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High cryo-resistance of SARS-CoV-2 virus: Increased risk of re-contamination at transplantation of cryopreserved ovarian tissue after COVID-19 pandemic

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ABSTRACT

Cryopreservation and re-transplantation of ovarian tissue after anticancer treatment is important medical technology. Today, during a pandemic, the risk of contamination of transplanted cells with SARS-CoV-2 virus is extremely high. Data about cryo-resistance (virulence and/or infectivity) of SARS-CoV-2 are limited. Analysis and systematization of literature data allow us to draw the following conclusions: 1) The cytoplasmic membrane of somatic cell, like envelope of corona viruses, consists of lipid bilayer and this membrane, like envelope of corona virus, contains membrane proteins. Thus, we can consider the cytoplasmic membrane of an ordinary somatic cell as a model of the envelope membrane of SARS-CoV-2. It is expected that the response of the virus to cryopreservation is similar to that of a somatic cell. SARS-CoV-2 is more poor-water and more protein-rich than somatic cell, and this virus is much more cryo-resistant. 2) The exposure of somatic cells at low positive temperatures increases a viability of these cells. The safety of the virus is also in direct proportion to the decrease in temperature: the positive effect of low temperatures on SARS-CoV-2 virus has been experimentally proven. 3) Resistance of SARS-CoV-2 to cryoprotectant-free cryopreservation is extremely high. The high viability rate of SARS-CoV-2 after freezing-drying confirms its high cryo-resistance. 4) The risk of SARS-CoV-2 infection after transplantation of cryopreserved ovarian tissues that have been contaminated with this virus, increases significantly. Our own experimental data on the increase in the viability of cancer cells after cryopreservation allow us to formulate a hypothesis about increasing of viability (virulence and/or infectivity) of SARS-CoV-2 virus after cryopreservation.

1. Introduction

A novel coronavirus (severe acute respiratory syndrome coronavirus 2, SARS-CoV-2), causing coronavirus disease (COVID-19) has recently produced unprecedented disaster for global health [55]. SARS-CoV-2 [21,54,61] is the third coronavirus to cross from an animal reservoir to infect humans in the 21st century, after SARS (severe acute respiratory syndrome coronavirus) [13,34] and MERS (Middle-East respiratory syndrome coronavirus) [59].

Cryopreservation of cells, tissues and organs for subsequent re-transplantation are important medical technology. Today, during a pandemic, the risk of contamination of transplanted cells with viruses, and especially the SARS-CoV-2 virus, is extremely high. Cryopreservation and subsequent transplantation of such contaminated cells is associated with the risk of transplantation of the SARS-CoV-2. It is because angiotensin-converting enzyme 2 (ACE2), as potential targets for SARS-CoV-2 infection, is widely expressed in different organs [32]. Taking into account this fact, the question of cryo-resistance of the SARS-CoV-2, the answer to the question “what happens with the virus during cryopreservation (freezing to deep temperatures, storage in cooling agent and thawing)?” is relevant. Aim of our review was an analysis and systematization of the respective published data.

2. Poor-water and protein-rich structure of SARS-CoV-2 virus: high cryo-stability

Viruses are complex “bimolecular capsules” with genomic nucleic acid (Deoxyribonucleic acid/Ribonucleic acid) and associated protein
SARS-CoV-2 viruses are spherical or ellipsoidal particle with size about 90 nm and total molecular weight about 40,000 kDa [34]. The internal content of the SARS-CoV-2 virus is protected from the adverse effects of the environment during the period when the virus is outside the cytoplasm of a host cell [43] by a shell, the so-called envelope [43]. This envelope embedded with a number of protein molecules [36]. The expected fact is that the viral envelope is structurally similar to the cytoplasmic membrane of a somatic cell. It is because envelopes are derived from the host cell membranes (phospholipids and proteins) (Fig. 1).

The aim of all cryopreservation protocols, without any exception, is to achieve the formation of cubic crystals of intracellular water, the size of which is commensurate with the size of smallest intracellular structures. Only in this case, a process of crystal formation, which is accompanying by a movement of growing crystals, will not be dangerous for intracellular structures. The formation of such small crystals can be achieved in three ways: using of permeable cryoprotectants, rehydrating of cells, or a combination of these both. Just the last method, when we use permeable cryoprotectants to achieve rehydration of cells and, at the same time, to bind the remaining after rehydration intracellular water, is sharply used now.

The membrane of somatic cells also consist a lipid bilayer with membrane, envelope and spike structural proteins [8,17,35]. Spike proteins are needed for interaction with the host cells.

The envelope proteins are 8.0–12.0 kDa in size and are composed of 76–109 amino acids are embedded in the lipid layer [41]. The spikes (each spike is about 20 nm long) are the most distinguishing construction of coronaviruses, and are responsible for the corona- or halo-like surface. On average a coronavirus particle has 74 surface spikes. Spike proteins are the most important components in the process of spread of infection and by this reason it is at present in a special focus of investigations [43]. Inside the envelope, there is the nucleocapsid, which is formed from multiple copies of the nucleocapsid protein bounded to the RNA genome [9,17].

It is generally known that water is the basis for an existence of biological objects. Without exception, all research in cryobiology is associated with the solidification of water at low temperatures. As a rule, water solidification is accompanied by formation of crystals. The task of applied cryobiology is to avoid the formation of crystals that would destroy intracellular structures during formation of these crystals. Theoretically ideal for cryopreservation of biological objects is the transition of water to a solid state without the formation of crystals, the so-called vitrification. However, it is practically impossible to achieve such state of water during cryopreservation of cells without losing of their viability.

The aim of all cryopreservation protocols, without any exception, is to achieve the formation of cubic crystals of intracellular water, the size

![Cell membrane and virus envelope with similar osmotic reaction and water (blue) inside](image_url)

**Fig. 1.** Schematic representation of somatic cell and SARS-CoV-2.
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cryoprotectants (Fig. 1). The cryoprotective properties of proteins are explained by the fact that they block the physicochemical receptors of water molecules. At cooling, these molecules cannot be formed into groups and clusters for formation of crystals. This is precisely what explains the increased cryo-resistance of the SARS-CoV-2 virus.

Study of structure of the SARS-CoV-2 virus, even without special cryo-investigations, suggests that this poor-water, protein-rich structure is very cryo-resistant.

3. Ovarian cells: positive effect of cooling

The exposure of somatic cells at low positive temperatures without increasing of their viability can be shown with the existing of technological protocols for transport of human ovarian tissue (Table 1). We analyze just this type of somatic cells because ovarian cells are the object of our investigations and we have respective experience in the field.

The Denmark group uses usual culture medium for 4–5 h transport of ovarian tissue at 4 °C [4,30,31,46,47]. German groups use medium to preserve the viability of the tissue by maintaining a stable temperature of 4–8 °C for 36 h [5,12,27–29,39,42,50,53]. Chicago group reported that cold temperatures are beneficial for the preservation on pranetal follicles [16,37]. The mentioned above data evidence about storage rate of viability of ovarian cells after cooling. This cooling was used for transportation of tissues from an operation room to a cryopreservation laboratory. These data are presented as a forced measure (Table 1).

What can be said about cooling of tissues for increase its vitality?

A positive effect of cooling of ovarian cells to low supra-zero temperatures on their future development after re-warming has been observed before and is not new. The routine protocol of cryopreservation of ovarian tissue in our clinic presupposes the obvious long-time precooling of tissue before freezing. It is because we have previously observed before and is not new. The routine protocol of cryopreservation of human ovarian tissue [23]. Also it was concluded that cooling of ovarian tissue to 5 °C for 24 h before cryopreservation decreased translocation of phosphatidylserine that evidences about increases the viability of the cells in the tissue after thawing [24].

It was described the positive effect of long-time pre-cooling of ovarian tissue before cryopreservation resulted to baby born after retransplantation [25,26] (Table 1).

4. SARS-CoV-2: positive effect of cooling

Temperature is critical factors in SARS-CoV-2 survivability [1].

The aim of experiments of Riddell et al. [45] was measurement of the survival rates of SARS-CoV-2 incubated at 20 °C, 30 °C and 40 °C. Viable virus was isolated for up to 28 days at 20 °C. It was established the half lives of SARS-CoV-2 virus between 1.7 and 2.7 days at 20 °C, and reducing to a few hours when temperature was elevated to 40 °C. Authors have detected a tendency of viability of SARS-CoV-2 virus to be increased with decreasing of storage temperature from 40 °C to 20 °C [45] (Table 1).

Guillir et al. [19] have summarized data from 3 publications [5,33,51] and presented the model for viability of SARS-CoV-2 by different temperatures: from 4 °C to 70 °C. This model demonstrates that a viability of SARS-CoV-2 is in direct correlation with the temperature of environmental: minimal at 70 °C and maximal at 4 °C (Table 1).

One data expected that the SARS-CoV-2 would be more stable at lower temperature conditions [10]. Authors have reported that virus is highly stable at 4 °C, but sensitive to heat. At 4 °C it was observed only partial reduction of infectious titer on day 14. With the incubation temperature increased to 70 °C, the time for virus inactivation was reduced to 5 min (Table 1).

After analyze of experimental data, La Rosa et al. [38] summarized that the titer of infectious virus is more stable at 4 °C than at 23 °C to 25 °C (Table 1).

Fisher et al. [18] have assessed the survival of SARS-CoV-2 after refrigeration of contaminated food. The samples were stored at 3 different temperatures (4 °C, −20 °C and −80 °C) and harvested at specified time points (1, 2, 5, 7, 14- and 21-days post-incubation). It was established that the titer of SARS-CoV-2 remained constant at 4 °C, −20 °C and −80 °C for 3 weeks in both the refrigerated (4 °C) and frozen (−20 °C and −80 °C) samples. It was shown that SARS-CoV-2 can survive the time and temperatures associated with transportation and storage conditions. SARS-CoV-2 to chicken, salmon and pork pieces there was no decline in infectious virus after 21 days at 4 °C (standard refrigeration) and −20 °C (standard freezing) [18] (Table 1).

Li et al. [40] checked the effect of SARS-CoV-2 storage temperature, and storage time after collection on SARS-CoV-2 by testing of nucleic acid. A total of 88 SARS-CoV-2-positive throat swab samples were obtained from hospital. The nucleic acid test was carried out within 2h after sampling with use of RT-qPCR. Tree temperature regimes were tested: 2–8 °C, 20 °C and 56 °C. It was established that viability of SARS-CoV-2 was maximal by storage of samples at 2–8°C (4 °C) [47] (Table 1).

Perchett et al. [44] have evaluated the stability of differing viral loads of SARS-CoV-2 over 28 days stored at room temperature, 4 °C, −20 °C, or −80 °C. Authors have used the first SARS-CoV-2-positive nasopharyngeal swab for spike-in material. For the high concentration of SARS-CoV-2, regardless of storage conditions, 100% of samples were detected by qRT-PCR through day 28. Storage at room temperature was associated with reductions of positivity beginning at day 7, and by day 28, 0% of samples were detected. At 4 °C, there was minimal change in positivity over time at the higher viral concentration. For lower titers, positivity increased over the 28 days. At −20 °C, lower titers of virus fluctuated slightly more. Storage of SARS-CoV-2 at −20 °C was the second least stable condition, accounting for 37.5% of negative PCR results. Storage at −80 °C showed the greatest stability, with all samples detected throughout the 28 days. Authors concluded that the stability of SARS-CoV-2 can be maintained at 4 °C for up to one month when −80 °C storage is not available [44] (Table 1).

Thus, we can draw a parallel between the cryo-resistance of ovarian

| Table 1  | Ovarian cells and SARS-CoV-2: effect of cooling. |
|----------|---------------------------------|
| Object   | Treatment                        | Effect                                      | References                  |
| Ovarian  | Transportation at 4 °C in culture medium for 4–5 h | Storage of viability | [4,30,31,46,47] |
| Ovarian  | Incubation at 20 °C, 30 °C, and 40 °C | Increasing of viability | [23–26] |
| Ovarian  | Cooling of ovarian tissue at 5 °C for up to 24 h | Storage of viability | [16,37] |
| Ovarian  | Cooling of tissue at 4 °C for up to 24 h | Increasing of viability | [23–26] |
| SARS-CoV-2| Incubation at 20 °C, 30 °C, and 40 °C | Minimal viability at 70 °C, maximal at 4 °C | [7,10,20,33] |
| SARS-CoV-2| Incubation at 23–25 °C | Viability at 4 °C higher than at 23–25 °C | [38] |
| SARS-CoV-2| Storage at 4 °C, −20 °C, and −80 °C | Viability is constant at 4 °C for 4 °C, −20 °C and −80 °C for 3 weeks in both the refrigerated (4 °C) and frozen (−20 °C, −80 °C) samples | [18] |
| SARS-CoV-2| Storage at 2–8 °C, 20 °C, 56 °C | Minimal viability at 56 °C, maximal at 2–8 °C | [40] |
| SARS-CoV-2| Storage at 20 °C, 4 °C, −20 °C, −80 °C | High viability at 4 °C | [44] |
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5. Resistance of SARS-CoV-2 to cryoprotectant-free cryopreservation

In experiments of Walker et al. [52] SARS-CoV-2 was experimentally inoculated into human milk samples. Each sample was layered onto uninfected cells after Holder pasteurization (56°C and 63°C for 30 min), or after 48h of storage at 4°C or –30°C. It was established that after heating to 63°C or 56°C for 30 min, SARS-CoV-2 was undetected. Cold storage of SARS-CoV-2 in human milk at 4°C or after freezing to –30°C have not significantly impact infections over a 48 h period. The demonstrated stability of SARS-CoV-2 in refrigerated or frozen human milk can evidence about a stability of this virus during cryopreservation without permeable cryoprotectants [52].

Experiments of Adrian et al. [2] indirectly shown a high cryo-resistance of viruses. These viruses in 1 μm of cryoprotectant-free suspension were cryopreserved by direct plunging into liquid nitrogen. After electron microscopy evaluation of viral particles it was noted that they were free from the kind of damage caused by dehydration and crystal formation [2].

An interesting view on the cross-contamination of cryopreserved cells by SARS-CoV-2 is recently presented by Arav [3]. In the opinion of the author, which we fully share, cryopreservation of cells by vitrification with direct contact with liquid nitrogen poses a particular danger. In the context of our review, it can be the vitrification of non-isolated ovarian fragments, which, for example, is recommended by some authors for performing of IVA- (in vitro activation of ovarian tissue) procedure. In this case, re-transplantation of SARS-CoV-2 during re-transplantation of ovarian tissue seems to be almost inevitable [3].

6. Risk of SARS-CoV-2 Re-Contamination through Re-Transplantation of cells

At present it was detected that a female reproductive tract is less impacted by SARS-CoV-2 than a male [48]. In the same time, angiotensin-converting enzyme 2 (ACE2), as potential targets for SARS-CoV-2 infection, is widely expressed in the ovary [20].

Mechanism of viral entry includes the binding of SARS-CoV-2 spike glycoprotein and the host receptor angiotensin converting enzyme 2 (ACE2). Protease of host cell, transmembrane serine protease 2 (TMPRSS2) is then involved to cleaving of the viral spike protein and fusion of the viral and host cell membranes [37,60].

In experiments of Stanley et al. [49] it was identified seven ovarian cell types, including oocytes and six somatic cell types (stromal cells, granulosa cells, smooth muscle cells, natural killer cells, macrophages, and endothelial cells. ACE2 and TMPRSS2 expression was restricted to germ cells. A moderate correlation between ACE2 and TMPRSS2 in ovarian cells was driven entirely by expression in oocytes [49].

7. Freezing-drying of SARS-CoV-2 as evidence of high cryo-stability

At present, technology of freezing drying (lyophilization) of viruses is used by biomedical industry very brightly. In fact, the use of this technology, can demonstrate an extremely high cryo-resistance of viruses. Results of original investigations performed on SARS-CoV-2 were published by Xu et al. [56]. Authors have described an optimized freeze-drying protocol that allows to transport SARS-CoV-2 PCR mixes and stored at ambient temperatures, without loss of activity.

Timely, most effective technology for diagnosis of COVID-19 is the real-time reverse transcriptase-polymerase chain reaction (rRT-PCR) [56]. However, transport and storage of PCR reagents for the following detection of virus RNA must be realized at a low temperature. Freezing-drying (lyophilization) can be a way for simplification of protocol for such transport and storage.

It was described the following materials and methods of experiments [56]. The collected specimens were stored in a 1.5-ml freezer tube at –80°C before nucleic acid extraction. RNA was extracted using the purification system. The rRT-PCR procedure was performed with encoding of nucleocapsid and spikes proteins of SARS-CoV-2. For freezing-drying the PCR mixes were supplemented with 10% trehalose, 1.25% mannitol, 0.002% BSA and 0.075% polyethylene glycol with mol. weight 20 KD and aliquoted into PCR tube strips.

The procedure of freezing-drying was performed as follows: –40°C for 720 min, –20°C for 60 min, 0°C for 60 min, 10°C for 60 min, and 25°C for 480 min. The pressure of the freeze dryer chamber was maintained at less than 100 mTorr throughout the freeze-drying. Once the freeze-drying was completed, the dried mix was packaged into an aluminium foil bag using a vacuum packaging machine. The sensitivity of the freeze-dried PCR reagents was tested by the SARS-CoV-2 plasmid. The freeze-dried PCR mixes were stored at ambient temperature, 37°C, and 56°C, and then reconstituted to their original volume with nuclease-free water. Authors have reported that the sensitivity, specificity, and repeatability of the freeze-dried reagents were similar to those of freshly prepared, wet reagents. The freeze-dried mixes retained activity at room temperature (18–25°C) for 28 days, and for 14 and 10 days when stored at 37°C and 56°C, respectively [56].

8. Cancer cells: increasing of viability after cryopreservation

In order to underline the “universality” of our hypothesis about the positive effect of cryopreservation on viability of different cells, we would like to consider such effect on cancer cells.

We have demonstrated an increase in the viability of cancer cells (in relation to these cells the term ‘malignancy’ is using) after cryopreservation. It was concluded that cryopreservation promotes breast cancer cells in terms of epithelial-mesenchymal transition and angiogenesis induction, thus increasing metastasis risk.

For short, in our experiments from cells of ZR-75-1 and MDA-MB-231 lines it was formed monolayer [15]. With aim to manipulate with a model of ovarian pieces, it was formed compacted fragments [14,15,22]. Then cancer cells were frozen and thawed by protocol of the ovarian tissue cryopreservation [23–26,28,29]. Cell proliferation, motility, and penetration were characterized by CCK-8, wound-healing, and transmembrane assay, respectively. The expression of Ki-67, P53, GATA3, E-cadherin, Vimentin, and F-Actin was captured by immunofluorescent staining and western blotting. The chorioallantoic membrane (CAM) xenotransplantation was conducted to explore angiogenesis induced by cancer cells [15]. It was established that some cryopreserved ZR-75-1 cells presented spindle shape with filopodia and lamellipodia and dissociated from the cell cluster after cryopreservation. Cell of both lines demonstrated increased cell migrating capability and invasion after cryopreservation. The expression of Ki-67 and P53 did not differ between the cryopreserved and non-intervened groups. E-cadherin and GATA3 expression downregulated in the cryopreserved ZR-75-1 cells. Vimentin and F-actin exhibited an upregulated level in cryopreserved ZR-75-1 and MDA-MB-231 cells. The cryopreserved MDA-MB-231 cells induced significant angiogenesis around the grafts on CAM with the vascular density [15].

It would be logical to assume (to formulate our hypothesis) that the viability of viruses (the terms “virulence and/or infectivity” are using for viruses) also increases after cryopreservation.
9. Hypothetical assumption about increasing of the SARS-CoV-2 viability after cryopreservation

In this review it was shown the positive effect of low temperatures (from 0 to 8 °C) as well as conventional cryopreservation (with a decrease of temperature at a rate of 0.3 °C/min) on somatic cells. After such influences, viability of ovarian cell was increased. It has also been shown that the viability of SARS-CoV-2 virus can be maintained at low positive temperatures for a long time.

It is known that at least five negative effects observed during cells cryopreservation: hypoxia, increasing of intracellular Ca²⁺, osmotic disruption of cellular membranes, generation of reactive oxygen species (ROS) and lipid peroxidation. All these knowledge were obtained on somatic cells. Data about cryo-stability of SARS-CoV-2 (the answer on the question “what is happened with viability of this virus after freezing and thawing?”) are limited. This question is actual because at presence of virus in tissue this virus “automatically” will be frozen and thawed.

It would be logical to assume (to formulate our hypothesis) that the viability of viruses (the terms “virulence and/or infectivity” are using for viruses) also increases after such cryopreservation.

Analysis and systematization of literature data allow us to draw the following conclusions:

1. The cytoplasmic membrane of somatic cell, like envelope of corona viruses, consists of lipid baitlayer and this membrane, like envelope of corona virus, contains membrane proteins. Thus, we can consider the cytoplasmic membrane of an ordinary somatic cell as a model of the envelope membrane of SARS-CoV-2. Somatic cells can be evaluated as a model of SARS-CoV-2. It is expected that the response of the virus to cooling and freezing is similar to that of a somatic cell. SARS-CoV-2 is more poor-water and more protein-rich than somatic cell, and this virus is much more cryo-stable.

2. The exposure of somatic cells at low positive temperatures increases a viability of these cells. The safety of the virus is also in direct proportion to the decrease in temperature: the positive effect of low temperatures on SARS-CoV-2 virus has been experimentally proven.

3. Resistance of SARS-CoV-2 to cryoprotectant-free cryopreservation is extremely high. This type of freezing is the first stage of freezing-drying. The high viability rate of SARS-CoV-2 after freezing-drying confirms its high cryostability.

4. The risk of SARS-CoV-2 infection after transplantation of cryopreserved cells and tissues that have been contaminated with this virus increases significantly.

10. Conclusion

The exposure of ovarian cells as well as SARS-CoV-2 virus at low positive temperatures increases a viability of these cells. Cryo-resistance of SARS-CoV-2 is extremely high. Our own experimental data on the increase in the viability of cancer cells after cryopreservation allow us to formulate a hypothesis about increasing of viability (virulence and/or infectivity) of SARS-CoV-2 virus after cryopreservation. The risk of SARS-CoV-2 infection after post-cancer transplantation of cryopreserved ovarian tissues that have been contaminated with this virus increases significantly.

Declaration of competing interest

The authors declare no conflict of interest.

Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| ACE          | Angiotensin-converting enzyme |
| BSA          | Bovine serum albumin |
| EBOV-GP      | Ebola glycoprotein |
| FSH          | Follicle stimulating hormone |
| MERS         | Middle-East respiratory syndrome coronavirus |
| RNA          | Ribonucleic acid |
| ROS          | Reactive oxygen species |
| RT-qPCR      | Real-time quantitative polymerase chain reaction |
| SARS-CoV-2   | Severe acute respiratory syndrome coronavirus 2 |
| TEM          | Transmission electron microscopy |
| Tmprss       | Transmembrane serine protease |
| ZR-75-1      | Human breast cancer cell line |

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Authors’ information

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