

Effect of the Supplementation of L-aspartic Acid, L-ascorbic Acid, and Silver Nanoparticles (AgNPs) to the In Vitro Fertilization (IVF) Media on the NMRI Mouse IVF Rates

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Abstract

Numerous lifestyle factors contribute to the incidence of infertility, which is a major concern for couples throughout the globe. The effectiveness of *in vitro* fertilization (IVF) is highly dependent on the characteristics of culture media used. The present research aimed to determine the effects of L-aspartic acid, L-ascorbic acid, and silver nanoparticles (AgNPs) on the success rate of IVF in NMRI mice. The solutions of L-aspartic acid, L-ascorbic acid, and synthesized silver nanoparticles (AgNPs) were prepared. The 6–8 weeks-old NMRI male mice were used as sperm donors, and 6–8 weeks-old NMRI mice were used for oocyte donation. In addition, the IVF procedure was performed in 50 μ L drops of KSOM + 15%BSA under mineral oil. Seven different experimental groups were investigated in addition to the control group. Oocytes were exposed to sperm for 4–6 h in the KSOM medium as a control group. According to the results, the groups containing supplements "AgNPs", "L-aspartic acid", and "AgNPs + L-ascorbic acid" were not suitable for the 2PN stage. In contrast, the group containing "L-aspartic acid + L-ascorbic acid" was the best group for the 2PN stage. In addition, supplementation of the mixture of L-aspartic acid and AgNPs solutions in KSOM + BSA culture medium can be considered as the most suitable group for the 2Cell stage. Gaining knowledge on how particular additives affect the success rates of IVF may lead to the development of advanced compositions, which will enhance assisted reproductive technologies (ART) throughout time and improve the lives of infertile couples.

1. Introduction

Nowadays, because of advancements in cleanliness, better living circumstances, and medicine, we are better able to avoid diseases. Even while infectious illnesses are still a major concern, non-communicable diseases (NCDs), also called lifestyle diseases, are currently the most prevalent causes of death in the United States. In contrast to chronic illnesses, which manifest earlier in life, lifestyle diseases tend to worsen with age and appear to be more common in developed nations and among those with higher life expectancy [1, 2]. The development of NCDs is linked to four modifiable lifestyle behaviors: smoking, eating an unhealthy diet, not exercising, and drinking alcohol. Long-term courses and a combination of behavioral, physiological, genetic, and environmental factors define chronic diseases, also known as NCDs. The main types of NCDs include diabetes, cancer, heart disease, and chronic respiratory problems [3]. Non-communicable diseases disproportionately affect people in nations with low or middle incomes, accounting for approximately 75% of all deaths from NCDs [4]. Numerous studies have demonstrated that unhealthy lifestyle behaviors, including stress, alcohol use, smoking, obesity, and eating habits, may have a long-term impact on the physiology of women. These lifestyle decisions have been found to dramatically reduce the probability of conception and have an impact on an individual's overall health and capability for reproduction [5, 6].

The argument that negative trends in the state of male reproductive health are primarily related to changes in individual lifestyles has received increasing attention recently. These changes include the prevalence of high-calorie dietary habits, a diet that is unbalanced, lack of physical activity, an elevated level of social stress brought on by urbanization, smoking, drinking, late fatherhood, uncontrolled drug use, and the use of mobile phones and laptops as the sources of electromagnetic radiation [7–17]. Research on the effects of prolonged exposure to these factors on reproduction has demonstrated that, even if each of them has a little detrimental effect, the combined effect of these factors may significantly worsen the reproductive health of individuals.

Furthermore, although different circumstances might have differing degrees of influence on a man's ability to reproduce, these effects often coexist and are amplified by one another [7, 16, 18]. Therefore, the importance and need to research the reasons behind a decline in male fertility center on various aspects of personal lifestyle and how they affect reproductive health [7]. Infertility is defined as the failure to conceive after one year of regular, unprotected sexual activity. There is a known reason for infertility in approximately 85% of cases [19, 20]. Typical infertility assessment consists of a hysterosalpingogram, an examination of the semen, an evaluation of ovulation, and, if necessary, testing for ovarian reserve and laparoscopy. Practitioners diagnose unexplained infertility when a conventional infertility examination yields normal findings. Estimates vary, but generally speaking, 15–30% of infertile couples' test results will be normal, meaning that their infertility is unexplained [19, 21, 22]. The main therapeutic options for unexplained infertility involve expectant observation with scheduled intercourse as well as lifestyle changes, controlled ovarian hyperstimulation (COH) with IUI, *in vitro* fertilization (IVF), and clomiphene citrate and intrauterine insemination (IUI) [19]. While IVF initially emerged to prevent irreversible tubal disease, it is now often used to treat infertility caused by unexplained infertility, male factors, and endometriosis. *In vitro* fertilization with donor oocytes has made it possible for women who are unable to conceive naturally because of primary ovarian insufficiency (POI) or age-associated decrease in oocyte quantity to become pregnant [23].

Culture conditions affect pre- and post-implantation development, as well as the future health of the offspring, and there is increasing evidence that these factors significantly affect the success rate of IVF [24, 25]. Nevertheless, the data is often disputed and inadequate to support the role of the media culture of IVF in these results [26]. In an interesting patent presented by Salvatore D'Aniello and Enrico D'Aniello, an experiment on male seminal samples indicated that the L-aspartic acid improved the parameters of seminal liquid (number and motility of sperms) and is effective in the treatment of male infertility [27]. Additionally, it has been observed that adding L-ascorbic acid to IVF culture and/or vitrification medium increases the survival rates of porcine blastocysts, which may be related to HSPA1A expression [28]. Silver nanoparticles (AgNPs) have many biomedical applications, including biosensing, drug delivery, tissue engineering, etc [29–35]. Some studies have reported that AgNPs have negative effects on sperm, oocytes, and IVF success rate [36–38]. However, Pérez-Duran et al. (2020) conducted an investigation regarding the effects of adding low concentrations of AgNPs on swine semen and concluded AgNPs' potential as a replacement for traditional antibacterial agents [39].

Due to these effects of L-aspartic acid, L-ascorbic acid, and AgNPs, the present study aimed to supplement these materials into the KSOM culture media to investigate their effect alone and in combination on the IVF rate of mice. A brief schematic of the procedures employed in the present work is presented in Scheme (1).

2. Materials and Methods

2.1 Materials Used

The materials employed in the present investigation are presented in Table (1).

Table 1
List of materials employed in the current research.

Material	Chemical Formula	Molar Mass (g/mol)	Purity (%)	Company
Silver nitrate	AgNO ₃	169.87	≥ 99.95	Sigma-Aldrich
Trisodium citrate dihydrate	C ₆ H ₅ Na ₃ O ₇ ·H ₂ O	294.10	≥ 98.00	Sigma-Aldrich
L-aspartic acid	C ₄ H ₇ NO ₄	133.1	≥ 99.98	Sigma-Aldrich
L-ascorbic acid	CH ₃ COCH ₃	176.12	≥ 99.00	Sigma-Aldrich
Bovine serum albumin (BSA)	---	66430.3	≥ 98.00	Sigma-Aldrich
Mineral oil	---	---	---	Sigma-Aldrich
EmbryoMax® Advanced KSOM Embryo Medium	---	---	---	Merck
Pregnant mare serum gonadotropin (PMSG) for mice	---	---	---	Sigma-Aldrich
Human chorionic gonadotropin (hCG) for mice	---	---	---	Sigma-Aldrich

2.2 Preparation of Silver Nanoparticles (AgNPs) Colloidal Solution

An amount of 0.1 g of AgNO₃ was dissolved in 250 cc of distilled water, and the resulting solution was placed on a heater stirrer until it reached boiling point. Subsequently, 0.1 g of C₆H₅Na₃O₇·H₂O was dissolved in 10 cc of water. The AgNO₃ solution, upon reaching a boil, was slowly introduced dropwise into the C₆H₅Na₃O₇·H₂O solution. The solution exhibited a transition to a grayish-green color, indicating the formation of colloidal silver. The temperature source was then disconnected, and the solution was allowed to stir for 15 min. Following this, it was transferred into a container and left at room temperature for 10 min. Then, it was moved to the refrigerator, and after 24 hours, an assessment was made to determine if silver had settled. It was desirable that the AgNPs would not settle and remain stable in the solution. The colloidal silver solution prepared by the method described contains approximately 0.5885 mM of AgNPs, and its molar mass is approximately equal to 258.972 g/mol.

2.3 Preparation of L-ascorbic Acid, L-aspartic Acid, and Silver Nanoparticles (AgNPs) Solutions

For preparing a 4 mM solution of L-aspartic acid, the following protocol was performed. The volumetric flask was shaken until the L-aspartic acid was fully dissolved in 100 mL of distilled water, resulting in a solution containing 0.5324 grams of L-aspartic acid. Then, 2 mL of this solution was transferred to another clean

volumetric flask capable of holding 50 mL of solution using a pipette. The 50 mL volumetric flask was subsequently filled with water up to the mark on the neck of the flask. The 50 mL volumetric flask was inverted or shaken multiple times to ensure thorough mixing of the solution. Furthermore, 1 μ L of the 4 mM L-aspartic acid solution was taken from the 50 mL volumetric flask using a new sterile pipette and added to 1 mL of IVF culture. A similar protocol was followed to prepare a 4 mM solution of L-ascorbic acid. Initially, 0.35224 g of L-ascorbic acid was dissolved in a volumetric flask with 100 mL of distilled water. The volumetric flask was then shaken until the L-ascorbic acid was fully dissolved. Subsequently, 2 mL of this solution was transferred to another clean volumetric flask capable of holding 50 mL of solution using a pipette. Water was added to the 50 mL volumetric flask up to the mark on the neck of the flask. The 50 mL volumetric flask was then inverted or shaken several times to ensure thorough mixing of the solution. Then, 1 μ L of the 4 mM L-ascorbic acid solution was taken from the 50 mL volumetric flask using a new sterile pipette and added to 1 mL of IVF culture.

Furthermore, in order to prepare 0.1 mM of 100 mL of AgNPs colloidal solution with a molar mass of 258.972 g/mol, 10 mL of the colloidal solution was measured out and diluted with enough DI water to make a final volume of 100 mL. Then, 1 μ L of the 0.1 mM AgNPs colloidal solution was taken from the 100 mL volumetric flask using a new sterile pipette and added to 1 mL of IVF culture.

2.4 Animal Operations

2.4.1 Animals

All protocols used in the present study were approved by the ethics committee of Gonabad University of Medical Sciences, Gonabad, Iran (Ethics Code: IR.GMU.AEC.1402.012). Mice were kept according to the standard protocol of a light/dark cycle (12/12 h) in 26°C, all mice received in the daily feed crispy pellets (Versele Laga, Belgium) and water, and finally euthanized by the cervical dislocation method. In the present work, 6–8 weeks-old NMRI male mice were used as sperm donors, and 6–8 weeks-old NMRI mice were used for oocyte donation.

2.4.2 Sperm Preparation

Fertile male mice were euthanized, and the tail of the epididymis was immediately transferred to a 1500 μ L drop of culture medium covered with mineral oil. The contents of the epididymis were removed. and the remaining tail tissue was discarded. Then, the petridish containing sperm was kept for 1 h at 37°C in 5% CO₂ in humid air for capacitation process.

2.4.3 Mature Oocyte Collection

Ovulation stimulation of female mice was performed using the i.p method, which consists of injecting a substance into the peritoneum so that the ovulation process takes place. First, 7.5 international units (IU) of PMSG was injected, and after 48 h, 7.5 IU of HCG was injected. Afterward, the oocytes were collected 14 h after HCG injection by removing the oviduct of the female mouse and placing them in the culture medium at 37°C. Then, cumulus-oocyte (COC) masses were collected by rupturing the oviduct using an insulin needle, the oocytes were singled, and finally, every five oocytes were placed in 50 μ L drops of the culture medium.

2.4.4 *In Vitro* Fertilization (IVF)

The IVF procedure was performed in 50 µL drops of KSOM + 15%BSA under mineral oil. Then, a pre-incubated sperm suspension was slowly added to the collected oocytes for the capacitation process. The combined suspension was incubated for 4–6 h, and then the fertilization rate was evaluated via recording the quantity of 2PN embryos. Afterward, by transferring 2PN embryos to each drop of 25 µL in KSOM + 4%BSA culture medium, the 2Cell rate was evaluated after 24 hours.

2.5 Studied Groups

Seven different experimental groups were investigated in addition to the control group. Oocytes were exposed to sperm for 4–6 h in the KSOM medium as a control group. The groups considered for the present study, along with their sample sizes and types for each stage (2PN and 2Cell), are presented in Table (2).

Table 2
Studied groups in the present study along with their sample sizes at 2PN and 2Cell stages.

Group	Composition	Stage	Sample Type	Sample Size
A	(KSOM + BSA) + L-ascorbic acid	2PN	Oocyte	75
		2Cell	2PN	9
B	(KSOM + BSA) + AgNPs	2PN	Oocyte	75
		2Cell	2PN	6
C	(KSOM + BSA) + L-aspartic acid	2PN	Oocyte	75
		2Cell	2PN	7
D	(KSOM + BSA) + L-aspartic acid + L-ascorbic acid	2PN	Oocyte	75
		2Cell	2PN	12
E	(KSOM + BSA) + AgNPs + L-aspartic acid	2PN	Oocyte	75
		2Cell	2PN	10
F	(KSOM + BSA) + AgNPs + L-ascorbic acid	2PN	Oocyte	75
		2Cell	2PN	7
G	(KSOM + BSA) + AgNPs + L-aspartic acid + L-ascorbic acid	2PN	Oocyte	75
		2Cell	2PN	10
Control	(KSOM + BSA)	2PN	Oocyte	75
		2Cell	2PN	10

2.6 Statistical Analysis

The analysis was carried out using SPSS (version 24) and PRISM (version 9.0) software. In the variables, first, the normality of the data was evaluated by employing the Kolmogorov-Smirnov test, and since they were normal, analysis of variance (ANOVA) and descriptive statistics of the mean ± standard deviation (SD) were employed. The significance level in the present study was considered to be $P < 0.05$. The bar chart is drawn

considering the "criterion error" as its error, and the stars on the chart indicate the significance of the two groups compared with each other.

3. Results

Table (3) is the list of results for the 2PN and 2Cell stages.

Table 3
Results related to 2PN and 2Cell stages presented as mean + SD.

Variable	Group									
	A	B	C	D	E	F	G	Control	Statistic	P-value
2PN	3.133 ± 1.302	2.2 ± 0.862	2.4 ± 1.242	4.067 ± 0.704	3.533 ± 1.06	2.467 ± 1.187	3.467 ± 0.915	3.4 ± 1.121	5.736	∅0.001
2Cell	2.6 ± 1.14	0.2 ± 0.447	2.8 ± 0.837	1.6 ± 1.14	5.0 ± 0.707	0.8 ± 0.837	0.8 ± 0.837	6.2 ± 0.837	29.9	∅0.001

As indicated in Figure (1), in terms of 2PN, there is no statistical significance between the control group and the other groups. Although Groups B, C, and F had no significant difference with control, we cannot consider them appropriate for developing 2PN. Groups A, E, and G, along with being in an acceptable range for 2PN, did not show any statistical significance compared to the control, and they can be taken into account as suitable groups for 2PN. Among all groups, Group D had a higher rate of 2PN compared to others, even control, and these groups indicated significant differences relative to Groups B, C, and F, which are considered to be improper groups. Therefore, we can consider Groups D, E, G, and A as the best supplements, in respective order, for the KSOM + BSA culture medium in order to develop 2PN.

Figure (2) and Figure (3) demonstrate that Groups B, F, and G were the most destructive groups for the 2Cell stage. On the other hand, although Group D demonstrated the most favorable results for the 2PN stage, there was statistical significance between Group D and the control at the 2Cell stage, indicating that this group was not appropriate for this stage. Moreover, it can be seen that Groups A and C (L-ascorbic acid alone and L-aspartic acid alone) had significant differences with the control with approximately identical 2Cell rates. Finally, Group E, with significant differences relative to all groups except control, was the best group in terms of developing 2Cell.

Discussion and Conclusion

With infertility affecting 85% of couples, unexplained cases prompt varied treatments, including in vitro fertilization (IVF) [19, 20]. While IVF culture conditions influence outcomes, the role of specific components remains debated [24–26]. Noteworthy, L-aspartic acid, L-ascorbic acid, and silver nanoparticles show potential effects on male reproductive health and IVF outcomes [27, 28, 36–39]. The present work aimed to supplement these substances into KSOM culture media, exploring their individual and combined impact on NMRI mouse IVF rates.

According to the results, the groups containing supplements "AgNPs" (Group B), "L-aspartic acid" (Group C), and "AgNPs + L-ascorbic acid" (Group F) were not suitable for the 2PN stage. In contrast, the group containing "L-aspartic acid + L-ascorbic acid" (Group D) was the best group for the 2PN stage. As can be observed in Figure (2) and Figure (3), Groups A and C, which respectively contained L-ascorbic acid alone and L-aspartic acid alone, the rate of 2Cell development from 2PNs was approximately 30% and 40%, in respective order. In fact, the positive effects of L-aspartic acid alone were greater than the positive effects of L-ascorbic acid alone. However, it can be seen that Group B, in which the culture medium contained only AgNPs additive, was the most destructive group among all groups. Although a low concentration of AgNPs solution was used in the present work, its toxic effects on embryos are still evident, which is in line with the literature [36–38]. Furthermore, supplementation of the mixture of two amino acids (Group D) did not indicate appropriate results on the success rate of 2Cell development, which is probably due to the increase in the acidity of the environment with the combination of these two amino acids. Similarly, in Groups F and G, no proper result was evident in terms of the development of 2Cell embryos. However, Group E had the best result after the control group. Indeed, since the AgNPs solution employed in the present work was 40 folds more diluted than the L-aspartic acid solution, the positive effect of L-aspartic acid has overcome the negative effect of AgNPs. Moreover, it can be declared that due to the alkaline nature of the AgNPs solution, the acidity of the environment has been neutralized. Finally, Group E (supplementation of the mixture of L-aspartic acid and AgNPs solutions in KSOM + BSA culture medium) can be considered as the most suitable group for the 2Cell stage.

Since Groups D, E, and G increased the rate of 2PN, it seems that it is better to first use these supplements for the development of 2PN and then employ the medium without supplements as the cleavage medium. The authors will attempt to follow this protocol in their future study. Considering that no study has reported the combined effects of these supplements, it is not possible to discuss precisely the reasons for the obtained findings. It is recommended that future studies investigate the biochemical, physicochemical, and biological effects, as well as the impact of gene expression factors on the media containing the studied groups.

Declarations

Conflict of Interest

The authors declare that there is no conflict of interest.

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Ethics Statement

All animal-handling protocols were approved by the ethics committee of Gonabad University of Medical Sciences, Gonabad, Iran (Ethics Code: IR.GMU.AEC.1402.012).

Data Availability

The data used in the present study are available from the corresponding author upon reasonable request.

Author Contributions

A.M.: Conceptualization, Formal analysis, Investigation, Methodology, Resources, Validation, Visualization, Writing - original draft, and Writing - review and editing. H.J.: Formal analysis, Investigation, Methodology, Resources, Writing - original draft, and Writing - review and editing. S.M.G: Investigation, Methodology, Resources, Software, and Writing - review and editing. H.S.: Visualization, Writing - original draft, and Writing - review and editing. J.M.: Project administration, Supervision, and Writing - review and editing.

Declaration of Generative AI and AI-assisted Technologies in the Writing Process

During the preparation of this work, the authors used ChatGPT, Quillbot, and Grammarly in order to translate from Persian to English, as well as rephrase and edit the text grammatically and in terms of typo. After using these tools/services, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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Scheme

Scheme 1 is available in the Supplementary Files section.

Figures

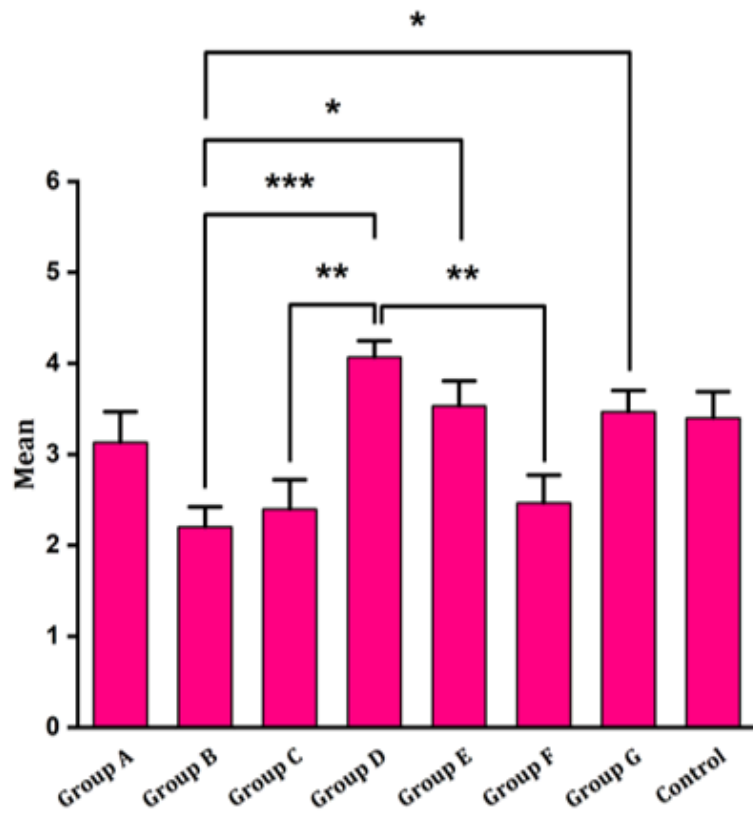


Figure 1

Mean number of 2PN embryos developed 6 hours after the IVF procedure.

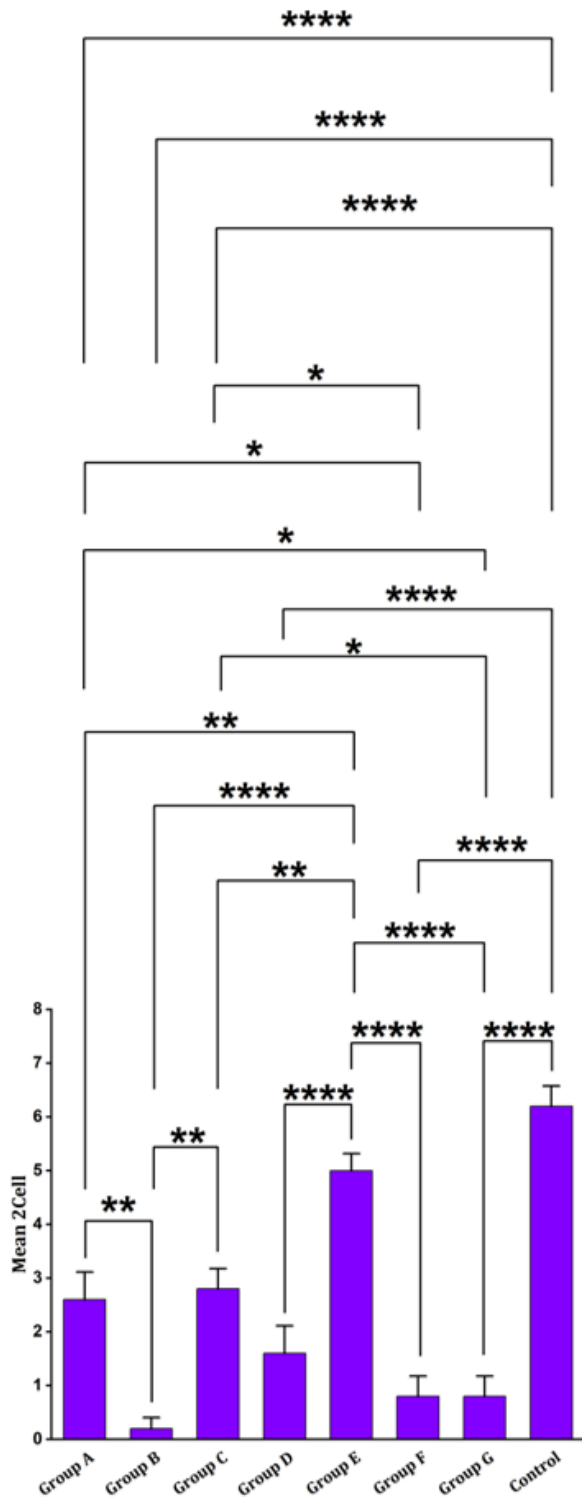


Figure 2

Mean number of 2Cell embryos developed 24 hours after the IVF procedure.

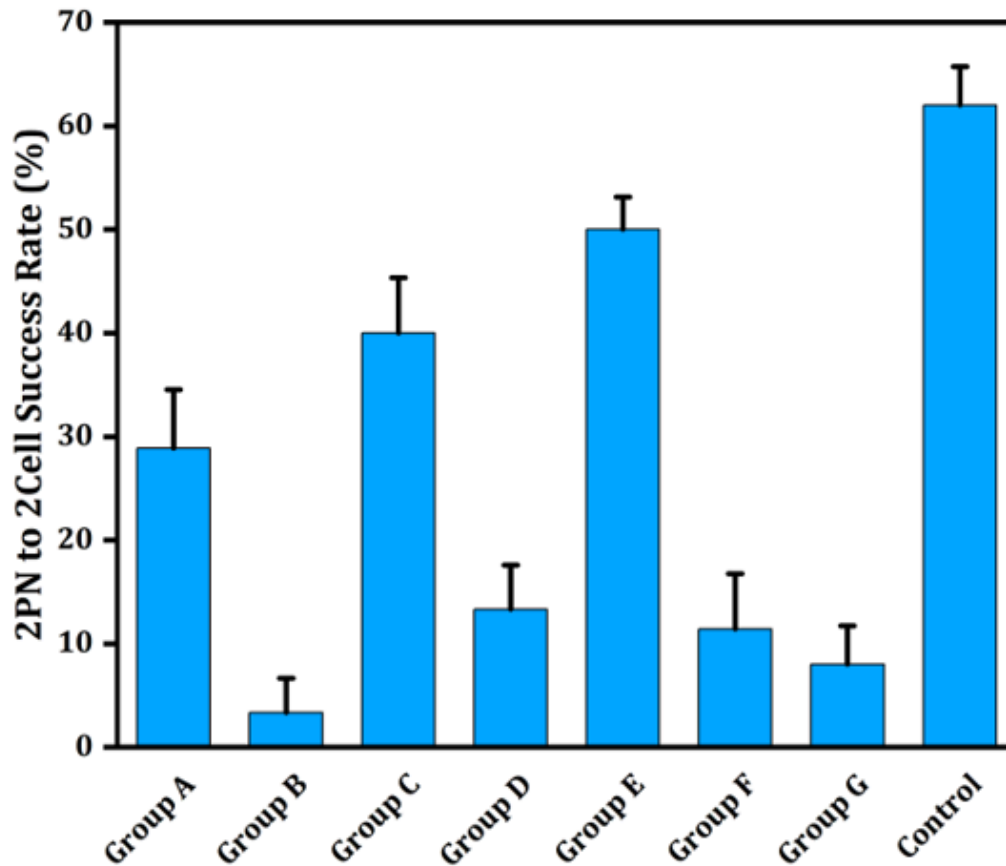


Figure 3

Success rate (%) of the development of 2Cell(s) from 2PN embryos after 24 hours.

Supplementary Files

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- [Scheme1.png](#)