



For successful long-term cryopreservation, studies compare the effects of tris-citric acid and tris-based extender (egg yolk-free) on a number of semen characteristics.

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ABSTRACT

Aim: To find the best qualified novel semen extender for a longer duration of cryopreservation technique between the two extenders that is, tris citric acid extender and tris based extender (egg yolk free). **Materials and Methods:** Sample size by ClinCalc with the alpha error threshold of 0.05, g power was 80%, enrolment ratio was 1 and 95% confidence interval. The research was repeated 14 times (N = 14) and measured the statistical Independent Samples Test for the semen samples. The research had two different groups such as tris citric acid extender and tris based extender (egg yolk free) with 28 semen samples. Semen sample in a collection tube and separated the sperm cell then added those two extenders separately and mixed it and then a small amount of the diluted semen samples were dropped on to the makler counting device and analyzed with the CASA software. **Results:** Tris citric acid based novel semen extender showed better results than tris based egg yolk free based novel semen extender. Evaluation of the sperm count and motility proved to be greater than the P <0.05, therefore it is insignificant. **Conclusion:** Tris citric acid extender showed better results for both the sperm count and sperm motility when compared with tris based egg yolk free extender for post thawed cryopreservation. **Keywords:** Semen, Cryopreservation, Novel semen extender, Tris citric acid extender, Tris based extender (egg yolk free), Assisted Reproductive Technology

INTRODUCTION

Cryopreservation is an approach where they have stored below -196°C in order to arrest any biological process, physical process and chemical process of the biological components such as semen samples, red blood cells, plant tissue (Jang et al. 2017). Tris citric acid was prepared by the addition of citric acid to the buffer which was used for quality improvement for the cryopreserved samples. Egg yolk free extender is useful for non-human primates preservation of sperm (Yan et al. 2016). Cryopreservation is used for cancer

patients and fertility in freezing the semen preservation (Fu et al. 2019). Computer Assisted Sperm Analysis (CASA) is an electronic visualizing system which shows the accurate evaluation of the various individual sperm motility (Giaretta et al. 2017). Assisted reproductive technology helps in the rectification in the minor and major defaults in the sperm for overcoming the male infertility. Artificial insemination (AI) is considered to be one of the assisted reproductive technology and the most traditional reproductive biotechnology used for conserving the

semen samples for fertilization. IVF, frozen embryo transfer, GIFT are also examples of assisted reproductive technology (Omran, Bakhiet, and Ehemann 2021).

44 articles were found in pubmed and 26 articles in academia.edu based on the title. When the semen sample is diluted in tris citric acid and kept in storage for three days, the sperm motility is reduced. Sperm motility for tris citric acid, skimmed milk, and sodium citrate on day 1 is 55%, 53%, and 52%. On day 2, it is 49%, 40%, and 32% for tris citric acid, skimmed milk, and sodium citrate, respectively. On day 3, it is 38%, 22%, and 15% for tris citric acid, skimmed milk, and sodium citrate (Rakha et al. 2013). Two unique tris Citric acid semen extenders are used to measure sperm motility; the values for chicken and duck egg yolks in tris-citric acid are respectively 60.0 and 19.36 percent and 57.0 and 19.36 percent (Khatimah et al. 2021). The total number of sperm counts is between 35 and 50 percent when a tris-based extender (egg yolk-free) is suspended with -carrageenan at concentrations of 0.1 percent, 0.2 percent, 0.3 percent, and 0.5 percent. As the concentration of -carrageenan increases, the sperm motility percent and viability decrease (Kim et al. 2021). Tris citric acid and arachidic acid are added in varying concentrations to improve sperm motility; these concentrations are 0 ng/ml, 5 ng/ml, 10 ng/ml, and 20 ng/ml, which are represented by $32.2 \pm 0.533\%$, $46.7 \pm 1.934\%$, 51.1 ± 1.474 and 55 ± 0 correspondingly (Ejaz et al. 2014). The motility percentage in egg yolk free extender is $95.82 \pm 0.25 \%$, triladyl is $91.73 \pm 1.06 \%$, and tris egg yolk based is $88.12 \pm 0.76 \%$, according to CASA

programme. 200 sperm in all were found in the sample of semen, and a fluorescent microscope was used to see them (Naz, Umair, and Iqbal 2018). The tris citric acid-based chicken and duck semen extender exhibits an improvement in motility and also maintains the characteristics of the bull's semen samples (Khatimah et al. 2021). The viability percentage of the *Bubalus bubalis* semen samples after being diluted with Tris citric acid extender and resveratrol at different concentrations of 10 mM, 20 mM, 50 mM, and 100 mM for post-thawing cryopreservation was 55.75, 58.58, 58.83, and 62.83, respectively, and the motility percentage was 44.58, 45.42, 48.33, and 49 (Hussain Ahmed, Jahan, Ullah, et al. 2020).

In the present article cryopreservation shows no improved results by using the suitable semen extender for the post thawed cryopreservation method. To improve the cryopreservation technique and also to maintain their features have worked on the semen extenders. Cryopreservation cannot be utilised to keep for a very long time because there is no technique that can handle a temperature of roughly -196°C . For the past ten years, authors have specialised on the treatment of male infertility and cryopreservation. Our goal was to develop a selective semen extender that could tolerate cryopreservation for an extended period of time.

MATERIALS AND METHODS

This research has been handled in the Biochemistry laboratory at Saveetha School of Engineering, Chennai. Authors have received ethical acceptance in order to work with the male semen samples. Tris citric acid and tris based egg yolk free

based semen extenders were used. Tris citric acid was labeled as group 1 and tris based extender egg yolk free has been labeled as group 2. The sample size was optimised using ClinCalc with alpha error threshold of 0.05, g power was 80%, enrolment ratio was 1 and 95% confidence interval.

According to the World Health Organization, the samples for the first study group's Tris citric acid-based semen extender were collected (2010). The tools employed to examine the semen samples included computer assisted semen analysis (CASA). It has also accurately evaluated the sperm motility and total number of sperm present in the sample, and it has produced video visuals. A substance known as a cryoprotectant prevents cell damage, aids in cryopreservation, and serves as an antifreeze

The second study group consisted of tris-based, egg yolk-free semen extenders, and samples were gathered in accordance with World Health Organization guidelines (2010). Computer assisted semen analysis (CASA) was utilized to quickly analyse the semen samples and provide the accurate sperm value in a thorough report (Schleh and Leoni 2013). Cryoprotectant is a liquid which resolves the ice crystal formation in samples that are stored in the cryocan. Previously our team has a rich experience in working on various research projects across multiple disciplines (Manikandan et al. 2021; Kulandaivel et al. 2020; Rajesh et al. 2020; Vimalraj et al. 2020)

Cryocan is a pressurized storage container can with liquid nitrogen which has been used to preserve the semen samples. To dilute the raw sperm before using the cryopreservation procedure to

freeze it, novel semen extenders were used. The semen samples were taken using sterilised plastic containers. The volume, pH, sperm count, sperm concentration and sperm motility was used to analyze the sample. A little amount of the diluted sample was placed onto a Makler counting chamber, which was compact and simple to use, after the sperm cells were removed from the semen samples and added the tris citric acid and tris based egg yolk free extenders separately. With the aid of the CASA software, it was possible to assess the sperm motility, count, and concentration using the Makler counting apparatus. Sperm motility in percentage (percent), sperm count in millions/ml, volume in ml, and potential of hydrogen (pH) were measured in the evaluated samples.

Statistical analysis

The sperm count and sperm motility in tris citric acid-based semen extender and tris-based egg yolk-free based semen extender were statistically analysed using IBM SPSS version 26 software. The retrieved results for volume, sperm count, sperm motility, sperm concentration, and group statistics for standard deviation, mean, independent sample T-test, significance, and the standard error mean were independent variables. There are no dependent variables discovered (McKenna et al. 2021).

RESULTS

A novel semen extender based on tris citric acid performs better than one based on tris egg yolk free extender. Sperm motility and sperm count were evaluated, and both results were more than the $P < 0.05$, making them insignificant.

Table 1 shows the comparison between tris citric acid semen extender and tris based extender (egg yolk free). The greatest value for tris based extender (egg yolk free) was 8.85gm in concentration per litre, while the lowest value for tris in tris based extender was 2.88gm in concentration per litre (egg yolk free). For both extenders, the pH has been kept at 7.2.

Table 2 displays the sperm count and motility prior to cryopreservation in million/ml and percentage, respectively (percent). Sperm count and sperm motility both reached their maximum values at 69.8 million/ml and 49.7 percent, respectively.

Table 3 displays the sperm count in million/ml and sperm motility in percentage (%) for the tris citric acid semen extender and tris based extender following cryopreservation (egg yolk free). Sperm count and sperm motility showed the greatest values for tris citric acid semen extender at 60.2 million/ml and 32.5 percent, respectively. Sperm count and sperm motility measurements for tris-based extenders (semen extenders free of egg yolk) showed the highest results at 60.2 million/ml and 31.7 percent, respectively.

Table 4 shows the values for the N, standard deviation, standard mean error and mean for the comparison of sperm count and sperm motility of tris citric acid semen extender and tris based extender (egg yolk free). Tris citric acid semen extender had a mean value of 37.4000 and tris based extender (egg yolk free) had a mean value of 27.9171 for sperm count. Tris citric acid semen extender had a mean value of 27.5714 and tris based extender (egg yolk free) had a mean value of 21.4143 for sperm motility, respectively.

Table 5 shows the independent sample T- test for sperm count and sperm motility which were found to be insignificant, that sperm count value was 0.365 and sperm motility was 0.418 because of $P < 0.05$.

Bar chart shows the tris citric acid semen extender was superior to the tris based extender (egg yolk free) in terms of sperm count in Fig. 1 and the tris citric acid semen extender appeared to be superior to the tris based extender (egg yolk free) in terms of sperm motility in Fig. 2. It shows that the sperm count and motility of the tris citric acid semen extender were higher than those of the tris based extender (egg yolk free) by a difference of 3 million/ml and 8%, respectively.

DISCUSSION

Based on sperm count and motility measurements made using the CASA software, tris citric acid performed better than tris-based egg yolk free extender. When the p value is set to $P < 0.05$, the mean values for sperm count and sperm motility are insignificant at 0.365 and 0.418, respectively.

According to Ahmed et al, a similar article based on our study shows that tris citric acid shows better results with addition of L-tryptophan at different concentrations. At 0 μ M, 25 μ M, 50 μ M, 75 μ M and 100 μ M the corresponding percentages are $46.25 \pm 2.39\%$, $47.50 \pm 1.44\%$, $53.75 \pm 2.39\%$, $56.25 \pm 1.25\%$, 60.00 ± 2.04 respectively. L-tryptophan concentration at 100 μ M concentration increases the motility rate (Hussain Ahmed, Jahan, Khan, et al. 2020). According to Raheja et al., Tris citric acid extender was diluted with a bovine semen sample, and the results showed that the

viability of the sperm increased to 89 percent from 64.83 percent when Tris based extender (egg yolk free) was used. This suggests that the increase in tris citric acid extender was caused by an enzymatic reaction with the GSH (2.0 mM) additive (Raheja et al. 2018). According to Ansari et al., the buffalo semen samples were diluted with tris citric acid extender and exogenous glutathione supplementation at concentrations of 0.0, 0.5, 1.0, 2.0, and 3.0 mM at 0, 3, and 6 hours after post-thaw cryopreservation. As a result, the sperm motility test at 2.0 mM glutathione concentration revealed an increase in sperm motility and viability of 56.7 ± 2.9 , 41.7 ± 2.9 , 28.3 ± 2.9 and 89.0 ± 2.0 , 75.3 ± 2.5 , 59.3 ± 4.9 (Ansari et al. 2010). For biological activity to achieve high conception rates, the sperm motility and alive sperms are crucial. In order to determine the best extender based on sperm motility and viability, *Ovis vignei punjabiensis* (Punjab ural) semen samples were diluted with Tris citric acid extender, skim milk, and sodium citrate extenders separately, as stated by Rakha et al. Tris-citric acid extender appears to be more effective than sodium citrate and skim milk extenders after post-thawing for three days at 5° (Rakha et al. 2013)

Based on our findings on sperm count and sperm motility, it can be inferred from our results that tris citric acid was more successful than tris-based egg yolk free extender. The study has been constrained by long-term cryopreservation due to its poor stability and low percentage of viable sperm. Semen extenders based on tris citric acid and tris egg yolk free are designed to preserve semen samples for a longer period of time while maintaining their original characteristics.

CONCLUSION

When compared to a tris-based egg yolk-free extender for post-thawed cryopreservation, the tris citric acid extender performed better in terms of sperm motility and count.

DECLARATIONS:

Conflict of interests

No conflict of interests in this manuscript

Authors Contributions

Author RNN was involved in data collection, data analysis, manuscript writing. Author VAS was involved in conceptualization, data validation, and critical review of manuscript.

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Figures and Tables

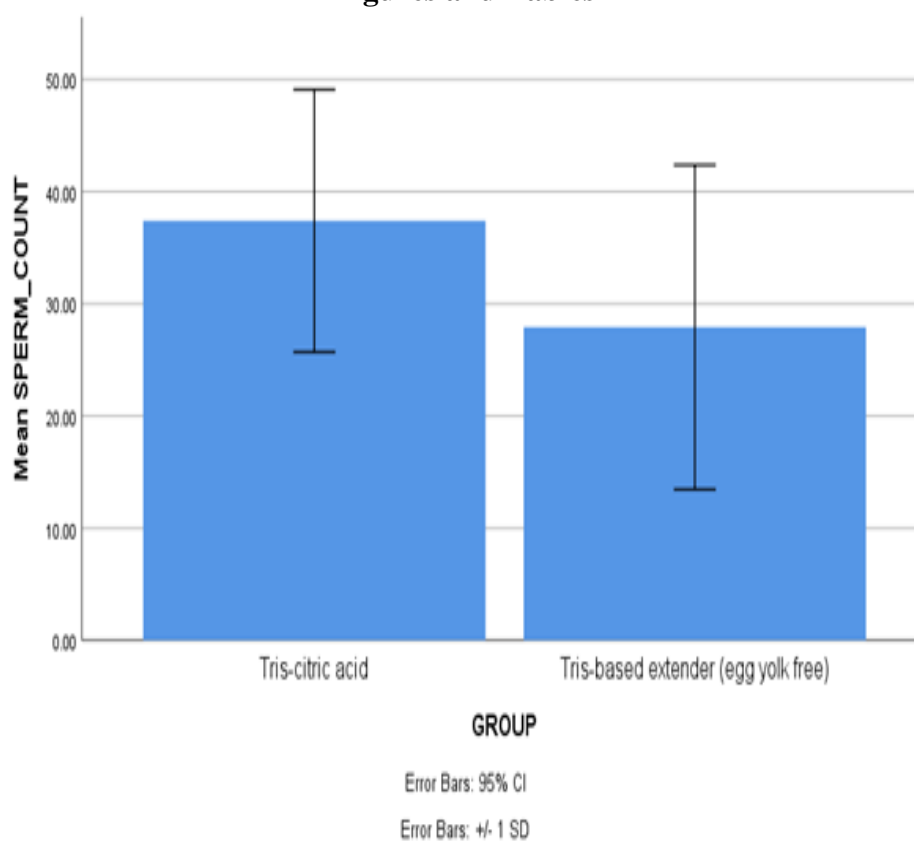


Fig. 1. Bar chart compares the mean sperm counts of two different extenders, tris citric acid extender and tris based extender (egg yolk free) to determine the optimal extender. With a difference of 6 million/ml, tris citric acid extender is shown to be the superior extender to tris based extender (egg yolk free). $P = 0.365$, the research's significance level, indicates that it is not significant. Comparison of Tris citric acid extender and Tris based extender (egg yolk free) on the X axis; mean sperm count ± 1 SD on the Y axis.

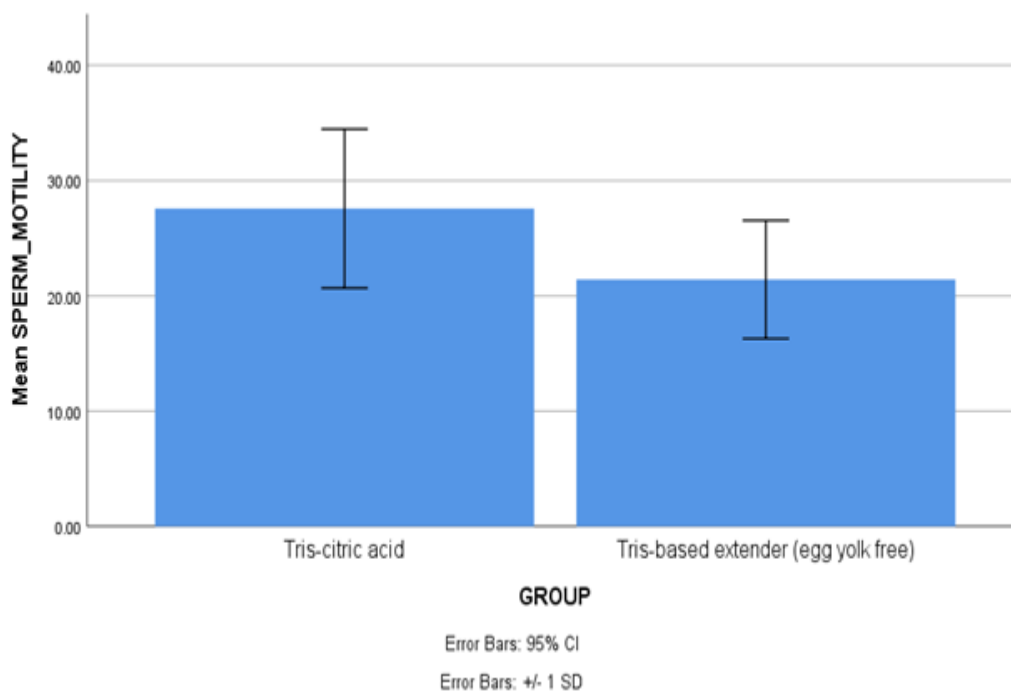


Fig. 2. Bar chart compares the mean sperm counts of two different extenders—tris citric acid extender and tris based extender (egg yolk free) in order to choose the best extender. Tris citric acid extender was shown to be the most appropriate extender when compared to tris based extender (egg yolk free) by an 8 percent margin. $P = 0.418$, the research's significance level, indicates that it is not significant. Comparison of Tris citric acid extender and Tris based extender (egg yolk free) on the X axis; mean sperm count \pm 1 standard deviation (SD) on the Y axis.

Table 1: Components present in the tris citric acid extender in concentration per liter and tris based extender (egg yolk free) in concentration per liter.

Tris-citric acid	Concentration per Litre	Tris-based extender (egg yolk free)	Concentration per Liter
Tris	2.88 gm	Tris	8.85 gm
Glycerol	7%	Glycerol	7%
Glucose	0.71 gm	Glucose	0.71 gm
Egg Yolk	20 ml	Egg Yolk	-
Sodium Citrate	-	Sodium Citrate	-
Ethylene diamine tetraacetic acid	0.07 gm	Ethylene diamine tetraacetic acid	0.07 gm
Trehalose	0.21 gm	Trehalose	0.21 gm
Cystine	0.1 gm	Cystine	0.1 gm

Citrate	-	Citrate	-
HEPES	0.15 gm	HEPES	0.15 gm
Sodium Bicarbonate	1.45 gm	Sodium Bicarbonate	1.45 gm
Potassium Chloride	0.32 gm	Potassium Chloride	0.32 gm
Catalase	0.1 gm	Catalase	0.1 gm
Vitamin E	-	Vitamin E	0.25 gm
Penicillin	45000 IU	Penicillin	45000 IU
Taurine	0.05 gm	Taurine	0.05 gm
Potassium Acetate	0.21 gm	Potassium Acetate	0.21 gm
pH	7.2	pH	7.2
Fructose	-	Fructose	-
Citric Acid	2.8 gm	Citric Acid	-

Table 2: The sperm count in million s/ml and sperm motility in % before cryopreservation.

Samples	Sperm count (Millions/ml)	Sperm Motility (%)
1	60.6	34.6
2	67.9	38
3	69.8	31.5
4	49.6	32.5
5	56.6	49.6
6	49.8	37.8
7	49.6	39.5
8	49.8	31.6
9	51.6	37.8
10	48.6	37.8
11	49.7	39
12	56.7	44.5
13	60.4	49.7

Vickram A S.*et.al.*, For successful long-term cryopreservation, studies compare the effects of tris-citric acid and tris-based extender (egg yolk-free) on a number of semen characteristics.

14	55.4	44.9
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Table 3: Comparison for tris citric acid extender and tris based extender (egg yolk free) of sperm count and sperm motility which gives its mean value, standard deviation and standard error mean value.

Sample s	Sperm count (Millions/ml)	Sperm Motility (%)	Sperm count (Millions/ml)	Sperm Motility (%)
1	45.7	22.6	40.67	18.4
2	54.6	28	51.67	22.5
3	60.2	21	60.2	17.8
4	36.5	26.5	31.5	21.6
5	45.5	45.4	28.9	31.7
6	31.2	29.5	21.7	21.8
7	40.3	32.5	21.9	25.6
8	27.8	17.8	34.5	11.8
9	16.8	29.5	12.6	21.7
10	22.6	31.6	14.5	27.5
11	31.5	31.6	16.7	25.4
12	34.7	26.7	15.6	20.8
13	37.7	21.6	18.9	17.8
14	38.5	21.7	21.5	15.4

Table 4: Shows the group statistics of sperm count and sperm motility for tris citric acid extender and tris based extender (egg yolk free) which gave its mean value, standard deviation and standard error mean value. The mean value of tris citric acid extender was found to be higher than the tris based extender (egg yolk free) after cryopreservation for both sperm count and motility.

Group Statistics					
	GROUP	N	Mean	Std. Deviation	Std. Error Mean
SPERM_COUNT	Tris-citric acid	14	37.4000	11.69089	3.12452

	Tris-based extender (egg yolk free)	14	27.9171	14.45330	3.86281
SPERM_MOTILITY	Tris-citric acid	14	27.5714	6.89285	1.84219
	Tris-based extender (egg yolk free)	14	21.4143	5.12818	1.37056

Table 5: Shows the independent samples test for analysis of sperm count and sperm motility which showed its statistical insignificance with P value = > 0.05

Independent Samples Test										
		Levene's Test for Equality of Variances							t-test for equality of Means	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
SPERM_COUNT	Equal variances assumed	.852	.365	1.909	26	.067	9.48286	4.96829	-.72961	19.69532
	Equal variances not assumed			1.909	24.912	.068	9.48286	4.96829	-.75136	19.71708
SPERM_MOTILITY	Equal variances assumed	.677	.418	2.682	26	.013	6.15714	2.29611	1.43742	10.87686
	Equal variances not assumed			2.682	24.016	.013	6.15714	2.29611	1.41838	10.89591