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Creatine - A Wonder Molecule: A Comparison Study to Identify the Best In Vitro Capacitating Agents for Sperms

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***Abstract*: This paper presents a comparative study to identify the best in vitro capacitating agent for sperms.**

***Keywords*: sperms, human spermatozoa, capacitation, creatine, human sperm capacitation.**

1. Introduction

Reproductive medicine aids infertile couples in achieving their dreams of having a child and so making the dream of parenthood come true. There are several reasons why couples choose ART which includes female infertility, male factor infertility, and other idiopathic reasons. Male infertility is caused by the decline in sperm count, motility, or morphology, which is assisted using Intracytoplasmic sperm injection (ICSI). WHO recommends three fundamental procedures for separating seminal plasma and spermatozoa: (a) direct swim-up, (b) sperm washing (by centrifugation), and (c) density-gradient centrifugation (DGC). Muratori *et al.*, 2019, observed that using DGC and the swim-up technique to separate spermatozoa from semen increased spermatozoa DNA fragmentation (sDF) in viable spermatozoa (40). The typical characteristics of sperm concentration, motility, and morphology, as determined by regular semen analysis, do not guarantee intact spermatozoa DNA. However, sDF may affect other spermatozoa parameters, such as spermatozoa motility. As a consequence, fertilization occurs even when DNA is damaged, resulting in pregnancy failure after ICSI treatment.

Now-a-days, different kinds of media containing various capacitation agents are utilized in ART to activate spermatozoa. Currently, *in-vitro* capacitation is accomplished using progesterone, Ca^{+2} ionophore, Coenzyme Q10, follicular fluid, and human tubal fluid. Progesterone is the primary regulator of sperm capacitation, chemotaxis, and acrosomal reaction. Progesterone has a dose and time-dependent impact on sperm; when sperm are incubated in a progesterone-containing medium, it first becomes more motile, then hyperactive, and eventually begin the acrosomal reaction [Fig. 1.]. Coenzyme Q10 (ubiquinone) is another in-vitro capacitating agent that is involved in ATP production. Coenzyme Q10, in general, can be used as an oral supplement as well as a media content for in-vitro sperm capacitation. Oral supplementation of coenzyme Q10 protects semen from oxidative damage caused by reactive oxygen species (ROS) [Fig. 2.]. Ca^{+2} ionophore is a di-cationic carrier molecule added to the medium to improve sperm motility. Ca^{+2} ionophore bypasses all ion channel mechanisms and raises calcium ion intracytoplasmic concentration [Fig. 3.]. Creatine, according to some embryologists, enhances sperm motility and fertilization rate in in-vitro fertilization [Fig. 4.]. There was no experiment trial done with human spermatozoa, however, creatine was used to reduce the period of in vitro capacitation for mice spermatozoa.

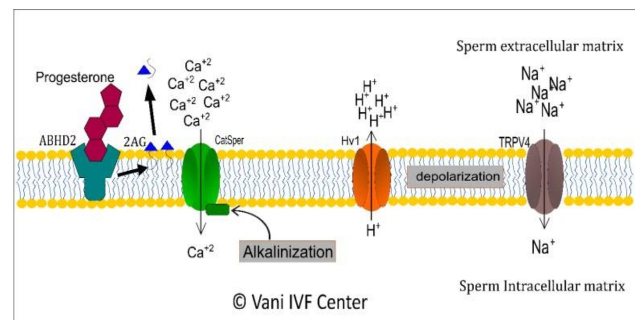

Fig. 1. Molecular mechanism of CatSper Ca +2 ion channels via ABHD2 receptors. *Source: Meet Patel, Vani IVF Center*

Progesterone-induces Ca^{+2} signaling at three different locations: (i) Progesterone-induced Ca^{+2} signaling in the acrosome revealed only an initial transient Ca^{+2} signal but no sustained Ca^{+2} signal. (ii) The midpiece, on the other hand, demonstrated both the initial response and a long- lasting Ca^{+2} signal, and (iii) the principal piece demonstrated a delayed Ca^{+2} response but not a sustained Ca^{+2} signal. In the non-genomic pathway, progesterone binds directly to the CatSper channel or binds to ABHD2 and hydrolyzes the 2-arachidonoylglycerol (2AG), and produces glycerol and arachidonic acid (AA). Because ejaculated human spermatozoa retain a significant

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amount of 2AG, P4 activation of ABHD2 is required for 2AG clearance. Simultaneously Na+ ion channel (TRPV4) transport Na+ ions inside the cytoplasm and inhibition of the potassium ion channel, causing membrane depolarization, which activates the proton ion channel Hv1, and increases pH in sperm plasma, which activates the CatSper Ca^{+2} ion channel. The activated CatSper Ca^{+2} ion channel allows the Ca^{+2} ion to enter the cell which initiates kinase activity and is followed by capacitation of spermatozoa.

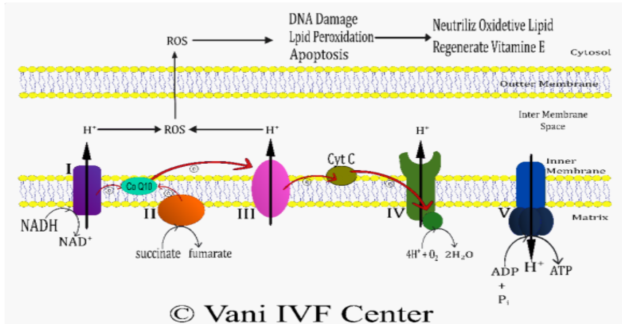


Fig. 2. Molecular representation of electron transport chain and role of Coenzyme Q10. *Source: Meet Patel, Vani IVF Center*

Coenzyme Q10 has two distinct properties: It is an antioxidant agent against ROS and a cofactor in the production of ATP. The spermatozoa's midpiece contains a large number of mitochondria, as sperm motility requires a lot of energy. in oxidized form, CoQ10 (Ubiquinone) is involved in the activity of ATP synthase, which affects sperm motility. CoQ10 is responsible for electron transport in the respiratory chain from the protein I complex (NADH dehydrogenase) to the protein II complex (succinate dehydrogenase), and from complex II to complex III (bc1 complex). It remains in its reduced form as ubiquinol after receiving electrons from both complex I and complex II, and it returns to its oxidized form as ubiquinone after transferring electrons to complex III. In its reduced form, CoQ10 (Ubiquinol) acts as an antioxidant, protecting the biological membranes against oxidation, inhibiting lipid peroxidation. CoQ10 is the only endogenously synthesized liposoluble antioxidant that can participate in redox reactions, preventing DNA and protein damage as well as lipid peroxidation, and indirectly stabilizing calcium channels by preventing calcium overload.

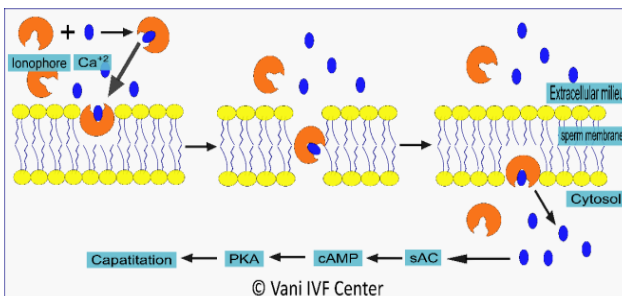


Fig. 3. Molecular representation of Ca +2 ion influx by Ca+2 ionophore in human spermatozoa. *Source: Meet Patel, Vani IVF Center*

Divalent ionophores, such as Ca+2 ionophore A23187, are lipid-soluble and reversible binders. Ca+2 ion binds to the ionophore and transfers across the membrane, and release into the cytoplasm, increasing intracytoplasmic Ca^{+2} ion concentration. Increased intracellular Ca^{+2} ion concentration stimulates cAMP production and kinase activity. Protein kinase A regulates the phosphorylation pathway, which causes hyperactivation and an acrosomal reaction.

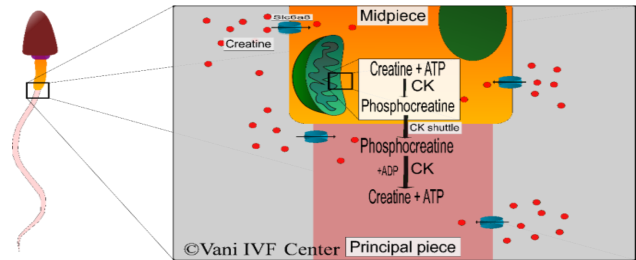


Fig. 4. Graphical representation of creatine phosphagen flux wave in spermatozoa. *Source: Meet Patel, Vani IVF Center*

Creatine is an amino acid derivative of the nitrogenous organic complex 2- (carbamimidoyl- methylamino) acetic acid. Slc6a8, a creatine transporter protein, is found in both the midpiece and the principal piece of human sperm. In the midpiece of spermatozoa, creatine kinase (CK), present the outer side of the inner mitochondrial membrane, phosphorylate creatine in presence of ATP. Phosphocreatine travels across the mitochondrial membrane via the CK shuttle in the principal piece. Where phosphocreatine binds with creatine kinase (CK), which is a reversible enzyme and produces ATP and creatine in presence of ADP. The CK shuttle produces a phosphagen flux wave that travels faster than the diffusion of other reactants such as ATP (11). These ATPs in principle piece involve in the protein kinase activity and induce the hyperactivation of spermatozoa.

Males with oligoathenoteratozoospermia (OAT) are not able to fertilize the ovum by conventional methods. Before fertilization, many female reproductive agents act on spermatozoa to activate them for fertilization of the ovum. Commercially available capacitating agents such as Progesterone (P4), Ca^{+2} ionophore, coenzyme Q10, and creatine are currently utilized to improve sperm motility. In this experiment, we use four capacitating agents mainly Ca^{+2} ionophore, Coenzyme Q10, and creatine in media with different concentrations for different incubation times for sperm motility. Our goal is to find out which of these four capacitating agents is the most effective for in-vitro sperm capacitation.

2. Methods

A. Patient Selection Criteria and Sample Processing

This project was carried out at Vani IVF Center in Ahmedabad, Gujarat. We have used two criteria to short-list the sample type for our project.

1) Exclusion criteria

- Patients with ages under 25 years and more than 35 years were excluded.
- Patients with the condition of Azoospermia were excluded.

2) Inclusion criteria

- Patients with the condition of Normozoospermia and Oligoasthenoteratozoospermia (OAT) were included.
- Cryopreserved semen samples also were included.

B. Participants were informed that their participation in the study was entirely voluntary and that they would not be compensated for their participation. The patient signed the consent form in presence of the hospital ethics committee

C. The semen samples were collected and analyzed for seminal parameters as per WHO guidelines (WHO laboratory manual for the examination and processing of human semen sixth edition 2021)

D. Treatment of Semen Sample

Sperm preparation is done by a centrifugation method using a flushing medium (ORIGIO, Knardrupvej 2, Dk-2760 Malov, Denmark) at 1200 r.p.m. for 10 min. The supernatant was discarded, and the resulting sperm pellet, after washing, was overlaid with the same medium to give a final volume of 4 mL. Afterward, the resuspended sperm-washed samples were divided into two portions, the first aliquot being the control. The volume for the second aliquot was adjusted to be 0.490 ml, and for sperm capacitation, 10 µl of stock solution of the calcium ionophore (V-IONOPHORE; VITROMED, Hans-Knöll-Str 6, 07745 Jena, Germany) was added to the resuspended sperm wash to produce a final concentration of 10 µmol/l Calcium ionophore and incubated for 10 minutes on the dry heating block at 37°C. Following a 10-minute incubation period, both the control and treatment samples were centrifuged and resuspended in 1 mL of flushing media. In the second treatment, the resuspended sperm samples were divided into two portions, the first aliquot being the control. The second aliquot was adjusted to be 1.96 ml and added 3.7 µl of stock solution of Creatine Monohydrate (Nutrabay Retail Pvt. Ltd, Okhla Industrial Area, New Delhi, 110020, Indi), dissolved in distilled water, to produce a final concentration of 850 µmol/l and incubated for 120 minutes (2 hours) on the dry heating block at 37°C. To analyze the third compound, we added the oral supplementation of Coenzyme Q10 (Q- MF 300, KAVIVA Life Science, Hir Asha Arcade, Science City, Sola, Ahmedabad, 380060, India) directly to the patients for 3 months.

E. Analysis of Sperm DNA Fragmentation Index

Experiments designed to study the influence of calcium ionophore, creatine monohydrate, and coenzyme Q10 on sperm DNA fragmentation were performed on normozoospermic and OAT samples. Sperm DNA fragmentation was measured by the CAN frag (CANDORE Bioscience, Prime Industrial Park-2, Santej - Vadsar Road, Santej, Gujarat - 382721, India) in control and treated samples after treatment. Firstly, agarose was melted at 90 °C by keeping it in the water bath for 2 minutes and then maintaining it at 37 °C by keeping it on a dry heating block for 5 minutes. Simultaneously, we assessed the concentration of each sample and diluted it with melted agarose by pipetting 40 µl of the resuspended sample into agarose vials. The sperm cell suspension was placed immediately onto the pre-treated slides, covered with the cover-slip, and kept at 4°C for 5 minutes. After 5 minutes, the coverslip was removed and the slides were incubated in an acid denaturation solution for 7 minutes. Then the slides were incubated in a lysis solution for 10 minutes and rinsed with distilled water. After that, we placed the slides in 70% ethanol, followed by 90%, and finally in 100% ethanol, each for 2 minutes, then allowed them to dry at room temperature. During that, the dye solution was mixed with the buffer (1:1) provided in the kit and deposited on the slides for 10 minutes. The slides were gently washed with distilled water and allowed to dry at room temperature. After that, slides were observed under the phase-contrast microscope using 20x magnification.

F. Immunofluorescent labeling of Sperm Mitochondria

MitoTracker Red CMXRos M7512 (Thermo Fisher Scientific) was used to detect mitochondrial activity in spermatozoa, which is a membrane-permeable dye that directly binds with the mitochondrial membrane (when the membrane is depolarized). The package contains 20 vials of 50 µg powdered dye. 94.1 µL distilled water was added to the vial for stock solution preparation, pipetted carefully, and kept at -20 °C in a dark place. The working solution was made by mixing 40 µL of stock solution in 2 mL of flushing medium. Before labeling, the semen samples were washed in a centrifuge at 1200 R.P.M. for 10 minutes. For labeling, the pellet was resuspended in a 2 mL flushing medium and 4 µL of stock solution was added to make the final concentration of 20 µM of dye, then incubated for 10 minutes at room temperature in a dark room.

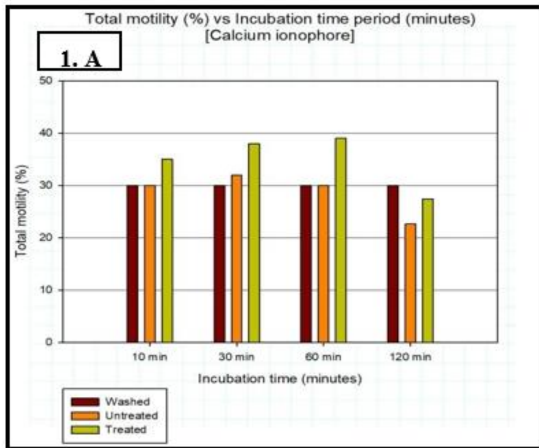
G. Statistical Analysis

Statistical analysis was done using SigmaPlot 11.0 software for windows. The mean and standard error of the data from all of the experiments were computed and statistical differences between control and test were performed by T-test. The graphs were plotted using the mean value of the experimental data in SigmaPlot 11.0. The significance of the results was observed with $P < 0.05$.

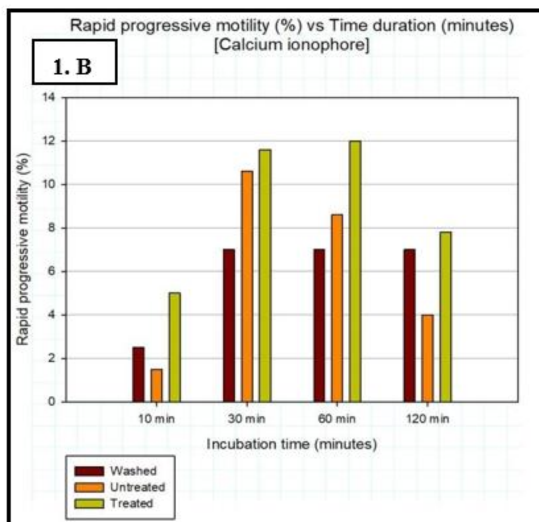
3. Results

A. Calcium Ionophore

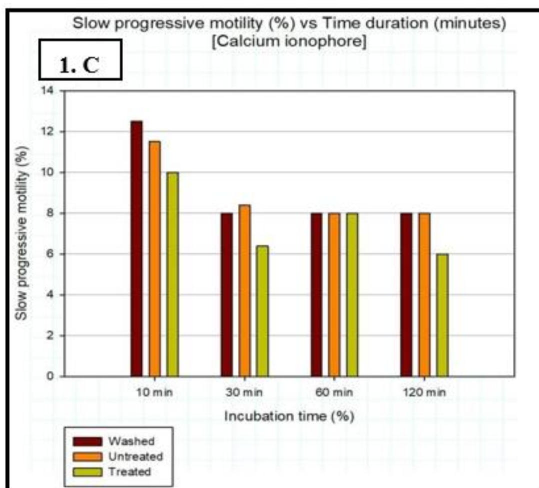
The first correlation was done between Motility and Calcium Ionophores, where the incubation time with calcium ionophores was from 10 min, 30min, 60min, and 120 min. We tried different time periods to achieve the maximum results for motility. We analyzed the fold increase of the motility in all the phases. The concentration of calcium ionophores was fixed (10micromolar) which was taken from the previous studies done on human sperms. From the previous studies, it was observed that 10 micromolar is the perfect concentration for the sperm's motility increment. Below mentioned are the graphs of total motility, rapid progressive, and slow progressive.



Graph 1. A. Represent the relation between total motility and time of incubation with calcium ionophores



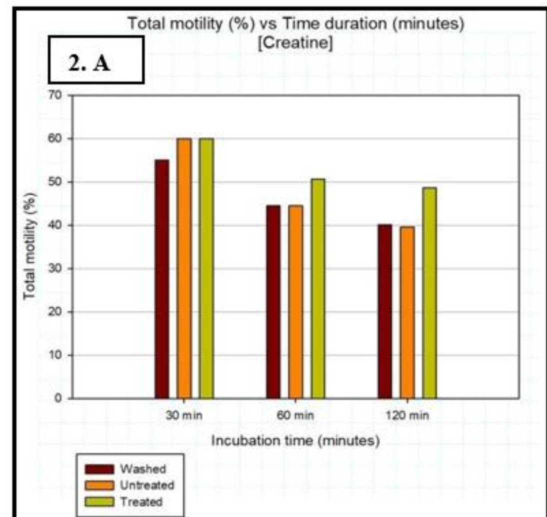
Graph 1. B. Represent the relation between Rapid Progressive Motility and time of incubation with calcium ionophores



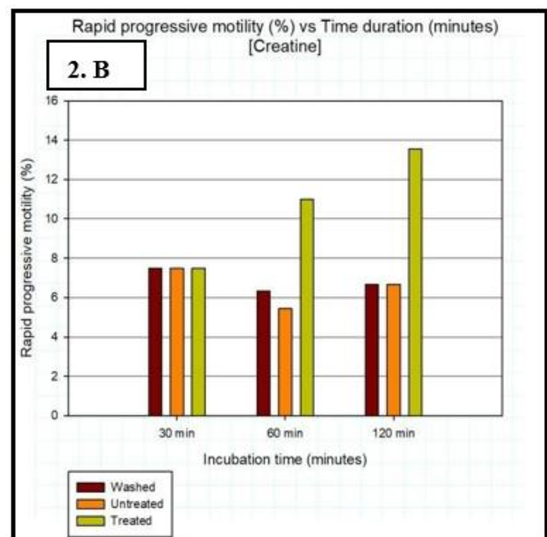
Graph 1. C. Represent the relation between Slow Progressive Motility and time of incubation with calcium ionophores

B. *Creatine*

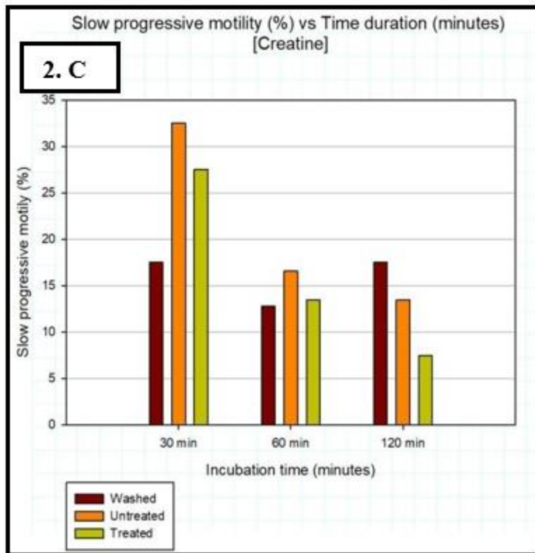
1. We took the same protocol for semen wash for creatine incubation as well. As this is one of the first trials for using creatine for human sperms we had to check different doses of the creatine. We finalized our dose to 850 micromolar as it gave us the best results in terms of sperm progressive motility. Below mentioned are the graphs which show us the correlation between Total Motility, Rapid Progressive motility, and slow progressive motility in terms of the time of incubation.
2. The best results that we obtained were at the incubation time of 60 min, where the dead sperm and live sperm ratio were unaffected. So, the conclusion is the best time of incubation is 60 min and the dose of creatine is 850 micromolar. With these mentioned details the total motility increased by 1.227x, rapid progressive motility increased by 2.02x and slow progressive motility decreased by 0.5 to 0.8x.



Graph 2. A. Represent the relation between total motility and time of incubation with ionophores



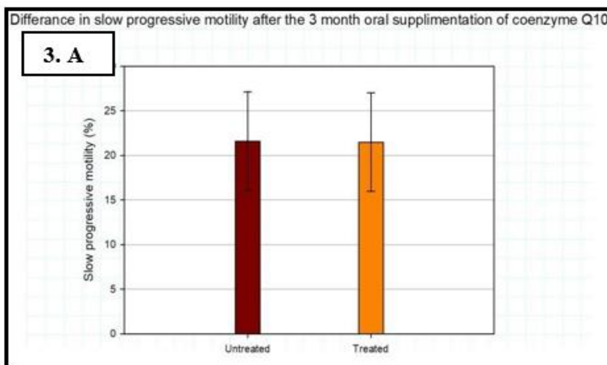
Graph 2. B. Represent the relation between Rapid Progressive Motility and time of incubation with ionophores



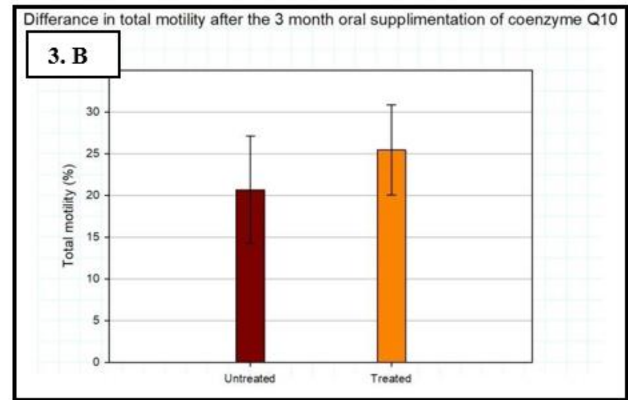
Graph 2. C. Represent the relation between Slow Progressive Motility and time of incubation with ionophores

C. *Co-Q Enzyme*

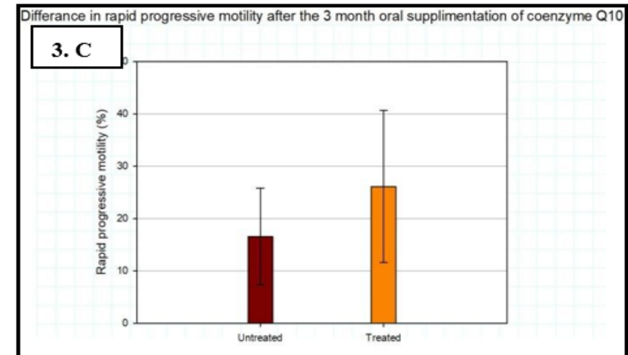
1. Co-Q enzyme has been used in the field of infertility to increase sperm parameters and decrease the antioxidant levels in male infertility cases. It is a well-established drug combination given to males suffering from OAT, Severe OAT, or AT. They are given in the combination of either 200mg/day or 400mg/day for 3 or 6 months depending.
2. For this study, we have used the dose of 200mg/dose (twice a day) for 3 months. The graphs mentioned below suggest total motility, rapid progressive, and slow progressive vs before and after treatment results. The total motility was increased by 1.23x, rapid progressive increased by 1.57x and slow progressive motility remained the same.



Graph 3. A. Represents the total motility of the sperms vs. before and after treatment values

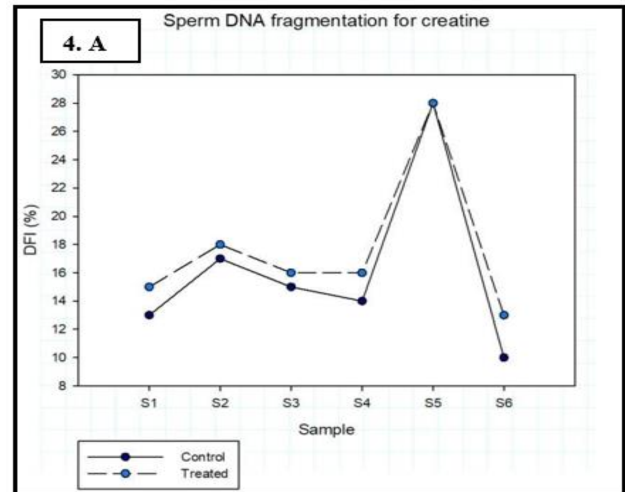


Graph 3. B. Represents the rapid progressive motility of the sperms vs. before and after treatment values



Graph 3. C. Represent the slow progressive motility of the sperms vs before and after treatment values

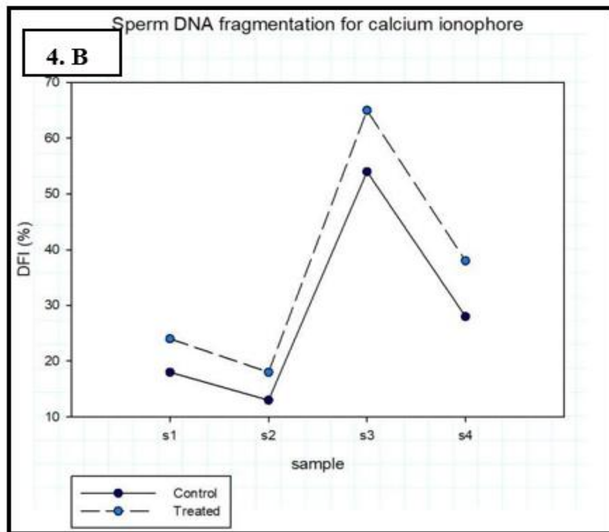
D. *Sperm DNA Fragmentation Index*



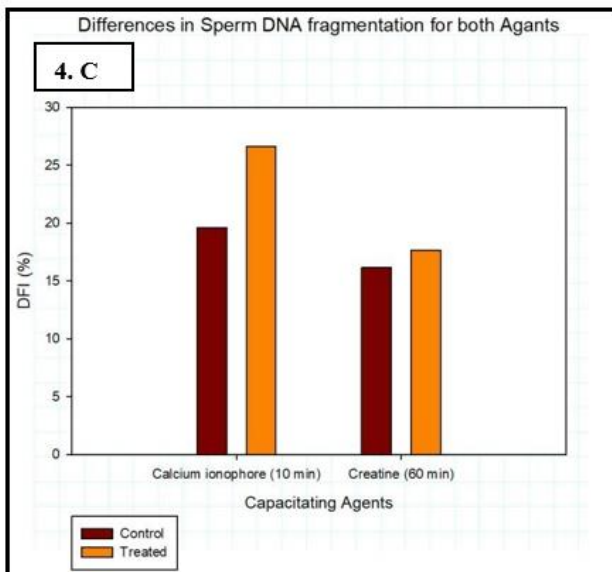
1. We observed that the calcium ionophores and creatine both gave us the motility rise in a rapid progressive manner. The difference in rapid progressive motility if the calcium ionophore treated sperms was vigorous while the sperms treated with creatine were linear progressive. As per the data and previous studies it has shown that in *in vivo* and *in vitro* conditions the sperms with linear progressive motility were able to fertilize the oocytes most effectively. Calcium ionophore treated sperms showed immediate change after 10 min

of incubation and creatine had the maximum impact of incubation after 60 min of incubation. To identify the difference in the motility and to check if these capacitating agents and incubation time had any impact on the sperm DNA, we did DFI (DN A Fragmentation Index).

2. The graph 4A shows the DFI of Calcium ionophores and creatine incubated sperms.



Graph 4. B. DFI of 4 (representative samples) for the sperms incubated with creatine



Graph 4. C. DFI difference in calcium ionophores and creatine

E. DNA Fragmentation Index

1. We observed that the DNA fragmentation had increased by 2 folds even after 10 minutes of incubation with calcium ionophores. On the other hand, it was observed that the DFI was only increased by 0.8 to 1.0% after 6o min of incubation with creatine. Also, the sperm quality in terms of living and dead was not much of a difference in the creatine incubated sperms. Whereas in the sperm incubated with calcium ionophores abnormal hyperactivity (vigorous movements of the sperm head) and an increase in the dead sperm ratio were observed.
2. Below mentioned are the images of the DFI for the calcium ionophores treated sperms and Creatine treated sperms. The halo formation around the sperm head represents the intact DNA and the sperm head without any halo around it suggests the fragmented DNA. We observed an increase in the DNA fragmentation of the sperms incubated with calcium ionophores, whereas the sperms incubated with creatine had a negligible change in the DNA Fragmentation Index.

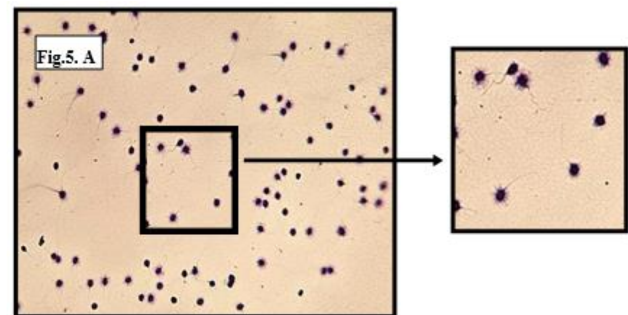


Fig. 5. A. Shows the sperms without any treatment of calcium ionophores with an incubation time of 10 min

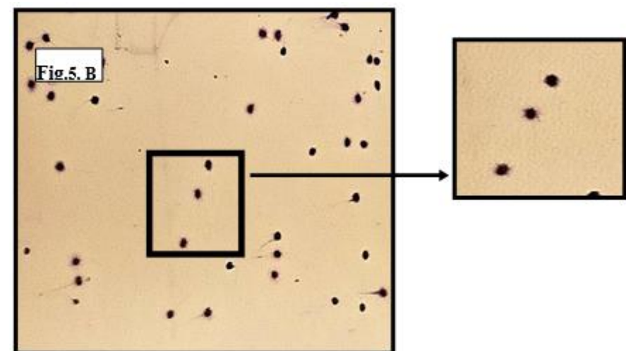


Fig. 5. B. Shows the sperms with calcium ionophores treatment with an incubation time of 10 min.

F. DFI with Creatine

Below mentioned are the images from the DFI of the sperms incubated with creatine. As compared to the calcium ionophores treated sperms the DFI of creatine-treated sperms was not increased even after 60 min of incubation. This was a very good sign as the motility of the sperms (rapid progressive) was increased by 5% with a good linear curve and the DFI was also not affected. The halo seen here is also big and perfectly surrounded by the head region which suggests the intact DNA of the sperms.

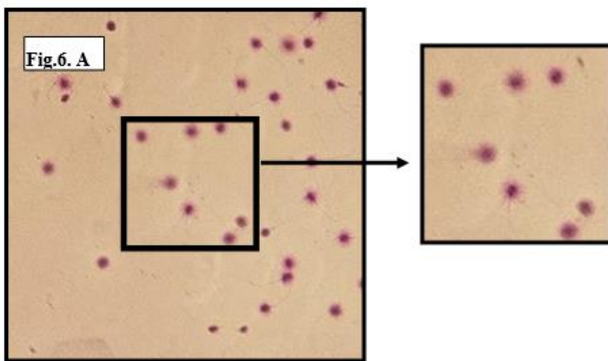


Fig. 6. A. Shows the sperms without the incubation of creatine for 60 min.

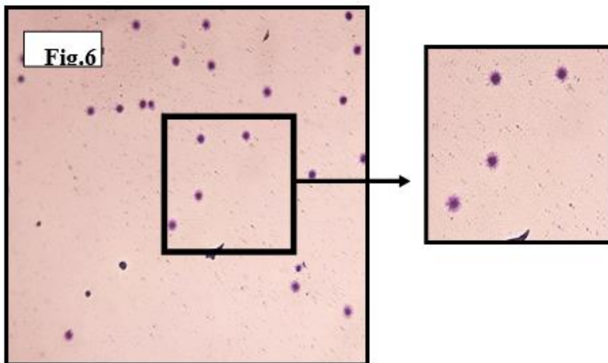


Fig. 6. B. Shows the sperms with the incubation of creatine for 60 min.

G. *Immunofluorescence*

1. We took up the immunofluorescence test to check the mitochondrial activity of the sperm. In the literature review, it is explained that increased motility of the sperms is linked with increased sperm mitochondrial activity.
2. Below mentioned are the images of the Mito tracker-tagged sperms treated with calcium ionophores (10 min incubation time) and creatine (60 min incubation time). We can see here that the control sample shows a very minimal activity of the mitochondria, calcium ionophores treated sperms a little increased activity of the mitochondria but this minimal increase was not correlated with the increased sperm motility. This means that the sperm capacitation process using the calcium ionophores was not fully completed. Despite the vigorous sperm motility of the sperm, the mitochondrial activity was not up to the mark.
3. In comparison to this, the sperm mitochondrial activity treated with creatine showed increased mitochondrial activity after 60 min of incubation. These results correlated with the increased motility of the sperms after the treatment. According to the literature review, the capacitation process includes hyperactivation of the sperms, increased mitochondrial activity, increased linear motility, and opening up the channels for the absorption of macromolecules. Creatine in *in vivo* structure is also known for its capacitation and also to prevent premature acrosomal reaction.

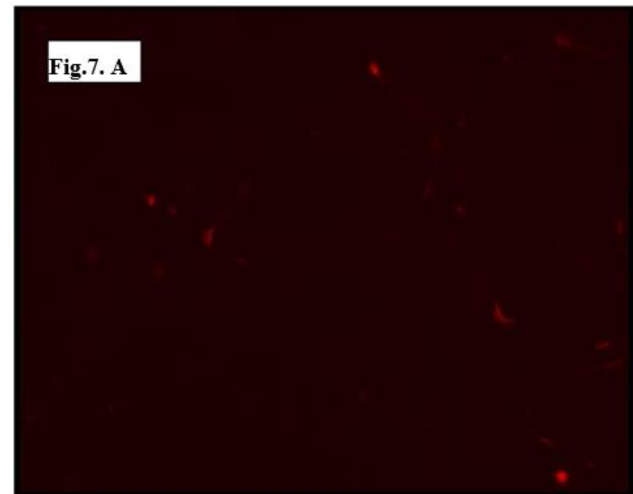


Fig. 7. A. Control without any treatment with MitoTracker Deep Red

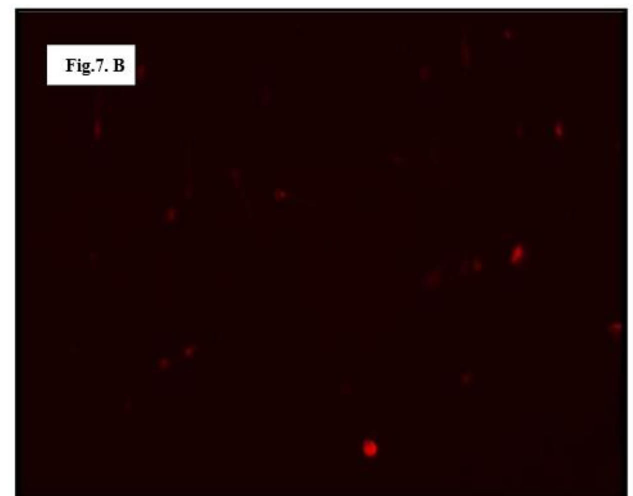


Fig. 7. B. Shows the sperms with calcium ionophores with 10 min. of incubation

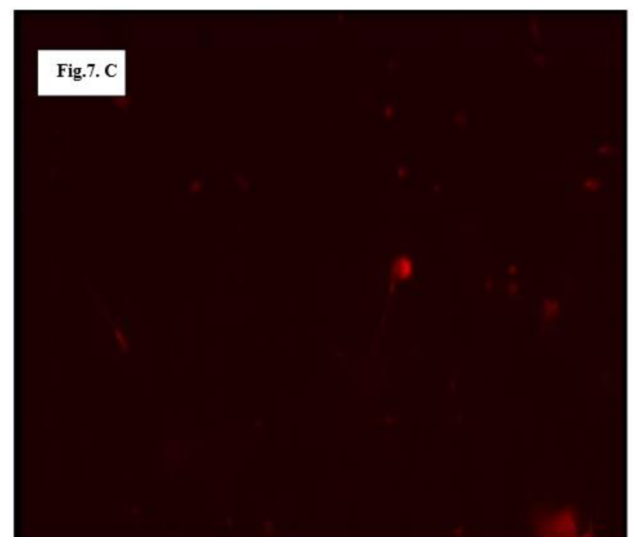


Figure 7. C. Shows the sperms with creatine with 60 min of incubation

4. Discussion

1. This study examined the in vitro capacitating compounds that can improve the motility of human spermatozoa once a

certain concentration of them was added to the washing media, without affecting other criteria like DNA integrity or sperm morphology. In our experiment, we used three compounds, such as Ca^{+2} ionophore, Creatine monohydrate, and Coenzyme Q10 (Ubiquinone), in which Creatine monohydrate was used for the first time on human spermatozoa. Our findings confirm that semen samples with asthenozoospermic conditions exhibit increased motility after incubation with the aforementioned compounds, especially creatine monohydrate, without affecting sperm DNA integrity.

2. Ca^{+2} ionophore is the divalent cation ionophore, selective for Ca^{+2} ions, which is a lipid-soluble molecule that transports Ca^{+2} ions across a cell membrane (46-47). Spermatozoa motility is related to the increasing intracytoplasmic concentration of Ca+2 ions (48). However, the Ca^{+2} ionophore readily increases the [Ca+2]i by forming a complex with Ca^{+2} ions and transporting them into the cytoplasm, which increases the motility of the spermatozoa. Total motility was not significantly increased in spermatozoa treated with Ca^{+2} ionophore (P = 0.327) but showed a significant increase in progressive motility (p = 0.002) with changes in the pattern of the movement. After 10 minutes of incubation, spermatozoa treated with 10 μM Ca^{+2} ionophore exhibit an asymmetric beating pattern as well as robust movement. This kind of Fullagar beading exhibits hyperactivated motility during capacitation. In asymmetric beating, the sperm head moves in an uneven direction and forms a "C" shaped pattern in flagellar movement. Earlier studies show that the increasing $[Ca^{+2}]i$ of the spermatozoa resulted in a more asymmetrical flagellar beating (50). On the other hand, we examine the effect of Ca^{+2} ionophore on the DNA integrity of the asthenozoospermic semen sample. After 10 minutes of incubation with 10 μM Ca^{+2} ionophore, we perform a sperm chromatin dispersion (SCD) assay to check the sperm DNA fragmentation index. We identified that the Ca^{+2} ionophore induces sperm DNA damage and shows ~10% increased Sperm DNA fragmentation even after 10 minutes of incubation. The value of the Sperm DNA fragmentation was significantly increased (p = 0.012) after treatment of Ca^{+2} ionophore. We hypothesize that this occurs as a result of robust movement caused by an overwhelming influx of Ca^{+2} ions into the cytoplasm of sperm. However, in this study, increased Ca^{+2} ions influx hindered sperm motility and increased sperm DNA fragmentation, resulting in a low fertilization rate during IVF/ICSI (49).
3. Creatine is a conditionally essential nutrient in the body (62), that has gained interest as a male fertility enhancing agent due to its function in sperm energy metabolism. Creatine metabolism in spermatozoa is a complicated pathway that involves the phosphocreatine shuttle, which transports ATP (adenosine triphosphate) from the mitochondria to the contractile machinery to fuel spermatozoa movement. Creatine is a naturally occurring molecule in both the male and female reproductive tracts that increases the viability and motility of spermatozoa during in vivo fertilization (63). Past studies on mice suggest that the addition of creatine in IVF media enhances the viability for up to 4 hrs and increases motility in order to get a high fertilization rate in IVF (61). However, in our study, we used creatine for the first time to analyze the changes in human sperm motility. The administration of 850 μM creatine in flushing media for 60 minutes enhances the motility of the spermatozoa by changing the flagellar beating from a symmetric to an asymmetric pattern. The asymmetrical pattern of flagellar beating is associated with hyperactivation of spermatozoa that positively correlates with the fertilization rate of human sperm under IVF conditions (64). The total motility of the spermatozoa treated with creatine significantly increased in comparison with control samples. The treatment of creatine also induces rapid progressive motility significantly (p = 0.005). Hyperactivated spermatozoa after 60 minutes of incubation, in the creatine-treated group, were shown liner movement with lateral head movement. A past study also suggested that creatine enhanced VCL, VSL, and ALH, in which the flagellar bend amplitude remains unchanged in mice sperms (61). However, in our study, we analyzed the sperm DNA fragmentation index and identified that creatine did not increase sperm DNA fragmentation significantly (p = 0.0). Unlike Ca^{+2} ionophore, Creatine did not damage sperm DNA integrity. It might happen due to the metabolic activity of creatine in spermatozoa which is involved in the ATP transportation pathway during capacitation instead of changing the cytoplasmic environment of spermatozoa.
4. Co-Q enzyme has been used in the field of IVF/Infertility for many years. It has been noted that the use of Co-Q enzyme for the dose of either 200mg/day or 400mg/day for 3/6 months has observed that the sperm quality increased and also the fertilization rate has been increased. It is our trial that by any means we can come up with something different that would take up a little less time and would give us better or at least the same results. With the use of the CO-Q enzyme in our study for 3 months with the dose of 400mg/day, we achieved a 1.57x rise in the rapid motility of the same and the same levels of DFI. In some cases, we were able to see a decrease in the DFI (by 2%) due to the anti-oxidant properties of the CO-Q enzyme.
5. We compared our results to the creatine and we were able to get an increase of 2.02% in the rapid motility. DNA integrity is also not affected by Creatine. Creatine turns out to be the wonder molecule for sperm capacitation. This study will be taken further in terms of embryo formation and clinical pregnancy rate.

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wonder molecule for sperm capacitation. This study will be taken further in terms of embryo formation and clinical pregnancy rate.

5. Conclusion

1. Comparison of the different capacitating agents has been a study of interest as capacitation is one step that is missed in the *in vitro* fertilization process. Thus, it becomes important to understand if by any means this step can make a difference in the fertilization process, or can increase the embryo formation rate or increase the clinical pregnancy rate or not. We took up three main capacitating agents to use for this study which includes Calcium ionophores, creatine, and Co-Q Enzyme. Calcium Ionophores have been used for some time in the field of infertility for cases where the male parameters are at fault. The main conditions where the calcium ionophores are used are OAT samples or severe OAT samples or the sperm aspirated from the TESA in some cases. In this study, we found out that though the sperm's rapid progressive motility is increased the sperm's DNA integrity is damaged. Even the increased motility is not linear and the head movement after adding calcium ionophores showed to be tremendously vigorous.
2. The next step was to take up the creatine and use it for the capacitation process. The capacitation of the sperms using creatine was undertaken. This study showed that the motility increase of the sperms was significant and also that linear motility has increased. Linear motility of the sperms is the best chosen for the successful fertilization process. Also, the best part was that the DFI of the creatine-capacitated sperms had not increased. DFI, remarks on the DNA integrity test which means that DNA Integrity remained the same even after using the creatine for sperm capacitation. There are multiple tests done before using the Co-Q 10 which also suggests that DNA integrity improved or remained the same with improved sperm quality after giving the treatment for either 3/6 months depending on the condition. The last stage to notify the capacitation process is the mitochondrial activity which was observed in the sperm treated with creatine. Co-Q enzyme and creatine have a similar effect on sperm motility and DFI also. Creatine is helpful as it can sometimes skip the 3/6 months of prolonged treatments to increase the rapid progressive motility. Co- Q Enzyme increases about 1.57(x) of rapid progressive motility, whereas creatine increases sperm rapid progressive motility by 2.02%.
3. This study takes the main credit as the creatine used in this study is for the first time used for the capacitation of the human sperms. This study will be further taken to the Embryo formation rate, clinical pregnancy rate, and successful delivery of the child. Creatine molecules can be a big revolution in the field of *in vitro* fertilization.

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Abbreviations

ADCY10 - Adenylyl cyclase 10
ART - Artificial Reproductive Technology
ATP - Adenosine triphosphate
[Ca+2]i - Intracytoplasmic concentration of Ca ion
C/PL - Cholesterols/Phospholipid ratio
cAMP - Cyclic Adenosine Monophosphate
CAS - Carbonic anhydrases
CatSper - Cation channels of Sperm (specific for calcium ions)
CCL20 - Chemokine (C-C motif) Ligand 20
CSF2 - Colony-Stimulating Factor-2
DC - Dendritic Cells
DGC - Density Gradient Centrifugation
EPAC - Exchange Proteins Activated Directly by Cyclic AMP
GM-CSF - Granulocyte-Macrophage Colony-Stimulating Factor
HDL - High-Density Lipoprotein
Hv1 - Voltage-gated Proton Channel
Hsper - H+ current of sperm (Proton Ion Channel on sperm)
ICSI - Intracytoplasmic Sperm Injection
IL1A - Interleukin-1A
IL6 - Interleukin-6
IL8 - Interleukin-8

IL10 - interleukin-10
IVF - In Vitro Fertilization
LTP-I - Lipid Transfer Protein-I
NETs - Neutrophil Extracellular Traps
NEH - Na+/H+ Exchanger protein
OAT – Oligoasthenoteratozoospermia
OECs - Oviduct Epithelial cells
oEVs - Oviductal Extracellular Vesicles
OF - Oviduct Fluid
PDE - Phosphodiesterase
pH - Potential of Hydrogen ions
pHe- Extracellular Potential of Hydrogen ions
PKA - Protein Kinase A
PMNs - Polymorphonuclear Neutrophils
PSA - Prostate-Specific Antigen
PTGS2 - Gene responsible for Prostaglandin-endoperoxide synthase 2
ROS - Reactive Oxygen Species
sDF - Sperm's DNA Fragmentation
Siglec - Sialic acid-binding Immunoglobulin-like Lectins
SLC - Solute Carrier protein family
STD - Sexually Transmitted Disease
TGF-β - Transforming Growth Factor-Beta
Treg - Regulatory T Lymphocyte
UECs - Uterine Epithelial Cells
ULV - Uterosome-like Vesical
UTJ - Uterotubal Junction
WHO - World Health Organization