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RESEARCH ARTICLE

Good Tissue Practices (GTP) for the Cryopreservation of Reproductive Tissues in an Era of Pandemic-mania: What is the Best Practice Policy?

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ABSTRACT
The global COVID-19 pandemic of 2020 to date has altered all our lives and how clinical medicine is practiced. Faced with a highly infectious coronavirus, specifically known as Severe Acute Respiratory Coronavirus-2 (SARS-CoV-2), in our homes, clinical environment and laboratories, we have applied preventative measures to potentially control and contain this mysterious pathogen. Although the concepts of frequent handwashing/ disinfection, social distancing and face mask wearing were rigidly applied with regional variation of acceptance in the USA, clinics and reproductive biology/IVF laboratories adapted strict global policies for a safer workplace for our staff, patients, and the specimens we handled. We address the concerns that resurfaced with the COVID -19 pandemic, regarding potential disease transmission between hosts, reproductive tissue (sperm, oocytes and embryos), recipient uteri and the fetus. To what extent were preventative measures sufficient and is there a need for adopting "best practices" above and beyond established "good tissue practices". In fear of future pandemic disease events ("pandemic-mania") impacting fertility treatments, this paper addresses the rationale and benefits of adhering strictly to best practices, like the use of secure closed system biocontainment in cryostorage as an important preventative measure. Additionally, historical and current perspectives are discussed and the ability to change established practices under the guise of commercial influences.

Keywords: Covid-19, Reproductive Medicine, Oocyte, Embryo, Sperm, Cryopreservation

Introduction

In our Assisted Reproductive Technologies (ART) laboratories we are faced with the inevitable question "What is the risk of disease transmission to and between human embryos, gametes and reproductive tissue in their production, cryostorage and use? Though it had been widely accepted for decades, as reviewed by Pomeroy and colleagues [1], that the viral transmission of pathogens between gametes, embryos and patients was historically deemed negligible, the global COVID -19 pandemic has had us take a fresh look at the biosecurity and effectiveness of our standard operating procedures (SOP's). In particular, the implementation of new ART practices in the last decade, like laser zona dissection and embryo biopsying, have destabilized the protective nature of the zona pellucida [2]. It is one thing to adhere to the principles of the Food and Drug Administration's Good Tissue Practice (GTP) or the guidance of the European Union and the European Society for Human Reproduction and Embryology (ESHRE), but what are the best practices that should be implemented for the safe and secure storage of gametes (sperm and oocytes), embryos and reproductive tissues communally cryopreserved in liquid nitrogen (LN2) storage tanks.

SARS-CoV-2 is an insidious, infectious airborne virus that is easily spread to any surface for susceptible touch transmission. However, good hand hygiene practices effectively eliminate possible touch transmission as this virus is readily susceptible to disinfection by soapy detergents. Yet, this killer virus has had our attention for over two years as several strains have mutated by eluding eradication through strict vaccination and prevention programs. In contrast to deadly hemorrhagic disease viruses, like Ebola, that possess low transmission rates due to their bloodborne nature, respiratory viruses (like Covid-19 and seasonal influenza) are highly infectious. By mid-July 2022, there had been nearly 550 million confirmed global cases of COVID -19, with over 6,350,000 global deaths (1.2%; see Fig.1 WHO update). Death is not the only harm a disease can cause, we must also consider morbidity and aspects of SARS-CoV-2 –related disorders that are just being realized. How many people will experience "long-Covid" and be impaired for life after SARS-CoV-2 infection? The focus of this paper is on how COVID -19 can impair the fertility of our patients undergoing treatment, the health of all developing fetuses and how to minimize any potential adverse effect that could arise.

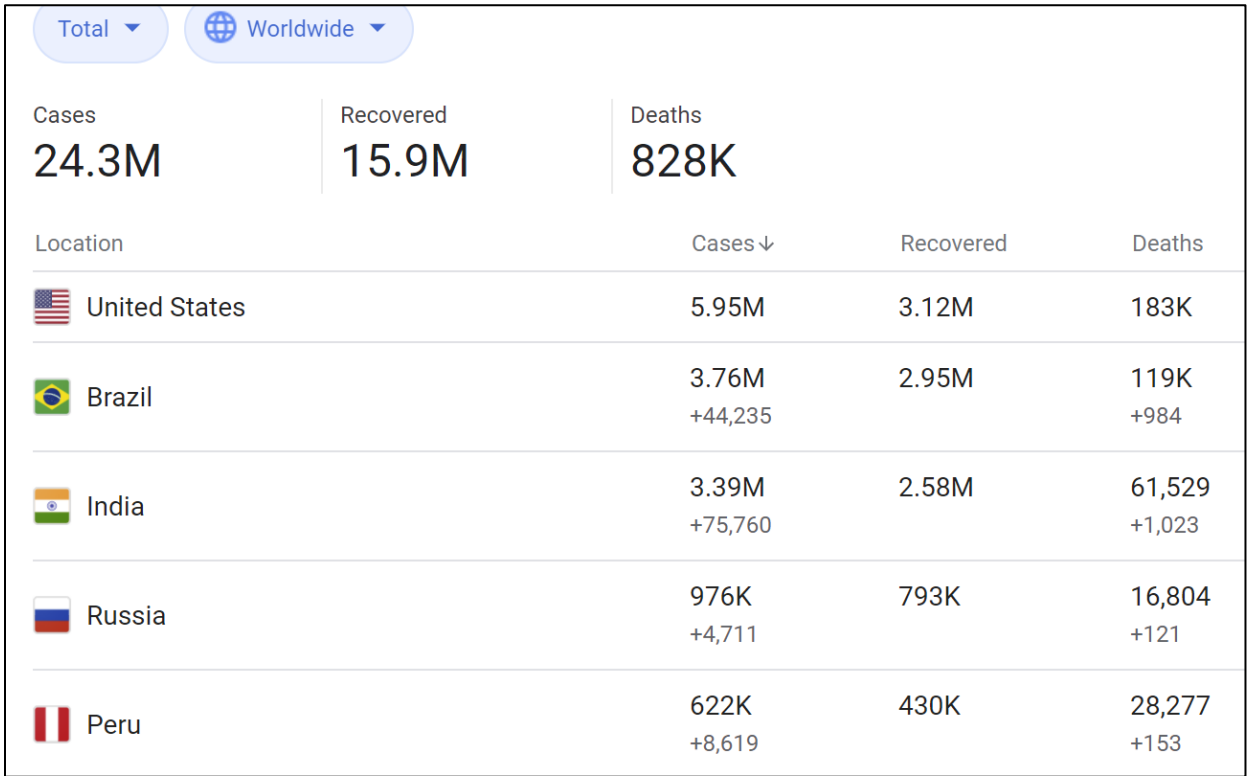

Figure 1. July 15, 2022: Update on the actual COVID -19 transmission event rates to the top 5 most impacted countries as calculated by the World Health Organization (WHO).

Like the Spanish flu and H1N1 influenza [3], SARS-CoV-2 has proven that global pandemics are not hypothetical events. They create fear and turmoil (i.e., mania) that can significantly influence society as a whole. With all new pathogens there is a learning curve to understanding its transfection, treatment and long-term impact on society. In our recent experience with Covid-19, the important questions we sought to answer, as it relates to reproductive treatments, was: 1) can ova, sperm or embryos vertically transmit COVID -19 to recipients of these tissues and how we can minimize those risks?; 2) can SARS-CoV-2 from the tissue of an infected patient be passed on to that patient or their offspring after transfer?; and 3) can the presence of SARS-CoV-2 in frozen reproductive specimens cross-contaminate tissue stored in the same storage tank?

How should we handle potential infectious microorganisms in ART laboratories today?

With the onset of COVID-19, we implemented strict containment, disinfection and risk reduction practices to our staff and patients [4]. Emergency staffing plans, including rotations and skeleton crews, were needed to mitigate peak exposure periods, while following PPE, temperature/symptom monitoring and frequent handwashing practices to effectively reduce direct vertical transmission interactions. Alternatively, the concept of transmitting pathogens via gametes, embryos and other reproductive tissue (i.e., ovarian, testicular) to both hosts and recipients has long been a concern in the animal agriculture industry, fearing the global economic consequences of an epidemic. Historically, embryo transfer technologies have been used to by-pass the vertical transmission of diseases between live animals. As highlighted by Pomeroy and Schiewe [2], such experiences included recovering embryos from donor cattle in Canada in the mid-1970's and transferring them to recipient cows in the USA via live rabbit couriers whose ligated oviducts temporarily incubated the embryos. By avoiding live-animal importation of cattle, veterinarians and ranchers circumvented a 7-year USDA quarantine for farm animals harboring potentially deadly diseases. Based on controlled studies with various infectious diseases of concern to farm animals and related non-domestic species in the 1980's, international organizations (e.g., OIE, USDA) began developing regulations and SOP's. This included a specific 10-step embryo wash procedure, incoporating a trypsin exposure/wash step, to minimize the risk of potential disease transmission. The scientific background and related procedural guidelines have been published by the International Embryo Transfer Society (IETS Manual) [5]. Thus, developed countries have long been concerned about triggering an epidemic in their animal agriculture industry that could financially and physically disrupt the food chain and economy. The Covid-19 pandemic has reinforced those concerns, by indirectly disrupting global logistics and socio-economic stability.

In the context of human IVF, the presence of infectious organisms is not new to reproductive laboratories. Any patient performing IVF and cryobanking specimens is required to perform infectious disease screen (IDS) panels for HIV-1/HIV-2, Hepatitis B and C, Syphilis, Chlamydia, Gonorrhea, CMV, as well as HTLV I/HTLV II (per some State regulations, e.g., California requirement for cryobanking semen). Furthermore, other novel viruses, like West Nile Virus, have become an FDA requirement for third party gamete and/or embryo use. Other regional viruses may be of concern when screening for other possible patient exposures. In reality, we have been living with viruses in our environments (home, work, laboratory) for many decades without particular regulatory concern (e.g., measles, rabies, tuberculosis, salmonella, meningococcal diseases, pneumococcal infections, Bordetella pertussis, and herpes).

The question arises whether we should in fact be concerned with all environmental pathogens in laboratory air and, in particular, cryostorage areas? Bielanski has extensively studied animal embryo-pathogen interactions [6], previously detailing the cross-contamination cycle between the environment, cryostorage tanks, specimens and ultimately our in vitro culture systems or in vivo transfection [7]. In short, the air around us contains pathogens, and we ourselves shed skin cells that contribute to the emission of bacterial and fungal genome product [8]. Although most human IVF/ART laboratories try to control and minimize the latter exposures by use of dedicated HVAC/air purification systems with at least 6 air exchanges/hour, cryostorage areas may be particularly vulnerable to contamination over time as each opening of a cryotank presents an accumulation opportunity. While the viability of gametes and embryos are freeze-preserved indefinitely, so too are some pathogens in their surrounding LN2.

What special precautions do we currently take to avoid contaminating embryos, ova, nitrogen storage tanks or infecting patients? Up to the recent COVID -19 pandemic starting in 2020, zero cases

have ever been identified where IVF-derived oocytes and embryos (e.g., culture of embryos, cryopreservation or storage of embryos/ova) have resulted in the production of a disease in a patient or recipient of donor reproductive tissue, as reviewed by Pomeroy and colleagues [1,2]. It is commonly agreed that the methods used today for IVF and cryopreservation essentially help avoid pathogen transmission simply by repeated washing steps (i.e., significant dilutions) reducing potential vector transmission to a negligible level of risk.

As stated earlier, veterinarians, reproductive biologists and epidemiologists in animal agriculture have long been concerned about controlling potential epidemic spreads by employing strict embryo and oocyte handling practices. Thus, when a global pandemic occurred in 2020 to date, it heightened our senses in the clinical community relative to the probability of a SARS-CoV-2 contamination between host, reproductive tissue and recipients. What impact would Covid-19 have on fertility treatment either in the short-term (i.e., acute patient exposures) or long-term (i.e., laboratory exposures and cryo- storage)? We entered a new dimension of "Pandemic-mania", where strict new SOP's were established for screening patients and staff (e.g., temperature checks, exposure survey), reducing clinical exposures by limiting onsite visits (e.g., couple consults, partner participation), mandatory preventative measures enforced (e.g., handwashing, face masks, social distancing), and rigorous decontamination practices [4].

In looking at the potential source of infectious diseases affecting patients undergoing IVF we needed to look at the three major tissue sources: sperm, oocytes and embryos.

Sperm

Sperm are ejaculated with a non-sterile fluid that is often contaminated with white blood cells, red blood cells, bacteria and viruses. Although sperm is often purified from semen using filtration techniques, it can still contain microorganisms. What makes this of concern with SARS-CoV-2 is that men that have been infected with COVID-19 have been shown to have the virus in their semen [9]. The presence of the virus in the semen of sick men created some concern regarding its possible sexual transmission, however 2 years later there is no evidence to support that concern. Thus, fears that sperm donors with repeated negative PCR viral testing may harbor SARS-CoV-2 in their testicular tissue/semen due to a minimum threshold of sensitivity after healthy men recover from COVID-19 are negated [10].

It has long been known that the seminal plasma of neat semen harbors the vectors for the possible viral transmission of disease, including HIV [11]. A simple sperm wash procedure that separates progressive motile sperm from the seminal supernatant reduces HIV levels greater than 10,000-fold among infected patients, simply by dilution [12]. Density gradient centrifugation combined with sperm swim-up further separates seminal components (e.g. lymphocytes) from the motile sperm but does not completely eliminate HIV-1 or HCV RNA. Loskutoff and co-workers [13] designed a double lumen centrifugation tube with a side port to load gradient columns and specimen, as well as a central channel to directly sample the pellet cleanly without exposure to gradient contaminates. They later added a middle trypsin layer to effectively lyse surface proteins on the HIV and HCV viruses, followed by soybean trypsin inhibitor in the 90% layer to deactivate the enzymatic reactions. This device/methodology proved to significantly eliminate viral RNA below detectable levels when combined with a two-step wash (i.e., additional dilution effect). Ultimately, the Proinsert device by Nidacon was developed for clinical use [14] to recover viable, motile sperm pellet fractions while minimizing any risk of viral contamination. Therefore, the use of this device and method should logically be applied to all patients suspected or known to be a viral carrier, including SARS-CoV-2.

Oocytes

The zona pellucida (ZP) is an acellular protective coat that surrounds oocytes and early embryos to the blastocyst stage. While there is no biological reason for SARS-CoV-2 binding ACE-2 receptors to reside on the ZP or vitelline membrane of the oocyte, the cumulus cells of the cumulus-oocyte complex likely possess receptors to the virus. Post oocyte retrieval, the stripping of cumulus cells should be performed to facilitate intracytoplasmic sperm injection (ICSI) as well as reduce the viral exposure risk prior to fertilization. If viral binding receptors were verified on the outer ZP, presenting a viral risk, the concept of a 10-step trypsin wash as adopted by the International Embryo Transfer Society [5] over two decades ago for the exportation of livestock embryos, would be an advisable preventative measure. Brief exposure of a ZP-intact ova or embryo to trypsin followed by a neutralizing soybean trypsin inhibitor wash step would pose negligible risk to developmental competence.

Embryos

The answer to whether embryos can become infected by the SARS-CoV-2 virus is still unknown. There is evidence though that blastocysts likely have receptors for the virus on their outer trophoectodermal cells [15], yet it remains to be seen whether the embryo can be infected by the virus.

In the last several years, PGT-A and the biopsying of embryos has become quite popular. One must examine the increased risks of infecting an embryo with a virus in a system where the protective coating of the ZP is commonly breached during embryo micromanipulation. Fortunately, this breach is often made after several passages through solutions, resulting in a significant dilution factor. Embryologists should consider increasing dilution steps to avoid the possible transfer of viruses when handling embryos before biopsy and vitrification. Since not performing biopsies may not be a realistic possibility, other risk mitigating measures have been proposed. In addition, practical liquid nitrogen sterilization methods have been developed and might be useful at minimizing viral exposure risks during vitrification, cryostorage and warming as described by Parmegiani [16,17]. Note, the practicality of this method was tested on bacteria and fungi, as opposed to viruses.

Concerns regarding gametes and embryos as disease vectors

Although there have been conflicting reports whether SARS-CoV-2 may be transmitted across the placental barrier from mother to fetus [18,19], there has been no evidence over the past 2 years that embryos could transfect their maternal host. Following the COVID-19 pandemic, we seem to have safely navigated disease transmission concerns with pregnancy, implantation and healthy live birth outcomes being unaffected (Fig. 2). However, the question remains whether a guidance aimed at establishing a zero-tolerance toward the risks of embryo disease transmission be seriously entertained and enforced knowing that future viral pandemics will undoubtedly reoccur?

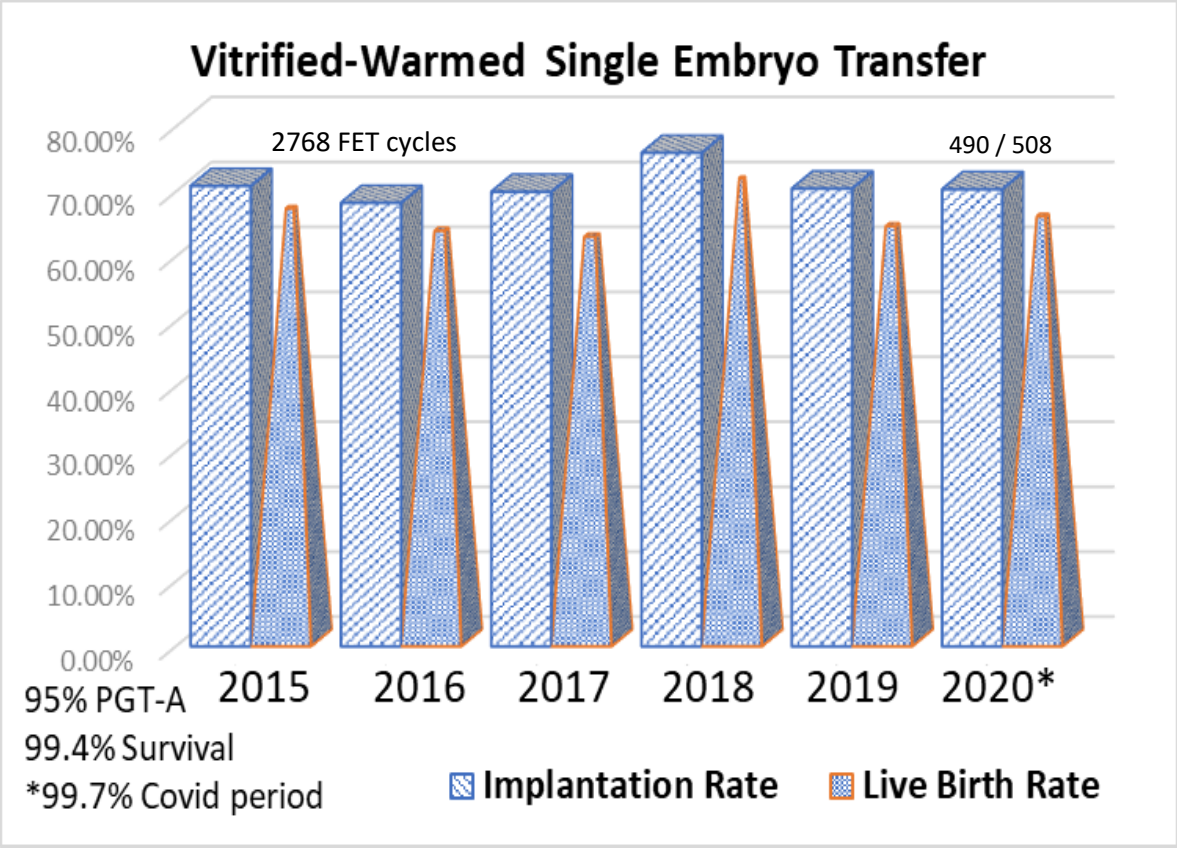

Figure 2. A comparison of pregnancy outcome success rates of 2768 Frozen Embryo Transfer (FET) cycles at one top performing IVF Lab in the USA (Ovation Fertility-Newport Beach) between 2015 and 2018 and 998 FET cycles during the pandemic (2019-2020) reflect no significant effects caused by SARS-CoV-2 or the preventative measures used to minimize its transmission potential.

Researchers have examined the risks of transferring diseases via embryos. One study looked at the potential for passing on virus via IVF from cattle that were infected with the flavivirus, BVDV [20]. Despite finding virus in the follicular fluid and on the cumulus-oocyte complexes after in vitro maturation of the ova and fertilization via IVF, no virus was detected 7 days later in the developing embryos. A similar study was performed in 24 patients undergoing IVF who were infected with HIV, HBV and HCV [21]. Despite some patients having high viral titers and the use of open vitrification devices for cryopreservation, virus was not detected in follicular fluid, culture media, liquid nitrogen used for vitrification or liquid nitrogen used for storage of the embryos. The authors concluded that their "findings provide evidence of the lack of a risk of cross-contamination while handling oocytes or embryos from seropositive patients, even when using an open device for vitrification." It is worth noting that the latter study involved ZP intact oocytes and embryos and was conducted by authors involved in the commercial egg banking industry. Since the latter study was influential is changing ART best practices as published by ESHRE [22] to allow the use of open vitrification devices as an acceptable ART practice, it also exemplifies possible influential commercial bias.

For decades we have known that the biggest defense against the potential vertical transmission of disease among embryos and oocytes has lied in simple dilution processes inherent to repeated pipetting steps. As described by Pomeroy [2], human IVF involves many steps where a small volume (~1ul) is added to a larger volume (~25 ul) in simple rinsing steps for fertilization, embryo culture, and cryopreservation procedures. With an estimated dilution factor of greater than 1 billion to 1, free floating virus are effectively eliminated from being passed along with oocytes or embryos. Though the risk is negligible, we should remember that the Cobo et al.[21] study and other historic livestock studies were performed on oocytes and embryos with an intact ZP. There is little knowledge on the effect of cracked zonae or ZP-free embryos on disease transmission. Today's common ART practices for blastocyst biopsying and or vitrification (i.e., blastocoele collapsing) involves laser zona and trophectoderm ablation. Thus, can the former classic disease transmission studies accurately predict what effect breaching of the protective ZP layer may have on disease transmission potential. In the case of SARS -CoV-2, the trophectoderm of blastocysts possesses ACE-2 receptor capable of binding pathogens. In the 1990's, the French government established a zero-tolerance standard toward the packaging and storage of semen by requiring the strict implementation of CryoBioSystem (CBS) straws made of a unique plastic resin that created "weld" seals upon automated heating [23]. These weld seals are a proven fail-safe, full-proof system of eliminating pathogens into or out of the straw storage containers [24]. Other European countries and Sperm Banks followed the French lead. Meanwhile, sperm banks in the USA continued their use of open system cryovials being unwilling to change a two decade tradition (i.e., standard practice), prioritizing commercial profitability (i.e., high cost to reform SOP's) over negligible risks. Do we need to experience a catastrophic event to be convinced that implementing a safeguard is best practice?

In the 21st Century, as vitrification was becoming the best practice standard for cryopreserving embryos and oocytes, the European community again led the way in adopting guidelines that supported and strongly advised the strict use of closed device systems (e.g., CBS-High Security Vitrification), to minimize disease transmission concerns [25]. However, following the published reports of Pomeroy et al. [1] and Cobo et al. [21], as well as the commercial influences of Egg Banks and disposable supply vendors promoting open device systems (e.g., Cryotop, Cryolock, Cryotech), standards were reduced to accepting negligible risks associated with the use of open vitrification devices as reflected in revised ESHRE guidance standards [22]. This was primarily mediated by a misconception that ultra-rapid cooling of oocytes achieved by the direct plunging of oocytes and embryos into liquid nitrogen before protective capping (not sealed closed) was absolutely necessary to optimize a vitrified state that insured post-warming survival/viability [26]. It has since been shown in prospective, randomized studies that different closed system devices (e.g., HSV, Vitrisafe) can indeed achieve comparable oocyte survival and developmental competence rate to that of an open system [27-29], and high blastocyst survival/live birth rates [30-31], while maintaining complete security from vertical disease transmission [32].

Negligible Risk of Cross-contamination

Historically, oocytes and embryos are considered poor vectors for diseases. In fact, there is no factual evidence that ZP-intact oocytes and embryos are capable of transmitting a disease acquired during the collection of embryos, in vitro fertilization/culture or while in cryostorage as previously reviewed by Pomeroy and others [1,2]. As

mentioned above, today's ART practices and the unknown future of new viral pandemic events leave us questioning best lab practices with renewed perspective. Eventhough no real-life examples of cross-contamination of cryo-container systems used in the reproductive laboratory have ever resulted in disease transmission, risk management must be considered as a best lab practice. A conservative approach to avoiding potential cross-contamination has been the use of a quarantine tank until patients are determined to have negative viral test results. Once identified as a non-infected patient, the embryos or oocytes are typically transferred into primary storage tanks. Yet, if cross-contamination is a real risk, why would one expose all new specimens to potential viruses until passing quarantine? Could not cross-contamination occur during quarantine, so that the patient's tissue becomes contaminated before transfer to a main storage tank, then potentially contaminating other supposedly unaffected tissue? The use of aseptic, closed vitrification devices from the start completely alleviates the need for tank separation steps, while maintaining the security of the enclosed embryo or gametes from viral exposure at any level.

Although the risk of viral transfection is unproven, risk assessment potential is virtually eliminated in an "aseptic, closed vitrification system" like the high security vitrification (HSV) [28,33], microSecure vitrification (μS-VTF) [30-31] and Vitrisafe [27,29,32] approaches. Unlike other original sub-optimal closed designs approved by FDA, the latter systems are superior in that their carrier devices are inserted into CryoBioSystem (CBS) straws. The unique ionomeric resin material of these high security straws innovated the concept of disease prevention in cryostorage containers [23], literally transforming human semen storage practices in Europe in the mid-1990's. By 2002, CBS straws were the first embryo storage container to be FDA approved based on their ability to form 100% reliable weld-seals, tamperproof internalized labelling, and a variety of color-coding options. Only the μS-VTF system fully retained the unique benefits of the original CBS 0.3 ml embryo straw design and has been clinically validated to be highly effective, reliable and secure system for oocytes and embryos freeze-preservation since 2010 [34].

Recommendations: Potential Modifications to Reduce the Risks of SARS-CoV-2

Although it was difficult to provide hard recommendations to minimize risks at the start of COVID-19 when we knew so little about SARS-CoV-2, it appears that standard oocyte and embryo dilution pipetting procedures, semen processing safeguards and other standardized preventative measures (strict preventative PPE, hand hygiene and through surface decontamination measures) safely mitigated the transfection of COVID-19 among and between patients. Sustained high pregnancy/implantation rates, acceptable loss rates and excellent live births in 2020 into 2022 have validated our handling of this pandemic. Although the COVID-19 pandemic has greatly altered the way we manage clinical practices, lab environments and how we interact with our patient population [35,36], it has not adversely impacted their treatment outcomes (Fig. 1). But what steps should be considered to ameliorate the effect of future viral pandemics in patients undergoing ART treatments? Some suggested measures are highlighted below:

- Sperm purification systems aimed to process and minimize viral contaminants in semen of positive-tested or at-risk patients should be used in all patients. Furthermore, the use sperm pelleted samples from dilution wash/centrifugation of raw semen should be avoided.
- All sperm freezing should be performed in weld-sealed CBS straws, and the sealer surface disinfected with 6% H2O2 between patients.
- At retrieval, cumulus-oocyte complexes should be trimmed extensively as soon as possible (i.e., using a dish tilt, spread and cut technique) and then diluted and washed in large volumes of media (3-5ml) prior to cumulus cell removal/ICSI and subsequent culturing and cryopreservation procedures. To avoid a decline in fertilization rates, ICSI of mature oocytes should be proceed within 1 hour.
- All handling steps should involve repeated rinsing through multiple washes to dilute out potential viral contamination.
- Liquid nitrogen should not be shared among patients for vitrification and warming of open device systems, or alternatively, LN_2 bathes should be UV disinfected between uses.
- To eliminate the possibility of contamination with any pathogen, a safeguard would be to cryopreserve gametes and embryos in a closed CBS straw device with complete weld seals. This practice is especially vital for biopsied, hatching and hatched blastocysts where a risk of viral exposure is undeterred of the ZP layer.
- If an imminent viral risk exists, embryos with intact-ZP should be vitrified before any opening of the ZP protective layer, if closed device

systems are not routinely used. This may mean reconsidering the extensive use of embryo biopsy and PGT-A, if open vitrification devices are standard practice.

Conclusion

There were many predictions and recommendations during this pandemic aimed to minimize its costs to human life and suffering. Change to our current clinical and laboratory protocols were applied as safeguards to defend an unfamiliar pathogen, SARS-CoV-2. Ultimately, COVID-19 did have a negative impact of the global logistics supply chain and employment market (i.e., staff shortages), placing stress and strain on operations, yet clinical reproductive outcomes appeared to be unaffected. Implantation and healthy live birth success rates were undeterred, remaining high throughout 2019 to date [37]. While there was evidence of SARS-CoV-2 in testicular, uterine and placental tissues of infected individuals, there has been no evidence of vertical transmission to gametes and embryos and so on. Though potentially unnecessary, it appears that the risks we took to apply best GTP measures were not harmful? Just like those embryo handling /importation measures taken in the animal agriculture industry to prevent pandemic events [38], we must carefully assess worst-case scenarios of future pandemic events to protect future patient reproductive fitness and gamete/embryo well-being by adopting best practice protocols aimed to safeguard best-case scenarios. This pandemic has taught us that we do not know what viral insults lie ahead, and that previous guidelines and current ART practices make most laboratories and their patients susceptible to risk. Thus, we should reassess the full intent of FDA's and ESHRE's good tissue practices and adopt safer and more protective procedures.

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