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Laboratory and Clinical Outcomes of Single Sperm Cryopreservation in Patients Underwent Micro-TESE

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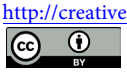

Abstract

For men with severe oligozoospermia, sperm cryopreservation can preserve surgically obtained sperm. How to cryopreserve single sperm in men is still a hot topic in assisted reproduction technology. Aim to analyze the laboratory and pregnancy outcomes of single sperm cryopreservation group, we retrospectively selected 38 cycles underwent single sperm cryopreservation and thawing as the study group and 618 cycles underwent conventional sperm cryopreservation and thawing as the control group, which were performed in the reproductive medicine center of the Sixth Affiliated Hospital, Sun Yat-sen University, from April 2014 to October 2023. All the sperm came from microdissection testicular sperm extraction (micro-TESE), and performed intracytoplasmic sperm injection (ICSI) for fertilization. Zygotes were cultured to Day 3 embryo, which were freshly transferred to female uterus. Surplus embryos were cultured to blastosphere and cryopreserved. There was no statistical difference in female/male age, female BMI, infertility duration and female basal sex hormone (FSH, LH E2, AMH), No. of oocytes retrieved per cycle, No. of ICSI oocytes per cycle and No. of embryos transferred per cycle between the two groups ($P > 0.05$). No significant difference was found in two-pronuclear oocyte fertilization rate (59.23% VS 58.84%), Day 3 available embryo rate (61.81% VS 63.55%), Day 3 good-quality embryo rate (45.73% VS 50.27%), blastocyst formation rate (47.83% VS 49.46%), the implantation rate (47.37% VS 52.16%), clinical pregnancy rate (36.84% VS 47.18%), miscarriage rate (14.29% VS 12.68%) and live birth rate (85.71% VS 81.70%) between two groups ($P > 0.05$). In conclusion, single-sperm cryopreservation was the optimal method to preserve sperm after micro-TESE. It can increase the utilization of each sperm and lead to clinical pregnancy.

Keywords

Microdissection Testicular Sperm Extraction (Micro-TESE), Single Sperm Cryopreservation, Severe Oligo-Astheno-Teratospermia, Non-Obstructive Azoospermia (NOA)

1. Introduction

45 years have passed since the birth of the first *in vitro* fertilization (IVF) case. In recent years, with the continuous development and improvement of assisted reproductive technology, more and more clinical challenges have been solved. However, continuing global industrialization and increasing pollution of various environments [1], the continuous accumulation of antibiotics and hormonal drugs, occupational factors [2] and lifestyle habits [3], and psychological stress have led to an increase in infertility problems worldwide. Male infertility now accounts for about 50% of fertility related problems [4]. In recent years, there has been a gradual decline in the quality of male semen, as well as a significant increase in the number of men with weak sperm, oligospermia, and azoospermia, with severe spermatogenic cell damage and low sperm quality, which seriously affects fertility levels [5] [6]. The birth of the world's first case of intracytoplasmic sperm injection (ICSI) *in vitro* fertilization (IVF) in 1992 marked a technological solution to the problem of infertility due to severe oligozoospermia in males [7]. For men with severe oligozoospermia, sperm cryopreservation can preserve surgically obtained sperm and reduce the need for repeat surgeries, alleviating the patient's pain and financial burden. In traditional sperm cryopreservation techniques, sperm loss may occur during the processing of sperm cryopreservation and contact with the cryopreservation carrier [8]. Therefore, how to cryopreserve single sperm in men is still a hot topic in assisted reproduction technology. In 1997, Cohen *et al.* cryopreserved sperm with empty zona pellucida, and they introduced the concept of single-sperm cryopreservation [9]. Subsequently, researchers proposed different carriers for single-sperm cryopreservation. Non-biological carriers such as empty ZP [10], polymerized alginic acid capsules [11], hollow-core agarose capsules, cultureoloops [12], cryoloops [13], cryozoos [14]. cryoloops [12], culture dish [15], cell sleeper [16], cryotop [17], cryoleaf [16], cryopiece [18].

The effects of single-sperm cryopreservation-resuscitated sperm on early embryonic developmental markers and clinical outcomes have been investigated [19] [20], and it has been concluded that human single-sperm cryopreservation is feasible and effective, and may be beneficial for patients with severe oligozoospermia. However, the effects of single-sperm cryopreservation methods on early embryonic development and pregnancy outcomes have not been studied and investigated compared to conventional sperm cryopreservation methods.

2. Method

2.1. Study Design

This single-center, retrospective cohort study was conducted to analyze the laboratory and pregnancy outcomes of single sperm cryopreservation and thawing of patients who underwent microdissection testicular sperm extraction (micro-TESE) at the Reproductive Medicine Center of the Sixth Affiliated Hospital, Sun Yat-sen University, from April 2014 to October 2023.

2.2. Patients

The inclusion criteria were: 1) male patients diagnosed as severe oligo-asthenoteratospermia or non-obstructive azoospermia (NOA); 2) male patients underwent micro-TESE; 3) sperms performed single sperm cryopreservation. The exclusion criteria were that cryopreserved sperms not performed thawing and fertilization. Consequently, a total of 38 cycles were selected as the study group. The control group should be matched more than a ratio of 4:1. We included 618 cycles, in which male patients underwent micro-TESE and conventional sperm cryopreservation. According to female/male age, female body mass index (BMI), AMH, number of oocytes retrieved/ICSI per cycle and number of embryos transferred per cycle, the control group were matched with the study group. All patients provided written consent to have their medical records used for research purposes. This study was approved by the ethics committee at the Center for Reproductive Medicine, the Sixth Affiliated Hospital of Sun Yat-sen University. This study was approved by the ethics committee at the Center for Reproductive Medicine, the Sixth Affiliated Hospital of Sun Yat-sen University (Program No. 2017ZSLYEC-016S).

2.3. Ovarian Stimulation and Oocytes Retrieval

Appropriate controlled ovarian stimulation program based on the ovarian reserve status of the female partner, and when follicular monitoring showed that two follicles reached ≥ 18 mm or three follicles reached ≥ 17 mm, 250 μg of recombinant human chorionic gonadotropin (HCG) (Azer, Merck Serono, American) was injected. And the oocytes were retrieved under B-ultrasound guidance 34 - 38 h after injection.

2.4. Testicular Sperm Extraction and Sperm Cryopreservation

The spermatogonial tubules containing spermatozoa were obtained by micro TESE before oocyte retrieval. The spermatozoa were searched using the microscope, then the spermatozoa were preserved by either the single-sperm cryopreservation method or the conventional cryopreservation method according to the number of spermatozoa.

Single-sperm cryopreservation in our center used cryopiece as frozen carriers. A 0.5 - 1 μl droplet of a 1:1 (v/v) mixture of Sperm Cryopreservation Medium (Sage *In-Vitro* Fertilization Inc. Trumbull, CT, USA) and Sperm Washing Me-

dium (Sage *In-Vitro* Fertilization Inc. Trumbull, CT, USA) was placed onto the center of the cryopeice, which was submerged into oil on the search plate. With the ICSI microsystem and microinjection needle, the spermatozoa were transferred from the collection droplets to the droplets on the cryopiece, remove the residual mineral oil on the cryopiece with a medical sterile gauze before put into the 2.0 mL cryogenic vials (Corning Incorporated, USA), followed by liquid nitrogen vapors for 15 min, and then stored in liquid nitrogen.

Conventional sperm cryopreservation: The sperm suspensions were diluted 1:1 with cryopreservation medium, incubated for 5 min at room temperature, followed by liquid nitrogen vapors for 15 min, and then stored in liquid nitrogen. This method is a simple, less difficult to operate, and does not require the use of a micromanipulator.

2.5. Sperm Thawing

1) Sperm thawing after single-sperm cryopreservation

A 35 mm dish with 3 ml of mineral oil was incubated at 37˚C for at least 1 h. The cryopiece was pulled out of the cryogenic vials in the liquid nitrogen, immersed immediately in the 37˚C mineral oil, then determine the location and motility of spermatozoa on the cryopiece. Remove the cryopiece to prewarmed mineral oil in the ICSI dish.

2) Sperm thawing after conventional sperm cryopreservation

The 2.0 ml cryogenic vials were removed from the liquid nitrogen, and put in a water bath at 37˚C for 10 min. Equal volumes of Sperm Washing Medium were added, and the mixture was centrifuged at 300 g for about 5 min, then the pellets were resuspended in 500 μl of sperm medium and incubated at 37˚C for ICSI.

2.6. Embryo Culture and Transfer

On the day of oocyte retrieval, select the active sperm after resuscitation under the microscope for ICSI, *in vitro* culture to observe oocyte fertilization/embryo culture, and then transfer 1 - 2 embryos under the guidance of ultrasound on the 3rd day after oocyte collection (D3). If the transfer is canceled in the fresh cycle due to various reasons, two embryos will be frozen and preserved in the D3, and the remaining embryos will be frozen and preserved if they are formed in the blastocyst culture, and then frozen embryo will be transferred later on.

2.7. Outcome Measures

Pronucleus of oocytes were observed in 14 - 19 hours after fertilization by ICSI. Two-pronuclear oocyte fertilization was considered as normal fertilization. Day 3 embryos with at least four cells that had even or slightly uneven blastomeres and less than 20% fragmentation were considered as day 3 available embryo, which could be transferred, frozen or further culture to the blastocyst stage. Day 3 good-quality embryos were embryos with at least six cells with even blastomeres and less than 20% fragmentation at day-3. Women's peripheral blood was

detected for β-HCG at 2 weeks after embryo transfer. The positive ones were judged as implantation. Clinical pregnancy is diagnosed when B-ultrasound was performed at 7 weeks after transfer and the pregnancy sac and primitive heart tube pulsation are seen in the uterine cavity. All patients were followed up until October 2023.

2.8. Statistics

Data were analyzed using SPSS statistical software (26.0, IBM, USA). Measurement data for continuous variables were expressed as mean ± standard deviation ($\bar{x} \pm s$).

The comparison of the mean between groups was performed by Mann-Whitney U test. And the enumeration data were presented as rate, using Pearson's chi-squared test or Fisher's Exact Test. P-value < 0.05 was considered as statistically significant difference.

3. Results

3.1. General Clinical Data of Patients

All the couples were of Han nationality in Southeast China. The male patients were diagnosed as severe oligo-astheno-teratospermia or non-obstructive azoospermia due to Klinefelter's Syndrome, sequela of orchitis, testicular dysplasia or Y chromosome microdeletion. There was no significant difference between single sperm cryopreservation group and conventional cryopreservation group in female/male age, female BMI, infertility duration and female basal sex hormone (FSH, LH E2, AMH) (P > 0.05) (Table 1). The numbers of oocytes retrieved per cycle, ICSI oocytes per cycle and embryos transferred per cycle in the two groups were (15.84 ± 8.00) VS (13.80 ± 7.65), (8.84 ± 4.01) VS (10.49 ± 6.18) and (1.78 ± 0.43) VS (1.69 ± 0.46) respectively, without significant difference (P > 0.05). The two groups were comparable according to the parameters.

3.2. Comparison of Laboratory and Clinical Outcomes between Two Groups

There was no significant difference in two-pronuclear oocyte fertilization rate, Day 3 available embryo rate, Day 3 good-quality embryo rate and blastocyst formation rate between the two groups (P > 0.05) (Table 2). It indicated single sperm cryopreservation could reach similar fertilization, embryo quality and developmental potential as conventional cryopreservation.

Among the 19 fresh embryo transfer cycles in single sperm cryo-preservation group, 9 cycles got successfully implanted. And the implantation rate (47.37%) was lower than that of conventional cryopreservation group (52.16%) with no significant difference (P > 0.05). And there was no significant difference in clinical pregnancy rate (36.84% VS 47.18%), miscarriage rate (14.29% VS 12.68%) and live birth rate (85.71% VS 81.70%) between two groups (P > 0.05). It indicated single sperm cryopreservation could reach similar clinical outcomes as conventional cryopreservation.

Table 1. Comparison of general data between two groups ($\overline{x} \pm s$).

Parameter	Single sperm cryopreservation group	Conventional cryopreservation group	Z	P
No. of patients	38	618	-	-
Female age (years)	29.08 ± 3.66	30.28 ± 4.75	−1.30	0.194
Male age (years)	31.79 ± 5.32	33.15 ± 6.54	−1.34	0.182
Female BMI (kg/m^2)	22.39 ± 2.58	21.97 ± 3.14	1.15	0.252
Infertility duration (years)	3.92 ± 2.78	4.11 ± 3.34	0.15	0.879
Basal FSH (U/L)	6.59 ± 1.75	6.84 ± 2.34	0.17	0.865
Basal LH (U/L)	6.17 ± 4.26	5.60 ± 2.49	−0.26	0.822
Basal E2 (pg/mL)	43.40 ± 22.63	49.27 ± 25.242	0.41	0.685
AMH (ng/mL)	4.31 ± 3.15	3.66 ± 2.40	0.81	0.417
No. of oocytes retrieved per cycle	15.84 ± 8.00	13.80 ± 7.65	1.66	0.097
No. of ICSI oocytes per cycle	8.84 ± 4.01	10.49 ± 6.18	−1.25	0.211
No. of embryos transferred per cycle	1.78 ± 0.43	1.69 ± 0.46	−0.751	0.452

BMI: Body mass index; Basal FSH: Basal follicle stimulating hormone; Basal LH: Basal luteinizing hormone; Basal E2: Basal estradiol; AMH: Anti-Müllerian hormone.

Table 2. Comparison of laboratory and clinical outcomes between two groups [% (n/N)].

Parameter	Single sperm cryopreservation group	Conventional cryopreservation group	P
Two-pronuclear oocyte fertilization rate	59.23 (199/336)	58.84 (3764/6397)	0.910
Day 3 available embryo rate	61.81 (123/199)	63.55 (2392/3764)	0.334
Day 3 good-quality embryo rate	45.73 (91/199)	50.27 (1892/3764)	0.217
Blastocyst formation rate	47.83 (33/69)	49.46 (733/1482)	0.807
Implantation rate	47.37 (9/19)	52.16 (157/301)	0.814
Clinical pregnancy rate	36.84 (7/19)	47.18 (142/301)	0.479
Miscarriage rate	14.29 (1/7)	12.68 (18/142)	1.000
Live birth rate	85.71 (6/7)	81.70 (116/142)	1.000

4. Discussion

Human assisted reproductive technology was an important technology to resolve the growing problem of infertility in the whole wide world. Especially the male factor was the main factor in infertility. Lots of men suffered from severe oligo-astheno-teratospermia or NOA. Repeated TESE for those patients was costly and invasive, and it had frequently adverse effects on the testis. Several studies had analyzed the successful sperm retrieval rate of micro-TESE, and demonstrated that micro-TESE was the optimum method for sperm retrieval in NOA, with the advantage of higher spermatozoa retrieval rate and minimal tissue loss,

which also avoided repeated TESE [21]. Conventional sperm preservation methods in which sperm were attached to larger carriers and subjected to centrifugation and washing procedures usually result in sperm loss. Hence, conventional methods were particularly problematic when there were only several sperms. Therefore, sperm cryopreservation was indispensable for sperm from micro-TESE. Single-sperm cryopreservation was the optimal method to preserve the small amount of sperm, with the advantages of preserving the intact sperm and reducing sperm loss after cryopreservation and thawing [19]. Single-sperm cryopreservation may avoid the risk of cycle cancelation and decrease the number of unnecessary oocyte retrieval procedures. Since the inception of single sperm freezing, research on freezing carriers and freezing methods has continued to improve sperm recovery and activity after sperm resuscitation. From the earliest empty ZP biocarriers to the current abiotic carriers, different types of carriers were available, suitable for cryopreservation of sparse quantities of spermatozoa under different laboratory conditions. Lots of materials were applied for single sperm cryopreservation, and cryopiece was used in our center. As a non-biological carrier, cryopiece was applied for single sperm cryopreservation, the search time for recovered sperm was short. The time used for searching sperm after thawing was reduced from hours in conventional sperm cryopreservation methods to minutes, and the recovery rate was close to 100%. Also, sperm cryopreserved in small volume droplets reduced sperm loss after thawing, allowing sperm to be transferred directly from the device to the ICSI plate without damaging the sperm. The procedure was easy to perform and the sperm recovery, mobility and fertilization rates were high. The fertilization rate and zygote cleavage rate using cryopiece were confirmed by Sun *et al.* [18].

In our study, 336 oocytes were performed ICSI using thawed-sperm after single-sperm cryopreservation, and 6379 oocytes used sperm after conventional cryopreservation. Single-sperm cryopreservation could reach similar two-pronuclear oocyte fertilization rate, Day 3 available/good embryo rate and blastocyst formation rate with conventional cryopreservation ($P > 0.05$). The results were also consistent with several studies [20]. And embryos from single-sperm cryopreservation could also reach similar implantation rate, clinical pregnancy rate, miscarriage rate and live birth rate with conventional cryopreservation ($P > 0.05$). The result showed single-sperm cryopreservation could reach similar laboratory and clinical outcomes with conventional sperm cryopreservation in patients underwent micro-TESE. Meanwhile, it could effectively protect the sperm intact and activity.

Most of studies focused on exploring the cryopreservation carriers for the single sperm cryopreservation method [10]-[18]. Although some studies had reported the spermatozoa revival rate and clinical pregnancy outcomes after single sperm cryopreservation and thawing, the reports were limited to a few case studies [20]. Our study reported 19 fresh embryo transfer cycle after single sperm cryopreservation and thawing, and enriched the laboratory and clinical outcomes data.

However, due to the limited number of cases in our study, we need a large additional number of cases to observe and further analyze.

5. Conclusion

Single-sperm cryopreservation was the optimal method to preserve sperm after micro-TESE. It could reach similar two-pronuclear oocyte fertilization rate, Day 3 available/good embryo rate, blastocyst formation rate, implantation rate, clinical pregnancy rate, miscarriage rate and live birth rate with conventional cryopreservation ($P > 0.05$). Hence, single-sperm cryopreservation using cryopiece was effective to protect the sperm intact and activity.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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