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Evaluation and improvement of the novel method for vitrification of a few human sperms

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ABSTRACT

Research question: The purposes of this study are to evaluate the performance of the novel SpermVD device on vitrification of a few human sperms, and determine whether PICSI dish and density gradient centrifugation can improve the quality of sperm after thawing.

Design and methods: In order to determine the suitable preparation methods, both washed sperm and neat sperm were selected in ICSI dish and PICSI dish, and frozen with the novel SpermVD device. The selected sperms were transferred to freezing droplets with 1 μl droplet of a 50/50 v/v mixture of QA Sperm Freezing Medium and QA Medium w/Hepes on the SpermVD wells. And the device was exposed to vapor of liquid nitrogen for 5 min and then placed into LN_2 immediately. The sperms were thawed in a 37°C oil filled dish which contained QA Medium w/Hepes droplets and they were searched and revaluated immediately. The frozen effects of sperm were evaluated by progressive motility, motility, viability and recovery rates of freezing-thawing sperm.

Results: After freezing-thawing, the overall sperm recovery rate was 94.2% with 70% viability, 20.7% progressive motility, and 36.2% motility. The progressive motility, motility, viability and recovery rates of washed sperm were lower than that of the neat sperm, with a significant difference ($P < 0.01$) at both viability and motility rates. In addition, the viability rate was significantly higher in PICSI dish group than that of ICSI dish group ($P < 0.01$) and the motility, recovery and progressive motility rates were not significantly different between the two groups ($P > 0.05$).

Conclusions: The spermVD device was one of the effective platforms for freezing a few human sperms and using PICSI dish to select mature neat sperms could improve the quality of sperm after thawing. Density gradient centrifugation might be not required or suitable sperm preparation methods before freezing.

Introduction

Currently, the intracytoplasmic sperm injection (ICSI) is considered as the most effective treatment for severe male factor infertility. Patients with obstructive azoospermia can obtain sperm by using surgically isolated sperm from their testis or epididymis [1]. Sometimes, the failure of surgical extraction of sperm always results in cancelation of the treatment cycle, and no pregnancies occur, repeated epidydimal or testicular collection procedures will be performed in next treatment cycle. Repeated operations not only increase the treatment cost, but also have negative consequences on epidydimal or testicular function and increase the risk of epidydimal or testicular damage [2]. As a result, freezing a few sperms is very important to avoid repetitive testicular aspirations or biopsies for the specific patients. cryopreservation is related to DNA damage and decreased motility and viability of sperm, especially in patients who have poor sperm quality [3,4]. Because of the characteristics of freezing method, only small count of sperm are frozen. Hence, it is necessary to select high-quality sperms for freezing, and the container used for sperm freezing are essential to prevent the loss of sperm during freezing-thawing.

In this study, a novel platform (SpermVD) and PICSI sperm selection device (PICSI dish) were used for freezing a few human sperms. PICSI dish is designed for selecting mature sperm for ICSI. Mature sperm may get a good frozen effect and provide better paternal contribution to the zygote. The novel SpermVD devices with or without PICSI dish for sperm selection were evaluated by the progressive, motility, viability and recovery rates of freezing-thawing sperm. The purposes of this study are to evaluate the performance of the novel SpermVD device on freezing a few

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human sperms, and determine whether PICSI dish can improve the quality of sperm after thawing, and ensure whether Density Gradient Centrifugation (DGC) is the suitable sperm preparation method for freezing a few human sperms.

Materials and methods

Evaluation of the sperm parameters

This study was carried out at Queen's Medical Centre at Nottingham University Hospital in June 2018. Samples were obtained from 6 donors who were oligozoospermia after appropriate consent was completed. Semen specimens were allowed 20 min for liquefaction at room temperature. Then, semen analysis was performed according to the World Health Organization (WHO) Laboratory Manual for the Examination and Processing of Human Semen (5th Edition). Immotile but viable sperms after thawing were determined by laser-assisted approach [5].

Sperm preparation for freezing

Samples were prepared by density gradient centrifugation, density gradient were prepared by dispensing 1 ml 80% lower solution and 1 ml 40% upper solution (SAGE In-Vitro Fertilization Inc. Trumbull, CT, USA) into a 15 ml centrifuge tube. 2 ml samples were added into centrifuge tube as top layer. The tubes underwent centrifugation for 20 min at 200 *g*. Then, further wash operation was carried out by mixing the pellet in 2 ml QA Medium w/Hepes (SAGE In-Vitro Fertilization Inc. Trumbull, CT, USA) and centrifuging for 5 min at 200 *g*. The supernatant was removed and the final pellet was mixed with 0.5 ml QA Medium w/Hepes. 20 ul of the washed sperm or liquefied neat semen were transferred to sperm selection dish for freezing.

Procedure of freezing

The ICSI dish (Corning, Durham, USA) and PICSI dish (PICSI® MidAtlantic Diagnoses-Origio, Denmark) were prepared by making a 10 μl QA Medium w/Hepes droplet and a 10 μl PVP droplet and covered with oil. Progressively motile sperms (PR) in ICSI dish and bound sperms in PICSI dish were selected by injection needle using inverted phase contrast microscope under X200 magnification and transferred into the PVP droplet. The SpermVD (Rafimed Inc., Yavne, Israel) was submerged into oil and about 10 sperms were transferred from the PVP droplet to the freezing droplet with about1 μl droplet of a 50/50 v/v mixture of QA Sperm Freezing Medium (SAGE In-Vitro Fertilization Inc. Trumbull, CT, USA) and QA Medium w/Hepes on the SpermVD wells. Then the SpermVD was placed inside a about 4 ml cryovial (Danyel Biotech Inc., Rehovot, Israel) and exposed to vapor of liquid nitrogen for 5 min (about 3 cm height from liquid nitrogen surface). After that the device was placed into LN_2 immediately.

Procedure of thawing

The cryovial was removed from LN_2 tank and opened. Then the SpermVD device was placed under 37°C oil on the prepared 60 mm dish (Corning, Durham, USA) which contained several 5 ul droplet of QA Medium w/Hepes for washing the sperm, and gently shaken to help the droplet of sperm thaw rapidly. Finally, the sperms were searched and revaluated under inverted phase contrast microscope immediately.

Statistical analysis

Statistical analysis was performed by SPSS 19.0 software. The differences between two groups were analyzed by independent samples *t*-test and $P < 0.05$ was considered to be significant. The data are given as mean percentage ± SD.

Results

A small number of sperm, 8 to 13 per SpermVD device (average 10.7) were frozen in each well and in total 664 sperms were frozen in 62 SpermVD devices from four groups followed by thawing, recovery and reassessment. After thawing, the rates of motility, PR, recovery and viable sperm for each group were showed in the Table 1. The overall sperm recovery rate after thawing was 94.2% with 70% viability, 20.7% PR and 36.2% motility. The results showed that the freezing-thawing recovery rate of sperm using the SpermVD device was high and stable, however, the other parameters were unsatisfactory compared with the other platforms showed in the Table 2. Comparison of the frozen effects between the washed sperm group and neat sperm group, the PR, motility, recovery and viability rates were higher in the neat sperm group, and the differences of viability and motility rates reached significant level ($P < 0.01$). The results indicated that neat sperm had a better frozen effect and density gradient centrifugation might be not the applicable sperm preparation method for freezing a few human sperms.

Comparison of the frozen effects between the two kinds of dish, the PR, motility, recovery and viability rates of PICSI dish were higher in the neat sperm group. The PR and motility rates of ICSI dish were higher, and the recovery and viability rates of PICSI dish were higher in the washed sperm group. In addition, the viability rate had a significant difference between the two dishes ($P < 0.01$). The PR (23.9%), motility (41.2%), recovery (94.9%) and viability rates (80.3%) of the PICSI dish in the neat sperm group were the highest in this study, So, the results indicated that PICSI dish showed a better performance at selecting small number of neat sperm for freezing.

Discussion

The different frozen effects of sperm between the two preparation methods

According to the results showed in the Figs. 1 and 2, it could be observed that the PR, motility, recovery and viability rates of washed sperm were lower than that of neat sperm, and the differences in motility and viability rates reached significant level ($p < 0.01$). Moreover, the difference in PR was significant ($p <$

Table 1
The sperm parameters after thawing in the each group.

Group	Repeat	Frozen	PR	Motility	Viability	Recovery
ICSI dish and washed	20	208	19.8 ± 7.1%	32.4 ± 8.8%	65.1 ± 3.7%	93.7 ± 7.4%
ICSI dish and neat	20	217	22.0 ± 5.8%	40.2 ± 8.5%	69.4 ± 4.6%	94.2 ± 5.9%
PICSI dish and washed	11	117	16.3 ± 4.8%	29.8 ± 8.4%	70.0 ± 3.0%	94.6 ± 5.5%
PICSI dish and neat	11	122	23.9 ± 5.2%	41.2 ± 7.1%	80.3 ± 5.4%	94.9 ± 5.6%
Total	62	664	20.7 ± 6.4%	36.2 ± 9.5%	70.0 ± 6.6%	94.2 ± 6.1%

Table 2
Different devices used for freezing a few sperms.

Device	Source of sperm	Motility rate (Post thaw)	Recovery rate (Post thaw)	Researcher
Empty zona pellucida	Testicular	26.6%	88.2%	[6]
Spherical algae Volvox globator	Testicular	60%	100%	[7]
Agarose gel microspheres	Ejaculate	70%	78%	[8]
5 ul drops in a plastic dish	Testicular	100%	2%	[9]
High Security Straws	Epidiymal, testicular and semen	73.7%	69.7%	[10]
LSL straws	Semen	38.5%	73%	[11]
ICSI pipette	Testicular and semen	52%	92%	[12]
Cryoloop	Semen	73%	68%	[13]
Sperm VD	Ejaculate	41%	94.3%	[14]
Sperm VD	Semen	36.2%	94.2%	This study
PICSI dish and Sperm VD	Semen	41.2%	94.9%	This study

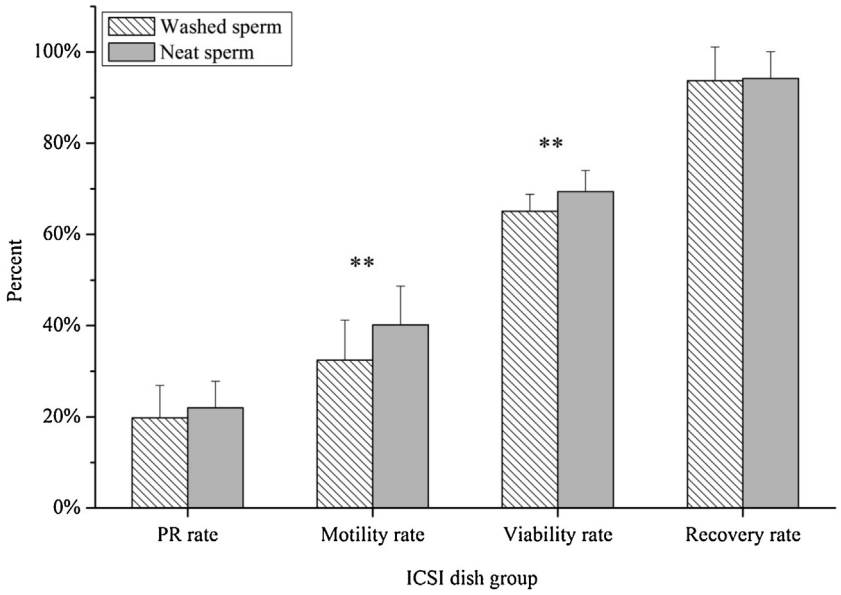

Fig. 1. The spermatozoa parameters in the ICSI dish group after thawing. ** respects the same volume were significantly different at 0.01 level.

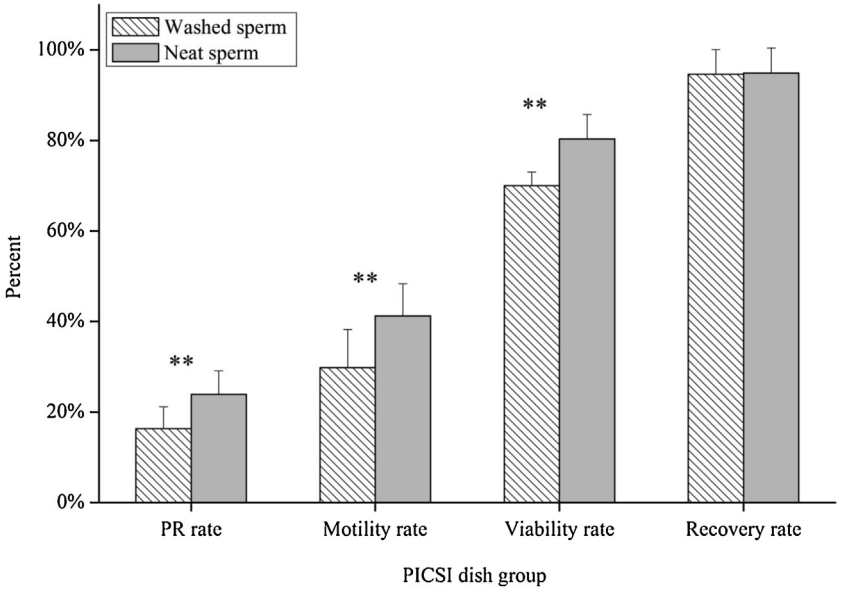

Fig. 2. The sperm parameters in the PICSI dish group after thawing. ** respects the same volume were significantly different at 0.01 level.

0.01) in the PICSI dish group. These results indicated density gradient centrifugation did not benefit for freezing a few human sperms.

PureCeption (SAGE In-Vitro Fertilization Inc. Trumbull, CT, USA), a commercially available density gradient centrifugation media, was used for sperm separation in this study, which is consisted of a colloidal suspension of silica particles and taurine as well as EDTA. density gradient centrifugation technique applies the principle that the density of morphologically normal sperm (1.10 g/mL) and that of morphologically abnormal sperm (1.06–1.09 g/mL) are different [15]. In theory, such processing procedure can help to select the highly motile and morphologically normal sperm, and the cellular debris as well as morphologically abnormal sperm will be removed [16]. However, the motility and viability rate of washed sperm were significantly lower than that of neat sperm. The results can be explained by the dilution effect [17], which will result in the irreversible loss of sperm viability after resuspension. In addition, the centrifugal forces entail the damage by mechanical stress and human sperm are sensitive to such stress [18]. Furthermore, centrifugation has been demonstrated to be related to oxidative damage, which have a negative impact in DNA integrity [19]. In this study, the positive effects of washing might not compensate the negative consequences.

The different frozen effects of sperm between the two kinds of dish

The PR, viability rate and motility rates of PICSI dish were higher than ICSI dish in the neat sperm group (Fig. 4) and the viability rate was significant difference ($p < 0.01$). Because, the sperm quality parameters such as motility and viability would be significantly decreased and DNA damage would be caused by freezing, especially in patients who had poor sperm quality [3,20]. In addition, PICSI sperm selection device (PICSI dish) was designed for the selection of mature sperm for intracytoplasmic sperm injection (ICSI), and mature sperms exhibited a high DNA chain integrity and developmental integrity, that ensure the frozen sperms are the optimal sperms from the samples [21] and the frozen effect was better after thawing. Since there was a negative correlation between levels of DNA fragmentation and sperm motility, theoretically, the motile ability of sperm in PICSI dish group should be higher than that of sperm in ICSI dish group before freezing, and the frozen effect in PICSI dish should be better than that in ICSI dish group. However, the PR and motility rates of PICSI dish were lower than that of ICSI dish in the washed sperm group (Fig. 3), that was not agreed with our expectation. The seasons might be that density gradient centrifugation had oxidative and mechanical damage which had a negative effect on the hyaluronan-specific receptor of mature sperm, so that mature sperms could not bind normally to the hyaluronan microdots of the PICSI dish.

The different platforms used for freezing a few sperms

There have been different types of containers (showed in the Table 2) used for freezing a few sperms. The frozen results of these carriers were different in different studies, and the reasons might be that different sperm quality, carriers, freezing solutions and methods of cryopreservation were different.

Though biological carriers can be used to store a small number of sperm, whose effectiveness has been proven in several studies [7,22,23], the application is limited due to the biological contamination. The process of zona preparation was labor-intensive and affected the retention of motile sperm [23]. Moreover, studies suggested lower motility, recovery and fertilization rates with human sperm cryopreserved in human zonae [24]. Though non-biological carriers can avoid the disadvantages of biological carriers, some of them aren't optimal for storing a small number of sperm. For example, once the sperm adhere to the vessel walls of the mini-straw, they will be lost. ICSI pipette has relatively high motility and recovery rates after thawing (Table 2), but ICSI pipette has a main problem that the tip of the ICSI pipette is fragile and can be easily broken.

In the study, The novel Sperm VD device used to freeze a few human sperms had the advantage of non-biological platforms and preventing biological contamination. The novel device is made of medical grade polycarbonate, including a handle and three sperms storage areas, so that it is simple and convenient. Because of the characteristics of freezing method, only small count of sperm be frozen, and the sperm quality parameters such as motility and viability will be significantly decreased and DNA damage will be caused by freezing, especially in patients who have poor sperm quality [3,4]. Hence, it was necessary to select high-quality sperm

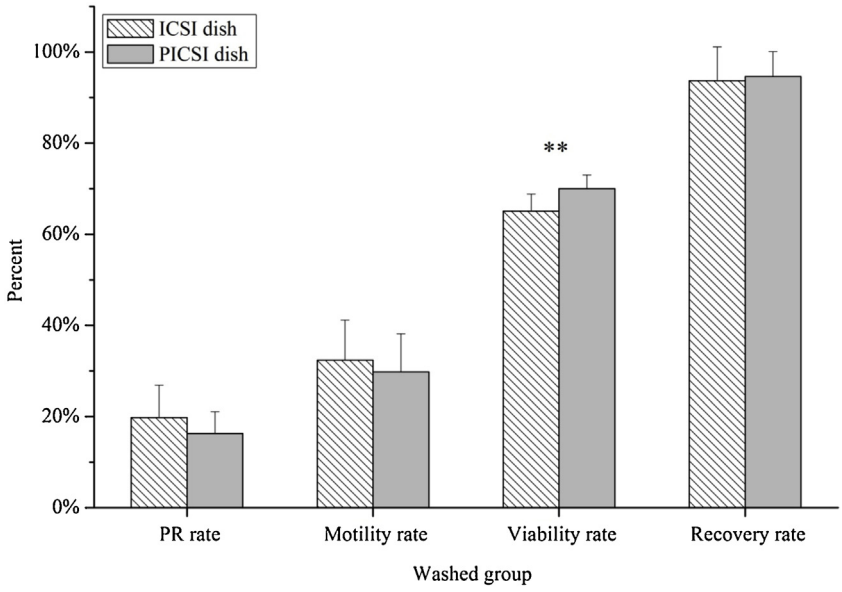

Fig. 3. The sperm parameters in the washed group after thawing. ** respects the same volume were significantly different at 0.01 level.

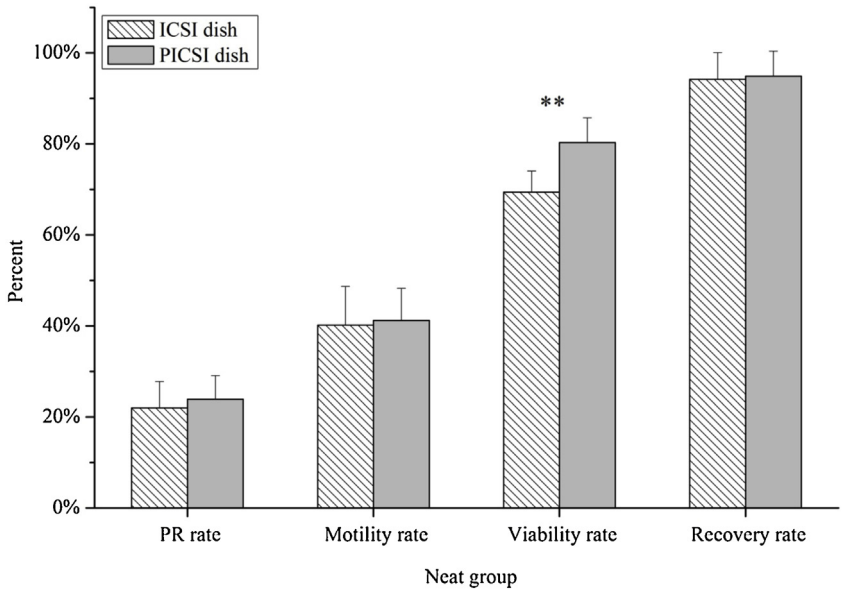

Fig. 4. The sperm parameters in the neat sperm group after thawing. ** respects the same volume were significantly different at 0.01 level.

for freezing. Compared to previous studies, the study used PICSI dish to selected small number of mature sperm for freezing and the performance was relatively good in freezing neat sperm, and the SpermVD device had a high and stable freezing-thawing recovery rate. It was reported that (Table 2) the motility rate (After thawing) of sperm stored in High Security Straws and CryoLoop were higher than that of sperm stored in traditional platforms, which demonstrated the effectiveness of High Security Straws (HSS) and CryoLoop for freezing a few sperms. However, no matter using Sperm VD with ICSI dish or using Sperm VD with PICSI dish, the motility rate of the sperm was not as good as HSS. The various motility rates might be caused by different sperm quality, freezing solutions and methods of cryopreservation used in the different studies. In addition, the advantage of PICSI dish is expected to be represents a natural alternative for selecting sperm, and hyaluronan-binding system favours selecting sperm with normal nucleus and intact DNA theoretically. Previous studies had demonstrated that the injection of HA-sperm could improved embryo quality, implantation capacity, fertilization rate, embryo quality and a reduction in the number of miscarriages when comparing HA-ICSI and conventional ICSI [25–30]. Therefore, DNA examination and clinical data are required to assess the effectiveness of Sperm VD and PICSI dish.

Conclusions

The SpermVD device is a novel and effective device in ART area for freezing a few human sperms. Density gradient centrifugation seems not to be the optimal semen preparation method for freezing, which needs further validation by assessing other sperm quality parameters except motility rate and comparing the effectiveness of different density gradient centrifugation techniques. Using PICSI dish to select neat sperm for freezing could improve the quality of sperm after thawing, and DNA fragmentation rate and outcomes of the fertility treatments (e.g., fertilization rate, high qualified embryo rate and ongoing pregnancy rate) should be used to assess the effectiveness of the Sperm VD with PICSI dish in the further study. In addition, the specific device needs to be continuously improved and different freezing solutions and methods of cryopreservation should be carried out to increase the motility rate of frozen-thawed sperm.

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