000 rpm for 15 mins. Then the samples were prepared as described by Mohammed and his colleagues.[9] Immediately after the centrifugation process was completed, the supernatant layers of all tubes were removed and only the pellets were kept by using a plastic Pasteur pipette.

The next step in the protocol was the addition of flushing media (0.5 mL) to each tube and repeat centrifugation at 2000 rpm for 5 min. After the second centrifugation was accomplished, all supernatant layers from the tubes have also been discarded and replaced by 1 mL of Sil-Select Plus™ washing media (FertiPro Company, Belgium) above the final pellets. In the last step, tubes were placed in the incubator for 20 min, and then sperm parameters were assessed after activation by taking 10 µL droplets from each tube and analyzing them with a light microscope.

Normozoospermic men were enrolled in this study as a control group for the purpose of evaluating the efficiency of PRP, Gg, the new combination medium and the control medium. Semen samples of this group were activated and handled totally just as asthenoteratozoospermic individuals.

DNA integrity evaluation with Acridine Orange assay

According to Dutta and his colleagues.[20] Briefly, a 10 µL semen sample was smeared on the slide and air-dried for 20 mins. The slide was then treated with Carnoy's solution for 2-24 hrs. A brief drying period followed before staining. All stain preparations were done in the dark at room temperature with a pH of 2.5. 3-5 milliliters of dye were applied to the slide. The slide was carefully washed with distilled water and allowed to dry.[21] The slide was seen using a fluorescent microscope with a 40X objective lens the same day it was stained. Each participant's 5 slides of spermatozoa DNA were tested before and after activation, and the results were reported as green, red, or yellow.[10-15] Normal spermatozoa were green-fluorescent, whereas abnormal sperm heads were yellow red as appear in Figure 1.

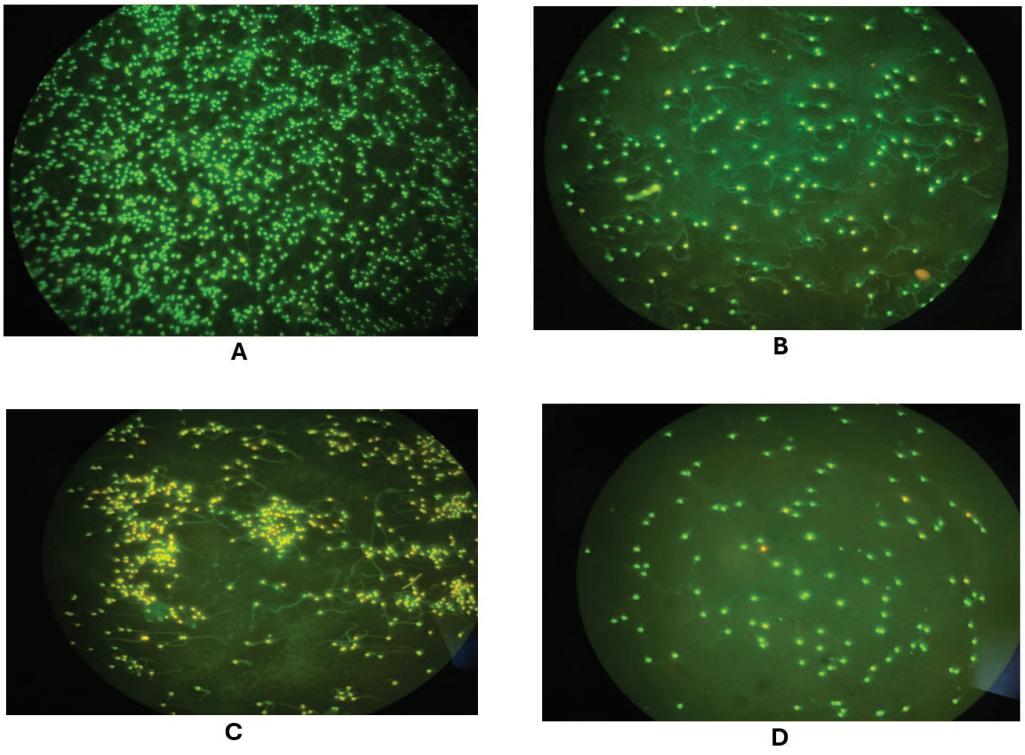


Figure 1. *DNA fragmentation assessment.*
A and B: DNA fragmentation assessment using Acridine Orange stain in normozoospermic men,
C and D: DNA fragmentation assessment using Acridine Orange stain in asthenoteratozoospermic men.

Statistical analysis

The data were analyzed using Statistical Package for Social Sciences (SPSS) version 23.0 and Microsoft Office 2010. The descriptive statistics including frequency, range, mean and standard error were measured to describe the data. The groups were compared by applying analysis of variance (ANOVA). The least significant test (LSD) was used to compare the results. $p \leq 0.05$ were considered statistically significant.

Ethical consideration

The current study was authorized by the ethical committee of the High Institute of Infertility Diagnosis and Assisted Reproductive Technologies/Al-Nahrain University, Baghdad, Iraq, with the approval code (0702-MF-2024N27). Informed consent was obtained from all participants involved in the study. Before gathering each sample, all participants provided their consent and were informed of the purpose of the questionnaire as well as the goal of the research.

Results

Basal (pre-activation) seminal fluids analysis parameters

The baseline seminal fluids analysis parameters were presented in Table 1., accordingly, the mean sperm concentration was 39.70±2.73 (10^6/mL), progressive motile sperms percent was 25.56±1.78, non-progressive motile sperms percent was 26.41±1.00, immotile sperm percent was 48.07±2.18, and morphologically normal sperm (MNS) percent was 22.70±1.32.

Comparison of certain sperm function parameters after activation between density gradient medium alone, Gg with and without PRP

Comparison of certain sperm function parameters after activation with density gradient medium was demonstrated in tables 2&3. In group 1 patients (Normozoospermia), the comparison between density gradient medium alone, density gradient mixed with Gg, density gradient mixed with PRP, and density gradient mixed with both Gg& PRP showed no significant ($p>0.05$) differences regarding sperm concentration, progressively motile sperms percent, non-progressively motile sperms percent, immotile sperms and DFI percentage respectively. However, there was significantly ($p=0.011$) higher MNS percent by using density gradient medium mixed with both Gg & PRP compared to using DG medium alone as shown in Table 2.

In Table 3., men with asthenoteratozoospermia had significantly ($p \leq 0.05$) higher sperm concentration (35.43±3.22), higher progressively motile sperms percent (73.24±1.77) and significantly ($p=0.031$) lower non- progressively motile sperms percent (15.80±1.15) after preparation by density gradient medium mixed with Gg; on the other hand, there was significantly ($p=0.032$) higher MNS(60.57±2.12) in density gradient media with PRP; however there were no significant ($p>0.05$) differences between all activation media regarding immotile sperms percent and DFI percent as presented in Table 3.

Table 1. Basal (pre-activation) seminal fluids analysis parameters and DFI (%).

Seminal fluids parameters	Range	Mean±SE
Volume (ml)	1.0-9.0	3.52±0.15
Sperm concentration (10^6/ml)	7.0 -116.0	39.70±2.73
Progressive motile sperms (%)	0.0-75.0	25.56±1.78
Non progressive motile sperms (%)	10.0-62.0	26.41±1.00
Immotile sperm (%)	5.0-85.0	48.07±2.18
Morphologically normal sperm (%)	5.0-75.0	22.70±1.32
DNA fragmentation index, DFI (%)	6.0-62.0	22.31±1.39

Table 2. Comparison of certain sperm function parameters after sperms activation by density gradient media alone and media contain *Gg,* PRP and mixed *Gg* with PRP in normozoospermic men.

Certain Sperm Parameters	DG alone	DG mixed with Gg	DG mixed with PRP	DG mixed with Gg and PRP	p value
Sperm concentration(m/mL)	33.57±4.90	24.14±3.21	38.93±7.24	22.21±5.46	0.106*
Progressive motile sperms (%)	74.21±2.72	81.07±3.03	77.21±3.30	78.57±3.29	0.470*
Non-progressivemotile sperms (%)	16.21±2.15	11.93±1.74	14.00±2.41	13.79±2.15	0.568*
Immotilesperm (%)	9.43±1.25	6.57±1.29	8.50±1.49	7.00±1.20	0.392*

Certain Sperm Parameters	DG alone	DG mixed with Gg	DG mixed with PRP	DG mixed with Gg and PRP	p value
Morphologically normal sperm (%)	62.36±2.59	70.93±2.11	71.43±2.86	73.29±1.99	0.011*
DNA fragmentation Index, DFI (%)	15.43±2.90	12.93±2.39	10.43±1.57	11.14±1.96	0.414*

Note: Data were analyzed with ANOVA and presented as mean±SE, *significant, DG: density gradient, Gg: glycyrrhiza glabra, PRP: platelet-rich plasma.

Table 3. Comparison of certain sperm function parameters after in vitro sperms activation by using density gradient media alone and media contain *Gg* with and without PRP in asthenoteratozoospermic men.

Certain Sperm Parameters	DG alone	DG mixed with Gg	DG mixed with PRP	DG mixed with Gg and PRP	*p* value
Sperm concentration (m/mL)	33.89±2.95	35.43±3.22	26.95±2.79	22.17±1.78	0.002*
Progressive motile sperms (%)	64.57±2.03	73.24±1.77	70.43±1.81	69.91±1.82	0.011*
Non-progressivemotile sperms (%)	21.41±1.55	15.80±1.15	18.74±1.35	19.22±1.22	0.031*
Immotilesperm (%)	13.48±0.87	10.61±0.86	11.26±0.95	10.85±0.95	0.103*
Morphologically normal sperm (%)	52.39±2.08	57.96±2.12	60.57±2.12	59.41±2.09	0.032*
DNA fragmentation Index, DFI (%)	15.09±1.24	13.89±1.09	12.48±1.22	13.07±1.28	0.458

Note: Data were analyzed with ANOVA and presented as mean±SE, *significant, DG: density gradient, Gg: glycyrrhiza glabra, PRP: platelet-rich plasma.

Discussion

The findings of present work demonstrated a significant enhancement in certain sperm parameters besides a highly significant reduction in DNA fragmentation index after *in vitro* stimulation using a single technique (Density gradient) that was applied to three distinct media types, plus as well as using it alone as a control method.

The comparative between basal semen parameters before activation as appear in Table 1. and semen parameters after activation in both studied groups as shown in Table 2. and Table 3. and Figure 2. and Figure 3., demonstrated that density gradient alone reduced sperm concentration, non-progressive motility, and immotility and increased progressive motility and morphologically normal sperm following activation.

In 2023, similar results were obtained by other study,[21] when used density gradients to isolate a considerable amount of structurally intact and actively moving human sperm from semen fluid. Waste, non-functional sperm, and non-sperm cells have been eliminated from these separated sperm.[22]

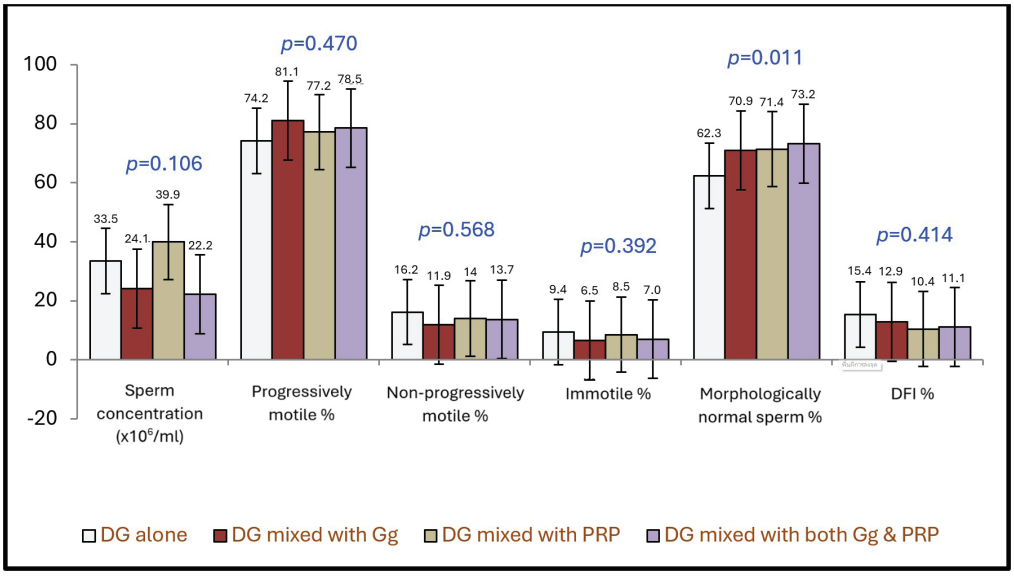


Figure 2. *Comparison of parameters in normozoospermic semen sample following the activation under various materials.* ***Open square:*** *density gradient media alone,* ***red square:*** *media contain glcyrrhiza glabra (Gg),* ***green square:*** *mixed with PRP,* ***purple square:*** *mixed with glcyrrhiza glabra and PRP.*

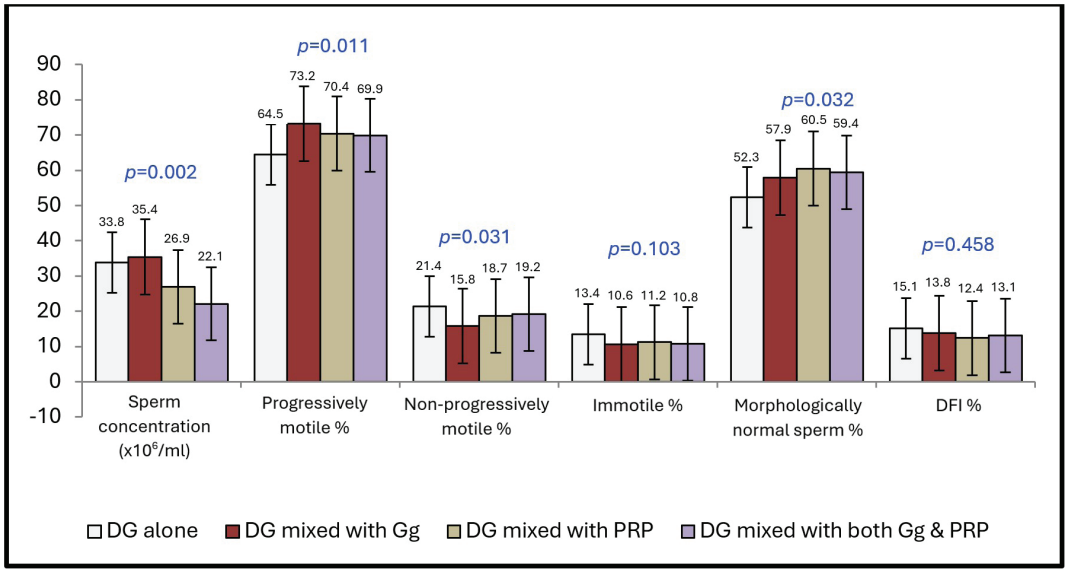


Figure 3. *Comparison of parameters in asthenoteratozoospermic semen sample following the activation under various materials.* ***Open square:*** *density gradient media alone,* ***red square:*** *media contain glcyrrhiza glabra (Gg),* ***green square:*** *mixed with PRP,* ***purple square:*** *mixed with glcyrrhiza glabra and PRP.*

Sperm activated with density gradient media and Gg have significantly improved progressive motile sperm and MNS percent, mainly because glabridin, a phytoestrogenic compound that binds to human estrogen receptors, has estrogenic activity. Estrogens increase sperm motility, grade activity, and hyperactivity.[19] Furthermore, Gg also contains calcium ions (Ca^{++}), potassium, glucose, fructose, vitamin E, vitamin C, zinc ions (Zn^{++}), sucrose, and amino acids. These nutrients boost sperm motility and forward migration *in vitro*.[23] Gg increases Ca^{++} in sperm medium to inhibit the phosphate diestrase enzyme, which limits cAMP breakdown, which increases sperm hyperactivation and motility.[9]

Sperms activated with density gradient media and PRP showed remarkable improvements in motility, morphology, and DFI. The various bioactive components of PRP are responsible for its benefits.[24] It has been noticed that PRP positively effects on goat semen parameters and found similar statistical findings for sperm motility and morphology improvement but a different finding regarding sperm concentration, which was significantly increased[25] whereas decreased in our findings. In the current study the reduction of sperm concentration is explained by the fact using the density gradient methodology which is known to lowers sperm concentration.[26] Like the other approaches, Gg coupled with PRP improves sperm parameters, however there is a slight difference in counts. This suggests that the newly developed medium did not enrich sperm parameters, particularly motility, as expected. In contrast, using Gg alone produces the maximum hyperactivation rating, as does PRP alone. Multiple researches suggested that PRP and Gg mode of intervention caused ineffective results. It has been postulated that platelet activation releases key granule components like P-selectin, ADP/ATP, GFs, and Ca^{++}.[27] In Gg, Glabridin may considerably inhibit activated platelet ATP release. Intracellular Ca^{++} increases platelet aggregation, while glabridin dramatically reduces it.

The first group i.e., normozoospermic was represented by thirty-four men with overall normal sperm parameters, including normal active motility and normal morphological sperm. Among all the certain sperm parameters that were analyzed after activation in the fourth culture media, there was no significant variation in the performance between these media across all parameters, with only one exception related to a significantly higher enhancement in sperm morphology when processed and cultured with both Gg and PRP. In contrast to the earlier studies, which demonstrated that Gg blocks platelet activity.[27] A study,[28] implies that the combination of PRP and Gg therapies in vivo has shown potential regenerative and healing effects, opening new therapy options.

The second group (asthenoteratozoospermic) expressed the largest population, with forty-six men. In addition, this group also exhibited the most diversity of variance in the decrease and increase of certain sperm function parameters throughout all activation media. Thus, after activation, this diversity involved significantly ($p \leq 0.05$) higher sperms concentration, higher progressively motile sperms percent and significantly ($p=0.031$) lower non- progressively motile sperms percent (15.80±1.15) at density gradient media with Gg, which confirmed the remarkable beneficial effects of Gg and the marvelous activation performance in the worst criteria group. However, according to Table 3, there were no significant differences ($p>0.05$) in terms of immotile sperms percent or DFI percent among all activation media. In the density gradient medium with PRP, there were significantly more morphologically

normal sperms than others.

The DFI percent were nearly equivalent in all media; however, for each specific method, the percent were statistically significantly decreased when comparing post-activation data to pre-activation data. These values indicate the high positive effectiveness of all methods that were used in eliminating the damaged cells and therefore reducing sperm DNA fragmentation.

Limitations

One of the study's limitations is its relatively small sample size, which would restrict how broadly the findings can be applied. Furthermore, the analysis was predicated on measurements and methodologies, which could cause biases in the measurements.

Conclusion

It is concluded that the application of Gg combined with PRP medium, along with the DG approach, demonstrates a significant enhancement in certain sperm function parameters as well as lowering DFI, in comparison to before activation in both groups of participants involved in this study. However, this combination is not as effective as using PRP medium alone or Gg medium alone.

Ethical approval

The study was approved by the Ethical Approval Committee with the approval code (0702-MF-2024N27).

Funding

There is no financial disclosure

Conflict of Interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Saad S. Al-Dujaily: designed, monitored, evaluated, and analyzed the result of the study; **Noor K. Khudhur**: performed the study; **Laith Amer Al-Anbary**: reviewed the article. All authors approved the final manuscript and take responsibility for the integrity of the data.

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