Harvest of sufficient motile sperms for ICSI in patients with severe oligoasthenoteratozoospermia

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Abstract

Background
Intra-cytoplasmic sperm injection (ICSI) can be a challenge in patients with severe oligoasthenoteratozoospermia (OAT). The method described herein permits the harvest of motile sperm from such samples.
Method
On an ICSI dish a thick wide rectangular streak of PVP was made and overlaid with oil. With a micropipette the middle portion of the pellet was loaded on to the bottom of the PVP streak and incubated at 37°C for 10 minutes. The PVP streak was pulled side-wards in 3-4 areas to form channels and then incubated at 37°C for 20 minutes. Motile sperm found inside the channels were immobilized for ICSI.
Results
Following ICSI, all the 9 patients' embryos had an average fertilization rate of >75%, an average subsequent blastulation rate of >35%. At least 1 blastocyst formed for each of the patients and was frozen on day 5 of embryo culture. For 1 of the 9 patients, sperm was retrieved via percutaneous epididymal sperm aspiration (PESA), and it led to 2 B grade blastocysts being formed and a subsequent live birth. The sample size in this study was too small for DFI assessment.
Discussion.
The methodology in this communication uses very fine channels of 7% polyvinylpyrrolidone as opposed to flushing media for sperm harvesting to (i) visualize progressively motile sperm from a pool of sperm with mixed motility that is surrounded by debris (ii) mimic a microfluidics sperm sorting maximizing the chances of finding motile sperm of good morphology. This method acts as a rescue for semen samples of severe OAT patients by attempting to separate morphologically normal motile sperm. This finding is preliminary until a larger cohort is investigated.
Conclusion
The technique described appears to assist in selection of morphologically better quality sperm. It mimics the principles of a microfluidic sperm sorting chamber which permits the visualization and isolation of motile sperm making ICSI faster and sophisticated. The present technique appears to improve fertilization and blastulation rates post ICSI for poor prognosis patients and for couples with severe male factor infertility.

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Introduction

Oligoasthenoteratozoospermia (OAT) is defined by the WHO semen parameter guidelines (WHO, 2020) as a sperm sample that has a count of less than 15 mill/ml, a normal morphology of less than 4%, and less than 30% progressive motility or less than 40% progressive plus non progressive motility. A borderline OAT sample does not pose a challenge for ICSI since only a handful of sperms are required. However, for semen samples whose count and motility are both less than 1% (WHO, 2020) or have only '1 or 2

twitching sperm per field' could pose a challenge to the ICSI operator as it is difficult to harvest motile sperm by standard sperm preparation methods. Furthermore, these sperms tend to have a higher DFI than sperm with better parameters (Quinn et al, 2018); but on the contrary, most sperm refinement techniques such as MACS, microfluidic sperm sorting via chambers, and PICSI cannot be performed on them due to extremely low count and motility. Such kind of sperm cannot be processed with density gradient centrifugation and need to be washed and spun with sperm wash media. This method concentrates too much debris in the preparation which causes motile sperm to be trapped in the debris and it becomes difficult to pick up the sperm by the injection pipette. The injection pipette may also get clogged with the debris.

Moreover, in some instances the technique for ICSI for such samples involves using flushing media as a sperm catching medium owing to its low viscosity (Torki-Bodalgi et al, 2016) so that sperms are not slowed down. If HEPES buffered flushing media is used to catch sperm, only 1 or 2 sperms can be held in the injection pipette at a time due to its low viscosity, which in turn causes the time taken for ICSI to be lengthened and oocytes are exposed out of the incubator for a longer period. It also affects the suction control of the microinjecting pipette and does not allow the embryologist to clearly observe the cytoplasmic resistance during oocyte injection. Due to the injecting speed being very high owing to the low viscosity of flushing media a large amount of flushing media enters the oocyte's cytoplasm which can damage the oocyte (Torki-Bodalji et al, 2016). Since Polyvinylpyrrolidone (PVP) has high viscosity to slow down motile sperm so they can be immobilized for ICSI, it is not the obvious choice as a sperm harvesting medium for a severe OAT patient sample. However, the present method described herein demonstrates how PVP can be used for poor semen samples such that sperm is sorted to select the morphologically best sperm, which can then be injected with minimal damage to the oocyte. It makes use of double incubation to lower the viscosity of PVP such that motile sperm trapped in semen debris can escape from it and be visualized. Using the principles of microfluidics where cells are manipulated to behave in a manner dictated by channels with flowing media (Figure 1; Samuel et al, 2018), PVP is used to create horizontal channels to sort sperm similar to what happens in a microfluidic sperm sorting chamber which contains micro-channels.

Material and methods

The materials used were standard media found in an ART/IVF laboratory. These included 7% PVP, Sperm washing media (Vitromed, SAR Healthline), HEPES buffered flushing media (Vitromed, SAR Healthline), Oil for embryo culture (Vitromed, SAR Healthline), and a 50 or 60 mm petri dish for ICSI (BD Falcon, SAR Healthline).

1 ml sperm wash media was added to the liquefied semen sample, and the mixture was centrifuged at 1600 RPM for 15 minutes. The supernatant was discarded and the pellet was concentrated, after which the sample was incubated at 37°C for 30 minutes. On a 50 mm or 60 mm ICSI petri dish, a thick wide rectangular streak of PVP was made, which was bigger in size than a PVP streak made for a patient with normal sperm parameters. Droplets of flushing media were made next to the PVP streak in which the oocytes were to be placed. The ICSI dish was then overlaid with oil.

With a micropipette set to 10ul, the semen sample was taken from the middle of the pellet and was loaded on to the bottom-most part of the PVP streak (Figure 3). The streak was overloaded with washed semen so that maximum amount of sperm could be recovered. The dish was then incubated at 37°C for 10 minutes. After the dish was taken out of the incubator the thick PVP streak was pulled side-wards in 3-4 areas to form channels (Figure 2). It was then incubated at 37°C for a further 20 minutes, 6-8 oocytes were placed in the flushing media drops, and then the dish was placed on the micromanipulator for ICSI.

The PVP streak was viewed under 640X and the corners of the channels were focused. Any sperm found inside any of the channels was immobilized (Figure 2). If no sperm were found inside the channels, then any sperm found at the edges of the upper channels were immobilized. If no sperms were seen in these, then sperm were harvested from the lower channels. If no

Figure 1: Shows a typical microfluidic sperm sorting chamber. The arrows indicate the flow of media via which sperm are separated. After washed semen is loaded at point A, sperm that is dead collects at D, sluggish sperm collects at B, and the best sperm that can swim sideways and upwards collects at C and can be used for ICSI. Sperm in black are motile, white sperm are immotile/sluggish.

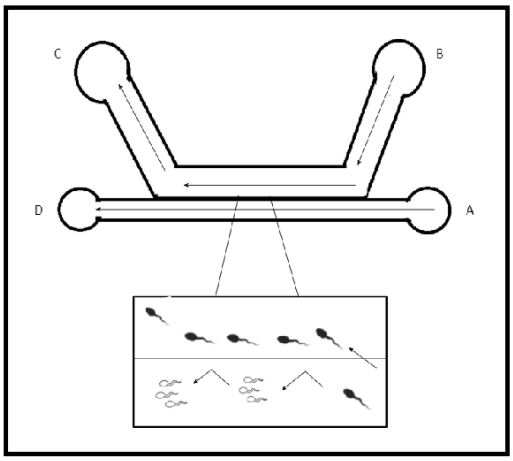


Figure 2: Shows the preparation of the dish for the technique. The PVP streak is made wide enough such that channels can be drawn from it. The semen is loaded at the bottom and allowed to move upwards. The white arrows indicate the points where the sperm should be viewed under at least 600x so that the morphologically best sperm can be selected, and these are also the points where the highest chances of finding motile sperm are in an OAT sample.

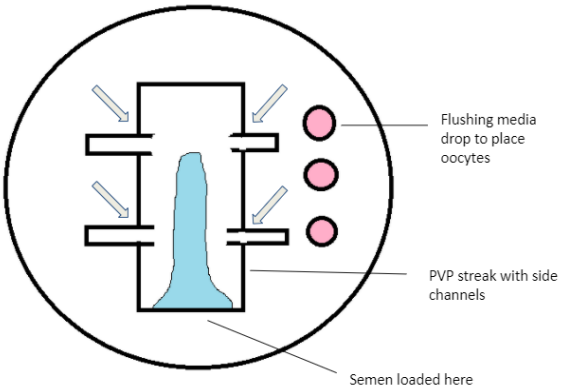
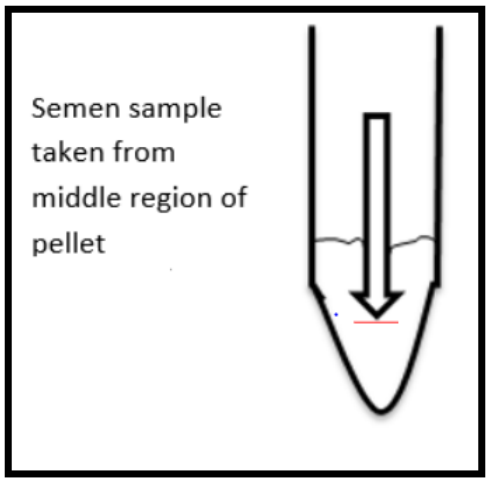


Figure 3: The semen sample to be loaded should be taken from the middle of the pellet as opposed to the top or extreme bottom of the test tube. This is because the top layer will not have enough sperm as they are not motile enough to swim up and the bottom most layer will contain a very high amount of debris.



sperm were found after these steps, the focus was shifted to the base of the streak where semen was loaded and the edges were zoomed in; any twitching sperm separated from the debris-filled semen in these areas were immobilized. This was repeated until the same number of sperms as oocytes were harvested and taken in the injection pipette, and ICSI was performed. Care was taken to space out sperm inside the injection pipette so as to not cause two sperm to enter a single oocyte at the time of microinjection. If two sperm were too close to each other in the injecting pipette at the time of injection, they were released out and the next sperm was taken for injection to avoid polyspermy. After the first set of 6-8 oocytes were injected with sperm, they were shifted to a culture dish containing fertilization media and the next set of oocytes were taken for ICSI. This was repeated till all the oocytes were injected.

Results

This technique was used for 9 patients whose sperm parameters were described as '1 sperm seen per field of view', or whose sperm count was <0.5 mill/mL with 0% progressive motility and between 1-2% non-progressive motility. Following ICSI, all the 9 patients' embryos had an average fertilization rate of >75%, an average subsequent blastulation rate of >35%. At least 1 blastocyst formed for each of the patients and was frozen on day 5 of embryo culture. For 1 of the 9 patients, sperm was retrieved via percutaneous epididymal sperm aspiration (PESA), and it led to 2 B grade blastocysts being formed and a subsequent live birth.

Since the sample size is very small and embryo transfers have not been done for all patients, a cumulative pregnancy rate cannot be calculated. The DFI of the sperm sample used for ICSI is not known because of extreme low count which made DFI assessment infeasible.

Discussion

Since density gradient centrifugation cannot be used to wash the semen of patients with severe OAT, a simple semen wash with sperm wash media followed by the isolation of motile sperm using the ICSI needle appears to be one common method in use in some centers. However, this leads to a high amount of debris being present in the sample and often the ICSI needle gets clogged with such debris. A variation to this method purposely overloads the washed semen at the bottom of the dish so that any sperm that are fully or partially motile will escape the debris-filled semen streak and collect at the corners of the PVP streak. Incubating the dish at 37°C after loading the semen and incubating it again after making the channels allows the viscosity of the PVP to continually decrease, which in turn allows for the free movement of motile sperm. Retaining the use of PVP as a sperm catching medium as opposed to flushing media allows for an optimum microinjection residue transfer from pipette to oocyte, ensuring that the oocyte does not contain any artificial remnants (Simopoulou et al, 2016).

Another method relied on the predilection of sperm to swim into narrow crevices. ICSI practitioners use streaks of canals radiating out from a droplet of buffered sperm medium depicting sunrays. A tiny portion of the pellet of the washed OAT semen is placed in the center of the sperm medium. The motile sperm in the pellet swam out of the pellet debris into narrow canals of medium which were harvested for ICSI (Ali, 2003). There are a number of permutations to this technique. In some instances the canal streaks are made of PVP instead of buffered sperm medium.

The process of using microfluidics for sperm sorting and selection is based on the principle that when sperm are manipulated with flowing media through channels, the better quality sperm are the ones that are able to swim upwards to a greater height, and also swim side-wards, hence overcoming the obstructions introduced via the micro-channels (Samuel et al, 2018). Since patients with OAT have very low sperm count and motility, a sperm sorting chamber or procedures such as MACS cannot be used to separate the good quality sperm from the bad quality ones. In addition, patients with poor sperm tend to have a higher DFI than patients with normozoospermia; and thus are in greater need of a method that can be used to extract the best of the sperm they have for an ICSI cycle (Nosrati et al, 2017).

In the above mentioned method described herein, after the washed semen is loaded onto the PVP, the sperm wash media mixed with the semen sample also spreads into the PVP streak

Table 1: Semen parameters of individual patient with the subsequent fertilization and blastulation rate. All patients had abnormal sperm morphology graded as '0% normal sperm' as per WHO sperm grading guidelines.

Serial No.	Sperm count (millions/ml)	Total sperm motility (%)	Fertilization rate (%)	Blastulation rate (%)	Outcome post embryo transfer
1	<0.1	2	90	32	Live birth -1
2	1	<1	70	35	Ongoing pregnancy
3	0.3	<1	70	30	Ongoing pregnancy
4	0.4	1	80	40	Ongoing pregnancy
5	1	<1	77	40	No pregnancy
6	<0.1	<1	73	36	No pregnancy
7	0.2	<1	75	33	Unknown
8	<0.1	<1	65	36	Unknown
9	<0.1	<1	80	55	Unknown

lowering its viscosity. This makes it easier for channels to be drawn on the sides, and sperm that have better motility are able to swim upwards and enter the channels, mimicking a sperm sorting chamber such as Qualis™. When sperm swim near the edges of a boundary, hydrodynamic sperm–boundary interactions result in surface accumulation and boundary-following behavior (Nosrati et al, 2017). Since the initial incubation post semen loading has caused the live sperm to separate from dead sperm and collect at the edge of the PVP streak, sperm that are capable overcoming the obstruction of a channel drawn at the edge of the PVP streak will enter the channel, and thus this sperm can be assumed to be of higher quality than those that could not swim upwards and side-wards. Observations from the results show that the sperm inside the channels when visualized under 640X at the time of ICSI, were morphologically better than the dead sperm stuck in the debris streak or the sperm that were twitching at the bottom of the streak.

Selection of this these sperm for ICSI in our study can appears to lead to better fertilization

and blastulation rates, improved embryo quality, and subsequently increase the chances of implantation and a healthy pregnancy. However the limitation of our study is its small sample size. Since sperm that are morphologically normal have a higher chance of having a lower DFI than morphologically abnormal sperm (Vingris et al, 2014), further research could involve testing the DFI of the sperm that have entered the channels as well as the sperm that are stuck in the semen streak debris post wash, along with pre-wash sperm in raw semen to observe changes in DNA integrity (Zhang et al, 2015). Another modification to this method could involve incubating PVP at two different temperatures - e.g. 35°C and 37°C and then making two streaks of different temperatures with a channel in between followed by loading the washed semen into the 35°C streak and only immobilizing sperm that has travelled to the 37°C streak.

Conclusion

The technique described herein appears to help select morphologically better quality sperm. It mimics the principles of a microfluidic sperm sorting chamber. The use of PVP for patients with severe OAT permits the visualization and isolation of motile sperm making ICSI faster and sophisticated. The present technique appears to improve fertilization and blastulation rates post ICSI for poor prognosis patients and for couples with severe male factor infertility.

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