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A Study On the Effect of Citrulline Concentrations On In-Vitro Sperm Motility in Diabetic Infertile Male

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Abstract: The worldwide prevalence of diabetes mellitus (DM) is gradually rising, which impacts reproductive systems, particularly sperm motility. Bio-molecules have been found to improve sperm motility in both in-vitro and in-vivo settings, thereby enhancing the effectiveness of Assisted Reproductive Technology (ART). Watermelon seeds naturally contain a significant amount of citrulline, which has been shown to improve sperm motility in animal models. Therefore, this randomized controlled study aimed to analyze the effect of watermelon seed extract and commercially available citrulline on in-vitro sperm motility in diabetic infertile males. Citrulline was extracted from watermelon seeds, and commercially available citrulline from Sigma was used. The extracted citrulline was quantified using HPLC. Infertile males were allocated randomly into two groups based on their sugar levels. Semen parameters, including motility, were assessed at baseline. The participants' sperm samples were treated with various concentrations (0.01-0.05 ml) of watermelon seed extract and commercial citrulline. Sperm motility was assessed in all semen samples treated with citrulline at 30-minute intervals for 2 hours. The HPLC analysis revealed that citrulline in the crude and purified watermelon extracts were 5 mg/g and 28 mg/g, respectively. The mean total sperm motility of group 1 and group 2 participants at baseline, was 41.5% and 37.7%, respectively. Sperm motility was improved in all concentrations of watermelon seed extract and commercial citrulline. However, the regression models showed that group 2 participants had a lower percentage of sperm motility than group 1 participants, even after treatment with watermelon seed extract and commercial citrulline solution. In conclusion, the study found that citrulline treatment improved in-vitro sperm motility in the study population, suggesting that citrulline could be a potential in-vitro sperm enhancer.

Keywords: Watermelon, Citrulline, Diabetes, Male infertility, Semen, Sperm motility.

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1. INTRODUCTION

Clinical infertility is the diagnosed condition characterized by the persistent inability of a couple to achieve conception, even after engaging in regular and unprotected sexual intercourse for a defined duration, typically one year, and nearly 30% of reproductive issues are male-specific. Various reasons, including autoimmunity, poor endocrine function and abnormalities, DNA fragmentation and infections in the genital tract, altered gene patterns, changes in healthy lifestyle habits, and exposure to gonadotoxic substances[1], cause male infertility. To lower the prevalence, it is required to assess the situation and offer treatment. There may be additional possibilities to become pregnant, such as assisted reproductive technologies (ART). Almost 8% of men in the reproductive stage require medical attention for reproductive problems. Along with population expansion, the infertility rate in India is gradually increasing, with a prevalence of 3.9 to 16.8%[2]. In India, estimates vary widely among states, ranging from 3.7% in Uttar Pradesh, Himachal Pradesh, and Maharashtra[3] to 5% in Andhra Pradesh[4] and 15% in Kashmir.[5] Furthermore, within the same region in India, the prevalence of primary infertility has been shown to differ across tribes and castes.[6] An Indian study reported the regional diversity in the prevalence of infertile couples due to male partners. According to the study, Kurnool and Jodhpur had the maximum prevalence of azoospermia, with 38.4% and 37.4%, respectively.[7] India's regional distribution reveals that the north has higher infertility rates than the south.[8] Systemic diseases can harm poor semen quality leading to male infertility. Results confirm that diseases like diabetes have a strong negative impact on male infertility.[9] Diabetes, especially when not under good control, can harm blood vessels and nerves and raise the risk of infection. Diabetes is consequently linked to several sexual issues, including erectile dysfunction, decreased sex drive (libido), ejaculation issues, and foreskin inflammation. Diabetes can potentially impact the modification of genes during spermatogenesis, leading to alterations in sperm that future generations can inherit. These genetic changes can potentially increase the risk of developing diabetes in the offspring.[10] Abnormal semen parameters have long been recognized as the most common cause of male infertility[11], and the only option is ART. Types of ARTs based on semen quality include Intra Uterine Insemination (IUI), In Vitro Fertilization (IVF), and Intra-Cytoplasmic Sperm Injection (ICSI).[12] The success rate of ARTs decreases due to poor sperm motility.[13]Artificial Insemination (AI) enables the enhancement of offspring quality through genetic improvements. It facilitates the propagation of hybrid germplasm by assisting in the delivery of semen into the female reproductive tract. Fresh spermatozoa motility decreases within an hour of collection in-vitro, reducing fertility potential.[14] During the handling and storage of sperm, the formation of lipid peroxides is a common occurrence. This leads to the impairment of spermatozoa plasma membranes and a subsequent decrease in sperm motility. Seminal plasma, conversely, contains an antioxidant system that plays a crucial role in protecting sperm cells by counteracting the detrimental effects of Reactive Oxygen Species (ROS) accumulation. However, it is worth noting that spermatozoa have a lower capacity for protection than somatic cells.[15] Diabetes in males has a detrimental effect on ART outcomes. It can negatively impact sperm parameters, including count, motility, and morphology, reducing the chances of successful fertilization during ART procedures. Erectile dysfunction, a common complication of diabetes, further hinders the success of ART. Lower success rates and increased risks of adverse pregnancy outcomes have been observed in diabetic males. Offspring of diabetic males may face a higher risk of congenital malformations. Further research is needed to fully comprehend the mechanisms underlying the impact of diabetes on male fertility and to develop effective strategies for optimizing ART outcomes in diabetic individuals.[16] Numerous herbal medications increase the semen parameters (count, viability, and motility), erection, and ejaculation and reduce the abnormal sperm percentage.[17] Watermelon (Citrullus vulgaris) seed contains proteins, omega-3 and omega-6 fatty acids, vitamins, zinc, and potassium. Various animal studies prove these seeds help reduce blood sugar levels[18-19] and improve sperm motility.[20] Citrulline, classified as a non-essential amino acid, is naturally abundant in watermelon seeds, and various environmental and physiological factors can affect citrulline concentration.[21] An animal study showed that citrulline supplementation for 70 days improves sperm motility and decreases dead sperm compared to the control group.[22] Another study on stallions showed significant improvement in sperm volume and motility with citrulline supplementation.[23] Most studies focus on citrulline supplementation on male fertility in animals. Only limited data are available on the effect of citrulline on human semen quality by supplementation. This project is the first of its kind in India. It aims to study the effect of watermelon seed extract and commercially available citrulline on in-vitro sperm motility in diabetic and non-diabetic infertile males.

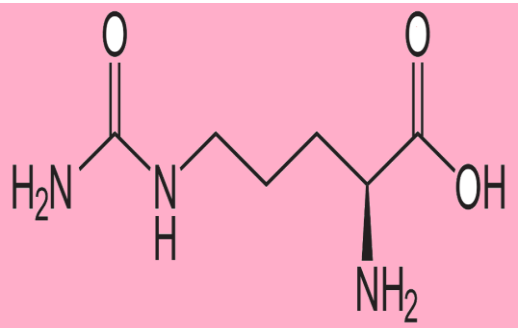

Fig 1: Structure of citrulline

Figure 1 shows the chemical structure of citrulline. Citrulline consists of a five-carbon chain with an amino group (-NH2) at one end and a carboxyl group (-COOH) at the other. It contains three nitrogen atoms and two oxygen atoms. The side chain of citrulline is a ureido group, which consists of an imidazolidine ring (a five-membered ring containing four

carbon atoms and one nitrogen atom) with a carbamoyl group (-NH-CO-) attached. The molecular weight of citrulline is approximately 175.19 grams per mole.

2. MATERIALS AND METHODS

2.1 Watermelon Seed Collection and Extraction

Watermelon seeds were chosen for the study and brought in one lot from the local market. Dust and waste particles were removed and cleaned from the ingredients. Seeds were dried in shadow for 3 days at room temperature to remove the moisture content and preserved in plastic airtight containers. 1g of seed powder was ground with 100 ml of extraction solvent (MeOH: 6M HCL, 9:1) in the water bath for 20 minutes and cooled at room temperature. The sample was then filtered using filter paper, and an additional 100ml of extraction solvent was used to wash the filter paper. Combined, 200ml of the filtrate was transferred to a 500-round bottom flask, and the solvent was removed using a rotary evaporator.[24] The impurities in the crude watermelon extract were removed by solvent fractionation. The dry extract was dissolved with 100 ml of MeOH and transferred to a separation flask. To this mixture, 100 ml of chloroform was added and mixed well for five minutes. The flask was then kept undisturbed for 2 hours to allow the separation of the polar and non-polar solvent layers. After the separation, the MeOH layer containing citrulline was carefully separated from the chloroform layer. The MeOH layer was filtered through filter paper to remove any remaining solid particles. The solvent was evaporated using a rotary vacuum, leaving behind the dry extract containing citrulline. Finally, the dry extract was weighed and stored for further use. Commercial citrulline [IUPAC name: 2-Amino-5-(carbamoyl amino) pentanoic acid] and 1.0M phosphate buffer solution (pH: 7.4) were purchased from Sigma Aldrich and were used in this study. 10 mg of watermelon seed extract was mixed with 100 ml of deionized water (Stock solution) in a sterile test tube, and similarly, 10 mg of commercial citrulline stock solution was prepared.

2.2 HPLC analysis of watermelon seed extract

High-Performance Liquid Chromatography (HPLC) was used to analyze the extracted citrulline. The standard citrulline solution was prepared by dissolving 5mg/ml of methanol in the standard flask, and various concentrations of the standard solution were prepared from the stock solution with methanol. The working sample was prepared by dissolving 2mg of watermelon seed extract with 1 ml of methanol. HPLC Agilent HP model with a C18 column with a temperature of 40 °C was used. A solvent system consisting of solvent A (ethanol: Acetonitrile: Water, 45:45:10 v/v) and solvent B (phosphate buffer, pH 7.5) was set in the ratio 80:20 with a retention time of 70 minutes. 2µl of the working sample was injected into HPLC and detected at 450nm by UV/VIS Detector.[25]

2.3 Ethical statement of the study

The study was approved by the institutional ethics committee, Fertility Foundation, SISU Hospital India Limited, Chennai, Tamil Nadu, India [Approval date: 04.10.2017], and all experiments in the study were conducted according to the ethical standards given in the Declaration of Helsinki. Participation consent was obtained from all participants after explaining the study in a regional language and clarifying their doubts (Annexure 1).

2.4 Inclusion and exclusion criteria

Infertile males aged between 25-45 years with a minimum of 1 year of medication for diabetes who visited for fertility treatment at the study site were approached and included in group 2. Participants without diabetes were included in Group 1. Participants with severe medications, erectile dysfunction, no semen production, uncertainty to visit the clinic again, and unwillingness to participate in the study were excluded.

2.5 Participant's Selection and sample size calculation

The study sample size for this randomized controlled study was calculated using an openEpi sample size calculator with a 35.1% prevalence rate of diabetes male infertility.[26] Twenty-four male infertile participants who visited the fertility center for ARTs were divided into groups 1 (non-diabetic) and 2 (diabetic) based on their blood sugar level.

2.6 Semen sample collection

The participants were instructed to collect the ejaculate by masturbation into a sterile wide-mouth container in a separate room close to the analytical lab to obtain the semen sample. The lab technician questioned participants about sample collecting procedures and any sample loss. The sample container was marked with the participant's name, the research ID, and the time and date the semen was collected. Semen analysis was done following WHO recommendations.[27]

2.7 Treatment of semen sample

Sperm cells were separated from seminal plasma using density gradient centrifugation for 8 minutes at 200 rpm, and the separated sperms were added to Ham's F-10 culture medium. 100µl of each semen suspension and 50µl of phosphate buffer solution (pH:7.4) were taken in ten sterile test tubes, and five concentrations (0.01ml, 0.02ml, 0.03ml, 0.04ml, and 0.05ml) of watermelon seed extract stock solution and five concentrations of commercial citrulline stock solution were added to the test tubes. All test tubes were incubated at room temperature, and sperm motility was assessed at 0.5h, 1.0h, 1.5h, and 2.0h.

2.8 Sperm motility

Immediately following the incubation of the samples, the sperm motility was tested. The samples were thoroughly mixed without air bubbles to prevent spermatozoa from sedimenting. Wet slides with a 20 mm coverslip were made using the homogeneous sample, allowing sperm to float freely. The slides were inspected under a microscope at 20X and 40X magnifications to determine the sperm motility expressed as a percentage. [27]

3. STATISTICAL ANALYSIS

In this study, the experiments were conducted in triplicates, and the data obtained were subjected to various statistical analyses using SPSS software (Ver: 25). The mean, standard deviation, and p-value (<0.05) significance were estimated, and the results were logically interpreted. A paired t-test was used

to assess the difference in sperm motility from baseline to different incubation times at various citrulline concentrations. The effect of watermelon seed extraction and citrulline concentration on in-vitro sperm motility in the diabetic population, compared to the non-diabetic population, was analyzed using a logistic regression model.

4. RESULTS

4.1 HPLC analysis of citrulline concentration

HPLC analysis was conducted to determine the presence of citrulline in the watermelon seed extracts. A chromatogram of the standard citrulline and the crude and purified samples was obtained. The retention time of the standard citrulline, crude watermelon seed extract, and purified watermelon seed extract was detected at 9.13 minutes, 9.58 minutes, and 9.29 minutes, respectively, in a 70-minute cycle (Figure 2-4). The difference between the retention time of the standard citrulline and the crude watermelon extract was 0.45 minutes (<5%), and the difference between the retention time of the standard citrulline and the purified watermelon extract was 0.16 minutes (<5%). The amount of citrulline in the crude watermelon extract was 5mg/g, while in the purified watermelon extract, it was 28 mg/g.

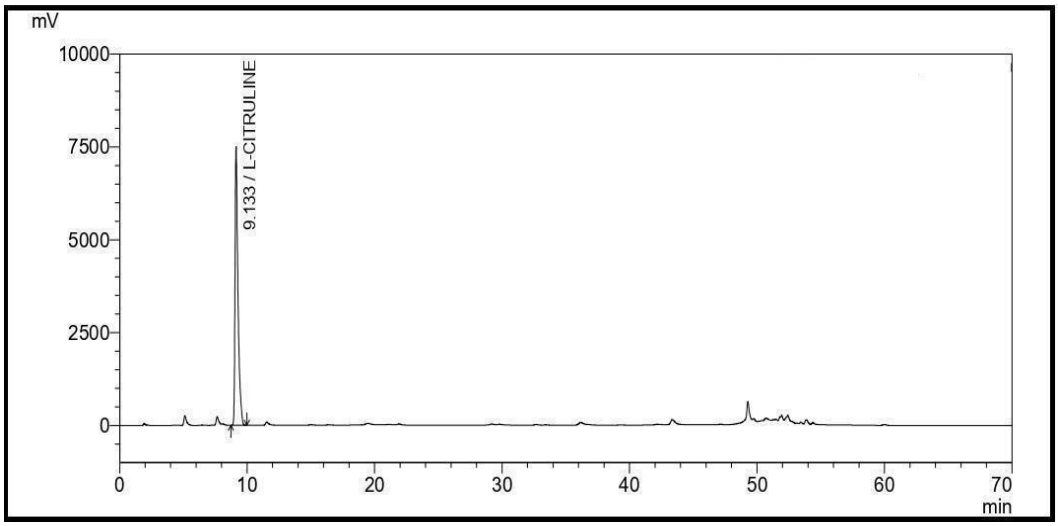

Fig 2: HPLC chromatogram of standard citrulline

Figure 2 depicts the chromatogram of the commercial citrulline used in the analysis. It is worth noting that the retention time of citrulline can vary based on the specific parameters of the high-performance liquid chromatography (HPLC) analysis, such as the type of column, composition of the mobile phase, flow rate, and detection method employed. In this study, the retention time for citrulline was observed at 9.13 minutes, which falls within the typical range of 8 to 12 minutes commonly associated with citrulline analysis.

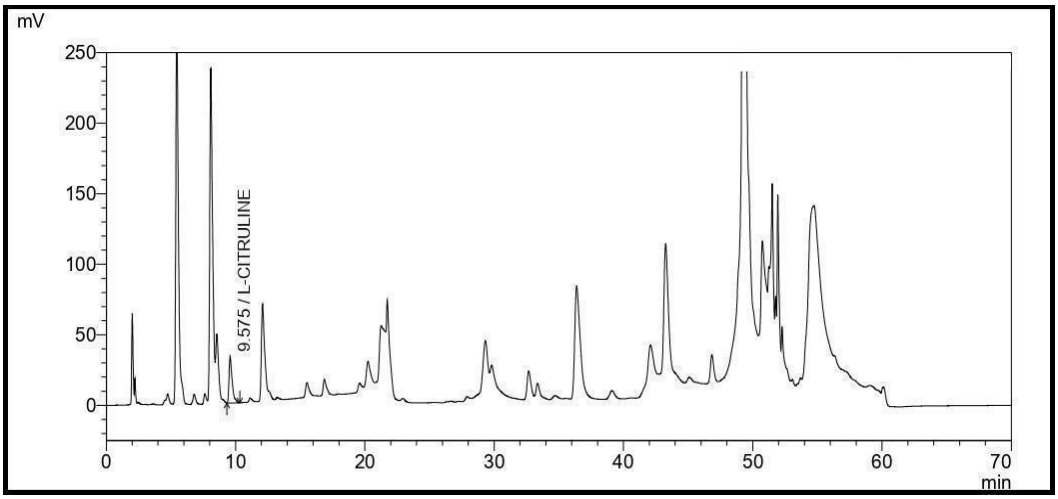

Fig 3: HPLC chromatogram of crude watermelon extract

Figure 3 displays the chromatogram of the crude watermelon extract analyzed in this study. Multiple peaks are observed, representing various compounds present in the extract. Among these compounds, citrulline is one of the constituents commonly found in watermelon extracts. Other compounds, such as amino acids, sugars, organic acids, and various phytochemicals, can also be present. The diverse composition of the watermelon extract contributes to variations in the size, shape, and retention time of the citrulline peak observed in the chromatogram.

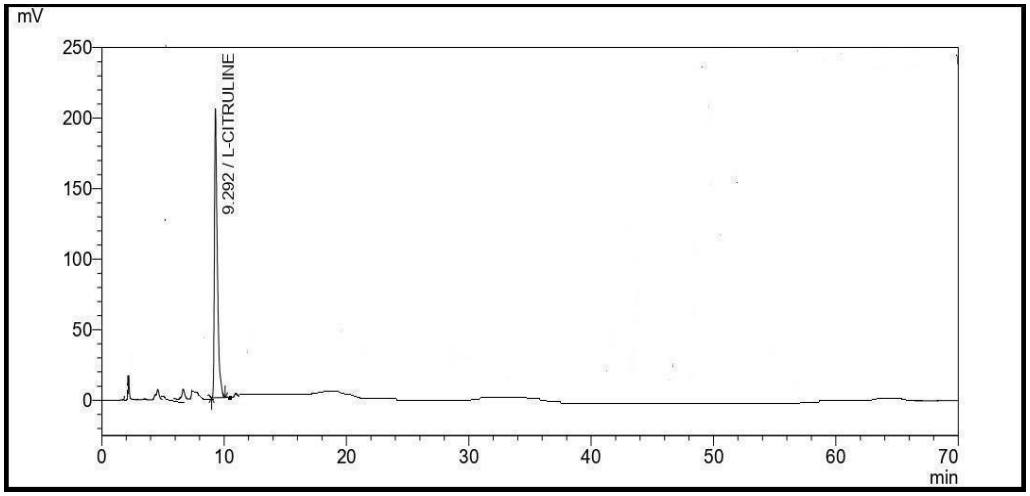

Fig 4: HPLC chromatogram of purified watermelon extract

Figure 4 illustrates the chromatogram of the purified watermelon extract obtained in this study. The purification process involved various techniques to isolate and purify the citrulline compound from the extract. A single, well-defined peak corresponding to the standard citrulline is observed in the chromatogram. This indicates that the purification process successfully resulted in a purified watermelon extract enriched in citrulline, with minimal interference from other compounds in the original extract.

4.2 Semen parameters of the study participants

The study participants had a mean age of 35.2 years, with Group 1 between 27-48 years and Group 2 between 26-51 years. All participants' mean random blood sugar value was 141mg/dl, with Group 1 between 86.0-124.0mg/dl and Group 2 between 200-280mg/dl. The participants had a mean marriage history of 6.7 years. The mean liquefaction time was 28.1 minutes, the mean pH was 7.8, and the mean semen volume was 2.95 ml. Compared to Group 1, Group 2 showed significantly lower mean pH, semen volume, sperm count, motility, viability, and percentage of normal sperm. Additionally, Group 2 exhibited significantly higher mean liquefaction time, sluggish sperm percentage, and dead sperm percentage than Group 1 participants.

4.3 Effect of citrulline from Watermelon Seed Extract on Sperm Motility

The effect of watermelon seed extract on sperm motility was assessed in the study. The average total sperm motility of the participants was 39.6%, with group 1 and group 2 having a mean total sperm motility ranging from 5% to 75% and 5% to 70%, respectively. The mean total sperm motility of the participants in group 1 and group 2 at baseline was 41.5% and 37.7%, respectively. The mean percentage change in sperm motility from baseline for each group treated with various concentrations of watermelon seed extract is presented in Table 1. For group 2 participants treated with 0.01ml of watermelon seed extract, the mean sperm motility improved to 40.9% (T1), 41.5% (T2), 42.2% (T3), and 42.6% (T4) from baseline. In group 1, the mean sperm motility improved to 45.1%, 46.3%, 47.0%, and 47.9% at T1-T4, respectively. With 0.02ml of watermelon seed extract, the mean total sperm motility increased to 41.1%, 42.6%, 43.4%, and 44.4% at T1-T4 in group 2 participants, while it improved to 45.4%, 46.4%, 47.2%, and 48.2% in group 1 participants. When treated with 0.03ml of watermelon seed extract, the mean motility of group 2 participants' sperm enhanced to 41.9% (T1), 42.5% (T2), 42.7% (T3), and 43.5% (T4). Similarly, for group 1 participants, it improved to 45.8% (T1), 47.0% (T2), 47.6% (T3), and 49.2% (T4). Treated with 0.04ml of watermelon seed extract, the mean motility of group 2 participants' sperm increased to 41.5% (T1), 41.2% (T2), 40.4% (T3), and 39.9% (T4), while for group 1 participants, it increased to 45.5% (T1), 45.1% (T2), 44.8% (T3), and 44.1% (T4). From Table 1, it is evident that when treated with 0.05ml of watermelon seed extract, the mean motility of sperm increased to 41.0%, 40.3%, 39.6%, and 39.0% at T1-T4 in group 2 participants, while in group 1 it increased to 45.0% (T1), 44.8% (T2), 44.2% (T3), and 43.7% (T4).

Table 1 Group-wise mean percentage change in sperm motility from baseline of the study participants by various conc. of watermelon extract

Watermelon extract conc.	Group 1Non-Diabetic (n=27)Mean±SD				Group 2Diabetic (n=27)Mean±SD				P**
	T1 (0.5hr)	T2 (1.0hr)	T3 (1.5hr)	T4 (2.0hr)	T1 (0.5hr)	T2 (1.0hr)	T3 (1.5hr)	T4 (2.0hr)	
0.01ml	3.6±0.2*	4.8±0.1*	5.5±0.3*	6.4±1.2	3.2±0.5*	3.8±0.5*	4.5±0.7*	4.9±0.6	0.001
0.02ml	3.9±0.9	4.9±0.9*	5.7±1.0*	6.7±1.0*	3.9±0.2*	4.1±0.3*	4.8±0.4*	5.5±0.7*	0.001
0.03ml	4.3±1.0*	5.5±1.0*	6.1±1.0	7.7±1.0	4.2±0.4*	4.8±0.4*	5.0±0.3	5.8±0.4	0.042
0.04ml	4.0±0.7*	3.6±0.9*	3.3±0.8*	2.6±0.9*	3.8±0.4*	3.5±0.6*	2.7±0.5*	2.2±0.5*	0.033
0.05ml	3.5±0.8*	3.3±0.8*	2.7±0.6*	2.2±0.6*	3.3±1.0*	2.6±1.0*	1.9±0.09*	1.3±0.07*	0.048

p (<0.05) between baseline and testing time, **p between groups

In Table 1, the group-wise mean percentage change in sperm motility from baseline was presented for both the diabetic and non-diabetic study participants who were exposed to various concentrations of citrulline from watermelon extract. Upon comparing the mean percentage change in sperm motility between the diabetic and non-diabetic groups for each concentration level, it was observed that the non-diabetic group exhibited a higher mean percentage change. This indicated that the watermelon extract had a more pronounced positive effect on sperm motility in non-diabetic individuals than in diabetic individuals.

4.4 Effect of Commercial Citrulline on Sperm Motility

In Table 2, the group-wise mean percentage change in sperm motility from baseline was presented for the study participants exposed to different concentrations of commercial citrulline solution. When treating the sperm with 0.01ml of commercial citrulline solution, the mean total sperm motility in group 2 participants increased to 41.9% (T1), 43.2% (T2), 43.8% (T3), and 44.5% (T4) from baseline. In group 1 participants, the motility improved to 47.5%, 48.4%, 48.8%, and 51.2% at T1-T4, respectively. For 0.02ml of commercial citrulline solution-treated sperm culture, the mean sperm motility in group 2 participants improved to 43.4% (T1), 44.5% (T2), 44.9% (T3), and 45.9% (T4) from baseline. In group 1 participants, the motility improved to 48.8%, 49.3%, 49.7%, and 52.6% at T1-T4, respectively. When treated with 0.03ml of commercial citrulline solution, the mean motility of group 2 (T1: 44.1%, T2: 45.6%, T3: 46.0%, and T4: 46.8%) and group 1 (T1: 50.0%, T2: 51.4%, T3: 52.0%, and T4: 54.3%) participants' sperm increased from baseline. Furthermore, when the sperm were treated with 0.04ml of commercial citrulline solution, the mean motility in group 2 increased to 45.5%, 45.9%, 47.1%, and 48.4% at T1-T4, while in group 1, it increased to 50.3% (T1), 51.7% (T2), 54.4% (T3), and 54.7% (T4). The mean motility of group 2 participants' sperm also improved from baseline at T1 (45.8%), T2 (47.0%), T3 (48.5%), and T4 (49.5%) when treated with 0.05ml of commercial citrulline solution. Similarly, in group 1 participants, the motility improved to 50.7% (T1), 53.2% (T2), 56.0% (T3), and 58.0% (T4).

Table 2 Group-wise mean percentage change in sperm motility from baseline of the study participants by various commercial citrulline concentrations.

Citrulline Conc.	Group 1Non-Diabetic (n=27) Mean±SD				Group 2Diabetic (n=27) Mean±SD				P**
	T1 0.5hr)	T2 (1.0hr)	T3 (1.5hr)	T4 (2.0hr)	T1 (0.5hr)	T2 (1.0hr)	T3 (1.5hr)	T4 (2.0hr)	
0.01ml	6.0±1.7*	6.9±1.0*	7.3±2.1*	9.7±2.9*	4.2±1.5*	5.5±1.6*	6.1±1.7*	6.8±1.4*	0.006
0.02ml	7.3±1.6*	7.8±1.2*	8.2±2.3*	11.1±2.3*	5.7±1.4*	6.8±1.8*	7.2±1.5*	8.2±1.5*	0.013
0.03ml	8.5±1.8*	9.9±1.8*	10.5±2.3*	12.8±3.0*	6.4±1.7*	7.9±1.5*	8.3±1.7*	9.1±1.6*	0.040
0.04ml	8.8±1.9*	10.2±1.2*	12.9±2.9*	13.2±2.2*	7.8±1.5*	8.2±1.5*	9.4±1.9*	10.7±1.6*	0.046
0.05ml	9.2±1.6*	11.7±1.5*	14.5±3.0*	16.5±3.3*	8.1±1.2*	9.3±1.6*	10.8±1.6*	11.8±1.6*	0.044

p (<0.05) between baseline and testing time, **p between groups

In Table 2, the group-wise mean percentage change in sperm motility from baseline was presented for diabetic and non-diabetic study participants exposed to various concentrations of commercial citrulline. Comparing the mean percentage change in sperm motility between the diabetic and non-diabetic groups for each concentration level, it was observed that the mean percentage change was higher in the non-diabetic group. This indicates that commercial citrulline had a more positive effect on sperm motility in non-diabetic individuals than in diabetic individuals. The regression models conducted in the study demonstrated a positive impact of both watermelon seed extract and commercial citrulline solution on the in vitro sperm motility of the study participants. Notably, group 2 participants had significantly lower odds for sperm motility compared to group 1 participants, even after treatment with both watermelon seed extract (as shown in Table 3) and commercial citrulline solution (as shown in Table 4), with a statistical significance of $p<0.05$.

Table 3 Logistic regression model on the effect of watermelon extract on sperm motility of the study participants.

Watermelon extract Conc.	Non-Diabetic (n=27)	Diabetic (n=27)Odd Ratio (CI)P			
	Standard	T1(0.5hr)	T2 (1.0hr)	T3(1.5hr)	T4(2.0hr)
0.01ml	1	0.89 (0.58, 0.98) 0.017	0.85 (0.44,0.99) 0.012	0.83 (0.41, 0.99) 0.026	0.82 (0.34, 0.90) 0.022
0.02ml	1	0.90 (0.52, 0.99) 0.002	0.88 (0.55, 0.92) 0.031	0.82 (0.61, 0.99) 0.001	0.80(0.62, 0.97) 0.002
0.03ml	1	0.93 (0.44, 0.99) 0.001	0.73 (0.39, 0.89) 0.001	0.69 (0.40,0.88) 0.001	0.60 (0.42, 0.86) 0.001
0.04ml	1	0.79 (0.40, 0.83) 0.001	0.70 (0.33, 0.89) 0.002	0.63 (0.29, 0.72) 0.001	0.58 (0.40, 0.73) 0.001
0.05ml	1	0.77 (0.39, 0.88) 0.002	0.68 (0.34, 0.85) 0.001	0.65 (0.34, 0.88) 0.001	0.60 (0.41, 0.80) 0.001

Upon examining the results presented in the table, a clear pattern emerges, indicating that the non-diabetic group consistently demonstrates a higher mean percentage change in sperm motility from baseline compared to the diabetic group across all concentrations of commercial citrulline. These findings strongly suggest that the citrulline present in the watermelon extract has a more pronounced positive effect on sperm motility in non-diabetic individuals compared to those with diabetes.

Table 4 Logistic regression model on the effect of commercial citrulline on sperm motility of the study participants

CitrullineConc.	Non-Diabetic (n=27)	Diabetic (n=27)Odd Ratio (CI)P			
	Standard	T1 (0.5hr)	T2 (1.0hr)	T3(1.5hr)	T4(2.0hr)
0.01ml	I	0.88 (0.51, 0.92) 0.001	0.86 (0.50, 0.92) 0.001	0.88 (0.52, 0.92) 0.001	0.71 (0.49, 0.87) 0.001
0.02ml	I	0.84 (0.55, 0.99) 0.002	0.89 (0.60, 0.99) 0.001	0.89 (0.54, 0.99) 0.001	0.71 (0.51, 0.88) 0.002
0.03ml	I	0.79 (0.44, 0.83) 0.001	0.80 (0.42, 0.97) 0.001	0.70 (0.51, 0.89) 0.002	0.68 (0.31, 0.94) 0.001
0.04ml	I	0.71 (0.49, 0.96) 0.001	0.75 (0.33, 0.87) 0.002	0.64 (0.36, 0.86) 0.002	0.60 (0.29, 0.89) 0.001
0.05ml	I	0.63 (0.39, 0.99) 0.001	0.61 (0.30, 0.86) 0.002	0.63 (0.34, 0.87) 0.004	0.53 (0.41, 0.99) 0.001

The logistic regression model provides estimates of the odds ratio, which indicates the effect of the commercial citrulline concentration on the log odds of experiencing improved sperm motility. The coefficients in the model reveal a stronger and statistically significant relationship between the commercial citrulline concentration and the likelihood of improved sperm motility in the non-diabetic group compared to the diabetic group. This suggests that the impact of commercial citrulline concentration on sperm motility is more pronounced and meaningful in individuals without diabetes.

5. DISCUSSION

The present study described the effect of citrulline from commercially available watermelon seed extract on sperm motility in diabetic and non-diabetic infertile males. The baseline data of this study had been previously published.[28] It was known that uncontrolled blood sugar in males affected many physiological systems in the body, including reproduction, leading to poor fertility.[29] Similar results were also found in the study, indicating that people with diabetes showed abnormal semen parameters, including impaired sperm motility. Studies [30-31] suggested that uncontrolled blood sugar levels could influence semen pH, with diabetic individuals often exhibiting lower pH levels than non-diabetic individuals, supporting the results of the present study. Participants with diabetes were observed to have lower semen volume. Studies [32-33] reported a negative correlation between diabetes and semen volume, influenced by sugar levels and lifestyle behaviors. A study[34] found that men with higher blood glucose levels had lower sperm counts, supporting the negative association between diabetes and sperm count observed in the present study. A review article analyzed multiple studies and concluded that diabetes was associated with impaired sperm parameters, including lower sperm count, and suggested that factors such as oxidative stress, hormonal abnormalities, and microvascular complications may contribute to this relationship.[35] Research suggested a higher prevalence of abnormal sperm morphology in individuals with diabetes than in those without diabetes. A systematic review reported that diabetes was associated with an increased prevalence of abnormal sperm morphology[36] The cross-sectional study findings indicated poor glycemic control in diabetic men was associated with abnormal sperm morphology.[37] Phytonutrients, including antioxidants such as lycopene and beta-carotene and amino acids like citrulline and arginine, were present in watermelon, with citrulline being a major bioactive compound found in all parts of the watermelon, including the seed.[38]In the study, it was observed that the retention time of the crude extract was 9.58 minutes, slightly deviating from the retention time standard due to other impurities in the mixture. As a result, the crude sample underwent further purification through the second extraction of the pellet, reducing the retention time to 9.29 minutes, which was the closest to the standard retention time (9.13 minutes). The concentration of citrulline improved after shortening the retention time of the crude sample. The study showed that the citrulline concentration in the seed was 28mg/g, contrasting with a previous study reporting a concentration of 0.1-0.03mg/g in watermelon seed and a higher concentration of 2-2.5mg/g in the flesh.[39] Citrulline played a role in the urea cycle and nitric oxide production. Although the direct effect of citrulline on sperm motility was not well-established, there was some evidence suggesting its potential benefits and multiple potential mechanisms of action.[40] Citrulline could be converted into arginine, serving as a precursor for synthesizing nitric oxide, which regulates blood flow and smooth muscle relaxation. Nitric oxide was believed to enhance sperm movement by promoting vasodilation and improving blood flow to the reproductive organs, thus impacting sperm motility.[41] Citrulline could also contribute to producing ATP, the primary energy source for sperm motility, thereby enhancing overall sperm function.[42-43] Furthermore, citrulline possessed antioxidant properties, reducing oxidative stress that could damage sperm cells and impair their motility. By reducing oxidative stress, citrulline could help protect sperm cells from damage and improve their motility.[44] Additionally, citrulline may help maintain the integrity and fluidity of the cell membrane of sperm cells, which is crucial for proper sperm function, including motility.[45] The study used watermelon seed extract to examine sperm motility. It was observed that total sperm motility improved in both groups after treatment with watermelon seed extract, although the improvement was lower in group 2 participants compared to group 1. In group 2, the maximum improvement was seen with 0.03ml treatment after 2 hours of incubation, while the minimum improvement was seen with 0.05ml treatment after 2 hours of incubation. It was also observed that total motility initially improved up to 0.03ml treatment, followed by a decline phase, possibly due to the presence of other active compounds or alterations in the pH of treated samples, but not due to citrulline toxicity, as the trend was not similar in the commercial citrulline treatment, where the motility improved up to 0.05ml treatment in both groups. A study primarily focused on the effects of L-citrulline supplementation on erectile function and reported improvements in sperm motility among the study participants.[46] Another study primarily investigated the effects of L-citrulline on oocyte quality and pregnancy outcome, also observing improvements in sperm motility as a secondary outcome.[47] A study in rams showed that supplementation of L-citrulline increased sperm concentration, linearly motile sperm, and viability while decreasing the quantity of dead sperm.[48] Another study found that supplementation with

combined pine bark extract, L-arginine, L-citrulline, and roburins improved semen quality in treatment groups compared to the control.[49] These studies suggested that citrulline supplementation may positively affect sperm motility. However, it's important to note that more research is needed to fully understand the specific mechanisms by which citrulline affects sperm motility and to establish its efficacy. Further research is required to investigate the specific mechanisms, optimal dosage and duration of supplementation, long-term effects, potential synergistic effects with other supplements or medications, and molecular pathways involved in citrulline's impact on sperm motility. Additionally, exploring the effects of citrulline supplementation on different types of male infertility and considering combinations with antioxidants, vitamins, or other amino acids could provide further insights into enhancing its efficacy. Lastly, investigating the impact of citrulline on mitochondrial function, energy metabolism, and intracellular signaling pathways related to sperm motility would contribute to a comprehensive understanding of its effects. The study's main limitations included using a single citrulline extraction method and not exploring the combinational effects of other antioxidants on in-vitro sperm motility.

6. CONCLUSION

This study found that people with diabetes have poor semen quality, including sperm motility. Treatment of sperm with citrulline from watermelon seed extract and commercially available citrulline shows that motility improved in diabetic and non-diabetic infertile males. Therefore, it is concluded that citrulline treatment improves in-vitro sperm motility in the study population and may show a better positive effect on ART outcomes.

7. AUTHORS CONTRIBUTION STATEMENT

Dr. Syed Ali designed the study, including extraction methods, study design, sample size calculation, and participant selection. Ms. Kalaiselvi did the laboratory experiments, including semen analysis, citrulline extraction, and treatment. Dr. Anuradha & Dr. Subhashini had done the data analysis of the study using SPSS software and prepared the manuscript. All the authors read and approved the final version of the manuscript.

8. CONFLICT OF INTEREST

Conflict of interest declared none.

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