Reproductive Biology 24 (2024) 100923

Contents lists available at ScienceDirect

Reproductive Biology

journal homepage: www.journals.elsevier.com/reproductive-biology

Review

Exploring the benefit of different methods to perform assisted hatching in the ART laboratory: A narrative review

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ARTICLE INFO

Keywords:
Assisted hatching
in-vitro fertilization
Assisted reproductive technology
Infertility
Laser assisted hatching
Zona pellucida

ABSTRACT

In the last decades, to enhance success rates in assisted reproductive technology (ART) cycles, scientists have continually tried to optimize embryo culture and selection to increase clinical outcomes. In this scenario, the application of laser technology has increased considerably worldwide and is currently applied across ART in several ways: for assisted hatching (AH) or thinning of the *zona pellucida* (ZP), embryo biopsy, to immobilize and select the sperm during intracytoplasmic sperm injection, as well as to induce artificial blastocyst shrinkage before cryopreservation. Laser-AH has been suggested as a procedure to improve embryo implantation: the concept is that drilling holes through or thinning of the ZP could improve the hatching process and implantation. The artificial disruption of the ZP can be performed by different approaches: mechanically, chemically and with the laser, which is one of the most favourable and easy methods to remove part of the ZP and to augment the possibilities of implantation in patients defined as having a poor prognosis of success, or when the ZP is too thick. However, in the current literature, there is not sufficient evidence about the potential risk or impairment that laser utilization might induce on embryo development; therefore, the main aim of the current review is to provide an overview of the existing knowledge on the ZP and the mechanisms of manipulating it to improve the effectiveness of ART. Also, it emphasizes the positive aspect of laser application as a powerful tool that might increase the chance of pregnancy for infertile couples undergoing ART cycles.

1. Introduction

The application of assisted hatching (AH) in assisted reproductive technology (ART) has notably increased during the last three decades. The artificial disruption or thinning of the *zona pellucida* (ZP) was described for the first time by Cohen and collaborators in 1988 [1], who reported the first pregnancy after partial zona dissection (PZD). The authors described the partial dissection of the ZP achieved on mature oocytes using mechanical force in couples undergoing ART cycles with male factor infertility. Several authors have reported beneficial effects of AH: a study by Valojerdi and colleagues [2] investigated if AH can increase pregnancy success rates of ART cycles in women with recurrent implantation failure, with advanced female age, or who are using frozen-thawed embryos. The authors found a benefit of AH in women having a frozen-thawed embryo replacement; the clinical pregnancy was statistically significantly higher in the AH group as compared with the control (31.2 % versus 11.1 %). However, in patients with advanced female age or recurrent implantation failure having a fresh embryo replacement, the clinical pregnancy and implantation rates were similar in both groups [2]. In agreement with Valojerdi's study, other groups have seen a beneficial effect in terms of clinical pregnancy and implantation rates following application of AH in frozen embryo transfer (FET) cycles, performed either using a laser or with Tyrode's solution [2–4]. However, there is still an active debate on the efficacy of the AH

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https://doi.org/10.1016/j.repbio.2024.100923
Received 6 May 2024; Received in revised form 21 June 2024; Accepted 27 June 2024
Available online 6 July 2024

procedure. A prospective randomized study published in 2006 reported increased pregnancy outcomes in couples undergoing FET treatments, but this improvement was not observed in fresh embryo transfer [5]. A Cochrane review of 39 randomized controlled trials (RCTs) including 7249 women and 2486 clinical pregnancies, reported that there was not enough evidence to recommend the use of AH to increase the live birth rate (LBR) [6]. Those results confirmed the data of a previous Cochrane analysis in 2012, concluding that the LBR was not proven to be raised by AH [7]. In addition, both reports announced increased multiple pregnancy rates in patients who receive the AH procedure compared to the control groups [6,7]. Therefore, further evidence is still missing on the routine use of AH for couples undergoing ART treatments. This conclusion agrees with data published by Hershlag and colleagues [8]. Indeed, more than three decades after the first application of AH on human embryos, proof of its efficacy is still lacking. It is important to mention that an appropriate interpretation of the evidence is required, considering that different methods can be employed to induce embryo hatching: mechanical, chemical and with laser technology [2–4]. In addition, even laser-assisted hatching (LAH) procedures might be applied in different ways and modes, and studies published are performed on different types of patients, with probably divergent results on the embryo hatching dynamics. Various methods have been suggested such as making holes in the ZP, or thinning of different sizes, drilling, cutting, digesting, or mechanically melting the ZP, chemically or with a laser beam; on FET or fresh embryos at different stages [9,10]. Finally, AH is currently recognized by the Human Fertilisation & Embryology Authority (HFEA) in the UK as a method that lacks sufficient moderate/high-quality evidence for its clinical effectiveness, which has been confirmed very recently by a paper produced by the European Society of Human Reproduction and Embryology (ESHRE) on good practice recommendations on add-ons in ART [11]. Given that worldwide this kind of procedure has been applied since 1988, it is critical to produce well-designed investigations to provide sufficient evidence on the efficacy and utility, as well as the risk associated with each method. Since the use of laser technology and AH are increasing in ART units, and since we still lack knowledge on the risk and efficacy of these procedures, the goal of the current manuscript will be to furnish an analytical and commentary review of the literature on the status of AH and LAH methods. It highlights the benefits of lasers as a powerful technology and aims to provide the necessary information for the refinement of AH and its possible incremental use in ART applications.

2. Search strategy

Relevant peer-reviewed studies were identified in the English-language literature using PubMed and were included in this narrative review. Search terms included the use of AH and LAH in the ART cycle and its correlation with pregnancy outcome and LBRs, also exploring any risk eventually associated with the method. Specifically, the following clinical outcomes were analysed: implantation, clinical pregnancy, abortion, multiple pregnancy, and LBRs. RCTs, prospective and retrospective studies were analysed where available. The search comprised mainly human studies, although for the first descriptive part, some studies in animal models were also included. Relevant published papers before March 2024 were selected and included in this review.

3. Zona pellucida structure and function

Mammalian embryos are surrounded by the ZP, which is an oocyte and embryo-associated acellular translucent protein structure composed of a sulphated glycoprotein matrix that is shaped enclosing the oocyte during its maturation within the follicle [12]. The human ZP is formed by four glycoproteins ZP1, ZP2, ZP3, and ZP4 and its thickness is around 15–20 μm. Repeated ZP2 and ZP3 units, cross-linked by ZP1, give rise to the filamentous nature of the ZP [12–15]. It plays a crucial role at the time of fertilization, during the binding of the spermatozoon to the oocyte, and alteration in genes encoding human ZP glycoproteins is one of the main causes of female infertility [15–17]. The ZP stimulates the acrosome reaction in the head of the spermatozoon [18] and soon after sperm binding and passage through to the oolemma, the structure of the ZP changes, and it becomes more difficult to be penetrated by other spermatozoa [19,20]. In effect, following fertilization, fusion of oocyte cortical granules with the oolemma and the discharge of their enzymatic contents into the perivitelline space (the cortical reaction) results in ZP "hardening" and represents an important secondary mechanism to reduce the incidence of polyspermy, which is when more spermatozoa penetrate the oocyte [21–23]. The ZP also has additional benefits: it surrounds both the unfertilized oocyte and the early embryo, and as a physical obstruction prevents damage to the oocyte and, post-fertilization, to the embryo within from immune cell invasion, or biochemical toxicity. The ZP is important for embryo compaction, which is the formation of structural gap and tight junctions between blastomeres. In humans, blastocoele expansion and thinning of the ZP are critically important for the hatching process. In mammalian species, the breach of the ZP is controlled by lysin "proteases", and a Na^+, K^+-ATPase which pushes sodium into the blastocoel cavity, inducing expansion and contraction of the blastocyst in preparation for hatching [23–26]. Some authors have investigated if there is a correlation between ZP thickness and maternal age or women's hormonal status, in patients undergoing ART treatments [27–29]. Balakier and colleagues measured the ZP thickness of 5184 cleavage stage embryos (day3) in 744 *in vitro* fertilization (IVF) patients, and found no significant correlation with the patient's age, stimulation protocol or infertility diagnosis, as well as no relation with their basal concentration of serum FSH. Interestingly, the authors reported a strong association with embryo quality, and good quality embryos exhibited a considerably thinner ZP compared with those embryos with poor morphology [27]. A study by Nawroth and collaborators found a slight association, which was not statistically significant, between ZP thickness and women's age in 843 metaphase-II oocytes from 100 ART patients [28]. Additional studies have investigated the variation of the ZP thickness during embryo development and correlated that feature with pregnancy outcomes. Lewis and co-authors analysed the variation in ZP thickness during embryo development and reported no correlation with implantation following day 3 transfer [29]. On the other hand, a trial published by Gabrielsen and co-workers reported that replacement of embryos which showed a degree of ZP thickness variation during their development represented a strong correlation with clinical pregnancy outcome following IVF cycles [30]. Finally, several authors seem to agree with the consideration that when an embryo possessed a thick ZP, this feature is generally associated with low-quality embryo development and poor morphology and, following embryo replacement, resulted in reduced pregnancy outcomes compared to embryos with a thinner ZP [27–33].

4. The rationale for assisted hatching

In vivo, proteolytic enzymes gradually and slowly digest the ZP. The embryo develops further until it hatches completely from the ZP, when it reaches the blastocyst stage, and eventually, the implantation process takes place [34,35]. However, *in-vitro* culture conditions, as well as cryopreservation procedures, might induce a thickness and hardening of the ZP, which may interfere with and disturb the hatching process [36,37]. Therefore, AH is a technique during ART in which a hole is made in the ZP, using a micromanipulator with a laser, and this has been proposed to improve the capacity of the embryo to escape from the ZP; thereby, the embryo should increase its implantation potential [26]. Over decades several protocols have been introduced, ranging from zona breaching, where a gap of different sizes is induced in the ZP, to zona thinning, where its thickness is reduced. This can be achieved with chemical solutions or by using a non-contact laser system [36–39]. However, the aperture within the ZP is thought to induce negative effects: it can impair the process of blastocyst expansion or allow blastomere loss through the breached ZP. Also, the embryo might be exposed to a higher risk of infectious or immunologic attack [38]. Events of embryo trapping have also been reported, however quite rarely following ZP breaching of the blastocyst. In animal studies, a correlation has been described between the diameter of the hole in the ZP and the achievement of the hatching process. Montag and van der Ven, in the mouse model, found that if the opening is less than 10 μm, full embryo hatching is impaired [32] while in cattle, breaches smaller than 40 μm induced trapping events compared to larger holes [40]. Advantages of zona thinning include the prevention of blastocyst hatching without full expansion and the reduction of embryo splitting to produce monozygotic twins [41]. Investigations performed on the mouse model have reported that during the hatching process, the blastocyst goes through contraction and re-expansion events [42]. The exact role of those contraction events, which can be multiple, is not completely clarified, but strong collapse with a reduction of the blastocoel cavity of more than 50 % of the original volume seems to impair the hatching and implantation processes compared to weak contractions [42]. Similar findings have been illustrated with the *in-vitro* human blastocyst in infertile couples undergoing ART cycles. Several authors have reported that a transfer of a spontaneously collapsing blastocyst results in reduced implantation potential and diminished pregnancy rates compared to those embryos that do not show any collapse events [43–46]. It is however hypothesized that AH might facilitate blastocyst hatching features and might prevent strong contractions, which might limit great energy expenditure.

5. Assisted hatching in fresh embryo replacement

The use of chemicals to induce zona drilling in human embryos has been investigated by several authors with controversial results. Some retrospective investigations showed no benefit of the procedure [47–49], except for some specific groups of patients: such as advanced maternal age [50], poor prognosis patients or those with previous failed IVF cycles [49–52]. A trial performed by Schoolcraft and co-authors [49] analysed the effect of AH in 76 poor prognosis patients undergoing IVF treatments. Those poor prognosis women were defined as having a high day 3 Follicle Stimulating Hormone (FSH) level; age > or = 39 years; and multiple prior IVF failures. AH was performed on the morning of day 3 of embryo development using acidified Tyrode's solution. Results found that the incidence of ongoing pregnancy in the AH group was 64 % compared with 19 % in the control group. The implantation rate per embryo transferred was 33 % in the AH group versus 6.5 % in the control group. In a retrospective study, Hurst and colleagues reported an impairment in embryo quality and implantation rate in a small group of young patients undergoing ART with AH compared with the control group. The authors performed the AH on the morning of day 3 of embryo development followed by embryo replacement on the same day [53]. In a randomized trial, Cohen and collaborators demonstrated that chemical zona drilling increased the percentage of clinical pregnancy in those embryos with a ZP thickness $\geq$ 15 μm, particularly in couples with a female age $\geq$ 38 and with high FSH levels [54]. In a prospective randomized study involving 100 couples, Tucker and co-authors [55] applied AH to cleavage stage embryos on day 3 using acid Tyrode's solution and found no beneficial effect on overall ICSI pregnancy outcomes. However, in those patients with a female age $\geq$ 35 years, AH was able to significantly increase the clinical pregnancy rate (45.2 % versus 16.9 %, $p < 0.05$). A prospective randomized trial published by Ma and colleagues [56] involving patients undergoing ART cycles with indication for ICSI found, regardless of age, a higher implantation rate in the AH group compared to the control group (16 % versus 8 %; $p < 0.01$). In a more recent prospective randomized double-blinded investigation in patients $\leq$ 38 years old, Hagemann and colleagues [57] analysed the effect of AH on pregnancy outcomes. Patients having any embryos with a ZP thickness $\geq$ 13 μm were eligible for randomization, and the study included 121 women. All hatching procedures were performed using acid Tyrode's solution on the morning of day 3, at least one hour prior to embryo replacement. Results found no differences between hatched and unhatched patient groups in rates of clinical pregnancy (47 % versus 50 %) or live birth (46 % versus 45 %). Furthermore, no significant differences were noted between hatched and unhatched groups in rates of spontaneous abortion, monozygotic twinning or dizygotic twinning. Some groups have also analysed the performance of chemical hatching compared to mechanical or LAH, and none of them demonstrated a superiority of one over the other two methods [58]. Lanzendorf and co-workers [59] did not find a difference in pregnancy outcomes using LAH or acid Tyrode's solution. Also, embryos from patients with a good prognosis were cultured without any AH and replaced at the blastocyst stage on day 5. The authors found a significantly greater implantation rate for day 5 non-hatched blastocysts than for both the LAH and chemical hatched groups. In contrast, Feng and collaborators [60] reported that LAH, mechanical AH, or with acid Tyrode's solution, increased the pregnancy rate when compared with the control group. In addition, the authors found that both chemical and LAH were more effective in enhancing clinical pregnancy compared to PZD. Chemical AH has also been suggested for thinning the ZP in fresh embryo transfers. In a prospective randomized trial, Tucker and co-workers [61] were not able to report any improvement in implantation rates in a cohort of about 200 couples undergoing ART with AH performed on day 3 embryos, prior to uterine replacement. The authors did not observe any improvement in clinical outcome: even in specific groups of patients, such as those with high FSH or embryos with a thick ZP; thus, they concluded that embryos with a thicker ZP implanted as well as those with a thinner ZP independently of the AH procedure. In another retrospective comparative study investigating four different AH procedures, including mechanical PZD, LAH, chemical breaching by acidified Tyrode's solution, and ZP thinning using pronase, Balaban and colleagues [62] reported no difference in terms of implantation and clinical pregnancy rates when compared with the control group. In contrast, in a randomized trial, Yano and co-authors [63] reported a superior efficacy in terms of implantation and clinical pregnancy rates with partial zona thinning compared to controls without AH. However, those data are inconclusive regarding efficacy and a correlation between ZP thinning and the incremental increase in implantation potential following fresh embryo replacement. Overall, these studies indicated a tendency to exclude the benefit of AH in all patients and advocated that better outcomes are only confined to subgroups of poorer prognosis patients.

5.1. Assisted hatching in frozen embryo transfer

In the 1990s, AH found its maximum diffusion and utilization, especially in cryopreserved human embryos, using acid Tyrode's solution as the method for AH. A retrospective trial by Check and collaborators [64] found that the clinical pregnancy and implantation rates were increased in the AH group compared to the non-AH group. In the non-hatching group, embryos were cultured in human tubal fluid + 0.5 % bovine serum albumin for 48 h after thawing and transferred. In the hatching group of patients, embryos were cultured in human tubal fluid + 10 % synthetic serum substitute for 72 h after thawing, then had AH and were transferred. Therefore, it needs to be mentioned that the culture conditions as well as protein supplementation were different between the two groups; thus, it was speculated that this improvement could be due to other factors and not only due to the AH procedure. Another prospective blinded randomized investigation performed on frozen/thawed cycles at the cleavage stage (on day 2) reported an increased implantation rate in the AH group compared with the control group (11.4 % versus 5.8 %; $p < 0.005$) [65]. However, different results have been reported following chemical zona thinning. In a prospective randomized study, Sifer and co-workers [66] cryopreserved surplus embryos at the cleavage stage (days 2–3), followed by partial enzymatic digestion of the ZP by pronase at the thawing step. Their results found no improvement in pregnancy outcome: implantation and clinical pregnancy rates were similar between the AH treatment and control groups. No benefits have also been reported with mechanical hatching and PZD in frozen embryo transfers by Tucker and co-authors [15]. An additional study by Edirisinghe and colleagues on PZD performed after thawing of frozen day 3 embryos, could not show any advantage in patients with advanced female age, or those patients with previous IVF failures [67]. In contrast with the previous studies, Vanderzwalmen and collaborators [68] found encouraging results in a retrospective investigation, in which artificial opening of the ZP was performed on blastocysts. The authors analysed 281 blastocysts after vitrification and warming, concluding that artificial opening of the ZP significantly increased implantation and pregnancy rates. A recent study published by Wei and co-workers [69] analysed 3535 FETs, out of which 2297 were non-LAH cycles and 1238 were treated with LAH. Their results found a higher LBR in the AH group compared to the non-LAH group (34.9 % versus 31.4 %, $p = 0.024$). Furthermore, the LAH group indicated a reduction in pregnancy loss and ectopic pregnancy rates, but those variations were not statistically significant ($p = 0.078$, $p = 0.063$ respectively). Finally, a recent study by Alteri and collaborators [70] investigated the effect of partial zona removal by AH in patients aged 18–39 years who underwent non-donor IVF cycles, having an elective single embryo transfer with a vitrified/warmed blastocyst. Overall, 698 participants were included in the study: 352 women were assigned to the AH group and 346 to the control arm. Their results showed a similar LBR (AH: 105 (29.8 %) versus non-AH: 101 (29.2 %). Following a further analysis considering women's age, cause of infertility, method of insemination, blastocyst quality, and day of blastocyst development, their results failed to report any clinical situation that could benefit from AH of blastocysts in FET [70]. Indeed, based on the findings presented above, there is insufficient evidence showing a clear benefit in terms of implantation potential and pregnancy outcomes in FETs after AH. Further studies, including well-designed and large RCTs, are still required to establish some benefit of AH in ART cycles.

6. Different hatching methods: chemical and mechanical

Thinning of the ZP using a chemical such as acidified Tyrode's solution at a pH of 2.5, or pronase, was first described for hatching in the mouse blastocyst [71–73]. The chemical approach was commonly used for zona thinning and biopsy before the advent of laser technology about 20 years ago (Fig. 1A, B). In this case, a holding pipette was used to firmly support the embryo which was then gently touched by an AH pipette filled with acid Tyrode's solution [3,9]. The acid Tyrode's solution was released gradually from the micropipette until the operator could observe thinning of the ZP or expelled until a trough ranging from 20–40 μm in length was obtained to perform the zona drilling [9,61,74–76]. However, the acid Tyrode's solution had to be released slowly and with caution and stopped immediately once desired thinning was achieved (Fig. 1A, B). Alternatively, AH can also be performed mechanically, using a micropipette and a micromanipulator. One of the first mechanical approaches applied was PZD, in which the embryo is held firm using a holding pipette and an opening was obtained by introducing an injection pipette through the ZP, followed by crushing the ZP smoothly against the holding pipette [76–78]. However, an important point and limitation to mention regarding mechanical zona dissection, is that the size of the hole cannot be controlled, and it may not be sufficient to achieve embryo hatching. In addition, there is a risk of mechanical injury during the manipulation, including squeezing or damaging cells of the embryo; also, there is a risk that the hydrostatic pressure might induce potential harm to the spindle, microtubules and the cytoskeleton [79,80]. Over the years, different micropipettes have been manufactured to carry out mechanical dissection with minimal risk of damaging the embryo. Generally, the pipettes used for PZD are the same as those applied for the intracytoplasmic sperm injection (ICSI) procedure [81]. Extra caution is required when this technique is

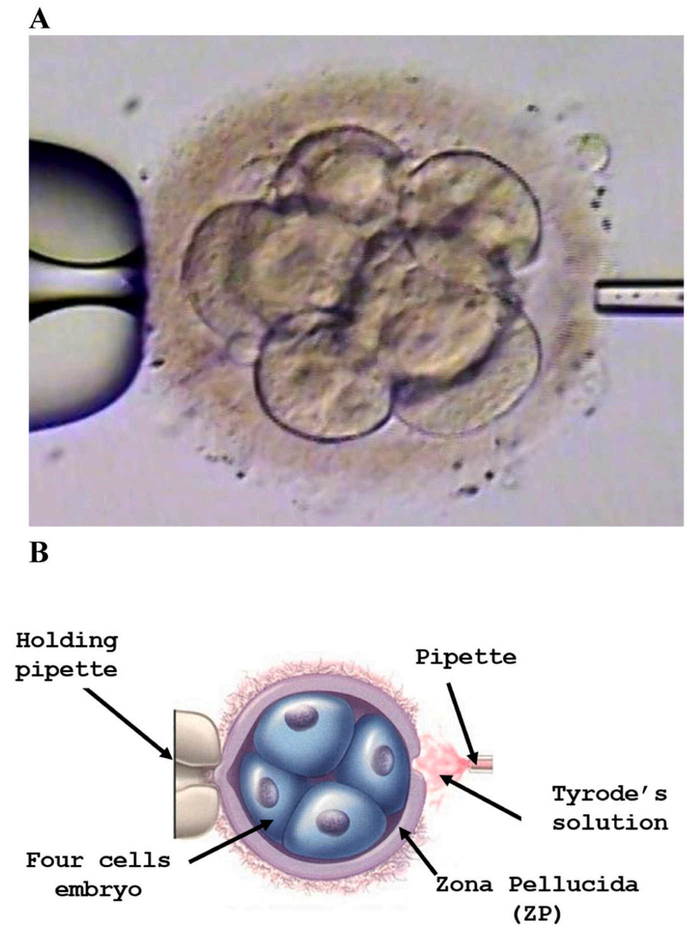

Fig. 1. (A, B): Use of acid Tyrode's solution or pronase for chemical thinning of the ZP. The embryo is held by a holding pipette and gently touched with a pipette filled with acidified/enzymatic solution.

performed on blastocysts, whereby, the hole in the ZP needs to be far away from the inner cell mass [80,81]. Another suggested approach is piezo micromanipulation, which involves a slight modification of the PZD method. The difference is the application of a vibratory motion of the piezo pipette produced by a piezoelectric pulse, and this is utilized so that ZP thinning, or a hole, can be obtained in a designated area [62].

6.1. Laser assisted hatching techniques

For the following reasons, even though it is the most expensive method, LAH is the most widespread technique used in ART nowadays: short exposure time, ease and accuracy of use, safety and efficacy [82]. The evolution of non-contact lasers utilizes infrared in the range of 800–1500 nm, which will not induce DNA damage, since the infrared range is well away from the DNA absorption peak of 260 nm. The first laser applied for human embryos in ART cycles was the indium-gallium-arsenic-phosphorus semiconductor diode laser, used by Rink and collaborators, who managed to drill mouse oocytes without the need for direct contact [83]. Laser pulses (10–20 ms), at a power of 60–70 mW, were applied to drill openings in the ZP that were 5–7 μm in diameter. They reported 70 % blastocyst formation in zona-drilled mouse oocytes with this method [83]. Interestingly, one of the main advantages of this laser was that it avoided the genetic mutation that had been described in other UV contact lasers, and this benefit contributed to making this type of laser a valuable tool for ART. With the assistance of this type of laser, the first live birth was described in 1995 by Germon and collaborators in Lausanne, Switzerland [84]. Practically, LAH can be delivered using three different methods as described in

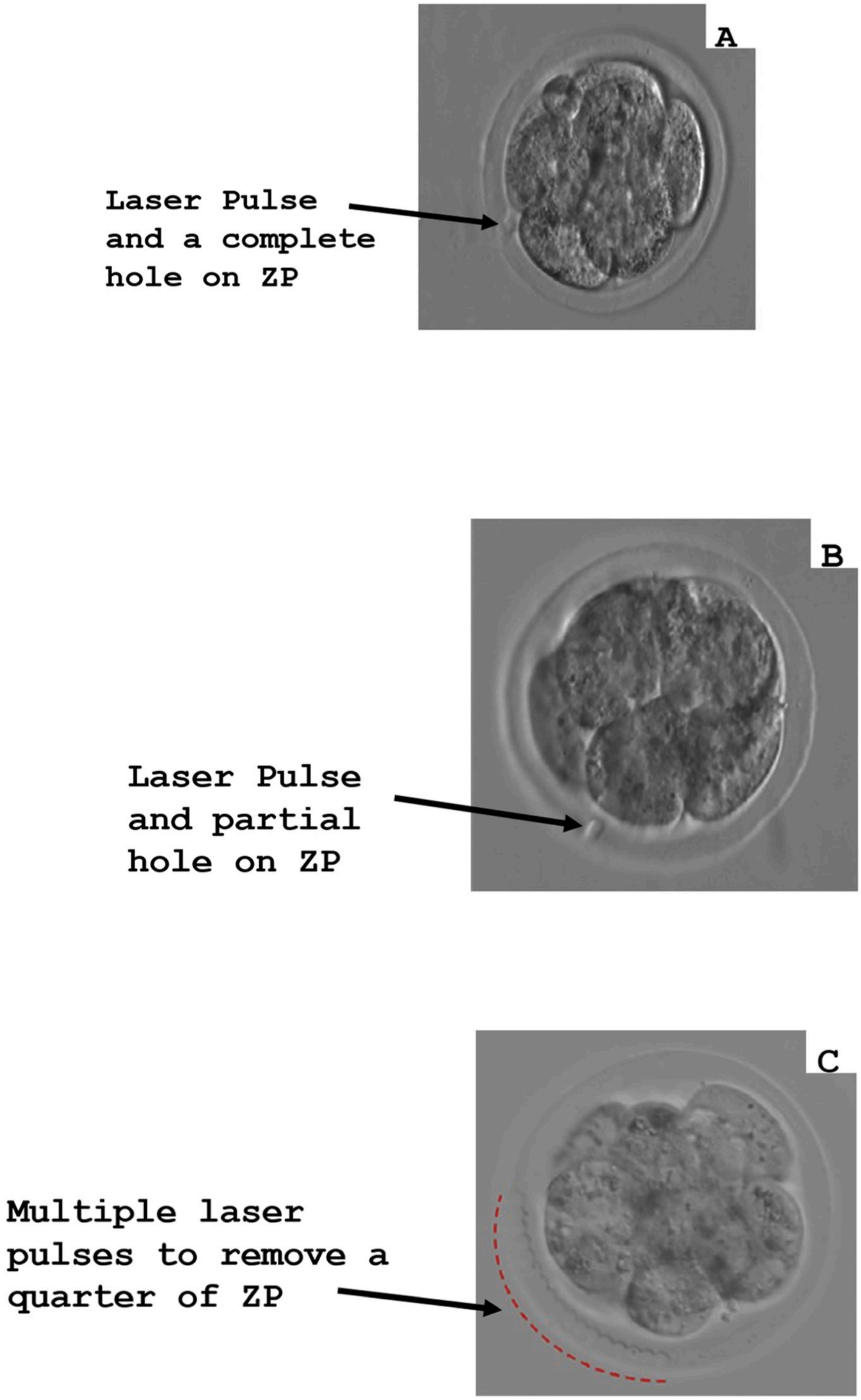

Fig. 2. Use of LAH. A: Complete LAH, in which a single hole is used to completely drill through the ZP. B: Partial LAH, in which a single hole is applied without breaching the inner membrane of the ZP (black arrow indicates the position of the laser pulse). C: Quarter LAH, in which around a quarter of the ZP is partially drilled (red interrupted line).

Fig. 2. A laser pulse can be used to induce a hole in one side of the ZP through most of its thickness, allowing an embryo to partially hatch (Fig. 3). Alternatively, one shot of the laser can be applied to make a hole completely penetrating both layers of the ZP, inner and outer. Finally, several multiple pulses of the laser can be utilized to reduce the thickness of the ZP around the embryo, to easily induce the hatching process. The specific effects of these three different applications of LAH remain controversial and it is not completely clear which method results in better pregnancy outcomes. However, an investigation conducted by Mantoudis and co-authors, comparing laser-assisted zona thinning, partial hatching, and full hatching methods [85], showed that zona thinning should be the favoured procedure to obtain a higher pregnancy outcome rather than total dissection. Similar results have been obtained recently in a large study by Wang and colleagues [86]. They systematically analysed the effects of two procedures for LAH: the drilling (D-LAH) group (694 patients) and the thinning (T-LAH) group (716 patients), in a total of 1410 patients undertaking a FET. Their results showed both implantation and clinical pregnancy rates to be significantly higher in the T-LAH group compared to the D-LAH group (32.73 % versus 29.09 %, $p < 0.01$ %, and 50.98 % versus 43.95 %,

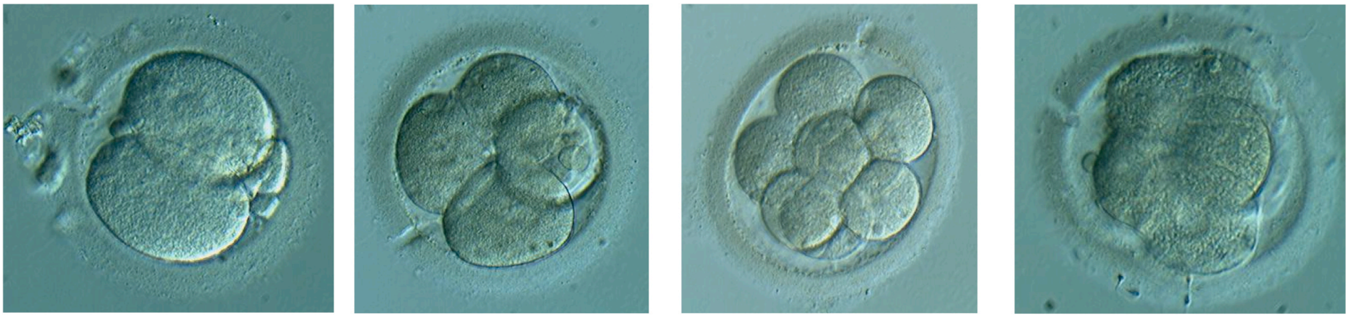

Fig. 3. Use of LAH at different embryonic development stages.

$p < 0.01$, respectively). The proportion of live births was also slightly higher in the T-LAH group, but this variation was not statistically significant (39.11 % versus 36.89 %, $p > 0.05$) [86]. Also, a meta-analysis and systematic review published by Chen and co-authors analysed the usage of laser AH and compared two methods: D-LAH and T-LAH, and correlated them with clinical pregnancy rates in women undergoing ART cycles [87]. The meta-analysis involved nine studies with 2405 clinical pregnancies from D-LAH and 2239 from T-LAH. Their results showed no difference in the clinical pregnancy rates between the two techniques. Even after subgroup analyses, the authors found no substantial differences between the two methods.

7. Clinical application of laser technology: blastocyst collapse and embryo biopsy

Laser technology is a simple, accurate and effective feature, and has been applied in different fields, including ART for about three decades. Prior to blastocyst vitrification, the use of artificial shrinkage induced by a laser pulse might facilitate the diffusion of cryoprotectants into the embryo, and thus, the embryo's exposure to the equilibration solution can be reduced to what would be adequate to obtain an efficient vitrification process [88]. Full and expanding blastocysts include a large amount of fluid in the blastocoel cavity, which during the process of vitrification could be easily converted into ice crystals and impair the efficiency of the vitrification procedure. With the application of a laser pulse, expanded blastocysts can be easily collapsed, lose fluid in a short time and be converted into a morula-like stage. A laser pulse at a minimal setting, orientated at the junction between two trophectoderm cells, away from the inner cell mass, can be applied to induce an artificial blastocyst collapse and optimize vitrification-warming outcomes [89]. This agrees with several investigations by other authors reporting an improvement in cryo-survival, implantation and clinical pregnancy rates using the laser collapse approach prior to vitrification [88,89]. An additional interesting application to mention regarding laser technology and ART is at the time of embryo biopsy (Fig. 4). Genetic testing of embryos has been increasingly applied worldwide to scrutinize chromosomal aberration or hereditary disorders. To complete such types of assessment, one or two cells from a cleavage stage embryo, or 5–10 cells from a blastocyst need to be collected and then sent to the genetics laboratory for analysis. Alternatively, genetic material can also be obtained by performing first and second polar body biopsies on the oocyte, which, however, involves genetic screening from only the maternal side. Historically, to perform embryo biopsy a hole in the ZP has been created using acid Tyrode's solution or mechanical methods utilising a sharp glass micropipette [90]. However, both procedures are quite variable and might result in inconsistent hole sizes or induce cell harm. In addition, with Tyrode's solution, there is a high risk that the embryo itself can be exposed to acidic levels of pH and be irreversibly damaged [90,91]. Currently, the use of a laser to perform embryo biopsy is considered the gold standard: laser pulses can easily induce a uniform hole in the ZP, whereby a biopsy pipette can pass through to remove the designated cells, without the potential risk of exposing the embryo to the

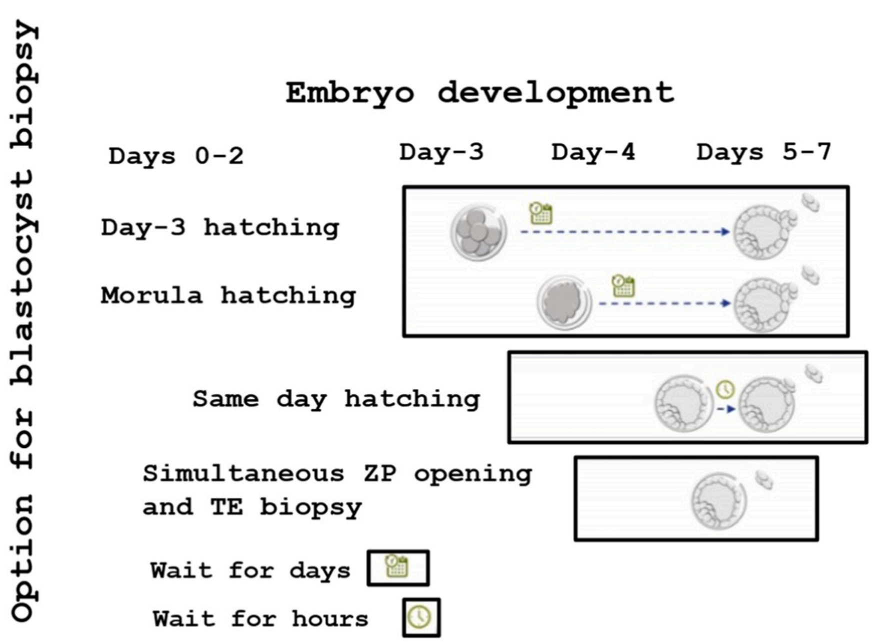

Fig. 4. Use of LAH to facilitate embryo biopsy. TE: trophectoderm; ZP: zona pellucida

acidic pH of Tyrode's solution. Investigations assessing the utilization of acid Tyrode's and laser zona drilling for human embryo biopsy have demonstrated that the use of the laser is easy and quick, ensuring a greater number of intact blastomeres, also consistently reducing the time the embryo stays outside the incubator [90–92].

8. Safety concerns and potential risks associated with AH and the use of lasers

It has been reported by several authors that AH might increase the risk of multiple pregnancies [6–8]. This is an important concern to take into consideration since the risk of complications is increased for both mothers and newborn babies. It is therefore critical to clarify this aspect with additional and well-designed large RCTs. Also, it has been reported that a complete hole through the entire ZP might negatively impact the process of blastocyst expansion or loss of blastomeres, as well as increase the risk of bacterial contamination or immunological attack [2]. Some authors have investigated the association between AH and ectopic pregnancy. A retrospective investigation of 623 clinical pregnancies obtained after standard IVF, found an increase in ectopic pregnancy of 5.4 % when AH was applied compared to 2.2 % in the group without hatching [93]. However, some other authors [85–87] have found no significant difference in the rates of miscarriage, multiple pregnancy, and ectopic pregnancy comparing LAH with non-LAH. Regarding the use of laser technology, since the first introduction of this procedure into ART, there were several concerns about safety, especially related to the risk of possible increased congenital alterations, DNA damage, as well as impairment of normal embryo development [94]. Laser wavelength, power and pulse length have been the main features scrutinized and, in particular, UV wavelength has always been investigated as inducing possible mutagenic outcomes and compromised DNA integrity. However, the wavelength normally applied by lasers used in the field of ART is specifically infrared, far away from those that can interfere with DNA integrity and thus induce harm [95]. Concerning the power of the laser, it can affect the size and width of the hole produced and the amount of heat created [95]. Lasers normally use high energy light, which contacts the medium surrounding the embryo, and thus, the heat produced will be quickly dissolved into the culture medium, therefore not affecting the embryo. The risk of thermal harm has been investigated by Wong and colleagues [96], and their conclusions failed to identify any significant harm to human embryo development after the use of laser procedures on vitrified-warmed blastocysts. Another study by Chailert and co-workers [97] showed that the utilization of a laser to completely open the ZP was positively associated with the number of hatched blastocysts in comparison with LAH thinning of the ZP and the control group; however, the number of trophectoderm cells and cells of the inner cell mass were reduced when both LAH and laser assisted zona thinning was applied in comparison with controls. Contrasting results have been reported by other authors: Honguntikar and colleagues [98] have reported an increase in DNA fragmentation at the blastocyst stage in the mouse model exposed to LAH. In a subsequent study, the same authors investigated the epigenetic dysfunction associated with the use of a laser in mouse embryos [99]. They reported that the expression of two *de novo* methyl transferases (*Dnmt3a* and *Dnmt3b*) decreased following LAH performed on day 2 of embryo development, whereas LAH at day 3 or day 5 did not induce any impairment in the equivalent genes. Also, the methylation of long interspersed nuclear element 1 (LINE-1), a marker of global genomic DNA methylation, did not alter following LAH at any embryonic stages (cleavage and blastocyst investigated) [99]. Also, an additional study by Fan and collaborators [100] investigated the effect of ZP removal by acid Tyrode's solution in mouse embryos. The authors found that the expression of differentiation-related genes in the inner cell mass and trophectoderm was significantly altered in ZP-free blastocysts compared with ZP-intact embryos [100]. To summarize, overall research involving both animal models and humans has not yet provided a clear answer. It seems that lasers might induce some effects on embryo physiology and development, but currently, there is insufficient evidence to support a clear link between the use of lasers and embryo viability and pregnancy potential. Finally, additional investigations and long-term studies of pregnancy rates and the health of offspring are needed to clarify and support the safety of laser techniques in the field of reproductive medicine.

9. Concluding remarks

This narrative review summarizes current knowledge of AH using various procedures to reduce the thickness of the ZP and illustrates technical aspects of zona manipulation to improve pregnancy outcomes and implantation potential in ART cycles. Many different methods are being used for AH, which may differ in both efficacy and risks. Evidence suggests that LAH is currently considered the most applied method as regards safety and efficacy. Improving AH techniques based on the data obtained from evidence-based medicine is critically important for the future. Finally, even though AH methods have been utilized worldwide for more than 30 years, they still lack consolidation from acceptable well-designed, large RCTs.

Authors' Contributions

R.S, S.F.; Contributed to data collection and curation. R.S., M.A., L. M., PF.G., E.G., S.F.; Contributed to conception and design. R.S.; Contributed to the interpretation of data, and drafted the manuscript, which was revised by S.F.; All authors read and approved the final manuscript.

Informed Consent Statement

Not applicable.

Human and animal rights

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institution and with the 1964 Helsinki declaration and its later amendments. For this type of study, formal consent is not required.

Funding

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

Declaration of Competing Interest

The manuscript has been seen and approved by all authors and is not under active consideration for publication. Finally, the authors declare that they do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted. SF is also an employee of CooperSurgical Fertility Solutions.

Data availability

No data are available.

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