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human reproduction update

Assisted hatching of human embryos: a systematic review and meta-analysis of randomized controlled trials

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TABLE OF CONTENTS

BACKGROUND: Assisted hatching (AH) is a manipulation of zona pellucida aiming to facilitate embryo implantation.

METHODS: Systematic review and meta-analysis of medical literature was used to evaluate the effect of AH on assisted reproduction outcomes: clinical pregnancy, live birth, multiple pregnancy and miscarriage. Additional analysis was performed in these subgroups: (i) fresh embryos transferred to unselected or non-poor prognosis women; (ii) fresh embryos transferred to women with previous repeated failure; (iii) fresh embryos transferred to women of advanced age; (iv) frozen-thawed embryos transferred to unselected or non-poor prognosis women. Analyses were based on risk ratio and 95% confidence intervals (RR, 95% CIs) using Mantel–Haenszel random effects model.

RESULTS: There were 28 studies (5507 participants) included. AH was related to a trend toward increased clinical pregnancy for all participants (RR = 1.11, 95% CI = 1.00–1.24), with a significant increase in subgroups 2 (RR = 1.73; 95% CI = 1.37–2.17) and 4 (RR = 1.36; 95% CI = 1.08–1.72, $P < 0.01$), but not for subgroups 1 and 3. For multiple pregnancy, a significant increase was observed for all participants (RR = 1.45; 95% CI = 1.11–1.90) and for subgroups 2 (RR = 2.53; 95% CI = 1.23–5.21) and 4 (RR = 3.40; 95% CI = 1.93–6.01). No significant heterogeneity was observed in subgroup analysis.

CONCLUSIONS: AH was related to increased clinical pregnancy and multiple pregnancy rates in women with previous repeated failure or frozen-thawed embryos. However, AH is unlikely to increase clinical pregnancy rates when performed in fresh embryos transferred to unselected or non-poor prognosis women or to women of advanced age. Due to the small sample evaluated by the pool of included studies, no proper conclusions could be drawn regarding miscarriage or live birth.

Key words: reproductive techniques / zona pellucida / embryo transfer / assisted hatching

Introduction

The embryo implantation rate in assisted reproduction procedures is ~20%, which leads to a low clinical pregnancy rate, ~35%, and even lower live birth rate, ~25%, per cycle started (Gunby *et al.*, 2010). Improving these rates is one of the main objectives of the new assisted reproduction technologies (ARTs). One of the most common methods to obtain better results is to obtain and to transfer multiple embryos, while freezing the spare embryos that can be used in future cycles (Jungheim *et al.*, 2009). However, this approach is associated

with the two main problems of ARTs: multiple pregnancy (Bissonnette *et al.*, 2007) and ovarian hyperstimulation syndrome (Nastri *et al.*, 2010). Low embryo quality and poor endometrial receptivity are frequently denoted as the main reasons for the low implantation rate in humans. Additionally, some researchers believe that difficulties during the blastocyst hatching process could also negatively interfere with the implantation process (Cohen *et al.*, 1990).

The human embryo is surrounded by a glycoprotein layer, named as the zona pellucida, which permits only acrosome-intact sperm to fertilize the oocyte and blocks the entry of multiple sperm. After fertilization, the zona pellucida compresses and shapes the embryo, facilitating the passage through the Fallopian tubes into the endometrial cavity and protects the embryo from micro-organisms and immune cells (Zhao and Dean, 2002). The embryo at the blastocyst stage then breaks out of this protective layer to start the deployment process, and failure at this stage can prevent implantation. The artificial rupture of the zona pellucida is known as assisted hatching (AH) and this technique has been used since the late 1980s (Cohen *et al.*, 1988), in attempts to improve the chances of implantation and clinical pregnancy during assisted reproduction.

There are three possible mechanisms by which AH could improve embryo implantation. (i) Zona pellucida hardening caused by IVF and cell culture (DeMeestere *et al.*, 1997) or cryopreservation (Carroll *et al.*, 1990) might make hatching difficult, which could be solved by AH. (ii) AH is associated with the anticipation of implantation in humans (Liu *et al.*, 1993), which is particularly relevant since the implantation window seems to occur 1–2 days earlier in women undergoing ovarian stimulation with exogenous gonadotrophins when compared with women during their natural cycles (Nikas *et al.*, 1999). (iii) Artificial opening could also serve as a channel for exchange of metabolites, growth factors and messages between the embryo and endometrium (Cohen *et al.*, 1992b). However, there is currently no recommendation to perform AH routinely in women undergoing assisted reproduction (ASRM, 2008), since no single study has been able to demonstrate sufficient evidence favorable to AH. However, no study has included a sufficient sample to properly evaluate the effect of AH on assisted reproduction outcomes; this sample could be easily obtained by a meta-analysis.

Methods

Eligibility criteria

Eligibility was confined to randomized controlled trials published as full articles evaluating the effect of AH human embryos compared with a control group in which embryos were not submitted to AH. Additionally, in order to reduce heterogeneity, analyses were also performed in these subgroups: (i) fresh embryos transferred to unselected or non-poor prognosis women; (ii) fresh embryos transferred to women with previous repeated failure; (iii) fresh embryos transferred to women of advanced age; and (iv) frozen/thawed embryos transferred to unselected or non-poor prognosis women. By non-poor prognosis women, we are referring to women evaluated by studies that used as inclusion criteria at least one of the following: a maximum age; a maximum FSH value; a maximum number of previous unsuccessful IVF attempts; a minimum number of embryos available to be transferred; the absence of uterine abnormalities.

The subgroup analysis was performed because there is a doubt about whether AH may be beneficial for every woman undergoing ARTs, regardless of any specific condition; this issue is assessed by subgroup 1 analysis. The clustering of women into the three other subgroups (previous repeated failure, advanced maternal age and frozen/thawed embryos) was based on the specific situations most investigated by RCTs, since a greater/better effect of AH is expected (ASRM, 2008; Ge *et al.*, 2008). However, the underlying mechanisms by which women would benefit from AH are different for each subgroup. In subgroup 2, we are considering that repeated unsuccessful attempts may be related to difficulties in the hatching process; in subgroup 3, we are investigating the hypothesis that maternal age has a negative impact on the embryonic hatching; and in subgroup 4, we are taking into account the possibility that the process of freezing/thawing could hinder embryo hatching, likely by increasing the rigidity of the zona pellucida.

Information sources

Studies were identified by searching electronic databases and scanning reference lists of articles by two independent reviewers (W.P.M. and I.A.R.). The search was last performed on 1 December 2010. The electronic searches were performed using both PubMed and OvidSP and included the following databases: MEDLINE, Cochrane Central Register of Controlled Trials and Cochrane Database of Systematic Reviews, Cochrane Methodology Register, Database of Abstracts of Reviews of Effects and Biological Abstracts. The following terms were used in electronic search: [(zona pellucida) or (AH)] and [(implantation) or (pregnancy)]. We did not search for ongoing trials. Eligibility assessment was performed independently in a non-blinded standardized manner by two reviewers (W.P.M. and I.A.R.). Disagreements between reviewers were resolved by consensus.

Data collection process

Data were collected in a sheet based on the Cochrane Consumers and Communication Review Group's data extraction template. One review author (W.P.M.) extracted the following data from included studies and the second author (I.A.R.) checked the extracted data. Disagreements were resolved by discussion between the authors.

Data items

The following data were extracted from included studies: (i) methods: aim of intervention, method of recruitment of participants, inclusion/exclusion criteria, and if informed consent was obtained as well as ethical approval; (ii) participants characteristics: number and age; (iii) intervention: method used for AH and time of AH; and (iv) results: number of women, clinical pregnancy, live birth, multiple pregnancy and miscarriage. Data regarding randomization, allocation concealment, blinding and other sources of bias were evaluated in the included studies.

Summary measures

All results were combined for meta-analysis with Review Manager 5 for Windows (Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2008). The meta-analysis was performed using Mantel–Haenszel random effects model. The number of women who were randomly allocated was considered as the total number of participants. Risk ratios (RR), 95% confidence intervals (95% CIs), and *P*-values were calculated. To evaluate the power, we arbitrarily defined as clinically relevant a RR $\geq$ 1.2 (a relative increase $\geq$20% in the evaluated outcome).

Analysis of heterogeneity

Heterogeneity between studies was assessed using τ^2, χ^2 and I^2. Currently, I^2 is the preferred test to evaluate inconsistency across studies, as this describes the percentage of the variability in effect estimates that is due

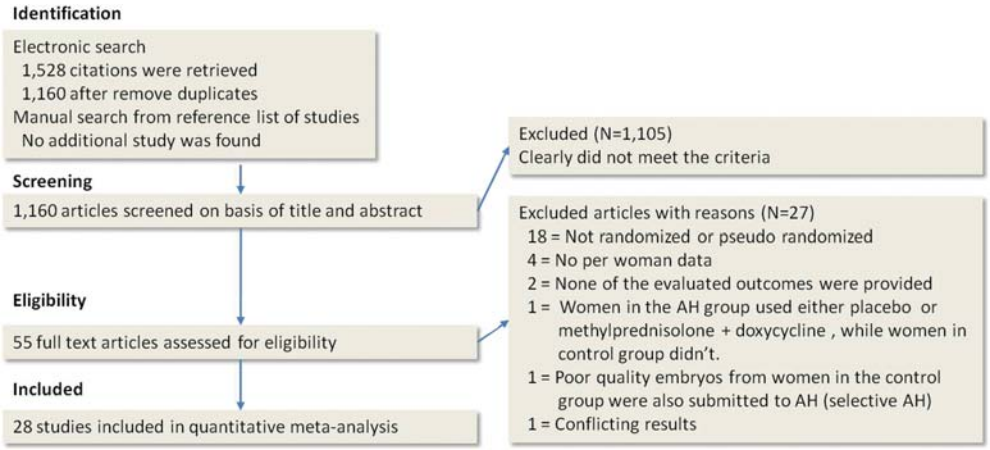

Figure 1 Flowchart of study selection.

to heterogeneity rather than sampling error (chance) (Higgins and Green, 2009). For interpretation of I^2: 0–40%: might not be important; 30–60%: may represent moderate heterogeneity; 50–90%: may represent substantial heterogeneity and 75–100% may indicate considerable heterogeneity.

Results

Study selection

The electronic search retrieved a total of 1160 citations (1528 before adjusting for duplicates). After reading titles and abstracts, 1105 studies were discarded because the articles clearly did not meet the criteria due to one of the following aspects: the study (i) did not evaluate the effect of AH performed in human embryos during assisted reproduction ($n = 997$); (ii) was not a randomized controlled trial ($n = 95$) or (iii) did not have a control group where AH was not performed ($n = 13$).

From the 55 fully evaluated articles, 27 were excluded with reasons: 18 studies were not randomized or were pseudo-randomized (Tucker *et al.*, 1991; Obruca *et al.*, 1994a, b; Takahashi *et al.*, 1994; Antinori *et al.*, 1996a; Bider *et al.*, 1997; Tao and Tamis, 1997; Edirisinghe *et al.*, 1999; Hershlag *et al.*, 1999; Mansour *et al.*, 2000; Horng *et al.*, 2002; Kinget *et al.*, 2002; Gabrielsen *et al.*, 2004; Frydman *et al.*, 2006; Yano *et al.*, 2007; Valojerdi *et al.*, 2008; Debrock *et al.*, 2009; Zhang *et al.*, 2009); four studies were excluded because they did not provide per woman data (Magli *et al.*, 1998; Nakayama *et al.*, 1999; Ebner *et al.*, 2002; Ma *et al.*, 2006); two studies did not provide any of the evaluated outcomes (Liu *et al.*, 1993; Wong *et al.*, 2003); in one study, women who were submitted to AH also used either placebo or methylprednisolone + doxycycline (Primi *et al.*, 2004), while women in the control group were not submitted to neither placebo nor methylprednisolone + doxycycline; in one study, the control group consisted of women whose embryos were submitted to selective AH (Cohen *et al.*, 1992a); and one study had conflicting results (Tucker *et al.*, 1996): in the group of AH the authors reported 21 clinical pregnancies but they also stated that only 16 embryo implantations were observed.

Therefore, 28 studies were included in this meta-analysis and the flowchart of study selection is shown in Fig. 1. We created two sections in three studies (Petersen *et al.*, 2005; Ge *et al.*, 2008; Kutlu *et al.*, 2010) and three sections in one study (Cohen *et al.*, 1992b), since completely different trials evaluating women and/or embryos with different characteristics were reported inside these studies. Considering these divisions, 33 trials were evaluated.

Characteristics of included studies

The characteristics of the included studies are described in Table I. Regarding subgroup analysis, 12 trials were included in the subgroup 'fresh embryos transferred to unselected or non-poor prognosis women'; five trials were included in the subgroup 'fresh embryos transferred to women with previous repeated failure'; four trials were in the subgroup 'fresh embryos transferred to women with advanced age' and six trials were in the subgroup 'frozen-thawed embryos transferred to unselected or non-poor prognosis women' (Table I). Additionally, we evaluated more than six trials from five studies which did not fit in any of the previously described subgroups (Table I). The first trial from the study by Cohen *et al.* (1992b) was included in the subgroup fresh embryos transferred to unselected or non-poor prognosis women; however, the second trial compared the selective AH (AH performed only in poor prognosis embryos) with a control group, both from woman with Day 3 FSH $\leq$ 15 IU/l, and the third trial evaluated AH performed in all fresh embryos from women with Day 3 FSH $>$15 IU/l. The study of Hagemann *et al.* (2010) included only women whose embryos had a zona pellucida thickness of $\geq$13 μm, and AH was only performed on these embryos. The study of Nadir Ciray *et al.* (2005) included only women with endometriosis. The study of Rufas-Sapir *et al.* (2004) included only women with repeated previous failure ($\geq$3) who had at least 3–4 grade A or B embryos. The study from Valojerdi *et al.* (2010) included only women submitted to vitrified–warmed embryo transfer.

Regarding the subgroup 'frozen-thawed embryos transferred to unselected or non-poor prognosis women', one may still consider a chance of bias by selecting other specific subgroups of women for

Table I Characteristics of the included studies.

Study	Method for allocation	Allocation concealed	Blinding	Informed consent	Ethical approval	Inclusion criteria	Exclusion criteria	AH	Time of AH	Participants AH/control	Age (mean ± SD) intervention /control
Fresh embryos transferred to unselected or non-poor prognosis women											
Balakier *et al.* (2009)	Computer-generated list	Yes	Yes, both participants and assessors	Yes	Yes	Age ≤37 years, Day 3 FSH baseline ≤10 IU/l, and no more than one previous unsuccessful IVF-ET attempt	None	Laser	Day 3 embryos, 1–3 h before embryo transfer	45/39	32.5 ± 3.8/ 33.8 ± 3.2
Baruffi *et al.* (2000)	Randomization table	Unclear	Unclear	Yes	Yes	Age ≤37 years, male infertility, first attempt	None	Laser	Day 2 embryos	51/52	31.8 ± 3.6/ 31.4 ± 3.6
Cohen *et al.* (1992b) (I)	Not stated	Unclear	Yes, both participants and assessors	Yes	Yes	Day 3 FSH levels ≤15 IU/l	None	Acid Tyrodés	68–72 h after oocyte retrieval, 4–8 h before embryo transfer	69/68	36.5 ± 3.3/ 36.7 ± 3.7
Ge *et al.* (2008) (I)	Sealed envelopes	Yes	Yes, both participants and assessors	Yes	Yes	Baseline FSH = 3–12 IU/l; <5 failed cycles of assisted reproduction treatment	Uterine abnormality or fertilization rate <20%	Laser	Days 2–3 embryos, immediately prior to embryo transfer	387/373	31.1 ± 4.7/ 30.4 ± 4.2
Hellebaut *et al.* (1996)	Computerized	Unclear	Unclear	Yes	Unclear	Women undergoing embryo transfer	None	Mechanical	Day 2 embryos	60/60	30.9 ± 4.3/ 30.8 ± 3.9
Hurst *et al.* (1998)	Computerized	Unclear	Unclear	Yes	Yes	Age ≤30 years; FSH ≤10 IU/l; normal endometrial cavity and semen; or Age ≤35 years; with six embryos resulted from the prior to IVF attempt; fertilization rate >50%; normal endometrial cavity	None	Acid Tyrodés	Day 3 embryos	13/7	30 ± 0.9/ 30 ± 0.8
Isik *et al.* (2000)	Table	Unclear	Unclear	Yes	Yes	>5 embryos on Day 3		Pronase	Day 5 embryos, 30–60 min before embryo transfer	24/22	29.1 ± 3.6/ 30.5 ± 5.2

Continued

Table I *Continued*

Study	Method for allocation	Allocation concealed	Blinding	Informed consent	Ethical approval	Inclusion criteria	Exclusion criteria	AH	Time of AH	Participants AH/control	Age (mean ± SD) intervention /control
Kutlu *et al.* (2010) (I)	Computerized	Unclear	Unclear	Yes	Yes	Age <35 years with at least four metaphase II oocytes	Severe male infertility or where testicular sperm were used, and cases with preimplantation genetic diagnosis indication, three or less embryos on Day 2	Laser	Day 3 embryos	73/66	29.9 ± 2.9/ 28.9 ± 3.4
Petersen *et al.* (2005) (I)	Table	Yes	Unclear	Yes	Yes	1 previous failure	None	Laser	Days 2–3 embryos	35/35	34.6 ± 4.6/ 34.1 ± 5.3
Sagoskin *et al.* (2007)	Computer-generated list 2:1	Unclear	Unclear	Yes	Yes	Viable embryos on Day 3; age ≤39 years; FSH ≤10 IU/l; first or second attempt; ovulatory menstrual cycles; no uterine abnormality or communicating hydrosalpinx	None	Laser	Day 3 embryos	121/82	34.0 ± 3.3/ 34.0 ± 3.2
Tucker *et al.* (1993)	Not stated	Unclear	Unclear	Yes	Unclear	Women undergoing embryo transfer	None	Acid Tyrodés	Day 3 embryos, 1–3 h before embryo transfer	110/108	34.1 ± 4.8/ 34.2 ± 4.1
Urman *et al.* (2002)	Table (computer-generated numbers)	Unclear	Unclear	Yes	Yes	Male infertility	Previous-failed fertilization	Pronase	Day 5 embryos	121/119	31.8/31.5
Fresh embryos transferred to women with previous repeated failure											
Antinori *et al.* (1996b)	Not stated	Unclear	Unclear	Yes	Unclear	≥2 previous failures	None	Laser	Immediately before embryo transfer	72/98	38.2 ± 1.3/ 37.8 ± 1.5
Chao *et al.* (1997)	Computer-generated list	Unclear	Unclear	Unclear	Yes	≥2 previous failures	None	Mechanical	Day 2 embryos, 4–6 h before embryo transfer	49/51	36.5 ± 5.2/ 34.0 ± 3.9
Jelinkova *et al.* (2003)	Not stated	Unclear	Unclear	Yes	Yes	≥2 previous failures; two or three embryos reaching morula or blastocyst stage after 5 days of the *in vitro* culture	None	Acid Tyrodés	Day 5 embryo, 20 min before embryo transfer	128/129	32.3 ± 4.2/ 32.1 ± 3.2

Petersen *et al.* (2005) (II)	Table	Yes	Unclear	Yes	Yes	≥2 previous failures	None	Laser	Days 2–3 embryos	40/40	35.7 ± 3.8/ 35.3 ± 5.1
Stein *et al.* (1995)	Not stated	Unclear	Unclear	Yes	Yes	>3 previous failures	None	Mechanical	1.5 h before embryo transfer	72/82	Not stated
Fresh embryos transferred to women with advanced age											
Frydman *et al.* (2006)	Sealed envelopes	Yes	Yes, both participants and assessors	Yes	Yes	Age ≥37 years; <3 previous failures; reached embryo transfer process	None	Laser	Days 2–3 embryo	49/54	39.0/38.5
Kutlu *et al.* (2010) (II)	Computerized	Unclear	Unclear	Yes	Yes	Age ≥35 years with at least four metaphase II oocytes	Severe male infertility or where testicular esperm were used, and cases with preimplantation genetic diagnosis indication, three or less embryos on Day 2	Laser	Day 3 embryos	58/55	38.0 ± 2.3/ 37.4 ± 2.4
Lanzendorf *et al.* (2007)	Sealed envelopes	Unclear	Yes, both participants and assessors	Yes	Yes	Age ≥36 years	None	Acid Tyrodés	Approximately 55 h after fertilization, 16–20 h before embryo transfer	41/48	38.0 ± 2.0/ 38.5 ± 1.8
Petersen *et al.* (2002)	Not stated	Yes	Unclear	Yes	Yes	Age ≥38 years; male infertility	None	Laser	Days 2–3 embryos, immediately before embryo transfer	50/50	39.8 ± 1.3/ 40.0 ± 1.9
Frozen-thawed embryos transferred to unselected or non-poor prognosis women											
Balaban *et al.* (2006)	Computer-generated list	Unclear	Unclear	Yes	Yes	Male or unexplained fertility submitted to ICSI in the last 24 months with frozen/ thawed embryos		Laser	Day 3 frozen/ thawed embryos	183/183	32.4 ± 3.3/ 32.7 ± 3.1
Fang *et al.* (2010)	Unclear	Unclear	Unclear	Yes	Yes	First frozen/thawed embryo transfer	Only one frozen/ thawed embryo	Mechanical expansion	Day 3 frozen/ thawed embryos, 3 h before embryo transfer	61/64	32.3 ± 3.4/ 32.1 ± 2.6

Continued

Table I *Continued*

Study	Method for allocation	Allocation concealed	Blinding	Informed consent	Ethical approval	Inclusion criteria	Exclusion criteria	AH	Time of AH	Participants AH/control	Age (mean ± SD) intervention /control
Ge *et al.* (2008) (II)	Sealed envelopes	Yes	Yes, both participants and assessors	Yes	Yes	Baseline FSH = 3–12 IU/l; <5 failed cycles of assisted reproduction treatment; survival frozen/thawed embryo number ≥1; total number of living blastomeres for embryo transfer ≥3 on Day 2 or ≥5 on Day 3	Uterine abnormality or fertilization rate <20%	Laser	Days 2–3 frozen/thawed embryo, immediately prior to embryo transfer	100/100	31.8 ± 3.9/ 30.7 ± 4.4
Ng *et al.* (2005)	Computer-generated list	Unclear	Yes, both participants and assessors	Yes	Yes	≥2 frozen embryos available for transfer	>3 stimulated IVF cycles; only one frozen embryo available for transfer; frozen embryos replaced in stimulated IVF cycles	Laser	Day 2 frozen/ thawed embryo	80/80	35.0/35.0
Petersen *et al.* (2006)	Not stated	Unclear	Unclear	Yes	Yes	Women with supernumerary embryo cryopreserved	None	Laser	Days 2–3 frozen/thawed embryo	110/110	31.7 ± 4.8/ 32.5 ± 4.4
Sifer *et al.* (2006)	Computer-generated list	Unclear	Unclear	Yes	Yes	Non-donor and first frozen/thawed embryo transfer	None	Pronase	Days 2–3 frozen/thawed embryo	61/64	32.3 ± 4.0/ 32.0 ± 4.4
Other situations											
Cohen *et al.* (1992b) (II)	Not stated	Unclear	Yes, both participants and assessors	Yes	Yes	Day 3 FSH levels ≤15 IU/l; only poor prognosis embryos submitted to AH (selective AH)	None	Acid Tyrodés	68–72 h after oocyte retrieval, 4–8 h before embryo transfer	80/83	36.7 ± 4.3/ 35.3 ± 4.2
Cohen *et al.* (1992b) (III)	Not stated	Unclear	Yes, both participants and assessors	Yes	Yes	Day 3 FSH levels >15 IU/l	None	Acid Tyrodés	68–72 h after oocyte retrieval, 4–8 h before embryo transfer	15/15	Not stated

Hagemann *et al.* (2010)	Opaque envelopes	Yes	Yes, both participants and assessors	Yes	Yes	Age <38 years, zona pellucida thickness ≥13 μm	Failure of all eggs to be fertilized or elective cryopreservation of all embryos	Acid Tyrodés	Day 3 embryos, 1–3 h before embryo transfer	43/48	32.1 ± 3.0/ 31.2 ± 3.5
Nadir Ciray *et al.* (2005)	Computerized	Unclear	Unclear	Yes	Yes	Age <40; presence of endometriosis; no other cause for infertility	Zona pellucida thickness ≥15 μm; embryo transfer cancelled	Laser	Day 3 embryos, 2–4 h before embryo transfer	76/38	33.1 ± 4.2/ 34.0 ± 3.7
Rufas-Sapir *et al.* (2004)	Not stated	Unclear	Unclear	Yes	Yes	Presence of 3–4 grade A or B embryos; ≥3 previous failures; regular menstrual cycle; normal endocrine profile; normal uterine cavity and endometrium	Male infertility; habitual abortion; clinically relevant systemic disease	Acid Tyrodés	Days 2–3 embryos, 1.5 h before embryo transfer	104/103	Not stated
Valojerdi *et al.* (2010)	Sealed envelopes	Unclear	Unclear	Yes	Yes	Women undergoing vitrified/warmed embryo transfer	None	Laser	Days 2–3 vitrified/ warmed embryo	200/200	30.86 ± 5.82/ 29.85 ± 5.14

the transfer of frozen embryos (e.g. advanced age and/or previous repeated failure). However, all RCTs evaluating frozen/thawed embryos included only unselected or non-prognosis women: the authors from these studies likely did not evaluate two or more specific conditions at a same time to avoid confusion when interpreting the results.

Clinical pregnancy

There were 33 trials from 28 studies (5507 participants) that reported clinical pregnancy as an outcome and were included (Fig. 2). We observed a trend toward increased clinical pregnancy considering the pooled data analysis (RR = 1.11; 95% CI = 1.00–1.24; P = 0.05); however, the observed increase was lower than we arbitrarily defined as clinically relevant (RR ≥ 1.2) and there was substantial heterogeneity between studies (I^2 = 49%). Considering single study data, only four trials reported a significant increase in clinical pregnancy rate (Fig. 2): two evaluating AH in fresh embryos transferred to women with previous repeated failure, one using laser (Antinori et al., 1996b), and the other using acid Tyrodés (Jelinkova et al., 2003); and two evaluating AH in frozen-thawed embryos transferred to unselected or non-poor prognosis women, one using laser (Balaban et al., 2006) and the other using mechanical expansion (Fang et al., 2010). However, there was one study demonstrating a significant decrease in clinical pregnancy rate when AH was performed in vitrified–warmed embryos using laser (Valojerdi et al., 2010).

When evaluating the subgroup 'fresh embryos transferred to unselected or non-poor prognosis women', 12 trials (2140 participants) were included (Fig. 2). AH was not associated with significant changes in clinical pregnancy in any of them and the same was observed in the meta-analysis (RR = 1.05; 95% CI = 0.95–1.15). No significant heterogeneity was observed (I^2 = 0%), although the method used for performing AH was different between trials (laser = 6, acid Tyrodés = 3 and pronase = 2, mechanical = 1). Considering the observed clinical pregnancy rate in the control group (447/1031 = 43.4%), the power to detect a RR ≥ 1.2 was very high (97.6%).

Considering the subgroup 'fresh embryos transferred to women with repeated previous failure', five trials (761 participants) were included (Fig. 2). AH was associated with a significant increase in clinical pregnancy rate in two of them and the same was observed as the result of our meta-analysis (RR = 1.73; 95% CI = 1.37–2.17). We considered the increase in clinical pregnancy rate as clinically relevant (RR > 1.2). No significant heterogeneity was observed (I^2 = 0%), although the method used for performing AH was different between trials (laser = 2, acid Tyrodés = 2 and pronase = 1).

Regarding the subgroup 'fresh embryos transferred to women with advanced age', four trials (405 participants) were included (Fig. 2). No significant heterogeneity was observed (I^2 = 0%) although different methods of AH were used (laser = 3 and acid Tyrodés = 1). AH was not associated with significant changes in clinical pregnancy rate in any of these four trials and the same was observed in the meta-analysis (RR = 0.96; 95% CI = 0.74–1.25). Considering the observed clinical pregnancy rate in the control group (73/207 = 41.8%), the power to detect a RR ≥ 1.2 was low (46.8%). However, we believe that AH is unlikely to relevantly improve clinical pregnancy in women with advanced age, since the observed clinical pregnancy was lower (although not significantly) when AH was performed (33.3 versus 35.3%; AH versus control, respectively; P = 0.77).

In the subgroup 'Frozen-thawed embryos transferred to unselected or non-poor prognosis women' (Fig. 2), six trials (1196 participants) were included. AH was associated with a significant increase in clinical pregnancy rate in two of them and in the meta-analysis (RR = 1.36; 95% CI = 1.08–1.72). The observed heterogeneity (I^2 = 14%) was not important, albeit the method used to perform AH was different between studies (laser = 4, mechanical expansion = 1 and pronase = 1).

In the six trials evaluating AH performed in other situations (1005 participants—Fig. 2), the pooled data analysis did not demonstrate any significant difference in clinical pregnancy rate, although data from one trial demonstrated a significant decrease. A substantial heterogeneity I^2 = 67% was observed between the results of these trials evaluating AH performed in different situations and using different methods (acid Tyrodés = 4 and laser = 2).

Live birth/ongoing pregnancy

There were 16 trials from 14 studies (2562 participants) which reported live birth or ongoing pregnancy as an outcome and were included (Fig. 3). No significant change in live birth rate was observed considering results from single trials or the meta-analysis (RR = 1.03; 95% CI = 0.91–1.16). No significant heterogeneity was observed (I^2 = 0%), although these trials evaluated AH performed in different situations and using different methods. Considering the observed live birth rate in the control group (337/1266 = 26.6%), the power to detect an RR ≥ 1.2 was 82.7%. In subgroup analysis, the only significant difference was observed in women with previous repeated failure: the pooled analysis from two studies (250 participants, Fig. 3) demonstrate an increased live birth rate (RR = 2.51; 95% CI = 1.06–5.96). In the other subgroups, no significant differences were observed; however, the power to demonstrate a 20% relative increase in live birth rate was low.

Multiple pregnancy

There were 17 trials from 15 studies (3551 participants) that reported on multiple pregnancy and were included (Fig. 4). A significant increase in multiple pregnancy rate was observed considering the pooled data analysis (RR = 1.45; 95% CI = 1.11–1.90). The increase in multiple pregnancy was higher (although with a small overlap in 95% CI) than that observed for clinical pregnancy rate (RR = 1.11; 95% CI = 1.00–1.24). The heterogeneity observed between studies may be considered as low to moderate (I^2 = 41%). Considering single study data, only two trials reported a significant increase in multiple pregnancy rate: one evaluating AH using acid Tyrodés in fresh embryos transferred to women with previous repeated failure (Jelinkova et al., 2003); and the other evaluating AH using laser in frozen-thawed embryos transferred to unselected or non-poor prognosis women (Balaban et al., 2006).

When evaluating the subgroup 'fresh embryos transferred to unselected or non-poor prognosis women', eight trials (1610 participants) were included (Fig. 4). AH was not associated with significant changes in multiple pregnancy rate in any of them, but a trend of increase was observed in the meta-analysis (RR = 1.20; 95%

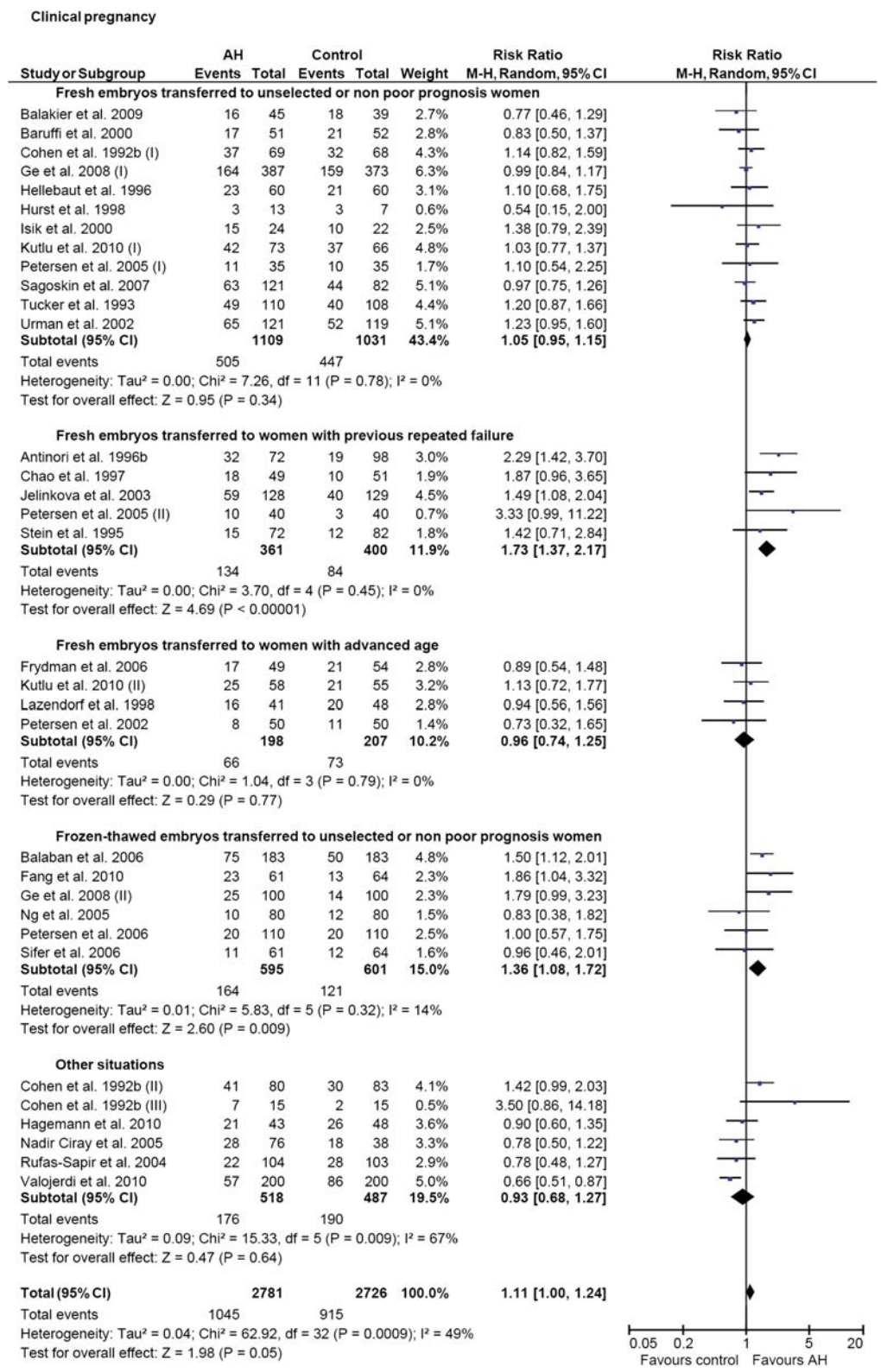

Figure 2 Effect of AH on clinical pregnancy.

CI = 0.97–1.48). Considering the observed multiple pregnancy rate in the control group (123/770 = 16.0%), the power to detect a RR ≥ 1.2 was only 34.6%. No significant heterogeneity was observed (I^2 = 0%), although the method used for performing AH was different between trials (laser = 3, acid Tyrodés = 2, pronase = 2 and mechanical = 1).

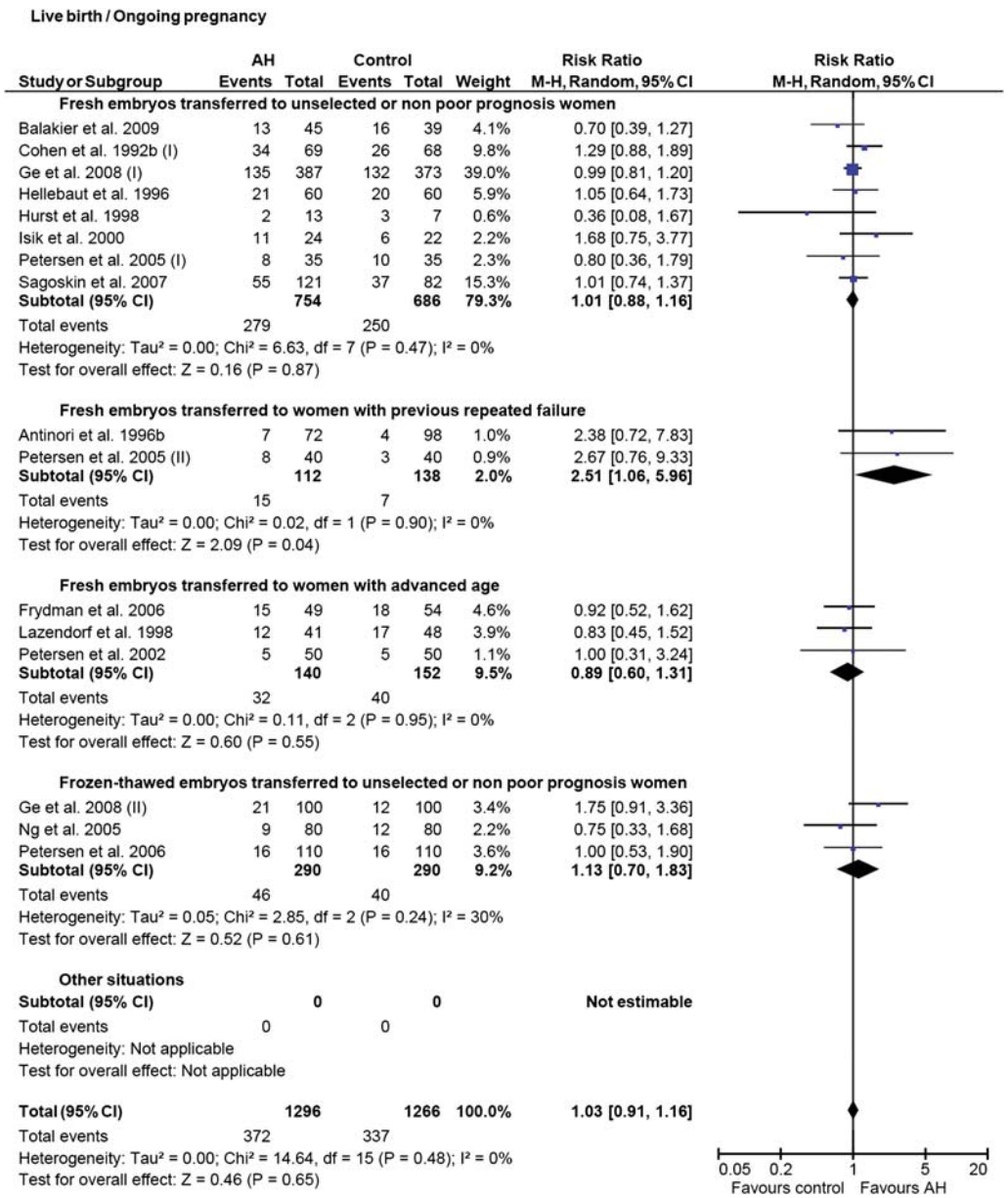

Figure 3 Effect of AH on live birth/ongoing pregnancy.

Three trials (527 participants) were included in the subgroup 'fresh embryos transferred to women with repeated previous failure' (Fig. 4). AH was associated with a significant increase in multiple pregnancy rate in one of them (Jelinkova *et al.*, 2003) and the same was observed as the result of our meta-analysis (RR = 2.53; 95% CI = 1.23–5.21). No significant heterogeneity was observed ($I^2 = 0\%$), although the method used for performing AH was different between trials (laser = 1, acid Tyrodés = 1and mechanical = 1).

No trial reported this outcome in the subgroup 'fresh embryos transferred to women with advanced age' (Fig. 4).

Four trials (851 participants) were included in the subgroup 'Frozen-thawed embryos transferred to unselected or non-poor prognosis women' (Fig. 4). AH was associated with a significant increase in multiple pregnancy rate in one trial (Balaban *et al.*, 2006) and in the meta-analysis (RR = 3.40; 95% CI = 1.93–6.01). No significant heterogeneity was observed ($I^2 = 0\%$), although the method used for performing AH was different between trials (laser = 3 and pronase = 1).

In the two trials evaluating AH performed in other situations (Fig. 4), there was considerable heterogeneity ($I^2 = 83\%$). One of these trials observed a trend of increased multiple pregnancy (RR = 1.59; 95% CI = 0.90–2.82) when AH using acid Tyrodés was performed on poor prognosis embryos from non-poor prognosis women (Cohen *et al.*, 1992b). In the other trial, a trend of decreased multiple pregnancy was observed (RR = 0.52; 95% CI = 0.26–1.06) when AH by laser was performed on vitrified–warmed embryos (Valojerdi *et al.*, 2010).

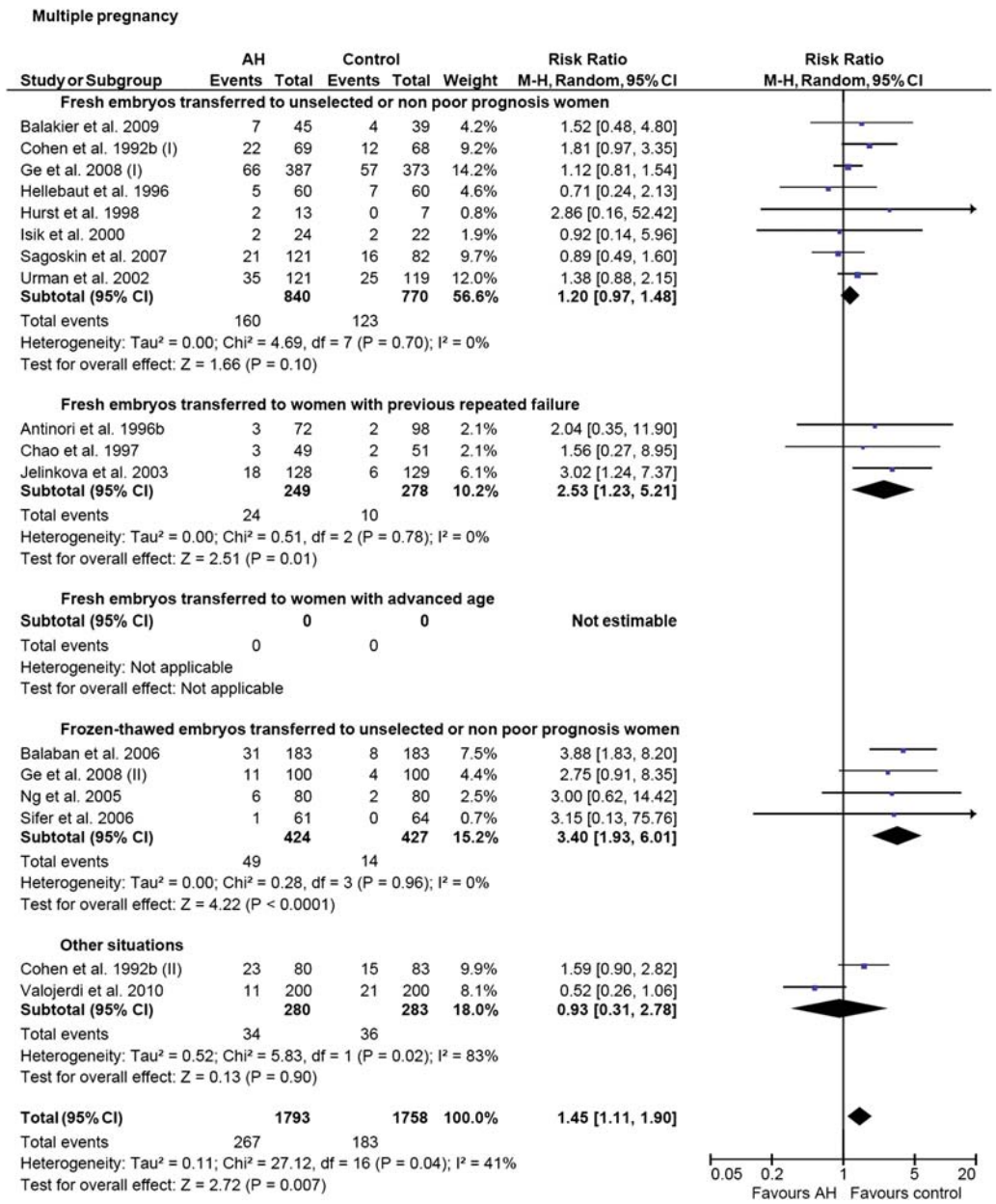

Figure 4 Effect of AH on multiple pregnancy.

Miscarriage

There were 17 trials from 16 studies (2398 participants) that reported on miscarriage and were included (Fig. 5). No significant heterogeneity was observed between the studies ($I^2 = 0\%$). No significant change in the risk of miscarriage was observed considering the result from any single trial or the pooled data analysis (RR = 1.02; 95% CI = 0.72–1.45). However, considering the observed miscarriage rate in the control group (56/1191 = 4.7%), the power to detect an RR ≥ 1.2 was only 17.4%.

Discussion

AH was associated with a trend toward increased clinical pregnancy when considering pooled data from all studies (RR = 1.11; 95% CI = 1.00–1.24, $P = 0.05$). However, a substantial heterogeneity was observed ($I^2 = 49\%$) and therefore this result should not be extrapolated for every situation. Moreover, the observed increment was small (only an 11% relative increase) and may not be considered important enough to justify the increment in costs and risks associated with AH. In subgroup evaluation, AH was associated with a significant and clinically relevant improvement in clinical pregnancy rate when performed in fresh embryos transferred to women with repeated previous failure (RR = 1.73; 95% CI = 1.37–2.17) or in frozen-thawed embryos transferred to unselected or non-poor prognosis women (RR = 1.36; 95% CI = 1.08–1.72). Contrarily, AH was not associated with any increment in pregnancy rate when performed in fresh embryos transferred to unselected or non-poor prognosis women.

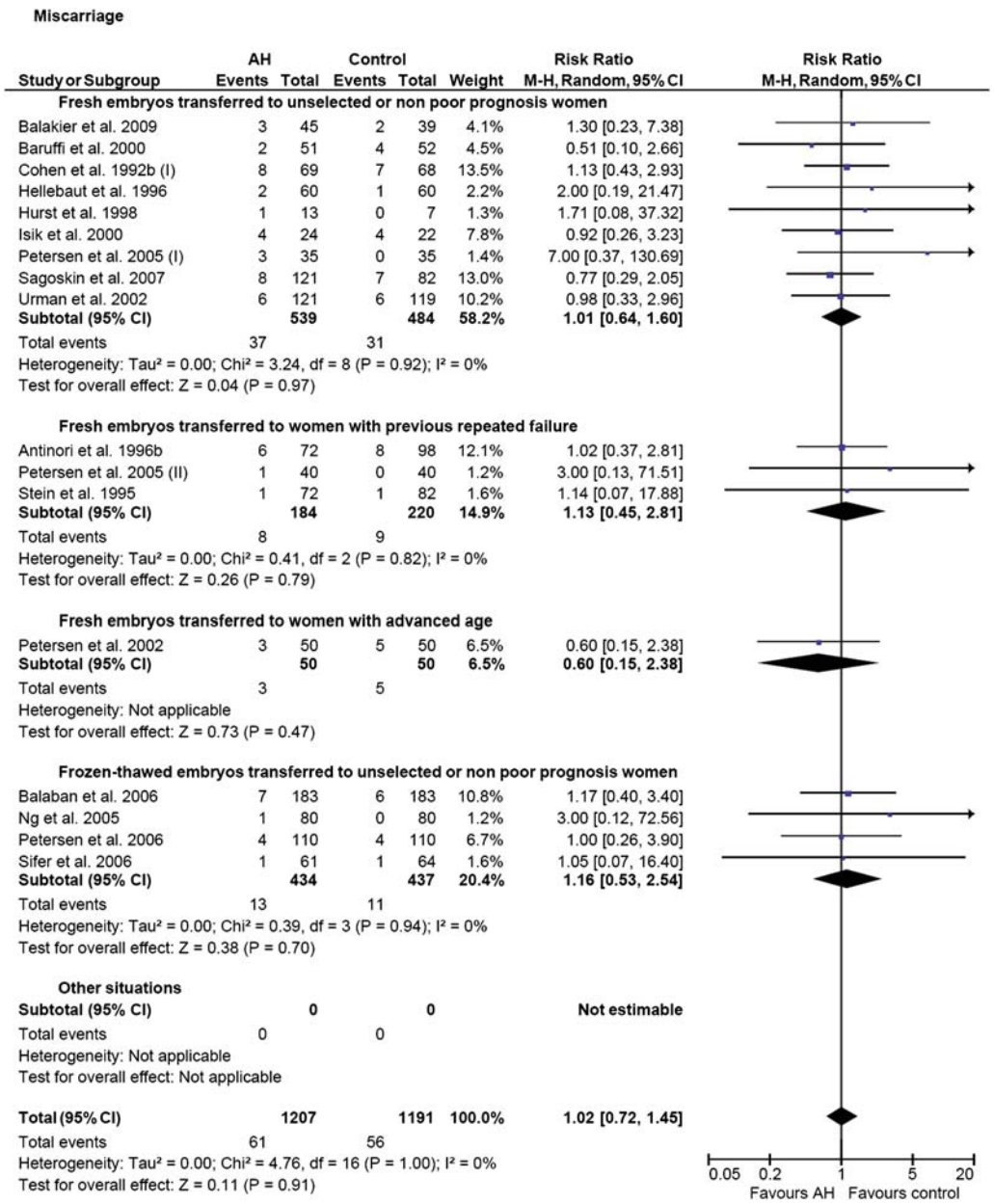

Figure 5 Effect of AH on miscarriage.

Moreover, a slight (but not significant) decrease in clinical pregnancy was observed when combining the results from the four trials evaluating women with advanced age. No significant heterogeneity was observed in any subgroup analysis, despite the fact that AH was performed by different methods.

Regarding multiple pregnancy, the observed results were not very different from those observed for clinical pregnancy. AH was associated with a significant and clinically relevant increase in clinical multiple pregnancy rate considering pooled data from all studies (RR = 1.45; 95% CI = 1.11–1.90), but a moderate heterogeneity was observed ($I^2 = 41\%$). In subgroup evaluation, AH was associated with a significant and clinically relevant improvement in clinical multiple pregnancy when performed in fresh embryos transferred to women with repeated previous failure (RR = 2.53; 95% CI = 1.23–5.21) or in frozen-thawed embryos transferred to unselected or non-poor prognosis women (RR = 3.40; 95% CI = 1.93–6.01). A trend toward increased multiple pregnancy was observed in fresh embryos transferred to unselected or non-poor prognosis women (RR = 1.20; 95% CI = 0.97–1.48). Similarly, no significant heterogeneity was observed in subgroup analysis, despite different methods used to perform AH.

Due to the small sample evaluated by the pool of included studies, no proper conclusions could be drawn regarding live birth or miscarriage. This is the main weakness of this meta-analysis: these two

outcomes are of utmost importance, since any increase in clinical pregnancy rate not followed by an improvement on live birth rate may not be considered clinically relevant at all.

Another issue that may rouse concern is grouping AH performed by different methods together (laser, chemical, mechanical and enzymatic). When evaluating the forest plot of all included studies, we realized that the observed heterogeneity among studies was more likely to occur due to differences in women and/or embryo characteristics than to the AH method. Additionally, two of the included studies (Petersen *et al.*, 2005; Ge *et al.*, 2008) pointed out that differences in women or embryo characteristics were more likely to cause the heterogeneity: they demonstrated that AH performed in two different situations, using the same method and performed in the same centre, provided significant improvements in only one of the situations. However, the difference (or the lack of difference) between AH performed by different methods on assisted reproduction outcomes is a debated issue in medical literature. Several studies have failed to demonstrate any difference when comparing different methods of AH in the same group of women (Cieslak *et al.*, 1999; Balaban *et al.*, 2002; Jones *et al.*, 2006; Makrakis *et al.*, 2006; Lanzendorf *et al.*, 2007; Yano *et al.*, 2007; Lan *et al.*, 2009), although some studies have shown otherwise: one demonstrated that half zona thinning was better than quarter zona thinning in vitrified–warmed embryos (Hiraoka *et al.*, 2009), and the other showed that laser was better than acid when performed in embryos from women aged $\geq$38 years (Hsieh *et al.*, 2002). Interestingly, these two studies were performed in situations where AH seems not to be beneficial.

Other weakness of this study was the lack of evaluation of long-term outcomes such as fetal malformations and neonatal development, which are as important as clinical pregnancy and live birth, but undoubtedly are more difficult to evaluate in clinical trials.

Despite the publication of a meta-analysis evaluating AH in 2009 (Das *et al.*, 2009), we decided to conduct this new meta-analysis considering the following points. (i) The previous meta-analysis considered the effect of AH in both fresh and frozen embryos, and in all women despite specific characteristics, which resulted in significant heterogeneity for most of the evaluated outcomes. Although they also performed subgroup analysis, they considered multiple aspects (first or repeated attempts, IVF or ICSI, method and depth of AH, etc.) resulting in almost 20 subgroups per outcome, which made the results harder to read. In the present meta-analysis, no significant heterogeneity was observed despite the use of only four subgroups. Therefore, we consider the present grouping and results to be more valid and easier to understand. (ii) The previous meta-analysis (Das *et al.*, 2009) used odds ratio assessed by Mantel–Haenszel fixed effect while we used RR assessed by Mantel–Haenszel random effect. Since all included studies were RCTs, RR is preferred because results are more intuitive. Additionally, the use of fixed effects was not the most appropriate since this model assumes that there is one identical true treatment effect common to every study (Higgins and Green, 2009). The random effects model assumes that the true treatment effect in any of the analyzed studies may be different from each other, which is the case of this meta-analysis, since AH was performed using different methods. (iii) In this meta-analysis, we have excluded one study with contradictory results (Tucker *et al.*, 1996) as the number of embryonic implantations in this study was lower than the number of clinical pregnancies. (iv) We have also included new studies (Ge *et al.*, 2008; Balakier *et al.*, 2009; Fang *et al.*, 2010; Hagemann *et al.*, 2010; Kutlu *et al.*, 2010; Valojerdi *et al.*, 2010) that greatly increased the power to indentify significant differences. These studies accounted for >25% of the weight when evaluating clinical pregnancy and >45% of the weight when evaluating live birth or ongoing pregnancy.

Based on our results, we consider that AH may be used as a strategy to improve clinical pregnancy when performed in embryos from women with previous repeated failure or in frozen-thawed embryos. Moreover, even with a small number of subjects included in the published trials, a significant increase in live births was observed when AH was performed in embryos of women with previous repeated failures. Interestingly, although AH was shown to improve clinical pregnancy when performed in frozen-thawed embryos using slow freezing, the results were the opposite when performed on vitrified–warmed embryos at cleavage stage: a significant and clinically relevant reduction on clinical pregnancy (28.5 versus 43%; AH versus control, respectively; $P < 0.01$) was observed in the only randomized controlled trial published until now (Valojerdi *et al.*, 2010).

Physicians as well as women undergoing assisted reproduction should also be aware, when performing AH, that there is an elevated increase in the risk of multiple pregnancy, including monozygotic twinning (Hershlag *et al.*, 1999; Schieve *et al.*, 2000). Dramatic cases of high order multiple pregnancies associated with AH are reported in medical literature, such as a quintuplet pregnancy consisting of a monochorionic triamnionic triplet associated with monoamniotic twins (Pantos *et al.*, 2009). Therefore, a reduction in the number of transferred embryos should be considered when AH is performed.

Despite the great number of trials published until now, more research is still required, since the evaluated sample from the included studies did not provide sufficient power to evaluate the effect of AH on miscarriage or even on live birth in the subgroup analyses. Other outcomes, such as risk of malformation, also need to be studied, since this was not reported by the included trials, although no significant increased risk has been demonstrated until now: one study evaluating 134 children born after laser AH did not find any increase in the major congenital malformation rate (2.2%) or minor congenital malformation rate (10.4%) when compared with all deliveries of the same institution (3.0 and 11.1%, respectively) (Kanyo and Konc, 2003). Other long-term outcomes, as neurodevelopment and fertility from offspring, should also be evaluated.

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