Analysis of post-warming degeneration & apoptosis following porcine ovarian tissue vitrification using the ohio-cryo device

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1. Purpose

Ovarian tissue cryobanking for cancer patients is now offered in many reproductive centers. The ovarian tissue is mainly intended for future transplantation for fertility preservation purposes. This is of highest importance in cancer patients who are planned to receive gonadotoxic treatment.(1,2) Ovarian tissue is currently conventionally preserved by slow freezing methods. Vitrification at its current standards is now being anticipated to at least match if not excel in oocytes' cryopreservation.(3,4) Optimum vitrification should enhance the post-thaw viability of the specimens by minimizing or eliminating ice crystals formation. This is especially important with oocyte cryopreservation. Also, it is more convenient and cost effective as it can be executed in much shorter time than slow freezing, with no need to expensive cryoplanner devices. However, to achieve this, specimens should be rapidly and effectively contacted and cleared from vitrification solution series, which have high concentrations of cryoprotectants. Also, a rapid cooling and heating rates should be achieved. The vitrification of any whole organ would be expected to come at the expense of slowing the heat transfer to the organ cores to be only equal to the organ temperature conduction. The Ohio-Cryo is a novel device that allows the vitrification of any kind of processed tissue or a big load of cells in a closed system (US provisional patent 61/073,392).

The device allows rapid handling of tissue with a proper control on contact time with different types of media, rapid and even heat transfer & the vitrification in a minimal volume of media.
The device at its current conception was designed to accommodate up to one processed hemiovary per device. This seems a mandatory requirement for fulfilling transplantation purposes. Currently, successful vitrification of ovarian tissue was mostly achieved with mice ovaries in cryotops. However, using tiny loading devices may not be applicable for human ovarian tissue vitrification as it will require an excessively large number of loading devices. Human ovarian tissue was successfully vitrified with solid surface vitrification. However, the method may not be considered as a closed method, nor does it handle the tissue contact with the media, and may not be practical for highly fragmented, better equilibrated specimen as compared to the ohio cryo handling the ovarian sand. The purpose of our study is to provide some preliminary evaluation on the use of the Ohio Cryo vitrification system, in vitrifying tiny ovarian tissue fragments. Evaluation parameters were focused on the apoptotic index and the histological degenerative changes. The use of the device was compared with the fresh tissue, tissue damaged by direct contact with liquid nitrogen with no cryoprotection and the spontaneously induced apoptosis by prolonged hypoxia.

2. Methods and Materials

Seven porcine ovaries from 4 different pigs were collected immediately post-mortem. After removal of the medulla, the ovarian cortex was carefully sliced into about 0.5 mm thick slices, then swiftly chopped at 0.5 mm intervals in 2 perpendicular planes using Mcwillian tissue chopper to make tiny ovarian cubes of almost 0.125 mm, or what we can refer to as the "Ovarian sand". From each ovary and after fragmentation, a sample was formaline fixed as a fresh control. Another sample was incubated in leibovitz media in a 4 degree C refrigerator for 5 days to spontaneously degenerate, then formaline fixed. Another sample was directly plunged into liquid nitrogen to induce cryo-damage. The remaining sample was vitrified using the Ohio-Cryo. After warming, tissues were incubated for 2 hours in culture media at 37 degree C then fixed. All samples were paraffin embedded & cut at 5um thickness. For each sample, one slide was stained with H&E for histological assessment of degenerative changes. Degenerative changes were judged according to Wood’s criteria with slight modification (7). In each section, each follicle was given a score between 0 to 2. Given scores were 0 for no degeneration, 1 for slight degenerative changes & 2 for severe degenerative. An adjacent section was assessed for apoptosis using TUNEL. In a defined visual field, all granulosa cell were given a score as 0 for absent Tunel signal, 1 for mild, 2 for moderate & 3 for intense signal. Ten different fields were randomly chosen in a given slide (8)

The Following animations explain the use of the Ohio-Cryo to vitrify the Ovarian sand:

[Assembly of the Filtration Member] 1- Assembly of the filtration member

[Vitrification] 2- Vitrification

[Warming] 3- Warming

3. Results

The histologic degeneration scores for primordial follicles was 0.15± 0.16 for fresh samples, 0.17±0.23 for vitrified samples, 0.56± 0.55 for the cryo-induced damage samples & 1.75± 0.08 for the degenerating samples. The degenerative scores for preantral follicles were 0.47± 0.4 for fresh samples, 0.36± 0.27 for vitrified samples, 1.00± 0.34 for the cryo-induced damage samples & 1.52± 0.23 for the degenerating samples. As for the TUNEL, the apoptotic index was 0.23± 0.08 for the fresh samples, 0.32± 0.05 for the vitrified samples, 1.01± 0.34 for the cryo-induced damage samples & 1.34± 0.59 for the degenerating samples.
TUNEL & Histological assessment

There was no significant difference between vitrification & fresh tissue, with a $p$-value of 0.14 for TUNEL, 0.82 & 0.64 for the degenerative scores of primordial & preantral follicles respectively. These indices were all significantly higher than fresh & vitrified tissues when cryo-damage or spontaneous degeneration were applied. Compared to fresh tissue, TUNEL showed a significant increase in the apoptotic index with a $p$-value of 0.006 for cryo-damaged samples & 0.036 for degenerating samples. Histologic degeneration assessment score showed an increase in primordial follicles degeneration compared to fresh samples, with with a $p$-value of 0.22 (NS) for cryo-induced damage & a $p$-value <0.001 for spontaneously degenerating tissue. Preantral follicles showed an increase in the same index that was significant for cryo-damage ($p$ = 0.043) & spontaneous degeneration ($p$=0.015) as compared to fresh control.

4. Conclusion

The vitrification of ovarian tissue in the Ohio-Cryo did not induce significant degenerative changes nor increased the apoptotic index in the ovarian tissue as evidenced by H&E
examination and TUNEL assay.

With the swiftness of its handling a relatively large volume of processed ovarian tissue for vitrification in a closed system, it may provide a suitable solution for a reliable ovarian tissue vitrification. The device together with the processing technique provide a unique approach that is not currently being proposed in the industry. The ovarian sand created is most suitable for rapid and even contact equilibration and removal of high cryoprotectants concentrations, if compared to larger unprocessed or less processed specimens. Current closed loading devices are either limited in volume so unable to accomodate a similar tissue volume or large with no specific adjustments to compensate for an anticipated compromise in vitrification outcome by low endogenous heat transfer. The device also allows direct contact of the specimen to a rapidly cooling solid surface in an even fashion. With the porcine ovarian volume similarity to human ovary, we would expect to accomodate one human ovary in at least 2 Ohio-Cryo devices.

5. References


Assembly of the Filtration Member

A disposable filter is brought to the front of the device and held in place by an O ring that comes in place with a special O ring applicator.

Ohio-Cryo device: Exploded view

patent# 61/073,392
TUNEL & Histological assessment

Vitrification

First in Equilibration media is brought into contact with the tissue. The filtration device is then used to remove it. The vitrification media is then added, and after 60 seconds, the filtration device is placed and the vitrification media is removed. The device is capped, and brought to the liquid nitrogen.
Warming

The device is brought out of liquid nitrogen, then immediately brought to contact a water bath at 37 C. After 15 seconds, the device is uncapped, the filtration device is removed and thawing media is added. After 1 minute, the filtration device is removed, dilution media is added. After 5 minutes, the filtration device removes the media and the tissue is brought in contact with the washing media for at least 10 minutes.