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Fate of human twin embryos generated unintentionally in *in vitro* fertilization: Clinical and ethical perspectives

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Abstract
In vitro embryo splitting or twinning is a method of reproductive cloning that involves dividing a single early-stage embryo into two or more individual cells and growing them as identical embryos. The first successful intentional *in vitro* human embryo splitting, reported in 1993, sparked a heated ethical discussion on the cloning of human embryos. Later, the intricacies of the ethical debate related to cloning through human embryo splitting were globally recognized. In response to this, several countries and international organizations have implemented prohibitions either through legal statutes, official decrees, or public statements. However, most of the studies reported the post-implantation spontaneous embryo splitting in utero that results into monozygotic twins or multiples and aren't fall under the prohibitions of cloning. Recently, rare cases of unintentional *in vitro* embryo splitting were confirmed during their culture mostly due to naturally zona-free oocytes or zona manipulations. Due to the ambiguity of current legislation to discriminate between intentional or unintentional *in vitro* embryo splitting, the fate of those embryos to transfer or not, remains a question. After assessing the potential risk associated with, there is a need to reconsider the prohibitions imposed on transfer of unintentionally generated split embryos in poor responders and advanced maternal age patients undergoing IVF treatment, where the use of donor gametes is also prohibited. This review paper describes the various IVF laboratory procedures that rarely and unintentionally generates split embryos. Moreover, the cellular and molecular changes occurring in split embryos and ethical considerations about use of split embryos to transfer are also deliberated.

Keywords: Unintentional, *in vitro* embryo splitting, twin embryo, *in vitro* fertilization, ethics, monozygotic twins

Introduction
Dividing embryo either manually or spontaneously into two or more genetically similar embryos is called embryo splitting or twinning. The frequency of twin pregnancies that occur naturally differs across the globe accounts 0.8-1.7% of births. The incidence of monozygotic twin (MZT) pregnancies is 0.35-0.4% [1-2]. Globally, the incidence of twin pregnancies has significantly increased after the widespread use of assisted reproductive technologies (ART). The occurrence of MZT pregnancies following ART is greater (0.9%) than the occurrence of spontaneous MZT pregnancies (0.35-4.0%) [3]. Findings of these studies suggested that IVF may lead to embryo splitting in utero or *in vitro*.

Although the exact reason behind the higher risk of MZT pregnancy after ART remains unclear, numerous factors inherent to women undergoing fertility treatment and ART have been hypothesized to be potential causes. These include maternal/oocyte age [4-5], embryo development at the time of embryo transfer (ET)/extended culture (i.e., blastocyst) [6-7], blastocyst morphology [8-9], zona manipulation in the form of assisted hatching (AH) and intracytoplasmic sperm injection (ICSI) [10], delayed implantation [11], embryo biopsy for preimplantation genetic testing (PGT) [12-14], frozen-thawed embryo transfer (FET) [4, 15], and exposure of the embryo to the blastocyst stage to the low concentration of Ca^{2+} in culture media.

Besides spontaneous embryo splitting in utero, several groups attempted to divide early embryo to increase their numbers [16-19]. The first ever intentional *in vitro* embryo splitting (IES) in humans was reported by researchers from George Washington University, Washington DC, USA, at the Joint American Fertility Association/Canadian Fertility and Andrology Society meeting in October 1993. They used 17 two- to eight-cell polyploid embryos, but the split embryos *in vitro* could not grow beyond 32 cells [20].

Based on the results of this study, Hall and coworkers suggested that the use of split embryos in infertility treatment could improve IVF outcomes. However, an in-depth analysis of their study methodology and results revealed that they conducted this study without institutional review board's approval. As a consequence, the authors were severely criticized for this act and were instructed to destroy their experimental data [21-22]. This incidence stimulated aggressive ethical discussion on human embryo cloning using IES, therefore new guidelines were framed and the establishment of a new ethics commission took place [23-28]. As a result of criticism from scientific experts, the American Society for Reproductive Medicine's (ASRM's) Ethics Committee framed an official declaration about embryo splitting and its application in infertility treatment, which came into effect in December 1995 [29].

There was fierce debate in the global community about the intricacies of the moral arguments associated with IES, and concerns about the probable use of this technique in human reproductive cloning were raised. Consequently, several international bodies and nations framed policies or bans on this technique through legislation, decrees or official declarations [30-34]. Intentional *in vitro* embryo splitting in humans has been studied by several groups, examined their suitability for clinical and research applications, and reviewed their ethical reflections [16-18, 35]. Recently, visualization of very rare cases of unintentional *in vitro* embryo splitting was confirmed using time-lapse cinematography (Bhor *et al*., unpublished data). The embryos generated by such way cannot be used for transfer because current legislation doesn't discriminate between intentional or unintentional embryo splitting *in vitro*. In reproductive age women undergoing IVF treatment, prohibiting use of split embryo to transfer if ample number of good quality embryos are available or possibility to obtain good quality embryos in the subsequent IVF cycle is considerable. However, current legislation doesn't allow to transfer split embryos in poor responders and advanced maternal age patients undergoing IVF, even though there is no embryo left over. In countries like Japan, where the use of donor gametes or donor embryo is banned, such women are being kept away from either treatment option. Therefore, there is a need to reframe policy or legislation that will conditionally allow to transfer of unintentionally generated split embryo (s) in an infertile woman with poor ovarian reserve, poor responders and advanced maternal age patients after carefully assessing potential risks and social, ethical and legal issues.

This review provides a comprehensive overview of the IVF lab procedures and/or add on that unintentionally results in *in vitro* embryo splitting. Although, the mechanism of zygotic splitting is not fully known, and most of them are speculations we have focused on molecular and cellular mechanisms altered in split embryos. Embryo splitting, either intentional or unintentional is considered as an act of human reproductive cloning by current legislation stipulated by various international and national level bodies and strictly prohibited. We emphasize that regulatory changes in existing legislation are needed to benefit poor responders, infertile women with poor ovarian reserve and advanced maternal age patients especially in countries where gamete and/or embryo donation is banned.

IVF laboratory procedures and/or interventions that unintentionally split embryos *in vitro*

Embryo splitting in naturally occurred zona-free oocytes in IVF

Complete absence of ZP in oocytes is rarely encountered during IVF procedures. ZP-free oocytes are incidentally obtained during the cumulus cells removal using standard treatment with hyaluronidase followed by stripping. However, the oocytes with damaged ZP, partially absent ZP or lost ZP is more common due to laboratory manipulations. Several factors leads to the occurrence of completely zona-free oocytes including ovarian stimulation, maternal age, increased zona fragility, genetic and immunological factors, etc. [36]. ZP is a protective layer that surrounds the oocyte and plays a role in fertilization and implantation. To achieve fertilization in zona-free oocytes, ICSI is required, as they cannot facilitate binding to sperm or prevent polyspermy. Moreover, it provides mechanical support by preventing blastomere separation during embryo development [37]. Several studies has reported the successful fertilization, embryo development, implantation, and live birth using ZP-free oocytes in IVF [36, 38-43]. However, there are several safety concerns about ZP-free oocytes such as mechanical damage during ICSI, loss of blastomeres during embryo culture until compaction, risk of mechanical damage during routine handling or embryo transfer, and comparatively less tolerant to vitrification. In rare cases, in ZP-free oocytes, on day 4 of culture, blastomeres fails to clasp into a single compact mass giving rise to two or multiple blastocoel cavities either conjoined (Fig. 1) or separated from each other. *In vitro* culture in microwells until day 5 or 6 develops into either conjoined or separate twin-blastocysts containing lesser number of cells in TE and ICMs (Fig. 1). Although, the occurrence of twin-blastocysts from natural ZP-free oocytes during *in vitro* culture is unintentional and rare incidence, but further research including developmental potential of those twin-blastocysts is required. Also, the recent study by Noli and co-workers has reported that embryos created artificially by blastomere biopsy/separation were not suitable for clinical purposes [16]. Moreover, there is a need to rethink about regulatory prohibitions imposed on twin-embryos in human especially twin-blastocysts generated unintentionally during *in vitro* culture.

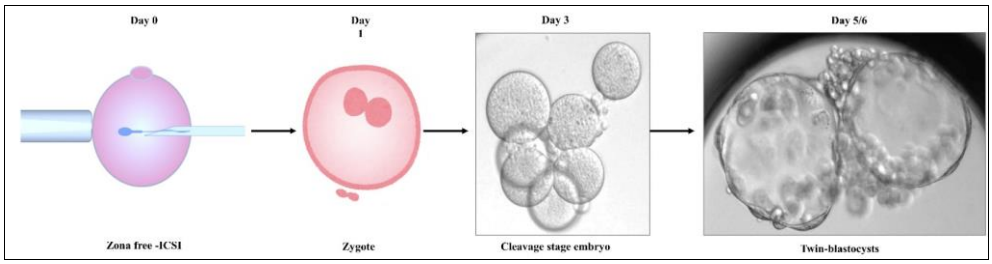

Fig 1: Embryo splitting in zona-free oocytes. The flow diagram showing the incidence of embryo splitting in zona-free oocytes in IVF. Oocytes lacking zona pellucida protective layer need to be fertilized by a single sperm using ICSI, commonly called as zona-free ICSI. 17 hrs after ICSI, two-pronuclear (2PN) stage embryo develops. Shortly after fertilization zygote undergoes cleavage. As the oocytes naturally missing ZP layer, the blastomeres from a cleavage stage embryo have lesser point of contact and possibly limited communication between blastomeres. These altered processes in zona-free embryos may give rise to twin-blastocyst in culture.

Embryo splitting in intentionally zona-freed zygotes in IVF

According to some studies, to decrease the cytoplasmic fragmentation from embryos before transfer, removal of ZP is suggested, which may improve their quality, viability, implantation potential and live birth rate [37, 44-48]. Cytoplasmic fragmentation occurs when parts of the cytoplasm break off from the main cell body and form small vesicles. These vesicles are called fragments, and they are usually devoid of DNA. Cytoplasmic fragmentation can occur at different stages (Cleavage or blastocyst) of embryo development and impair further embryo development and pregnancy outcomes. Therefore, in a prior study, 3PN zygotes were utilized, and their ZP was artificially removed at the pronuclear stage. Compared to those of zona-intact 3PN and 2PN/2PB embryos, a reduced rate of fragmentation was observed in zona-free embryos through time-lapse cinematography and culture systems [48]. However, the embryo development from 1-cell stage to compact morula stage is very critical and absence of protective layer of ZP may impose the risk of blastomere dispersal and separation. This may prevent maximum contact between blastomeres, aggregation and assembly to form the compact morula [49]. These altered early embryo developmental processes due to unavailability of ZP layer may lead to produce conjoined, twin or multiple blastocysts on day 5 or 6 of *in vitro* culture (Fig 2). These blastocysts are of same genetic make-up and gives rise to controversial issue of human cloning.

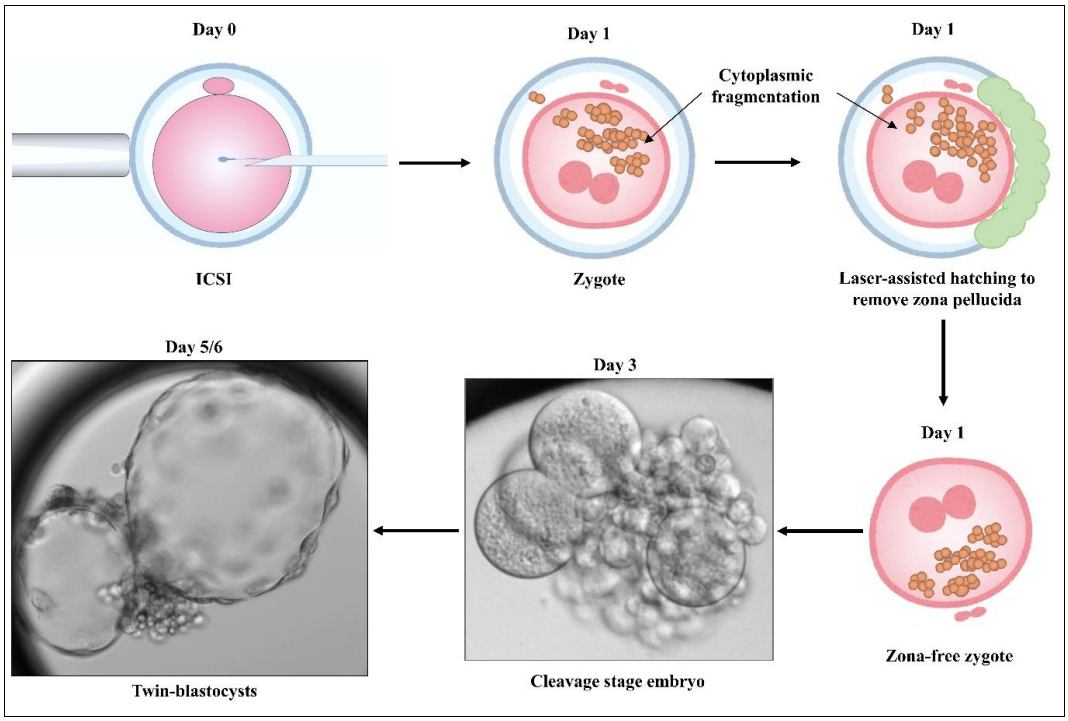

Fig 2: *In vitro* embryo splitting in zona-freed zygotes. The flow diagram of processes that leads to produce twin-blastocyst in artificially produced zona-free zygotes in IVF. To decrease the cytoplasmic fragmentation in 2PN stage embryos and to increase the quality and viability of blastocyst, artificially removal of ZP is suggested. Zona-free zygotes undergoes cleavage to produce blastomeres that remains separated from each other which may leads to produce twin-blastocyst.

As previously reported, the embryo splitting may affect morphokinetics of those blastocyst but not the chromosomal constitution [17]. Whereas, the results from another study suggested that human embryos generated by *in vitro* embryo splitting are not suitable for clinical or research purposes [16]. It is well-known that embryo splitting may have adverse effects on pregnancy outcomes, such as an increased risk of miscarriage, preterm birth, low birth weight, and complications such as twin-to-twin transfusion syndrome [50]. However, there is a need to reframe ethical and regulatory considerations about split embryos generated incidentally by removal of ZP to decrease cytoplasmic fragmentation after careful evaluation and management. This technique along with ethical and regulatory reforms will raise the hope to treat poor responders or patients with advanced maternal age in IVF.

Embryo splitting due to TE biopsy

TE biopsy is a procedure that involves making a small opening in the outer layer of blastocyst (Usually 4 or 5 days after fertilization) and removing few (about 5-10) cells. It is typically performed for preimplantation genetic testing of aneuploidies (PGT-A), that detects chromosomal abnormalities such as aneuploidy and mosaicism in the embryo before transfer [51]. This procedure aims to select the best embryo for transfer to achieve a successful pregnancy and reduce the risk of miscarriage or birth defects. TE biopsy may have some effects on the embryo itself, such as altering its gene expression, viability, implantation potential, or developmental competence [52]. One of the possible effect of TE biopsy is that it creates a physical gap or injury in the TE layer which divides embryo in to two (MZT) or higher-order multiples [16, 52]. Embryo splitting in *in vitro* culture due to TE biopsy has been reported in several animal species, such as sheep, cattle, horses, and pigs [16]. Previous studies has indicated that TE biopsy significantly increases the risk of monozygotic splitting in utero [12, 53-54]. However, there are very few studies on human embryo splitting *in vitro*, and most of them are based on *in vitro*-matured oocytes, which may not reflect physiological conditions [55]. The biological mechanisms and consequences contributing to embryo splitting in humans are poorly understood, and there are ethical and safety concerns regarding the potential risks of embryo splitting for pregnancy outcomes and the health of offspring [52].

As of now, there is no specific information or statistics available

for the occurrence of human embryo splitting solely due to TE biopsy. However, a few studies indicate the relevance of TE biopsy with embryo splitting [56-57]. In a mouse model, it was suggested that TE biopsy may increase the incidence of embryo splitting, especially when combined with other factors, such as blastocyst vitrification, ZP opening, or AH [58]. Moreover, embryo splitting may have implications for the clinical outcomes of PGT-A cycles, such as increasing the risk of multiple pregnancies, MZTs, or congenital anomalies [58]. Therefore, additional research is needed to elucidate the mechanisms, frequency, and consequences of embryo splitting after TE biopsy and to optimize biopsy protocols and culture conditions to minimize the occurrence of this complication. Assessing the impact of TE biopsy on embryo splitting in *in vitro* culture is a challenging and controversial issue that requires further research and regulation.

Cellular and molecular mechanisms altered in split embryos

The molecular processes occurring in human split embryos are not well understood, but some studies have suggested that embryo splitting may affect several mechanisms or processes in split embryos, including cellular composition, gene expression, and epigenetic modifications.

Cellular composition

Split embryos have fewer cells than intact embryos at the same developmental stage [17]. This may affect cell allocation to different lineages and cell–cell communication within the embryo. Compared with intact embryos, split embryos may have reduced or altered cell–cell communication, adhesion, and polarity, which can affect cell differentiation and morphogenesis [59]. In intact human embryos, the process of compaction, which usually occurs at the 8-16 cell stage, involves blastomeres tightly holding to each other, increasing the contact area between neighboring cells and further facilitating the acquisition of a polarized shape [59]. However, this property can be lost or severely affected in split embryos.

Gene expression

Recently, some studies have suggested that split embryos may have different patterns of DNA methylation, histone modifications, and imprinting than intact embryos. Alterations in these molecular processes lead to changes in gene expression and further development [16, 60]. A study by Velásquez and coworkers (2017) reported that *in vitro*-produced bovine split embryos had lower levels of expression of pluripotency markers such as OCT4, SOX2, TP1, and EOMES than IVF intact embryos [61]. Furthermore, a study comparing miRNA profiles of spent blastocyst medium (SBM) from human split embryos generated by blastomere biopsy with SBM from normal blastocysts that resulted in live births revealed that split embryos secreted six miRNAs in significantly greater amounts than intact blastocysts did, while 22.9% of the miRNAs secreted by split embryos were not detected in the SBM of blastocysts that led to live births or TE samples from normal blastocysts used for research purposes [62]. The results from this study suggest that the presence of miRNAs exclusively found in twin embryos may be due to differences in lineage commitment between the two embryos.

Epigenetic modifications

The epigenetic modifications in split human embryos are poorly understood. However, using mouse embryos, it is suggested that split embryos may have different patterns of DNA methylation, histone modifications, gene imprinting, and chromatin accessibility than intact embryos, which can affect gene expression and development [63]. DNA methylation of several specific genes in early embryos may affect blastocyst formation and the gastrulation process by altering the transcriptional regulation of lineage-specific genes [64]. In a mouse model, histone modifications, such as methylation, acetylation, and ubiquitination, play crucial roles in regulating gene expression and chromatin remodeling during early embryonic development [65]. However, the current understanding of comparative histone modifications in split and intact human embryos is incomplete. Additionally, another study in a mouse model suggested that laser-assisted hatching performed on preimplantation embryos either to assist in embryo splitting or to improve the implantation potential has an adverse effect on the methylation and expression profile of the imprinted gene IGF2/H19 in the embryo and fetus [66]. However, additional research is needed to understand the differences in epigenetic modifications between split and intact human embryos.

Ethical considerations of human twin embryos generated unintentionally in IVF

In vitro embryo splitting with or without specific purposes is considered as a form of human reproductive cloning and therefore it has been a topic of ethical, legal, and scientific debate. Several international bodies and nations framed policies or banned this technique through legislations, decrees or official declarations [30-34]. As of now there is no clear rules or legislation about human split embryos generated unintentionally during IVF and it falls under the same regulations as human reproductive cloning. Restricting the use of unintentionally generated split embryos in IVF for transfer in reproductive age women with ample number of good quality embryos available to transfer is considerable. However, after assessing the potential risk, ethical and practical challenges associated with transfer of split embryos, there is a need of special consideration for using split embryos generated unintentionally in poor responders and advanced maternal age patients with no embryos left over for transfer in countries like Japan, where use of donor gametes or embryos is prohibited. Although, the incidence of unintentionally generated split embryos in IVF is rare but there are no proper guidelines for their clinical management, genetic testing, embryo transfer, cryopreservation, patient counselling, and/or disposal from the international or national level regulatory bodies.

The ethical considerations of human split embryos generated unintentionally in IVF are multifaceted and may involve various perspectives, including those of bioethicists, religious groups, policymakers, and the general public. It may include the moral status of split embryos in comparison to their counterpart. Depending on one's view, the split embryos or IVF lab procedures that leads to generate them may be seen as a violation of the embryo's rights, a legitimate way of creating more lives, or a morally neutral procedure. Also, there are some unintended consequences and risks associated with it including reduced uniqueness and diversity, uniqueness and diversity of human beings, creating ethical dilemmas for the resulting children, posing potential health and psychological risks for the embryos, the parents, and society. Also, it may increase the possibility of developmental abnormalities and some unforeseen issues in children born using split embryos. Even though the regulatory bodies allow conditional use of split embryos generated unintentionally in poor responders and advanced maternal age patients undergoing IVF treatment, religious and

cultural perspectives and socio-legal issues may restrict their use.

Conclusion

Human embryo splitting in *in vitro* culture is a very rare incidence and there is no much scientific information available. Several factors that leads to post-implantation embryo splitting has been studied, however there is a lack of studies that assess the impact of embryonic factors, culture conditions, and IVF procedures on pre-implantation *in vitro* embryo splitting. The current legislations don't allow to transfer twin embryos generated unintentionally *in vitro* due to zona manipulations. Upon evaluating the potential risks and ethical dilemmas related to transferring split embryos, it becomes crucial to give special attention to unintentionally generated split embryos in cases of poor responders and advanced maternal age patients who have no surplus embryos available for transfer. This consideration is particularly relevant in countries like Japan, where the use of donor gametes or embryos is not allowed.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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