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The Influence of Density Gradient Technique and Autologous Platelet Rich Plasma Mixed with Glycyrrhiza Glabra Root Extraction Medium on DNA Fragmentation Index of Teratozoospermic Men

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ABSTRACT

Male infertility is a global health issue affecting over 20 million males. A spermatogenesis-related cell differentiation abnormality causes teratozoospermia (TZS). Extensive research has linked TZS to poor fertilization outcomes at assisted reproductive technology (ART) centers as well as to spontaneous pregnancies. This study used *Glycyrrhiza glabra (Gg)* and Platelet Rich Plasma (PRP) media to establish a culture medium that aimed to improve certain sperm functions of teratozoospermic men in vitro. A total of fifty semen samples were examined. The World Health Organization 2021 and 1999 standards were used to evaluate certain sperm parameters and the DNA fragmentation index. Platelet-rich plasma and Gg extract were utilized as a medium for in vitro preparation and activation of semen samples complaining of teratozoospermia factor.

Following activation by the utilization of the density gradient approach and the combination of Gg and PRP medium, there was a statistically significant (p <0.001) improvement in certain sperm function metrics. These improvements included a higher percentage of morphologically normal sperm, a rise in active motility, and a drop in the DNA fragmentation index. The density gradient technique, regardless of the medium used in this investigation, resulted in a significant enhancement of morphologically normal sperms with other certain sperm parameters and a decrease in the DNA fragmentation index.

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KEYWORD

Teratozoospermia, DNA fragmentation index, Density gradient technique, Platelet Rich Plasma, *Glycyrrhiza glabra*

1. *Introduction*

Infertility in couples is a growing widespread problem, with male factor infertility (MFI) accounting for almost half of the cases **(Salonia et al., 2020 [1]).** Moreover, epidemiological research has demonstrated a significant loss in sperm quality in the overall male population over the past few decades, with a 50-60% decrease in sperm morphology **(Candela et al., 2021 [2]).**

Teratozoospermia is a medical condition that occurs when a significant proportion of spermatozoa in semen have an irregular and abnormal shape **(Mehrparvar et al., 2020 [3]).** To be considered morphologically normal, a spermatozoon should possess a properly developed acrosome, an oval-shaped head measuring between 2.5 and 3.5 µm in width and 5 and 6 µm in length, a midpiece ranging from 4.0 to 5.0 µm, and a tail approximately 50 µm in length **(Ammar et al., 2020 [4]).** In a teratozoospermic situation, the aberrant form of spermatozoa can be seen in the head, mid-piece, or tail of the man's ejaculate **(Braham et al., 2019 [5]).**

Assisted reproductive technology is often used to treat male factor infertility. Intracytoplasmic sperm injection (ICSI), first used to treat male factor infertility with severely reduced spermatogenesis, is now a standard fertilization procedure. Over time, ICSI has been used to address non-male causes. Since gamete quality may affect spermatogenic success, semen quality may affect IVF success **(Del Giudice et al., 2022 [6]).**

The preparation of spermatozoa in ART procedures aims to choose the most optimal spermatozoa for insemination carefully. Various techniques are available to optimize the production of active sperm. It is.

Important to handle sperm processing processes with care in order to prevent the creation of free radicals **(Dover et al., 2016 [7]).**

Morphologically, normal spermatozoa have a higher density than abnormal spermatozoa. A normal mature spermatozoon has a density of at least 1.10 g/mL, whereas an immature and morphologically abnormal spermatozoon has a density between 1.06 and 1.09 g/mL. The density gradient centrifugation technique utilizes this change in densities to separate morphologically normal spermatozoa from abnormal ones **(Nayar et al., 2023 [8]).**

On the other hand, Glycyrrhiza glabra, commonly known as licorice, has been studied for its beneficial effects on male reproductive health **(Aldhahrani et al., 2021 [9])**. Research indicates that licorice extract can positively impact sperm parameters by ameliorating sperm. Abnormalities, increasing sperm motility, and improving sperm quality **(Ghorbanlou et al., 2020 [10]).** Additionally, licorice extract has protective effects against oxidative stress-induced testicular dysfunction, enhancing antioxidant enzyme activity and reducing inflammatory cytokines **(Al-Dujaily et al., 2013 [11]).**

Platelet-rich plasma (PRP) is a concentrated fraction of autologous blood plasma with a high number of platelets **(Berrigan et al., 2024 [12]).** Platelets are small fragments of cytoplasm that lack a nucleus. They are derived from megakaryocytes, which are specialized cells in the myeloid cell lineage. Platelets contain a variety of growth factors (such as platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and transforming growth.

Factor beta1 (TGF-B) coagulation factors and differentiation factors. These substances play important roles in processes such as angiogenesis, immunosuppression, and tissue regeneration **(Éliás et al., 2024 [13]).** Platelet-rich plasma (PRP) has been utilized in various disciplines, such as plastic surgery, pediatric surgery, cardiac surgery, ophthalmology, and reproductive medicine **(Collins et al., 2021 [14]).**

2. Patients and Methods

This study analyzed fifty semen samples collected from individuals diagnosed with teratozoospermia. Before collecting each sample, all patients granted their agreement, and the participants were educated about the purpose of the questionnaire and the objective of the research. The study was conducted at the High Institute for Infertility Diagnosis and Assisted Reproductive Technologies, Al-Nahrain University, and Al-Harithiya International Lab in Baghdad, Iraq. It took place from October 2023 to January 2024. The inclusion criteria consist of fertile normozoospermic males and infertile teratozoospermic men. Exclusion criteria encompass azoospermic males, oligozoospermia/asthenozoospermia, and patients with a history of undescended testes, hyperprolactinemia, genetic malformations, and diabetes mellitus.

1) Semen collection

Semen was collected in a sterile wide-mouth container after being obtained through masturbation. In an incubator maintained at 37°C, the specimen was subsequently delicately mixed. Macroscopic and microscopic measurements were conducted in accordance with the guidelines established by the World Health Organization (WHO) in 2021, following the liquefaction of the semen **(WHO, 2021 [15]).** Furthermore, the WHO Manual 1999 was strictly followed in the assessment of morphologically normal sperm **(WHO, 1999 [16)]).**

2) Preparation of *Glycyrrhiza glabra*

To prepare the working solution, 10 mg of Glycyrrhiza glabra 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. Glycyrrhiza glabra solution was then transferred to an appropriate container and stored in a cold, dark place away from direct sunlight and heat.

3) Platelet-rich plasma preparation

Platelet-rich plasma is obtained by processing the blood of patients. Two to three milliliters of platelet-rich plasma (PRP) were obtained from eight milliliters of venous blood. The speed and duration of PRP centrifugation were determined by the specificity of the protocol, the quality of the apparatus, and the concentration of platelets. The conventional criteria of 3200 RPM for 15 minutes were employed in this study. PRP that had been separated was frozen at -8°C **(Croisé et al., 2020 [17]).**

4) Control media preparation

The FertiCult™ Flushing HEPES buffered medium (FertiPro Company, Belgium) is a ready-to-use solution that contains bicarbonate, physiologic salts, glucose, lactate, pyruvate, and (4.0g/L) of human serum albumin. Furthermore, phenol red and/or gentamicin (10mg/L) were available. The medium was stored in accordance with the manufacturer's instructions, typically in a refrigerator at the stated temperature (4-8°C). 4.5 mL of flushing medium were taken from the refrigerator and transported to the laboratory workstation for worming and utilization. The temperature of the incubator is typically equivalent to that of the human, which is 37°C.

5) Density gradient centrifugation protocol

This 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:

First tube: Density gradient media + liquefied semen + control medium (0.5 mL)

Second tube: Density gradient media + liquefied semen + PRP (0.5 mL)

Third tube: Density gradient media + liquefied semen + *Gg* (0.5 mL

Fourth tube: Density gradient media + liquefied semen + PRP and *Gg* (0.5 + 0.5 mL)

As soon as the loading media and semen were finished, all tubes were moved to a centrifuge at 4000 rpm for 15 minutes. Then after, the samples were prepared as described by **(Mohammed et al., 2020 [18]).**

6) Acridine Orange staining strategy:

A 10 ul semen sample was smeared on the slide and air-dried for 20 minutes. The slide was treated with Carnoy's solution for 2-24 hours. A brief drying period followed before staining. All stain preparations were done in the dark at room temperature with a pH of 2.5. Three to five millilitres of dye were applied to the slide. The slide was carefully washed with distilled water and allowed to dry **(Dutta et al., 2021 [19]).** The Slide was seen using a fluorescent microscope with a 40X objective lens the same day it was stained. Each patient's five slides of spermatozoa DNA were tested before and after activation and the results were reported as green, red, or yellow. Al-Dujaily et al. (2015) reported that yellow-red-fluorescent sperm heads are abnormal but green-fluorescent spermatozoa are normal **(Al-Dujaily et al., 2015 [20]).**

3. 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 an analysis of variance (ANOVA). Least significant test (LSD) was used to compare between the results. The results were considered statistically significant when *p* value was equal to or less than 0.05.

4. Results

1) Basal seminal fluids analysis parameters

The baseline seminal fluids analysis parameters were presented in (Table 1). Accordingly, the mean sperm concentrations were 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 normal sperm morphology percent was 22.70 ± 1.32. Whereas , the DNA fragmentation index was 22.31 ± 1

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 sperm %	0.0 – 75.0	25.56 ± 1.78
Nonprogressive 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
DFI %	6.0 – 62.0	22.31 ± 1.39

(2) Comparisons of certain sperm function parameters between the four-activation media that are used for in vitro activation of teratozoospermic semen samples by density gradient technique .

There was significantly ($p=0.008$) higher progressively motile sperms percent after activation by density gradient media with Glycyrrhiza glabra (76.1 ± 1.37;), on the contrary, non-progressively motile and immotile sperms were significantly ($p \leq 0.05$) lower in the above media; (14.43 ± 0.86) and (9.04 ± 0.63;) respectively, on the other hand, density gradient media plus PRP had significantly ($p < 0.001$) higher morphologically normal sperms percent (62.66 ± 1.50;). There were also no significant ($p > 0.05$) differences regarding sperm concentration and DNA.

Table (2): **Comparison of certain sperm function parameters between four activation media**

Parameters	**Before Activation**	**DG alone**	**DG mixed with Glycyrrhiza glabra**	**DG mixed with PRP**	**DG mixed with both Glycyrrhiza. glabra & PRP**
Sperm concentration (10^6/ml)	39.70 ± 2.73	29.08 ± 2.11 A	30.68 ± 2.27 A	29.04 ± 2.12 A	29.08 ± 2.19 A
Progressive motile sperm %	25.56 ± 1.78	69.14 ± 1.53 B	76.1 ± 1.37 A	73.63 ± 1.43 A	73.43 ± 1.41 A
Non-progressive motile sperms %	26.41 ± 1.00	18.79 ± 1.09 A	14.43 ± 0.86 B	16.60 ± 1.01 A	16.89 ± 0.90 A
Immotile sperm %	48.07 ± 2.18	11.59 ± 0.66 A	9.04 ± 0.63 B	9.91 ± 0.71 B	9.33 ± 0.70 B
Morphologically normal sperm %	22.70 ± 1.32	54.13 ± 1.47 B	60.39 ± 1.54 A	62.66 ± 1.50 A	62.25 ± 1.49 A
DFI %	22.31 ± 1.39	15.03 ± 0.96 A	12.74 ± 0.87 A	12.31 ± 0.89 A	12.56 ± 0.91 A

Ɐ: ANOVA; S: Significant; NS: Not significant; A+B: Different capital letters mean there is a significant variation

5. Discussion

The results of this study showed significant improvements in certain sperm parameters and a significant decrease in DNA fragmentation index following in vitro stimulation using a single technique (Density gradient) applied to three different types of media. This technique was also used alone as a control method.

As shown in Table 2, the findings indicated that the density gradient by alone decreased the concentration of sperm, non-progressive movement, and immobility while enhancing progressive movement and the presence of morphologically normal sperm after activation. In 2023, a published scientific paper discovered comparable outcomes **(Fernandes et al., 2023 [21]).** This research employed density gradients to separate a substantial quantity of structurally intact and actively motile human sperm from semen fluid. These separated sperm underwent the removal of waste, non-functional sperm, and non-sperm cells **(Dai et al., 2024 [22]).**

The combination of density gradient media with Glycyrrhiza glabra to activate sperm has been found to greatly enhance the percentage of sperm that exhibit progressive motility and morphologically normal sperm. This improvement is mostly attributed to the presence of glabridin, a phytoestrogenic chemical that binds to estrogen receptors in the human body, hence exhibiting estrogenic effects. Estrogens enhance sperm motility, improve grade activity, and promote hyperactivity **(Al-Dujaily & Ali 2015 [23]).**

Spermatozoa that were treated with density gradient media and platelet-rich plasma (PRP) exhibited significant ($p<0.001$) enhancements in motility, morphology, and DNA fragmentation index. The diverse bioactive constituents of PRP are accountable for its beneficial effects **(Magalon et al., 2014 [24]).** In 2021, Abduljabar et al.

Conducted a study on the impact of PRP on semen characteristics. They discovered that PRP had a positive influence on sperm motility and morphology, yielding statistically significant improvements **(Abduljabar et al., 2021 [25])** despite the fact that they used a glass wool filtration technique instead of a density gradient. The outcome of using PRP as sperm activation media on DNA statues, on the other hand, showed a significant decline in DFI value (22.31 ± 1.39 vs. 12.31 ± 0.89). This result agreed with a previous study by **Bader *et al.* (2020) & Hamdan *et al.* (2021)**, which both explained these findings on the basis of the ability of PRP to hinder ROS through its antioxidant and anti-apoptotic properties (Hamdan et al., 2021 [26]). A substantial decrease in the level of DFI is due to the diverse array of growth factors present in PRP alpha granules **(Bader et al., 2020 [27]).**

Although there is a small difference in numbers, Gg combined with PRP enhances sperm parameters, just like the other methods. Evidently, the newly created medium failed to enhance sperm parameters, especially motility, contrary to expectations. The maximal hyperactivation rating is achieved when Gg alone or PRP alone are used in comparison. The results of several studies indicated that the PRP and Gg intervention modes were ineffective. Platelet activation releases critical granule components such as P-selectin, ADP/ATP, GFs, and Ca^{++}, according to **Ko et al. (2023).** Glabridin has the potential to significantly reduce the release of activated platelet ATP in Gg. Compared to glabridin, intracellular Ca^{++} causes platelet aggregation to rise **(Ko et al., 2023 [28]).**

The DFI percentages were roughly equivalent across all media. However, for each individual method, the percentages significantly fell when comparing post-activation data to pre-activation data. These data reflect the highly positive efficacy of all treatments employed in removing the damaged cells and thereby reducing sperm DNA fragmentation.

6. Conclusion

The study concludes that using *Gg* in combination with PRP medium, along with the DG method, significantly improves specific sperm function metrics and reduces DFI compared to the pre-activation state in men with teratozoospermia factor. Nevertheless, the efficacy of this combination is inferior compared to either PRP medium or Gg medium alone.

7. Limitation of the study

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 particular measurements and methodologies, which could cause biases in the measurements.

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Author Contribution

Noor Kadhim performed the study, and Saad S. Al-Dujaily Laith A. Al-Anbari supervised the work .

Conflict of Interest

The authors declare no conflict of interest .

Ethical Clearance

The study was approved by the Ethical Approval Committee.

Financial Disclosure

There is no financial disclosure.

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