nature portfolio

Peer Review File

Effect of single blastocyst-stage versus single cleavage-stage embryo transfer on cumulative live births in women with good prognosis undergoing in vitro fertilization: Multicenter Randomized Controlled Trial

Editorial Note: This manuscript has been previously reviewed at another journal that is not operating a transparent peer review scheme. This document only contains reviewer comments and rebuttal letters for versions considered at Nature Communications

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Comments on the revised version and answers to referees

Many questions and comments are responded in a satisfactory way.

1. However, I think the problem with the primary endpoint, livebirth after three embryo transfers, remains. As I said in my previous review it is known since man years that livebirth per embryo transfer is higher for blastocyst than for cleavage stage transfer. Blastocyst culture is a selection procedure where less good embryos will not survive while those surviving have a higher quality resulting in a higher implantation rate compared to cleavage stage embryos. The drawbacks are that a certain number of embryos will not survive resulting in no embryo transfer. In these cases we don't know if transfer on day 2 would have resulted in a livebirth. The selection procedure is most probably the main reason behind the better results after blastocyst transfers. The results in Cochrane are presented as live birth per embryo transfer but most probably holds also for the summary of three embryo transfers. These results are presented overall and also for good prognosis patients with advantage of blastocyst vs cleavage stage. Saying that the news of this manuscript is rather limited. On the other hand a true cumulative live birth rate, calculating LBR after all embryos after one oocyte retrieval had been transferred in both groups would have been very interesting and have a high scientific value. It would show how the benefit of embryo selection is counteracted by the risk of embryos not surviving long term culture, including all embryos from one be oocyte retrieval in the denominator. It is obvious from the calculation post study which the authors have performed that the difference in livebirth rate between groups when adding part of the remaining embryo transfers, is getting smaller, and I presume not longer significant (80.9% vs 77.6%). And with more livebirths added in the cleavage group than the blastocyst group. I think this calculation, despite limitations brought up by the authors, comes closer to the truth, if we wish to calculate cumulative live birth rate, compared to what is presented in main analysis in this ms. How many embryos are left in freezer in the two groups after this exercise? Overall and for patients not having achieved a livebirth?

2. I think the authors misunderstood my comment concerning pre-eclampsia. I meant you should compare PE in frozen vs fresh. (not cleavage vs blastocyst). Several observational studies (also RCT-

freeze all studies) have shown a higher rate of HDP (hypertensive disorders of pregnancy) including pre-eclampsia in pregnancies after frozen embryo transfers compared to fresh transfers. Doing the calculation myself I understand that there are 6 cases out of 456 in the fresh group, 1.1% and 13 of 231 in the cryo group, 5.5%. And cryo is more frequent in the cleavage group. Again the suggestion is to adjust for cryo.

Reviewers' Comments:

Reviewer 1, comment 1

A. Reviewer: Summary of key results

Whether cleavage-stage or blastocyst-stage embryo offer better pregnancy and live birth rates has remained controversial and basically unresolved. Here Chinese investigators in a multicenter, open label randomized trial attempted to offer an answer: 992 women (ages 20-40) were randomized at cleavage stage if they had at least 3 embryos for either single cleavage stage transfer (n=495) of extended embryo culture with blastocyst-stage transfer (n=497). Primary outcome was cumulative pregnancy rate after transfer of 3 first embryos in 3 transfers. The authors reported cumulative live birth rates on 328 (66.3%) of 495 cleavage stage transfers (66.3%) and on 372/497 blastocyst-stage transfers (74.8%). Absolute difference in favor of blastocyst-stage transfer was, thus, 8.6% (95% CI: 2.9-14.2; in non-inferiority producing a P<0.001 and in superiority a P=0.003). They also reported a significantly shorter time to pregnancy. At the same time, blastocyst-stage transfer was also associated with negative (secondary) outcomes, mostly perinatal/neonatal adverse outcomes: Premature rupture of membranes was increased with blastocyst-stage transfers (P=0.003), as were premature births (P=0.02) and neonatal hospitalizations(P=0.004).

The authors concluded that. in women under age 40 with at least 3 cleavage-stage embryo transfers blastocyst-stage embryo transfers were superior to single cleavage-stage transfers in improving cumulative live birth rates and reducing time to live birth, while the difference in pregnancy complications was low. They, furthermore, concluded that their study supported the use of single blastocyst transfer in this patient population. They also reported mildly higher preeclampsia rates with cleavage-stage transfers though could not explain the finding and the study was unable to confirm larger birthweights with blastocyst-stage transfers, previously reported in the literature.

Originality and significance

This is a widely anticipated and well-designed study by Chinese investigators who are to be congratulated on the organizational feat involved in conducting such a study.

B: Response: Thank you very much for your encouraging comments!

Reviewer 1, comment 2

A. Reviewer: ***Data & methodology; validity of approach, quality of data, quality of presentation***

The study protocol, especially the study of cumulative live birth rates, is important, as is the good number of study subjects. One point of concern is the fact that the large number of centers means that every center on average contributed only 90 patients, with no further details about individual center numbers provided. Theoretically 2-3 large center with varying practice patterns from other centers, therefore, could have significantly influenced outcomes.

B. Response: We provide below the details regarding the number of participants and outcomes at each center. The differences in cumulative live birth rates were generally consistent across centers. An analysis with the Cochran-Mantel-Haenszel test indicated results to remain robust after controlling for center (RR 1.13 [95% CI 1.04 to 1.22], P = 0.0026) (Response Table1 and Figure 1).

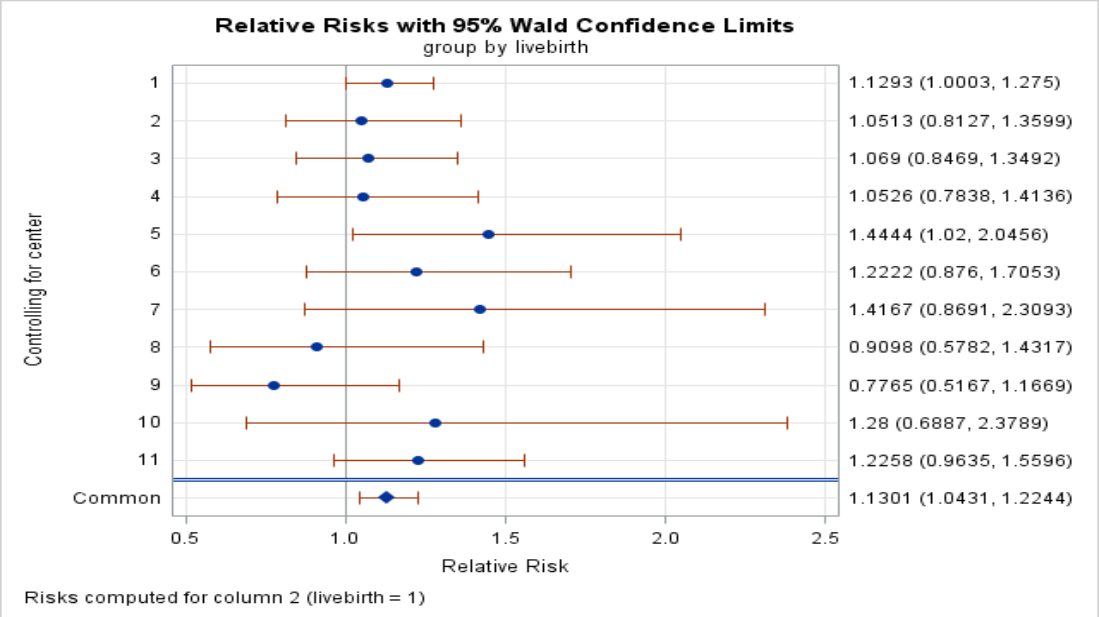
Response Table 1. The cumulative live birth rates stratified by centers

Center	Blastocyst-stage embryo transfer	Cleavage-stage embryo transfer	Absolute Difference (95% CI)	Relative Risk (95% CI)	P values
Cumulative live birth rate					
Center 1	131/161 (81.4%)	116/161 (72.0%)	9.3% (0.1 to 18.5)	1.13 (1.00 to 1.27)	0.0480
Center 2	41/61 (67.2%)	39/61 (63.9%)	3.3% (-13.6 to 20.1)	1.05 (0.81 to 1.36)	0.7031
Center 3	31/38 (81.6%)	29/38 (76.3%)	5.3% (-13.0 to 23.6)	1.07 (0.85 to 1.35)	0.5736
Center 4	20/25 (80.0%)	19/25 (76.0%)	4.0% (-18.9 to 26.9)	1.05 (0.78 to 1.41)	0.7328
Center 5	26/32 (81.3%)	18/32 (56.3%)	25.0% (3.1 to 46.9)	1.44 (1.02 to 2.05)	0.0310
Center 6	33/52 (63.5%)	27/52 (51.9%)	11.5% (-7.3 to 30.4)	1.22 (0.88 to 1.71)	0.2337
Center 7	17/25 (68.0%)	12/25 (48.0%)	20.0% (-6.8 to 46.8)	1.42 (0.87 to 2.31)	0.1520
Center 8	16/30 (53.3%)	17/29 (58.6%)	-5.3% (-30.6 to 20.0)	0.91 (0.58 to 1.43)	0.6826
Center 9	11/17 (64.7%)	15/18 (83.3%)	-18.6% (-47.1 to 9.9)	0.78 (0.52 to 1.17)	0.2076
Center 10	8/10 (80.0%)	5/8 (62.5%)	17.5% (-24.2 to 59.2)	1.28 (0.69 to 2.38)	0.4101
Center 11	38/46 (82.6%)	31/46 (67.4%)	15.2% (-2.2 to 32.6)	1.23 (0.96 to 1.56)	0.0919
Total [a]	372/497 (74.8%)	328/495 (66.3%)	8.6% (2.9 to 14.2)	1.13 (1.04 to 1.22)	0.0030
Cochran-Mantel-Haenszel [b]				1.13 (1.04 to 1.22)	0.0026

[a] The unadjust results.

[b] Controlling for centers.

Response Figure 1. The cumulative live birth rates stratified by centers



* Common indicates the cumulative live birth rates controlling for centers by Cochran-Mantel-Haenszel test.

* Red lines indicate 95% confidence intervals (CI); Blue dots indicate relative risk.

C. Changes made: We added **"The results for the primary outcomes remained robust after controlling for centers."** in the revised Results.

D. Location of changes: Please refer to the **Results**, page 7, line 161 in the revised manuscript with highlighted track changes.

Reviewer 1, comment 3

A. Reviewer: ***Appropriate use of statistics and use of uncertainties.*** *Yes, to statistics, and No to use of uncertainties. The authors completely failed to point out that their study population by young age and because of the fact that they had to have at least 3 days 3 cleavage-stage embryos for transfer, represented a clearly very-good-prognosis population for IVF. Their conclusions, however, extended to all of IVF. This is highly inappropriate.*

B. Response: Indeed, our study population represented IVF population with good prognosis only, and the conclusions cannot extend to all the IVF population. Although we tried to make this clear in the original manuscript by stating that our work was conducted among women ≤40 years undergoing IVF who had at least three cleavage-stage embryos available, we apologize for not having being clear initially.

C. Changes made: Per the reviewer's suggestion, we added the mean age of participants to the abstract, and clearly described that our study population represents women with a good prognosis throughout the revised manuscript and in the conclusion. Also, we have added a paragraph in the discussion in which we explained this.

> **Abstract**
>
> "992 women (aged 20 to 40 years with three or more transferrable cleavage-stage embryos undergoing IVF, **mean age 29.8 years**) were randomly assigned (1:1) to single blastocyst transfer (n=497) or single cleavage-stage transfer (n=495)."
>
> **Discussion**
>
> "First, we include women with good prognosis of no less than three cleavage-stage embryos **and a mean age of 29.8 years, with the age distribution ≤35 years accounting for 93% (924) of the women. As shown in extended data figure 2, the benefits of blastocyst transfer appear to diminish with advancing age.** Therefore, ~~we should be cautious~~ **our results may not be** ~~in~~ generaliz**able**~~ing the results~~ to **other populations including** women with ~~poor prognosis or advanced age~~ **older age, fewer oocytes retrieved and less than three cleavage-stage embryos available."**
>
> **Conclusion**
>
> "In conclusion, among infertile women **undergoing IVF <u>with good prognosis</u>** (≤40 years with at least three cleavage-stage embryos), single blastocyst transfer was non-inferior and even superior to single cleavage-stage transfer in improving cumulative live birth rates and reducing time to live birth."

D. Location of changes: We made changes throughout the revised manuscript. Please refer to the **Abstract**, page 3, line 60; **Discussion**, page 11, line 270; **Conclusion**, page 12, line 295 in the revised manuscript with highlighted track changes.

Reviewer 1, comment 4

A. Reviewer: *Conclusions, robustness, validity, reliability*
This is where this manuscript completely fails. There is nothing in the authors conclusions that really reflects suggested conclusions:

B. Response: We have tried to revise the manuscript according to the reviewer's suggested conclusions as detailed above and below. See our response to comment 3, and comment 5 to comment 7 of this reviewer.

Reviewer 1, comment 5

A. Reviewer:

(i) *As already noted, this study involves very young patients (ages 20-40), which denotes a very good- prognosis patient population for IVF. This conclusion is also supported by their mean ages of 29.6 and 29.9 years (Table 1). The original study of blastocyst-stage transfer (Gardner et al) was also reported after studying only good-prognosis patients. That paper then unfortunately made the same mistake as in here presented manuscript reflecting, and expanding conclusions to all IVF patients. Good-prognosis patients in most centers represent, however, only roughly 20% of all IVF patients (centers, of course vary in distribution). No wonder, therefore, that, to this reviewer's knowledge, not a single study of extended blastocyst-stage culture in unselected patients was ever able to demonstrate the outcome benefits Gardner et al reported in their original publication. This is, indeed, the likely main reason for the differences in opinion about this issue reflected in the literature. The authors did point out those differences well.*

B. Response: These comments were also ventilated under comment 3 by this reviewer. We refer to our response there. We agree with the reviewer's comments that the distribution of IVF patients with good prognosis varies across regions and centers.

We did a literature review of the global data. The **2020 US** National Summary Report from CDC reported **37%** of women undergoing IVF in America to be <35 years of age (the largest percentage),[1] and the **2018 ESHRE's** European Registry reported **45%** of women undergoing IVF in Europe to be <34 years of age.[2] Based on the report from **Chinese Society** of Reproductive Medicine in **2019**, the age distribution undergoing assisted reproductive technology (ART) from 221 centers in China was **71%** in women of < 35 years.[3] Furthermore, it was reported that in 2017 the age distribution of ≤34 years was about 43% in North American, 30% in Latin American, 57% in Africa, and 40% in Australia and New Zealand. In addition, a large part of the couples undergoing IVF is treated in China and Europe [**33.6% (1,075,788 treatments) in mainland China, 31.5% (1,007,598 treatments) in Europe, and 5.6% (180,406 treatments) in the USA**] based on data published in 2018.[4-6]

Therefore, we feel that our study population still represents a large proportion of the global IVF population, which varies by regions and centers, although limited to couples with at least 3 transferrable cleavage-stage embryos, and with an age distribution of 93% participants ≤35 years. In addition, we also anticipate high-quality trials focusing on other populations, especially women of advanced age and poor prognosis.

C. Changes made: Based on the reviewer's suggestions, we have thoroughly revised the description of the population in the manuscript to women with good prognosis, and have also added this limitation in the revised **discussion and conclusion** as follows.

Discussion

"First, we include women with good prognosis of no less than three cleavage-stage embryos **and a mean age of 29.8 years, with the age distribution ≤35 years accounting for 93% (924) of the women. As shown in extended data figure 2, the benefits of blastocyst transfer appear to diminish with advancing age.** Therefore, ~~we should be cautious~~ **our results may not be** ~~in~~ generaliz**able**~~ing the results~~ to **other populations including** women with ~~poor prognosis or advanced age~~ **older age, fewer oocytes retrieved and less than three cleavage-stage embryos available."**

Conclusion

"In conclusion, among infertile women **undergoing IVF with good prognosis** (≤40 years with at least three cleavage-stage embryos), single blastocyst transfer was non-inferior and even superior to single cleavage-stage transfer in improving cumulative live birth rates and reducing time to live birth."

D. Location of changes: Please refer to the **Discussion**, page 11, line 270; **Conclusion**, page 12, line 295 in the revised manuscript with highlighted track changes.

Reviewer 1, comment 6

A. Reviewer:

(ii) *This reviewer would further argue that in this study demonstrated outcome advantages of 8.6% absolute and 11.5% relative better live birth rates in infertility patients who already have the by far highest live birth chances represent a relatively minor gain (which has also been the conclusion for good prognosis patients in one of the by the authors' cited meta-analyses) and is more than adversely compensated by the demonstrated increased in perinatal/neonatal complications with blastocyst-stage transfers, both as clinical outcomes but also in terms of cost-effectiveness (prematurity is the by far biggest and most costly problem in obstetrical care worldwide).*

B. Response: We agree with the reviewer with regards to the pros and cons of reporting of absolute differences versus relative ratio, and followed the practice in related and recent published work [Yan et al. (2021)[7]; Dang et al., (2021)[8]]. We understand that both quantities may undermine or exaggerate the comparison depending on the benchmark. As described below, we added a comparison of cumulative complications.

We also agree that the risk occurrence of perinatal/neonatal complications should be fully considered in making clinical decision on embryo transfer strategies. Since the frequency of an individual perinatal complication tend to be very low and the relatively ratio can be unstable, we calculated the total cumulative obstetric/perinatal outcomes for women with at least one of these complications, next to the live births that occurred with/without complications (**Response Table2**).

As can be seen (Response Table 2), the total live birth rate from an oocyte retrieval cycle increased with an absolute 8.6%, with a 2.3% absolute increase of uncomplicated live births, while the number of live births from pregnancies with maternal and/or neonatal complications increased with an absolute 6.3%. The majority of these complications was mild, with severe complications like preterm birth < 34 weeks, placental abruption, placental accreta, fetal congenital anomalies, stillbirth, and neonatal death occurring in 12.7% (89/700) of all live births. Namely, if 100 couples undergo IVF, there will be with day 5 transfer 8.6 more children, of which 6.3 had a pregnancy with some (mostly mild) complications.

In addition, when calculating complications for participants not limited to live births, the risk of any complications is in a similar order of magnitude with the cumulative live birth rate. The results

showed that women after blastocyst transfer had a 7.1% higher absolute risk and 16% higher relative risk of developing at least one obstetric/perinatal complication, in contrast to an 8.6% absolute and 13% relatively higher cumulative live birth rates. Both the benefits and risk are important in making a clinical decision. Patients should be well informed of the information before deciding on the embryo transfer strategies.

Also, we agree that the interpretation of the benefit-risk ratio of blastocyst transfer strategy need to consider more aspects, such as the cost-effectiveness as the reviewer pointed out, which is our plan after this original manuscript.

Response Table 2: Cumulative obstetric and perinatal outcomes

No./total (%)	Blastocyst-stage group (n=497)	Cleavage-stage group (n=495)	Absolute difference (95% CI)	Risk ratio (95% CI)	*p* value
Cumulative live births	372 (74.8)	328 (66.3)	8.6 (2.9 to 14.2)	1.13 (1.04 to 1.22)	0.003
Live birth without a complication	130 (26.2)	118 (23.8)	2.3 (-3.1 to 7.7)	1.10 (0.88 to 1.36)	0.40
Live birth with at least one complication [a]	242 (48.7)	210 (42.4)	6.3 (0.1 to 12.5)	1.15 (1.00 to 1.32)	0.048
Participants with at least one of the maternal or neonatal complications [b, c]	253 (50.9)	217 (43.8)	7.1 (0.9 to 13.3)	1.16 (1.02 to 1.32)	0.03
Participants with at least one of the maternal complications [b]	134 (27.0)	122 (24.6)	2.3 (-3.1 to 7.8)	1.09 (0.89 to 1.35)	0.40
Participants with at least one of the neonatal complications [c]	201 (40.4)	171 (34.5)	5.9 (-0.1 to 11.9)	1.17 (1.00 to 1.38)	0.06

[a] Complications in live births include: Maternal complications include gestational diabetes mellitus, preeclampsia or eclampsia, gestational hypertension, premature rupture of membrane, preterm birth, placenta previa, placental abruption, placental accreta, other placental abnormality, and postpartum hemorrhage; and neonatal complications include neonatal hospitalization > 3 days, neonatal jaundice, neonatal infection, neonatal death among live newborns, congenital anomaly, low birth weight, macrosomia, small for gestational age, and large for gestational age.

[b] Maternal complications in all participants include: gestational diabetes mellitus, preeclampsia or eclampsia, gestational hypertension, premature rupture of membrane, preterm birth, placenta previa, placental abruption, placental accreta, other placental abnormality, and postpartum hemorrhage.

[C] Neonatal complications in all participants include: therapeutic abortion or fetal reduction due to fetal congenital anomalies during 12 to 28 weeks of gestation, stillbirth, neonatal hospitalization > 3 days, neonatal jaundice, neonatal infection, neonatal death among live newborns, congenital anomaly, low birth weight, macrosomia, small for gestational age, and large for gestational age.

C. Changes made: Accordingly, we revised the discussion and conclusion of our manuscript based on the reviewer's suggestions as follows.

Abstract

"Among women **with good prognosis** (≤40 years undergoing IVF who have at least three cleavage-stage embryos), single blastocyst transfer was non-inferior and even superior to single cleavage-stage transfer in improving cumulative live-birth rates and reducing time to live birth, while **resulted in a higher risk of perinatal and neonatal complications including preterm premature rupture of membranes, preterm birth and neonatal hospitalization** ~~the difference in pregnancy complications was low~~. **Patients need to be**

fully informed of the benefits and risks after blastocyst transfers. ~~These data support the use of single blastocyst transfer in our study population."~~

Results

"Women after blastocyst transfer had an increased risk of developing at least one of the maternal or neonatal complications compared with those after cleavage-stage transfer (50.9% vs 43.8%; AD 7.1%[95%CI 0.9% to 13.3%]; P=0.03)."

Discussion

"~~In our opinion, the higher cumulative live birth rate compensates for the relatively low, albeit statistically higher, rates of obstetrical-perinatal complications~~ **Our results showed that women after blastocyst transfer had a 7.1% higher absolute risk and 16% higher relative risk of developing at least one obstetrical-perinatal complication, in contrast to an 8.6% absolute and 13% relatively higher cumulative live birth rates. We evaluate the cumulative obstetrical-perinatal complications because each complication has a low frequency. Patients should be well informed of the information before deciding on an embryo transfer strategy. We will conduct a cost-effectiveness analysis after this original publication to further explore the benefit-risk ratio of blastocyst transfer in our study population.**"

Conclusion

"In conclusion, among infertile women **undergoing IVF <u>with good prognosis</u>** (≤40 years with at least three cleavage-stage embryos), single blastocyst transfer was non-inferior and even superior to single cleavage-stage transfer in improving cumulative live birth rates and reducing time to live birth. **However,** ~~while pregnancy complications were acceptable. These data support the use of single blastocyst transfer in women with three or more transferrable cleavage-stage embryos.~~ **the increased risk of preterm premature rupture of membranes, preterm birth and neonatal hospitalization after blastocyst transfer need to be fully informed of patients before deciding on an embryo transfer strategy. The cost-effectiveness of blastocyst transfer in this population and the long-term impact on the infants warrants further studies**."

D. Location of changes: Please refer to the **Abstract**, page 3, line 71; **Results**, page 6, line 148; **Discussion**, page 10, line 237; **Conclusion**, page 12, line 295; **Table 4**, page 24 in the revised manuscript with highlighted track changes.

Reviewer 1, comment 7

A. Reviewer:

(iii) *The authors also fail to consider/mention that when in a general population an intervention does not change outcome, evidence for a subgroup showing marginally better outcomes, automatically means that the remaining patients must reflect two other subgroups: patients who are not affected by the intervention and a compensatory group of patients that negates the better than average outcomes in the better prognosis group. In infertility, this would then have to mean that ca. 20% of good-prognosis patients will benefit from extended culture to blastocyst, ca. 60% will be unaffected, and ca. 20% of, likely, primarily poor-prognosis patients, will be negatively affected from blastocyst culture. The latter group is, of course, especially important.*

B. Response: We thank the reviewer's comment. As we clarified in response 3 and 5 to this reviewer, our study reports on couples with a good prognosis. We share the reviewer's thoughts that blastocyst transfer might result in favorable, unaffected or negative pregnancy outcomes in different subgroups, although we are not aware of data that indicate that "ca. 20% of, likely, primarily poor-prognosis patients, will be negatively affected from blastocyst culture".

As shown in extended data figure 2, the benefit of blastocyst transfer appeared to decrease with increasing age. Patients with younger age and hyper-responders, representing subgroups of women with very good prognosis, benefitted from single blastocyst transfer. Conversely, women with older age, diminished ovarian reserve and fewer oocyte retrieved did not appear to have between-group differences in cumulative livebirth rates. Given our study was not powered for post-hoc subgroup analysis and most of our participants were ≤35 years old, we cannot draw definitive conclusions on treatment effects in other subgroups. Thus, we are also anticipating further studies on specific subgroups, especially on the poor prognosis patients.

The rational for this trial was that there was lack of high-quality evidence on which embryo transfer strategy was better, even for women with good-prognosis. The importance of this trial is that we found higher cumulative live birth but also higher obstetrical-perinatal complications. This allows practitioners and patients to make an informative decision.

C. Changes made:

In response to the reviewer, we have added this point of view in the revised **Discussion** as follows.

> "**Our post-hoc subgroup analysis suggested the benefit of blastocyst transfer appeared to decrease with increasing age. Patients with younger age (≤30 years), representing subgroups of women with very good prognosis, benefited from single blastocyst transfer. Conversely, women with older age, diminished ovarian reserve and fewer oocyte retrieved did not appear to have between-group differences in cumulative livebirth rates. Given our study was not powered for post-hoc subgroup analysis and the majority of participants were ≤35 years, we cannot draw definitive conclusions on treatment effects in other subgroups. Further studies of specific subgroups with sufficient power are needed to support our exploratory findings in use of blastocyst transfer in different populations, especially in women with older age or poor prognosis.**"

D. Location of changes: Please refer to the **Discussion**, page 10, line 256 in the revised manuscript with highlighted track changes.

Reviewer 1, comment 8

A. Reviewer: Suggested improvements: experiments, data for possible revision

Based on all above made points, the authors must substantially revise their conclusions and, therefore, their discussion section.

B. Response: Thank you for the important points. We have tried to make substantially revisions including the conclusion and discussion sections as highlighted above.

Reviewer #2:

Remarks to the Author:

Reviewer 2, comment 1

A. Reviewer: *This is an important study which addresses a knowledge gap in clinical ART practice by comparing cleavage stage to blastocyst transfer in the context of a prospective RCT. The study is novel by focusing on cumulative live birth rate and pregnancy and neonatal health complications. The authors show that the former is slightly increased by a strategy of blastocyst transfer, which is also associated with increases in a number of adverse outcomes.*

B. Response: Thank you very much for the encouraging comments!

Reviewer 2, comment 2

A. Reviewer: *My major criticism of the study is the interpretation and messaging around the outcomes. The authors conclude that blastocyst transfer is superior to cleavage stage, and that "the difference in pregnancy complications was low." However while this is true in absolute terms, it is certainly not true in terms of relative risk. A reasonable person, such as a couple seeking treatment, might conclude from the same dataset that the improvement in overall success rate is quite small, at 8.4% points. Whereas the adverse events the authors report on, including premature rupture of membranes, spontaneous pre-term birth, and neo-natal hospitalisation, were increased by 200-300%. Although not mentioned in the abstract, neonatal infection was also increased by a similar amount, and there were many more cases of MZ twinning in the blastocyst group. Although this last one was not significant as the study design was not powered for this outcome, it does confirm existing literature and should be considered as a complication. This small increase in benefit but large increase is some risks should be clearly spelled out in the messaging from this study. Many couples might not accept a doubling of the risk of their baby being in hospital or with an infection, for such a small relative increase in success rate. Economic costs should also be taken into account, in many parts of the world the costs of ART complications and neonatal care in hospital is born by the society in the form of state medicine, so ART providers should take this into account when deciding policy.*

B. Response: We agree with the reviewer with regards to the pros and cons of reporting of absolute differences versus relative ratio, and followed the practice in related and recent published work [Yan et al. (2021)[7]; Dang et al., (2021)[8]]. We understand that both quantities may undermine or exaggerate the comparison depending on the benchmark. As described below, we added a comparison of cumulative complications.

We also agree that the risk occurrence of perinatal/neonatal complications should be fully considered in making clinical decision on embryo transfer strategies. Since the frequency of an individual perinatal complication tend to be very low and the relatively ratio can be unstable, we calculated the total cumulative obstetric/perinatal outcomes for women with at least one of these complications, next to the live births that occurred with/without complications (**Response Table2**).

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In addition, when calculating complications for participants not limited to live births, the risk of any complications is in a similar order of magnitude with the cumulative live birth rate. The results

showed that women after blastocyst transfer had a 7.1% higher absolute risk and 16% higher relative risk of developing at least one obstetric/perinatal complication, in contrast to an 8.6% absolute and 13% relatively higher cumulative live birth rates. Both the benefits and risk are important in making a clinical decision. Patients should be well informed of the information before deciding on the embryo transfer strategies.

Also, we agree that the interpretation of the benefit-risk ratio of blastocyst transfer strategy need to consider more aspects, such as the cost-effectiveness as the reviewer pointed out, which is our plan after this original manuscript.

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Participants with at least one of the maternal complications [b]	134 (27.0)	122 (24.6)	2.3 (-3.1 to 7.8)	1.09 (0.89 to 1.35)	0.40
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[a] Complications in live births include: Maternal complications include gestational diabetes mellitus, preeclampsia or eclampsia, gestational hypertension, premature rupture of membrane, preterm birth, placenta previa, placental abruption, placental accreta, other placental abnormality, and postpartum hemorrhage; and neonatal complications include neonatal hospitalization > 3 days, neonatal jaundice, neonatal infection, neonatal death among live newborns, congenital anomaly, low birth weight, macrosomia, small for gestational age, and large for gestational age.

[b] Maternal complications in all participants include: gestational diabetes mellitus, preeclampsia or eclampsia, gestational hypertension, premature rupture of membrane, preterm birth, placenta previa, placental abruption, placental accreta, other placental abnormality, and postpartum hemorrhage.

[C] Neonatal complications in all participants include: therapeutic abortion or fetal reduction due to fetal congenital anomalies during 12 to 28 weeks of gestation, stillbirth, neonatal hospitalization > 3 days, neonatal jaundice, neonatal infection, neonatal death among live newborns, congenital anomaly, low birth weight, macrosomia, small for gestational age, and large for gestational age.

C. Changes made: Accordingly, we revised the discussion and conclusion of our manuscript based on your suggestions as follows. Additionally, the rate of neonatal infection has been also added to the abstract. MZ twinning, which are considered as a complication, have been added to the discussion. Please refer to the details listed below.

Abstract

", and neonatal infection (4.8% vs 2.2%; AD 2.6%[95%CI 0.3% to 4.9%]; P=0.03)."

"Among women **with good prognosis** (≤40 years undergoing IVF who have at least three cleavage-stage embryos), single blastocyst transfer was non-inferior and even superior to single cleavage-stage transfer in improving cumulative live-birth rates and reducing time

to live birth, while **resulted in a higher risk of perinatal and neonatal complications including preterm premature rupture of membranes, preterm birth and neonatal hospitalization** ~~the difference in pregnancy complications was low~~. **Patients need to be fully informed of the benefits and risks after blastocyst transfers.** ~~These data support the use of single blastocyst transfer in our study population."~~

Results:

"Women after blastocyst transfer had an increased risk of developing at least one of the maternal or neonatal complications compared with those after cleavage-stage transfer (50.9% vs 43.8%; AD 7.1%[95%CI 0.9% to 13.3%]; P=0.03)."

Discussion

"~~In our opinion, the higher cumulative live birth rate compensates for the relatively low, albeit statistically higher, rates of obstetrical-perinatal complications~~ **Our results showed that women after blastocyst transfer had a 7.1% higher absolute risk and 16% higher relative risk of developing at least one obstetrical-perinatal complication, in contrast to an 8.6% absolute and 13% relatively higher cumulative live birth rates. We evaluate the cumulative obstetrical-perinatal complications because each complication has a low frequency. Patients should be well informed of the information before deciding on an embryo transfer strategy. We will conduct a cost-effectiveness analysis after this original publication to further explore the benefit-risk ratio of blastocyst transfer in our study population.**"

Discussion

"**The higher rate of monozygotic twins in blastocyst group is consistent with previous findings,[7] although not statistically significant in our study.**"

Conclusion

"In conclusion, among infertile women **undergoing IVF <u>with good prognosis</u>** (≤40 years with at least three cleavage-stage embryos), single blastocyst transfer was non-inferior and even superior to single cleavage-stage transfer in improving cumulative live birth rates and reducing time to live birth. **However,** ~~while pregnancy complications were acceptable. These data support the use of single blastocyst transfer in women with three or more transferrable cleavage-stage embryos.~~ **the increased risk of preterm premature rupture of membranes, preterm birth and neonatal hospitalization after blastocyst transfer need to be fully informed of patients before deciding on an embryo transfer strategy. The cost-effectiveness of blastocyst transfer in this population and the long-term impact on the infants warrants further studies**."

D. Location of changes: Please refer to the **Abstract**, page 3, line 70; **Results**, page 6, line 148; **Discussion**, page 10, line 237, line 248; **Conclusion**, page 12, line 295; **Table 4**, page 24 in the revised manuscript with highlighted track changes.

Reviewer 2, comment 3

A. Reviewer: *My second major point is the difference in the number of embryos remaining in freezing at the end of the study. The cleavage group had more than double the number of embryos left compared to the blastocyst group, in women who had not yet conceived. While I appreciate the reasons for limiting the trial to 1 year with a 2 year followup, the authors should do a life-*

course analysis using the frozen embryo data to predict the uplifts in live birth rate in both groups. This would make the results of the trial more applicable to real world clinical practice.

B. Response: We appreciate the comments of the reviewer. We fully agree to continue with the life-course follow-ups. We also think that one year follow-up plus the time to observe live birth is long enough, because most of the participants achieved a cumulative live birth during this study period (75.7% vs 68.9% for all embryo transfers). At the end of this period, the 8% difference in the advantage of blastocyst transfer after the first 3 SETs is explained partly by the higher number of embryos that remains in the cleavage-stage group (4.9 vs 2.2), but also by the 5% higher rate of couples in blastocyst group that has ended the trial without a live birth, and without embryos remaining (9.3 vs 4.4). The differences of cumulative live birth remain with 6.8% when we calculated all embryo transfers within this period.

We did the follow-ups until July 28th, 2023, but the embryo transfers after the study period did not follow our prespecified protocol. We found cumulative live birth rates of 80.9% in the blastocyst group and 77.6% in the cleavage-stage group. Among the deviations that occurred >1 year after randomisation, 86% of women who obtained an extra live birth in cleavage-stage group did not have this live birth through single cleavage-stage transfers (**Response Table 3**). As indicated, we prefer to keep the manuscript as it is.

If the editor wants extra data we can add it, but this was not according to our prespecified protocol. We will continue doing the life-course follow-ups as the reviewer suggested.

Response Table 3: Number and stage of embryos transferred leading to live births in the follow-up cohort after the study period.

Number and stage of embryos transferred No. (%)	**Cumulative live births in blastocyst-stage group (n=26)**	**Cumulative live births in cleavage-stage group (n=43)**
1 cleavage-stage embryo	0 (0.0)	6 (14.0)
1 blastocyst	16 (61.5)	14 (32.6)
2 cleavage-stage embryos	0 (0.0)	10 (23.3)
2 blastocysts	5 (19.2)	4 (9.3)
1 cleavage-stage and 1 blastocyst-stage embryos	0 (0.0)	3 (7.0)
Natural conception	5 (19.2)	6 (14.0)

C. Changes made: Per the reviewer's suggestion, we added the comments in the revised **Discussion** as follows:

> **"Since more frozen cleavage-stage embryos were left than frozen blastocysts in women who did not achieve a live birth (4.9 vs 2.2), we continued the follow-ups and will conduct a life-course analysis to reveal the results in real world practice."**

D. Location of changes: Please refer to the **Discussion**, page 9, line 216 in the revised manuscript with highlighted track changes.

Response to Reviewer 3:

A. Reviewer: *Many questions and comments are responded in a satisfactory way.*

1. However, I think the problem with the primary endpoint, livebirth after three embryo transfers, remains. As I said in my previous review it is known since man years that livebirth per embryo transfer is higher for blastocyst than for cleavage stage transfer. Blastocyst culture is a selection procedure where less good embryos will not survive while those surviving have a higher quality resulting in a higher implantation rate compared to cleavage stage embryos. The drawbacks are that a certain number of embryos will not survive resulting in no embryo transfer. In these cases we don't know if transfer on day 2 would have resulted in a livebirth. The selection procedure is most probably the main reason behind the better results after blastocyst transfers. The results in Cochrane are presented as live birth per embryo transfer but most probably holds also for the summary of three embryo transfers. These results are presented overall and also for good prognosis patients with advantage of blastocyst vs cleavage stage. Saying that the news of this manuscript is rather limited. On the other hand a true cumulative live birth rate, calculating LBR after all embryos after one oocyte retrieval had been transferred in both groups would have been very interesting and have a high scientific value. It would show how the benefit of embryo selection is counteracted by the risk of embryos not surviving long term culture, including all embryos from one be oocyte retrieval in the denominator. It is obvious from the calculation post study which the authors have performed that the difference in livebirth rate between groups when adding part of the remaining embryo transfers, is getting smaller, and I presume not longer significant (80.9% vs 77.6%). And with more livebirths added in the cleavage group than the blastocyst group. I think this calculation, despite limitations brought up by the authors, comes closer to the truth, if we wish to calculate cumulative live birth rate, compared to what is presented in main analysis in this ms. How many embryos are left in freezer in the two groups after this exercise? Overall and for patients not having achieved a livebirth?

B. Response: Thank for the reviewer's comments. We fully agree that cumulative live birth rates (CLBRs) are the most relevant outcome, as is shown from our choice for CLBR from 3 single embryo transfers (SETs) as the primary outcome, and all live birth rates from all embryo transfers within 1 year of randomization as the secondary outcome. However, it is generally impractical to design and conduct a clinical trial without a fixed time frame. In addition, a further stretch of this period to 18 months or 2 years would have been difficult to accept by patients, and led to more refusals to be randomized and more protocol violations.

To answer the reviewer's question, we expanded the effort beyond the original trial and calculated the CLBRs including the follow-up of embryo transfers until July 28th, 2023. This has been added as a non-prespecified secondary outcome. We found that the CLBR was not significantly higher in the blastocyst group than in the cleavage-stage group **(80.9% [402/497] vs 77.6% [384/495]**; **AD, 3.3% [95%CI -1.7 to 8.4]; P = 0.199) (Response Table 1)**. The absolute difference in CLBRs between the two groups did appear to become smaller with longer follow-up, compared with the main results.

However, it should be stressed that additional embryo transfers after the study period (e.g., from 1 year after the randomization to July 28, 2023) were not performed according to our prespecified study protocol. 41.3% of women in cleavage-stage group underwent blastocyst transfers **(Response Table 2)**, and 48.8% of women in cleavage-stage group obtained an extra live birth through blastocyst transfers **(Response Table 3)**. The extended data are therefore not suitable for comparing the CLBR between the two arms of our trial.

The total number of frozen embryos left was 5.2 in the cleavage-stage group versus 3.9 in the blastocyst group **(Response Table 1)**. Among women who have not achieved a live birth, the number of frozen embryos remaining in the cleavage-stage group was still higher than in the blastocyst group (2.8 vs 1.2). It is possible that CLBR will be the same in both treatment groups if women continue to transfer more cleavage-stage embryos. However, in this scenario, it is difficult to determine whether the catch-up in CLBRs among women in the cleavage-stage group is due to crossover to blastocyst transfer, more embryo transfer cycles, or both.

Our results provide robust evidence that for the first three SETs as well as for embryo transfers within 1 year of randomization, blastocyst transfers resulted in a significantly higher CLBR than did the cleavage-stage transfers, with the majority of women (more than 70%) achieving a live birth **(Table 3 in the main manuscript; Supplement Table 9)**; blastocyst transfer also significantly shortened the time to achieve a live birth.

Based on the reviewer's comment, we have added the results of long-term follow-ups as the non-prespecified outcome and discussions to the main manuscript.

Response Table 1: Non-prespecified outcomes for cumulative live births including the long-term follow-up cohort (Intention-to-Treat Analysis).

Outcomes, No./total (%)	**Blastocyst-stage embryo transfer group (n=497)**	**Cleavage-stage embryo transfer group (n=495)**	**Absolute difference (95% CI)** [a]	**Relative risk (95% CI)**	***P* value** [b]
Cumulative live births	402 (80.9%)	384 (77.6%)	3.3% (-1.7 to 8.4)	1.04 (0.98 to 1.11)	0.199
Singleton live births	391 (78.7%)	375 (75.8%)	2.9% (-2.3 to 8.1)	1.04 (0.97 to 1.11)	0.274
All twin live births	11 (2.2%)	9 (1.8%)	0.4% (-1.4 to 2.1)	1.22 (0.51 to 2.91)	0.658
No. of unused frozen embryos, mean (SD)	3.9 (3.4)	5.2 (4.1)	-1.3 (-1.8 to -0.9)	-	<0.001
No. of unused frozen embryos in women with a live birth, mean (SD)	4.5 (3.3)	5.9 (4.0)	-1.4 (-1.9 to -0.9)	-	<0.001
No. of unused frozen embryos in women without a live birth, mean (SD)	1.2 (1.9)	2.8 (3.8)	-1.6 (-2.4 to -0.7)	-	<0.001
No. of women without a frozen embryo	89 (17.9%)	65 (13.1%)	4.8% (0.3 to 9.3)	1.36 (1.02 to 1.83)	0.038
No. of women without a frozen embryo without a livebirth	58 (11.7%)	53 (10.7%)	1.0% (-3.0 to 4.9)	1.09 (0.77 to 1.55)	0.631
No. of women without a frozen embryo with a livebirth	31 (6.2%)	12 (2.4%)	3.8% (1.3 to 6.3)	2.57 (1.34 to 4.95)	0.003

Noninferiority p value <0.001 for cumulative live births (with margin = -0.1, $\alpha = 0.025$, one-sided);
SD, standard deviation.
[a] Absolute differences in percentages are indicated in percentage points, and absolute differences in other values are indicated in units of that value.
[b] All *P* values are for superiority.

Response Table 2: Characteristics of embryo transfers between the two groups in the long-term follow-up cohort after the study period (1 year of randomization).

Characteristics	Blastocyst-stage embryo transfer group (n = 51)	Cleavage-stage embryo transfer group (n = 92)	*P* Value
No of embryos transferred, No. (%)[a]			
One embryo	45/51 (88.2%)	58/92 (63.0%)	0.002
Two embryos	6/51 (11.8%)	33/92 (35.9%)	
Three embryos	0 (0.0%)	1/92 (1.1%)	
Stage of embryo transferred, No. (%)[a]			
Blastocyst-stage embryo transfer	51/51 (100.0%)	38/92 (41.3%)	<0.001
Cleavage-stage embryo transfer	0 (0.0%)	54/92 (58.7%)	

[a] Calculated based on the total number of embryo transfer cycles.

Response Table 3: Number and stage of embryos transferred leading to live births in the follow-up cohort after the study period (1 year of randomization).

Number and stage of embryos transferred No. (%)	Cumulative live births in blastocyst-stage group (n=26)	Cumulative live births in cleavage-stage group (n=43)
1 cleavage-stage embryo	0 (0.0)	6 (14.0)
1 blastocyst	16 (61.5)	14 (32.6)
2 cleavage-stage embryos	0 (0.0)	10 (23.3)
2 blastocysts	5 (19.2)	4 (9.3)
1 cleavage-stage and 1 blastocyst-stage embryos	0 (0.0)	3 (7.0)
Natural conception	5 (19.2)	6 (14.0)

C. Changes made: We have added **Response Table 1** as **Table 5** to the main manuscript (Page 25), and the **Response Table 2 and 3** as **Supplementary Table 13 and 14** into the supplementary information (Page 17). We also included the discussions of this issue in the revised **Methods, Results, Discussion** as follows:

Methods

"The non-prespecified outcome of cumulative live birth rate was also calculated, including follow-up of embryo transfers from day of randomization to July 28th, 2023. The treatments after the study period (1 year of randomization) did not follow our prespecified protocol."

Results

"Post Hoc Analyses of long-term follow-up outcomes

When analyzing follow-ups of embryo transfers from day of randomization to July 28th, 2023, cumulative live birth rate was not significantly higher in the blastocyst group than in the cleavage-stage group (80.9% [402/497] vs 77.6% [384/495]; AD, 3.3% [95%CI -1.7 to 8.4]; P = 0.199) (Table 5). Among the deviations that occurred after the study period (1 year after randomization), 41.3% of women in the cleavage-stage group underwent blastocyst transfers, whereas all women in the blastocyst group underwent blastocyst transfers (Supplementary Table 13). Furthermore, 48.8% of women in cleavage-stage

group obtained an extra live birth through blastocyst transfers (Supplementary Table 14).”

Discussion

“We conducted the long-term follow-ups from randomization day to July 28th, 2023, and found similar cumulative live birth rate between the two group. However, treatment after the study period (1 year of randomization) did not follow our prespecified protocol, as 41.3% of women in the cleavage-stage group had blastocyst transfers. Therefore, it is difficult to determine whether the catch-up in live birth rates among women in the cleavage-stage group is due to crossover to blastocyst transfer, more embryo transfer cycles, or both. In addition, the number of frozen embryos remaining in the cleavage-stage group, among women who have not achieved a live birth, was higher than in the blastocyst group (2.8 vs 1.2). The cumulative live birth rate might be the same in both treatment groups if women continue to transfer more cleavage-stage embryos. Of note, our results of increased cumulative live birth rate and reduced time to live birth after blastocyst transfer should be applied in the context of the first three SETs and embryo transfers within 1 year of randomization.”

D. Location of changes: Please refer to the **Methods**, page 28-29, line 683-686; **Results**, page 7, line 169-176; **Discussion**, page 9, Line 219-229; **Table 5**, page 25; **Supplement information, Supplementary Table 13,14**, page17, in the revised manuscript with highlighted track changes.

A. Reviewer: *2. I think the authors misunderstood my comment concerning pre-eclampsia. I meant you should compare PE in frozen vs fresh. (not cleavage vs blastocyst). Several observational studies (also RCT-freeze all studies) have shown a higher rate of HDP (hypertensive disorders of pregnancy) including pre-eclampsia in pregnancies after frozen embryo transfers compared to fresh transfers. Doing the calculation myself I understand that there are 6 cases out of 456 in the fresh group, 1.1% and 13 of 231 in the cryo group, 5.5%. And cryo is more frequent in the cleavage group. Again the suggestion is to adjust for cryo.*

B. Response: We apologize for misunderstanding the reviewer’s comment, and thank the reviewer for clarifying it again. Per the reviewer’s suggestions, we adjusted for cryo with a logistic regression model when analyzing the association between cumulative live birth and embryo transfer stage. As shown in the **Response Table 4**, the risk of preeclampsia remained higher in the cleavage-stage group than in the blastocyst-stage group after adjustment for frozen or fresh embryo transfer (aOR 0.35 [95% 0.12, 0.98]; P=0.046), which was consistent with the unadjusted results (OR 0.35 [95% 0.12, 0.98]; P=0.045).

We appreciate the reviewer’s calculation based on the livebirth from the different cycles in Table 3. It should be noted that among the 456 live births after the first embryo transfer, 235 were from fresh transfers and 221 from the first frozen transfers following the freeze-only strategy. Thus, there were 6 cases of preeclampsia out of 235 (2.6%) in the fresh group, and 13 of 452 (2.9%) in the frozen group (**Response Table 5**). Based on our data, we did not find an association between

preeclampsia and cryo. For clarity, we have added the footnote in Table 3 for the fresh or frozen after the first embryo transfer.

Response Table 4. Logistic regression model to determine the adjusted treatment effect of blastocyst or cleavage stage transfer on preeclampsia.

	Unadjusted Odds Ratio (95% CI)	P value	Adjusted Odds Ratio (95% CI)	P value
Treatment arm, blastocyst transfer vs. cleavage-stage embryo transfer	0.35 (0.12, 0.98)	0.045	0.35 (0.12, 0.98)	0.046
Frozen vs. Fresh embryo transfer cycles	N.A.	N.A.	0.80 (0.30, 2.14)	0.657

N.A. indicates that the variable was not included the corresponding model.

Response Table 5. The association between preeclampsia and fresh or frozen embryo transfers.

	Fresh (N = 235)	Frozen (N = 452)	P value*
Preeclampsia or eclampsia	6/235 (2.6%)	13/452 (2.9%)	1.000

* Fisher exact test

C. Changes made: Based on your comments, we added the **Response Table 4** into the supplementary information, included descriptions in the revised **Results and Discussion,** and added **footnotes** to Table 3 as follows.

Results

"In addition, more preeclampsia occurred after fresh cleavage-stage transfer (6/14 [42.9%] vs 0/5 [0.0%] for fresh cycles (Supplementary Table 11)**, and logistic regression analyses showed that the risk of preeclampsia remained higher in the cleavage-stage group than in the blastocyst group after adjustment for frozen or fresh embryo transfer (Supplementary Table 12)**."

Discussion

"Our study found more preeclampsia after fresh cleavage-stage transfer, however the mechanism is unclear. **Although frozen embryo transfer may be a confounder for the increased incidence of pre-eclampsia, the risk of pre-eclampsia remained higher in the cleavage-stage group after adjustment for frozen embryo transfer**."

Footnotes (Table 3)

"Livebirth after the first embryo transfers included 235 livebirths from 536 fresh embryo transfer and 221 livebirths from 441 first frozen embryo transfer (freeze-only strategy)."

D. Location of changes: Please refer to the **Results**, page 6, line 151-153; **Discussion**, page 10, Line 246-247; **Table 3 footnotes**, page 22, Line 530-531; **Supplement information**, **Supplementary Table 12,** page 16 in the revised manuscript with highlighted track changes.

Reference:

1. Centers for Disease Control and Prevention. *2020 Assisted Reproductive Technology Fertility Clinic and National Summary Report.* US Dept of Health and Human Services; 2022.

2. The European IVF Monitoring Consortium (EIM), for the European Society of Human Reproduction and Embryology (ESHRE), Wyns C, et al. ART in Europe, 2018: results generated from European registries by ESHRE. Hum Reprod Open 2022; 2022: hoac022.

3. Xiaodong Zhang, Yuan Shen, Juanzi Shi, et al. Annual report on assisted reproductive technology of Chinese Society of Reproductive Medicine in 2019. The Journal of Reproductive Medicine, 2022(008):031.

4. G.D Adamson and others, O-151 ICMART preliminary world report 2018, Human Reproduction, Volume 37, Issue Supplement_1, July 2022, deac105.057, https://www.icmartivf.org/wp-content/uploads/ICMART-ESHRE-WR2018-Preliminary-Report.pdf

5. Bai, F., et al. Assisted reproductive technology service availability, efficacy and safety in mainland China: 2016. Hum Reprod 35, 446-452 (2020).

6. De Geyter, C., et al. 20 years of the European IVF-monitoring Consortium registry: what have we learned? A comparison with registries from two other regions. Hum Reprod 35, 2832-2849 (2020).

7. Yan J, Qin Y, Zhao H, Sun Y, Gong F, Li R, Sun X, Ling X, Li H, Hao C, Tan J, Yang J, Zhu Y, Liu F, Chen D, Wei D, Lu J, Ni T, Zhou W, Wu K, Gao Y, Shi Y, Lu Y, Zhang T, Wu W, Ma X, Ma H, Fu J, Zhang J, Meng Q, Zhang H, Legro RS, Chen ZJ. Live Birth with or without Preimplantation Genetic Testing for Aneuploidy. N Engl J Med. 2021 Nov 25;385(22):2047-2058.

8. Dang VQ, Vuong LN, Luu TM, Pham TD, Ho TM, Ha AN, Truong BT, Phan AK, Nguyen DP, Pham TN, Pham QT, Wang R, Norman RJ, Mol BW. Intracytoplasmic sperm injection versus conventional in-vitro fertilisation in couples with infertility in whom the male partner has normal total sperm count and motility: an open-label, randomised controlled trial. Lancet. 2021 Apr 24;397(10284):1554-1563.

9. De Vos A, Van Landuyt L, Santos-Ribeiro S, et al. Cumulative live birth rates after fresh and vitrified cleavage-stage versus blastocyst-stage embryo transfer in the first treatment cycle. Hum Reprod 2016; 31: 2442-9.

10. Points to consider on switching between superiority and non-inferiority. London: European Medicines Agency, July 2000.

11. Mauri L, D'Agostino RB Sr. Challenges in the Design and Interpretation of Noninferiority Trials. N Engl J Med. 2017 Oct 5;377(14):1357-1367.

12. Stone GW, Sabik JF, Serruys PW, Simonton CA, Généreux P, Puskas J, Kandzari DE, Morice MC, Lembo N, Brown WM 3rd, Taggart DP, Banning A, Merkely B, Horkay F, Boonstra PW, van Boven AJ, Ungi I, Bogáts G, Mansour S, Noiseux N, Sabaté M, Pomar J, Hickey M, Gershlick A, Buszman P, Bochenek A, Schampaert E, Pagé P, Dressler O, Kosmidou I, Mehran R, Pocock SJ, Kappetein AP; EXCEL Trial Investigators. Everolimus-Eluting Stents or Bypass Surgery for Left Main Coronary Artery Disease. N Engl J Med. 2016 Dec 8;375(23):2223-2235.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This is one of the most well-described and detailed revisions I have seen as a reviewer in over 40 years and the authors are to be congratulated on this effort. They, however, in this truly extraordinary effort are missing the central point of my original criticism of their paper which basically is that nothing in their manuscript offers any new information regarding the utilization of PGT-A in IVF:

(i) It has been now for some time been accepted that PGT-A, for simply mathematically and biologically indisputable reasons, cannot improve the cumulative pregnancy and live birth chance of an in an IVF cycle generated number of retrieved oocyte cohort and the resulting embryo cohort. What happens after egg retrieval can only be influenced downwards by poor clinical or embryology practice.

(ii) (ii) What PGT-A, therefore, can influence regarding outcomes is only time to pregnancy, and even that only in good-prognosis patients. This is why the authors' acknowledgment in the revised manuscript that they studied a very good-prognosis patient population only is of such importance and is appreciated. But this is definitely NOT new information!

(iii) (iii) We also appreciate the authors' acknowledgement that potential adverse consequences of PGT-A in their original manuscript received short thrift. Offering new data on these adverse outcomes, as they did in the revised manuscript, is important but does not offer the reader an explanation why a marginal improvement in a rather small minority of overall IVF patients (their suggestion that good-prognosis patients in IVF centers exceed ca. 15-20% is, simply, incorrect unless a clinic, a-priori, discriminates in accepting average and especially poor-prognosis patients) renders the performance of PGT-A worthwhile, as their paper still is claiming.

(iv) The correct conclusion of their paper, therefore, is not, as their revised manuscript states, that in good-prognosis patients PGT-A improves IVF outcomes but that, even in good-prognosis patients, PGT-A – maybe - marginally improves IVF cycle outcomes. Considering that good-prognosis patients, however, even without PGT-A, already offer "best" IVF outcomes (except for donor egg cycles), whether performance of PGT-A in even good-prognosis patients is worthwhile, therefore, is quite questionable.

Reviewer #2 (Remarks to the Author):

I would like to thank the authors for their comprehensive answers to the points I raised.

The manuscript has now moved more towards a balanced view of the potential benefits versus risks of blastocyst transfer.

With respect to the issue of remaining frozen embryos, the analysis the authors include is very valuable and I do not think there is much more than can do in that respect, I take their point that they would be open to criticism for moving too far away from the original trial design.

However, having read through the responses to the all the reviewers, it seems to me that the authors now have an important opportunity to make some very clear and valuable points about blastocyst v cleavage stage transfer and the design of studies which compare these strategies. I do not think that this sort of analysis exists in the literature to date, and so this paper would be a very important contribution.

1) Success rates and risks can be expressed in both absolute and relative terms, and there are a number of metrics available for expressing these. The way these metrics are reported strongly influences the messaging around the study, and in particular the clinical message which is of course passed onto patients.

2) Clinical trial design tends to favour shorter term outcomes, as shown in the current trial design, where for pragmatic reasons the follow up period was limited. This may be considered a necessary evil, as a longer follow up would render the original trial out of date.

3) Considerable value can, however, be added by analysing potential real world scenarios after the trial has concluded. As the authors now point out, the greater number of frozen embryos remaining in the early cleavage group likely means that when all embryos have been used, the cumulative pregnancy rates will be similar between the two groups. However, this is turn assumes that couples continue to return for transfers.

Reviewer #3 (Remarks to the Author):

My main concerns with this paper remain and which deal with the design of the study. I think it is well known (Cochrane 2022, 15 studies, 2219 women) that blastocyst transfer is superior to cleavage stage transfer and that this is an effect of embryo selection. It is then quite obvious that also three blastocyst transfers should be superior to three cleavage stage transfers which is the result and the main conclusion from this trial. The message that blastocyst transfer is superior to cleavage stage transfer in terms of live birth rate is thus not new or unique.

The authors have done a secondary, post hoc analysis, including all transfers until July 2023 and by then delivery rates do not differ any longer between the groups. However, this analysis can not be basis for conclusion since not included in the protocol and a mix of transfers has taken place.

There is another thing that confuses me. You state that the primary outcome was “cumulative live-birth rate following first 3 single embryo transfers from one oocyte retrieval and

all embryo transfers within 1 year of randomization”.

I don’t think this is quite correct and it does not fit. First it is obvious from Suppl Table that a considerable portion of the patients, not achieving live birth after first ET, never go for a second transfer. The same pattern in both groups and the same pattern for third transfer. This is not strange . That is how it works, all patients do not get embryos in the freezer and thus don’t get further transfers but then the design should not say cumulative livebirth after three transfers but cumulative livebirth after a maximum of three transfers. Further, were there other reasons (than no embryos frozen or no embryos surviving freezer) for not getting ET 2 and ET 3?

I can’t understand either how it can be both three embryo transfers and within one year after randomization, How was it planned to be handled if one woman did her third ET one month after this year and that resulted in a livebirth? was only the first two ET (both within a year) included for that patient?

Similar if a patient did her forth ET within one year was the results of that ET excluded?

In summary I think the design should have been one of the following:

1.Cumulative livebirth after maximum of three transfers

2. All transfers within a year after randomization

I can’t understand the design otherwise.

Reviewer 1, additional comments

A. Reviewer:

"Blastocyst-stage culture in place of cleavage-stage culture was initially proposed with the (false) argument that blastocyst-stage culture through "embryo selection" improves pregnancy and live birth rates in IVF cycles. This has been proven incorrect since, overall, cumulative outcomes for cleavage-stage transfers have now in several studies been demonstrated to at least be equal and, likely, even marginally better than blastocyst-stage transfers (it, of course, is important to differentiate between outcomes in single cycles or cumulative outcomes for a complete oocyte cycle cohort).

A principal reason for the continued practice of extended embryo culture to blastocyst-stage (day-5-7) in IVF has, therefore, now become the almost universal (in my opinion again mistaken) routine utilization of PGT-A (chromosomal testing for aneuploidy of embryos before transfer in to the uterus) in IVF cycles based on the believe that, since only blastocyst culture allows for the performance of PGT-A, and PGT-A improves pregnancy and live birth rates (again a by now proven false assumption), all embryos should undergo extended blastocyst-stage culture.

Otherwise, there is no reason left for routine blastocyst-stage culture for everybody (there, of course, always exist some exceptions) since, as noted, once oocytes are retrieved in an IVF cycle, their cumulative pregnancy and live birth chance is determined, as egg quality represents ca. 95% of embryo quality and embryo quality determines most of pregnancy and live birth chances. For simple mathematical reasons, the argument that extended culture to blastocyst can or will improve cumulative IVF outcomes (pregnancy and/or live birth rates), therefore, is incorrect. What determines cumulative pregnancy chances for any cycle cohort of eggs in an IVF cycle are those eggs: Are they fertilized; do they produce embryos; what is the quality of the embryos. The maximal cumulative pregnancy rate can from that point on not be positively affected by either extended embryo culture to blastocyst, or PGT-A, but both can (in certain sub-groups of patients) negatively affect outcomes by poor laboratory performance or the simple fact that most embryo arrests happen between cleavage and blastocyst-stages.

What I was trying to say is that the paper in its current format, still does not fully address these complexities. The only potentially positive thing extended embryo culture to blastocyst stage can mathematically achieve in IVF, and even that only in so-called good-prognosis patients representing only max. 15-20% of average patient populations, is to minimally shorter time to pregnancy, as in such patients (and only in good-prognosis patients) those embryos that make it through extended in vitro culture to blastocyst have a minimally higher implantation chance than day-3 cleavage-stage embryos. But this, of course, raises the question whether it is worth to do automatically extended blastocyst-stage culture and/or PGT-A on everybody to achieve in 15-20% of patients a pregnancy maybe 1-2 months earlier? And in my opinion it does not - and not only for economic reasons: Both, extended embryo culture and PGT-A in patients at the opposite chance spectrum, will actually to a degree reduce pregnancy and live birth chances because a small number of embryos which don't make it in even very good labs to blastocyst, if transferred on day-3 at cleavage-stage, may still produce a pregnancy and/or delivery."

B. Response: We thank reviewer 1 for this third round of comments. We totally agree with reviewer 1 that blastocyst transfer or PGT-A should not be performed as a routine on everybody! Routine PGT-A might be (almost) routine in the USA, it is definitely not in many other parts of the world, including China and Europe (Please see the additional information on global PGT utilization at the end of this response). However, as stated before, our randomized clinical trial does not

address PGT-A, and couples undergoing PGT-A were excluded from our study. We also fully agree with the reviewer that the preferred outcome is cumulative live birth rate., which is the primary outcome from our study. Per reviewer 1's comments, to fully address these issues, we have added detailed descriptions to the discussion of the revised manuscript (Please see change made below).

Having said that, the reviewer does not make specific recommendations on what we should change. Here is our response on the main issues raised by reviewer 1:

"This has been proven incorrect since, overall, cumulative outcomes for cleavage-stage transfers have now in several studies been demonstrated to at least be equal and, likely, even marginally better than blastocyst-stage transfers"

> The reviewer does not provide references on this statement. As the best evidence should come from randomized clinical trials, we refer to Analysis 2.1 of the Cochrane review 2022 "Cleavage-stage versus blastocyst-stage embryo transfer in assisted reproductive technology". There are until now five RCTs with a sample size between 98 and 171 that overall, with a large confidence interval, show no difference between cleavage stage and blastocyst stage transfer (cumulative pregnancy rate: RR 0.89, 95%CI 0.64-1.22) (Please see the table below; page 6).[1]
>
> The latest Cochrane review and the European IVF Monitoring Consortium for the European Society of Human Reproduction and Embryology clearly state, [1,2] that there is a lack of evidence on the effect of blastocyst transfer versus cleavage-stage transfer on the cumulative live birth rate. In our humble opinion, this is exactly the question we try to address. In fact, we performed the largest multicenter RCT on the subject with a sample size of 992 women. We tried already to describe this in the Introduction on page 4, line 90-102, and Discussion on page 8, line 190-208.

"The only potentially positive thing extended embryo culture to blastocyst stage can mathematically achieve in IVF, and even that only in so-called good-prognosis patients representing only max. 15-20% of average patient populations, is to minimally shorter time to pregnancy, as in such patients (and only in good-prognosis patients)"

> As stated in our previous rebuttal, this 15-20% patients with a large embryo yield might be true for the USA (age <35: 36.2%, reported by CDC 2021)[3], this percentage is much larger elsewhere in the world (for example China: age <35 71%, 2019[4]; Europe: age <34 45%, 2018[2]; Africa: age ≤ 34 57%, 2017; Asia: age ≤ 34 24.4%, 2017; Latin American: age ≤ 34 30%, 2017; Australia and New Zealand: age ≤ 34 40%, 2017[5]; see page 7-9 of the response).
>
> It is also very important to stress that of course the results of our study should not be applied to all couples undergoing IVF, but just to those with a large embryo yield (maybe 15-20 % in the USA and a much larger part elsewhere). The results of our study will inform the couples and their doctors.

"For simple mathematical reasons, the argument that extended culture to blastocyst can or will improve cumulative IVF outcomes (pregnancy and/or live birth rates), therefore, is incorrect."

> Consistent with the reviewer, we originally hypothesized that the cumulative live birth rate of blastocyst-stage transfers would be non-inferior to that of cleavage-stage transfers. We also added the point to the Discussion that mathematically cumulative live birth rate might in the end be the same in both treatment groups, if women in the cleavage-stage group would continue to return for embryo transfers.

However, while mathematically correct, we want to stress that medicine and mathematics are different disciplines in real life after conducting the trial. Our study is by far the largest randomized clinical trial on this topic and shows that 1 year after randomization the cumulative live birth rate after day 5 transfer is 75.7% versus 68.9% after day 3 transfer (absolute difference 6.8, 95%CI 1.2 to 12.3; RR 1.10, 95%CI 1.02 to 1.19; P=0.02; Please see Supplementary Table 9). Indeed, not only is the time to pregnancy "*maybe 1-2 months earlier",* but as our study shows, some couples give up. That is a reality reflecting everyday life in the clinic.

Also, according to our follow-up of real-world data after the trial, 41.3% of the transfers after the study was ended in the cleavage-stage group were blastocyst transfers (Please see Supplementary Table 13), which suggests that many physicians and patients can't adhere to transfers at cleavage-stage embryos, due to their lower implantation potential per transfer than blastocysts.

Our study, limited to couples with three or more cleavage-stage embryos, demonstrated a higher cumulative live birth rate and a one month shorter time to live birth (Median time to live birth: 344 days vs 373 days; HR 1.26[95% CI 1.09 to 1.47]; P=0.002) after day 5 blastocyst transfer after a 12-months period. While this is maybe a consequence of embryo selection, and couples in the day 3 group giving up, that is also a reality reflecting everyday life in the clinic.

We want again express our gratitude for reviewer 1's comment. Based on reviewer 1's previous-round comment, we provided comprehensive description on representativeness of our study population and interpretation of our findings, and substantially revised our manuscript with respect to the interpretation and generalizability of the results and conclusions, which have greatly improved the quality of our manuscript. Based on this round comment by reviewer 1, we propose to change the title of our paper, and added more descriptions to fully address the complexities of the issues.

C. Changes made: To fully address the complexities of our study results, we revised the title and added the following to the discussion.

Title

"Effect of single blastocyst-stage versus single cleavage-stage embryo transfer on cumulative live births in women **with good prognosis** undergoing in vitro fertilization: Multicenter Randomized Controlled Trial**"**

Discussion

"**The number and quality of embryos derived from an oocyte-retrieval cycle are key determinants of cumulative pregnancy and live birth rates. Thus, mathematically, the cumulative live birth rate might in the end be the same in both treatment groups,** ~~if women continue to transfer more cleavage-stage embryos,~~ **assuming that women in the cleavage-stage group would continue to return for embryo transfers**."

"Of note, our results of increased cumulative live birth rate and reduced time to live birth after blastocyst transfer should be applied in the context of ~~the~~ **a maximum of** the first three SETs and embryo transfers within 1 year of randomization **in good-prognosis patients**. **From the perspective of cumulative transfers, extended embryo culture to blastocyst may negatively affect the pregnancy outcomes due to poor laboratory performance or the fact that most embryos are arrested between cleavage and blastocyst stages in certain subgroups of women (e.g., women with low prognosis), which would produce a pregnancy if transferred at cleavage stage. Therefore, we should**

not perform routine blastocyst transfer on everybody, similar to the recommendations for the utilization of PGT-A."

"**Furthermore, our study had for pragmatic reasons a follow-up period of 1 year after randomization. While this might favour blastocyst transfer, as the cleavage stage group has more unused embryos left, we also think that a 1-year follow-up reflects the reality of clinical practice."**

D. Location of changes: Please refer to the **Discussion**, page 9, line 234-245; page 11, line 286-289 in the revised manuscript with highlighted track changes.

Reviewers' Comments:

Reviewer 1, comment 1

A. Reviewer:

This is one of the most well-described and detailed revisions I have seen as a reviewer in over 40 years and the authors are to be congratulated on this effort. They, however, in this truly extraordinary effort are missing the central point of my original criticism of their paper which basically is that nothing in their manuscript offers any new information regarding the utilization of PGT-A in IVF.

B: Response: We are grateful for these encouraging comments! We wish to respectfully clarify that our study investigated the utilization of blastocyst culture and transfer in IVF (we compared blastocyst culture (day 5 transfer) versus cleavage stage culture (day 3 transfer)), and that we did not study PGT-A in this trial. In fact, in this study we excluded women who underwent preimplantation genetic testing for aneuploidy (PGT-A) (please refer to Methods in the manuscript, page 27). This was why this trial does not offer new information regarding the utilization of PGT-A in IVF. We are a bit puzzled by the comments of reviewer 1 on PGT-A.

C. Changes made: None.

D. Location of changes: Not applicable.

Reviewer 1, comment 2

A. Reviewer:

(i) It has been now for some time been accepted that PGT-A, for simply mathematically and biologically indisputable reasons, cannot improve the cumulative pregnancy and live birth chance of an in an IVF cycle generated number of retrieved oocyte cohort and the resulting embryo cohort. What happens after egg retrieval can only be influenced downwards by poor clinical or embryology practice.

B. Response: We fully agree with this comment that PGT-A may not improve the cumulative live birth after one oocyte retrieval, and the outcomes can only be influenced by poor practice. However, as stated above our trial compares cumulative live birth rates after blastocyst-stage versus cleavage-stage embryo transfer, and does not compare PGT-A versus no PGT-A.

C. Changes made: None.

D. Location of changes: Not applicable.

Reviewer 1, comment 3

A. Reviewer:

(ii) What PGT-A, therefore, can influence regarding outcomes is only time to pregnancy, and even that only in good-prognosis patients. This is why the authors' acknowledgment in the revised manuscript that they studied a very good-prognosis patient population only is of such importance and is appreciated. But this is definitely NOT new information!

B. Response: We agree that the results of PGT-A in patients with a good-prognosis are completely not new, and this is exactly what we have reported in our previously published trial (Yan et al., N Engl J Med, 2021)[6]. However, as indicated our trial compares blastocyst stage to cleavage stage embryo transfer and not PGT-A, and in fact patients undergoing PGT-A are explicitly excluded. This was why our trial does not provide new information on the use of PGT-A.

C. Changes made: None.

D. Location of changes: Not applicable.

Reviewer 1, comment 4

A. Reviewer:

(iii) We also appreciate the authors' acknowledgement that potential adverse consequences of PGT-A in their original manuscript received short thrift. Offering new data on these adverse outcomes, as they did in the revised manuscript, is important but does not offer the reader an explanation why a marginal improvement in a rather small minority of overall IVF patients (their suggestion that good-prognosis patients in IVF centers exceed ca. 15-20% is, simply, incorrect unless a clinic, a-priori, discriminates in accepting average and especially poor-prognosis patients) renders the performance of PGT-A worthwhile, as their paper still is claiming.

B. Response: We sincerely thank the reviewer 1 for the encouraging comments on adverse outcomes data. However, PGT-A is not the intervention we studied.

C. Changes made: None.

D. Location of changes: Not applicable.

Reviewer 1, comment 5

A. Reviewer:

(iv) The correct conclusion of their paper, therefore, is not, as their revised manuscript states, that in good-prognosis patients PGT-A improves IVF outcomes but that, even in good-prognosis patients, PGT-A – maybe - marginally improves IVF cycle outcomes. Considering that good-prognosis patients, however, even without PGT-A, already offer "best" IVF outcomes (except for donor egg cycles), whether performance of PGT-A in even good-prognosis patients is worthwhile, therefore, is quite questionable.

B. Response: We thank the reviewer's comments. We totally agree that the use of PGT-A is highly questionable even in good-prognosis women, as stated in our previously published trial (Yan et al., N Engl J Med, 2021)[6]. However, PGT-A is not the intervention we studied. In this trial, we studied a different, unsettled, and important issue: the timing of embryo transfer by comparing blastocyst stage (Day 5) versus cleavage stage (Day 3) transfer, and excluded women undergoing PGT-A.

C. Changes made: None.

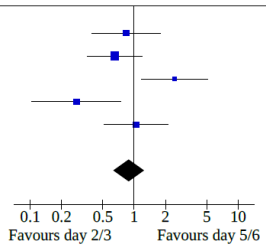
D. Location of changes: Not applicable.

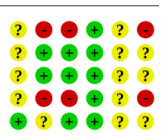
Table 2.1 (Page 91, Cochrane review 2022 "Cleavage-stage versus blastocyst-stage embryo transfer in assisted reproductive technology")[2]

Analysis 2.1. Comparison 2: Blastocyst- versus cleavage-stage transfer: cumulative pregnancy rate following fresh and frozen transfer, Outcome 1: Cumulative pregnancy rate from fresh and frozen transfers

Study or Subgroup	Day 5/6 Events	Day 5/6 Total	Day 2/3 Events	Day 2/3 Total	Weight	Odds Ratio M-H, Fixed, 95% CI
Brugnon 2010 (1)	24	55	25	52	18.2%	0.84 [0.39 , 1.79]
Emiliani 2003	43	82	56	89	32.0%	0.65 [0.35 , 1.20]
Fernandez-Shaw 2015 (2)	33	60	20	60	11.3%	2.44 [1.17 , 5.12]
Rienzi 2002	31	50	41	48	19.9%	0.28 [0.10 , 0.75]
Van der Auwera 2002	24	70	22	66	18.6%	1.04 [0.51 , 2.12]
Total (95% CI)		**317**		**315**	**100.0%**	**0.89 [0.64 , 1.22]**
Total events:	155		164			

Heterogeneity: Chi² = 13.76, df = 4 (P = 0.008); I² = 71%
Test for overall effect: Z = 0.75 (P = 0.46)
Test for subgroup differences: Not applicable





Footnotes
(1) Study had policy of single embryo transfer
(2) Both cumulative pregnancy and live birth rates given, same numbers except for one voluntary termination in blastocyst group due to anomaly after VET

ADDITIONAL INFO

1. EXTRA INFORMATION ON THE UPTAKE OF BLASTOCYST CULTURE AROUND THE WORLD

According to data from European countries in 2018, blastocyst transfers were applied in 50.1% of fresh transfers and 73.9% of frozen transfers, varying considerably by countries. However, PGT only represent 7.1% among the total number of treatment cycles (initiated IVF+ICSI and FET cycles).[2] The percentage of cycles for blastocyst transfer is much higher than for PGT suggesting other reasons for blastocyst transfer besides PGT-A, although PGT-A is performed by blastocyst biopsy.

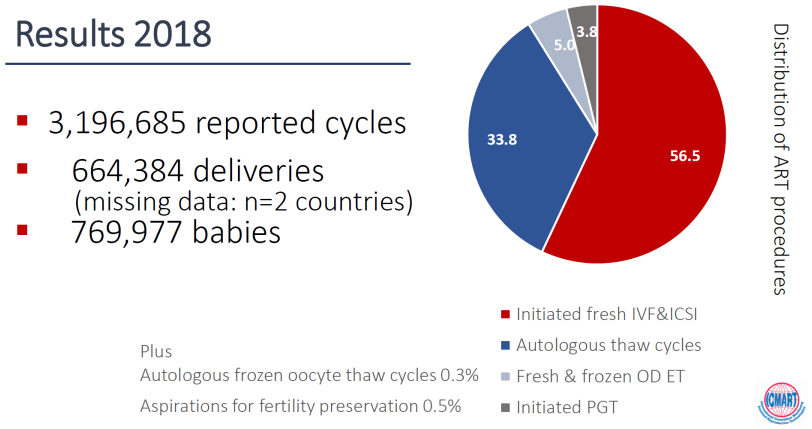
Another main reason for practice of extended embryo culture and blastocyst transfers is to increase the success rate per single embryo transfers (SETs), thereby reducing number of embryos transferred and the rate of multiple pregnancies. With the increasing use of blastocyst culture and transfer, there was a dramatic increase in SET.[7] In the USA, from 2012 to 2021, SET increased from 24.8% to 82.9% of all transfers, and cycles that resulted in twins decreased from 8.3% to 2.1%.[3] However, we fully agree with the reviewer that we need consider the efficacy of blastocyst transfer in terms of cumulative live birth rate, rather than a single transfer, which is the primary outcome from our study. There has been an ongoing debate about whether extended embryo transfer will benefit cumulative live birth rates and which population will be benefited. Our trial aims to answer this important question for women with a good prognosis.

We also share the view with the reviewer that extended culture may negatively impact the pregnancy outcomes due to poor lab performance or waste of potentially pregnant embryos when used in certain groups of women (e.g., those with a poor prognosis).

2. UTILIZATION OF PGT-A WORLDWIDE

Additionally, we would like to present data on global utilization of PGT-A based on literature search. The 2018 ESHRE's European Registry included "PGT for monogenic disorders and structural rearrangements (PGT-M/SR) and PGT for aneuploidies (PGT-A) activities, which were reported from 24 countries. The total number of treatment cycles was 48,294 representing 7.1% of initiated IVF+ICSI and FET cycles together (37,303)".[2] Data from ICMART reported 3,196,685 cycles from 79 countries in 2018, of which PGT cycles accounted for 3.8% (Please refer to the Figure below).[8] In addition, the U.S. 2021 National ART Summary reported PGT as 17% of cycles using assisted reproductive technology; [3] data from China reported PGT as 2.6% of cycles in 2019.[4] As can be seen from the global data, PGT is not used for all patients in most regions, and that PGT represents only a small proportion of the total cycles, which are performed based on clinical indications (not preclude overuse in some areas).

Figure: Data from ICMART reported 3,196,685 cycles from 79 countries in 2018[8]



3. AGE DISTRIBUTION OF WOMEN UNDERGOING IVF WORLDWIDE

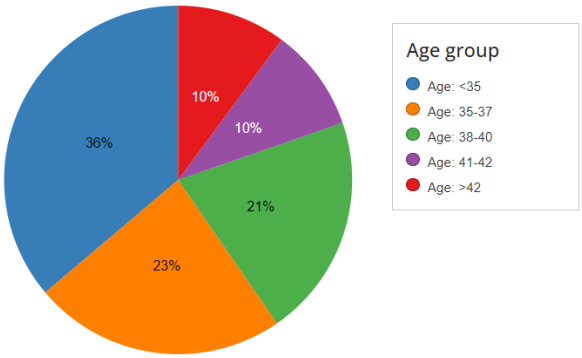
We did a literature review of the global data. The **2021 US** National Summary Report from CDC reported **36.2%** of women undergoing ART in America to be <35 years of age (the largest percentage),[3] and the **2018 ESHRE's** European Registry reported **45%** of women undergoing IVF in Europe to be <34 years of age.[2] Based on the report from **Chinese Society** of Reproductive Medicine in **2019**, the age distribution undergoing assisted reproductive technology (ART) from 221 centers in China was **71%** in women of < 35 years.[4] Furthermore, it was reported that in 2017 the age distribution of ≤34 years was about 43% in North American, 30% in Latin American, 57% in Africa, 24.4% in Asia and 40% in Australia and New Zealand.[5] In addition, a large part of the couples undergoing IVF is treated in China and Europe [**33.6% (1,075,788 treatments) in mainland China, 31.5% (1,007,598 treatments) in Europe, and 5.6% (180,406 treatments) in the USA**] based on data published in 2018.[8,9,10]

Therefore, we feel that our study population still represents a large proportion of the global IVF population, which varies by regions and centers, although limited to couples with at least 3 transferrable cleavage-stage embryos, and with an age distribution of 93% participants ≤35 years.

Figure: ART use by age group, United States, 2021[3]

Figure 1 shows the distribution of the 413,776 ART cycles started in 2021 in the United States, by patient age group. The largest percentage of ART cycles performed was among patients younger than age 35. This age group represented 36.2% of all cycles, compared to 23.4% among patients aged 35–37, 20.7% among those aged 38–40, 9.5% among those aged 41–42, and 10.1% among those older than age 42. The average age of patients using ART services in 2021 was 36.3 years.

Figure 1
ART Use by Age Group, United States, 2021



Download Data (CSV)

Data Table

Age group	Number	Percent
Age: <35	149,686	36.2%
Age: 35-37	96,991	23.4%
Age: 38-40	85,658	20.7%
Age: 41-42	39,487	9.5%
Age: >42	41,954	10.1%

Figure: Data from ICMART reported 1,955,908 cycles from 79 countries in 2017[5]

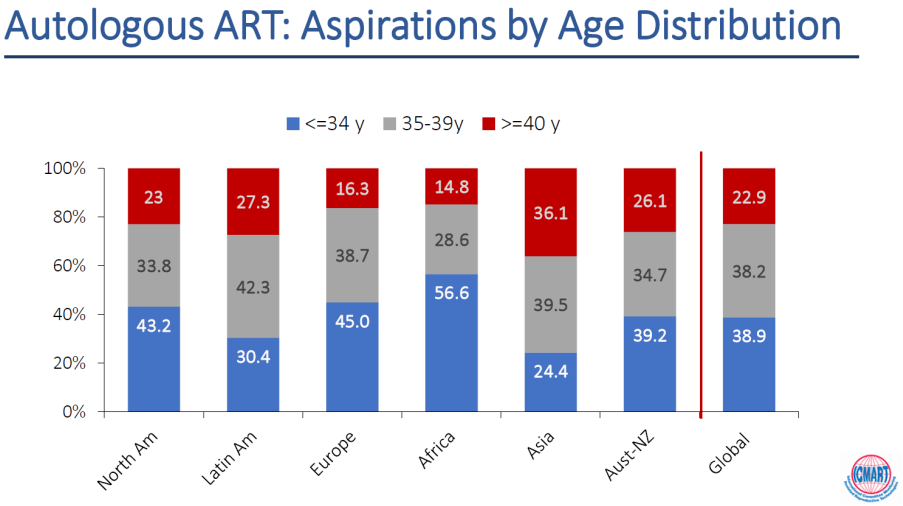


Figure: Data from Chinese Society of Reproductive Medicine reported from 221 centers in 2019[4]

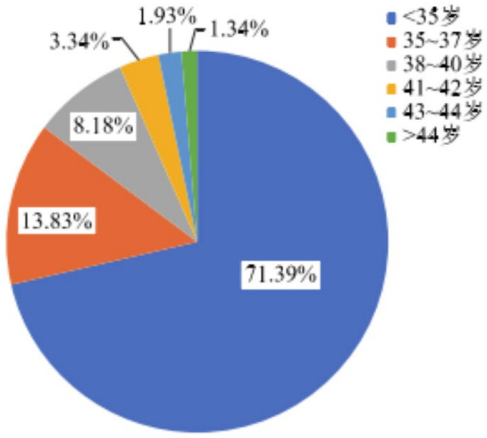


图 3　2019 年 ART 周期女性年龄分布

Reviewer #2:

Remarks to the Author:

Reviewer 2, comment 1

A. Reviewer: *I would like to thank the authors for their comprehensive answers to the points I raised. The manuscript has now moved more towards a balanced view of the potential benefits versus risks of blastocyst transfer.*

With respect to the issue of remaining frozen embryos, the analysis the authors include is very valuable and I do not think there is much more than can do in that respect, I take their point that they would be open to criticism for moving too far away from the original trial design.

However, having read through the responses to the all the reviewers, it seems to me that the authors now have an important opportunity to make some very clear and valuable points about blastocyst v cleavage stage transfer and the design of studies which compare these strategies. I do not think that this sort of analysis exists in the literature to date, and so this paper would be a very important contribution.

B. Response: We appreciate that reviewer 2 agreed that our manuscript has a more balanced view of the potential benefits versus risks of blastocyst transfer. We thank Reviewer 2 for pointing out that we provided very valuable points on stage of embryo transferred and related study designs, and that we have this important opportunity to discuss these points, which do not exist in the current literature. To ensure clarity of our important contributions, as suggested by reviewer 2, we have added the points of discussion to the revised manuscript as detailed below.

C. Changes made: We added *"***In addition, our study informs the discussion on blastocyst versus cleavage-stage transfer and the design of such studies. We use both absolute and relative terms in expressing success rates and risks, which strongly contributes the clinical message conveyed to clinicians and patients. Furthermore, our study had for pragmatic reasons a follow-up period of 1 year after randomization. While this might favour blastocyst transfer, as the cleavage stage group has more unused embryos left, we also think that a 1-year follow-up reflects the reality of clinical practice***."*.

D. Location of changes: Please refer to the **Discussion**, page 11, line 284-289 in the revised manuscript with highlighted track changes.

Reviewer 2, comment 2

A. Reviewer: *Success rates and risks can be expressed in both absolute and relative terms, and there are a number of metrics available for expressing these. The way these metrics are reported strongly influences the messaging around the study, and in particular the clinical message which is of course passed onto patients.*

B. Response: We thank reviewer 2 for the valuable comments. We agree on the importance of expressing the clinical messages in both absolute and relative terms. Per reviewer 2's comment, we made revisions as follows.

C. Changes made: We have added relative risks to the abstract and results section of the revised manuscript. We also added **"We use both absolute and relative terms in expressing success rates and risks, which strongly contributes the clinical message conveyed to clinicians and patients."** in the revised Discussion.

D. Location of changes: Please refer to the **Discussion**, page 11, line 285-286 in the revised manuscript with highlighted track changes.

Reviewer 2, comment 3

A. Reviewer: *Clinical trial design tends to favour shorter term outcomes, as shown in the current trial design, where for pragmatic reasons the follow up period was limited. This may be considered a necessary evil, as a longer follow up would render the original trial out of date.*

B. Response: We agree with Reviewer 2 on the two-sided feature of the follow-up duration in clinical trial design. We have added the comments to the discussion.

C. Changes made: We added **"Furthermore, our study had for pragmatic reasons a follow-up period of 1 year after randomization. While this might favour blastocyst transfer, as the cleavage stage group has more unused embryos left, we also think that a 1-year follow-up reflects the reality of clinical practice."** in the revised Discussion.

D. Location of changes: Please refer to the **Discussion**, page 11, line 286-289 in the revised manuscript with highlighted track changes.

Reviewer 2, comment 4

A. Reviewer: *Considerable value can, however, be added by analysing potential real world scenarios after the trial has concluded. As the authors now point out, the greater number of frozen embryos remaining in the early cleavage group likely means that when all embryos have been used, the cumulative pregnancy rates will be similar between the two groups. However, this is turn assumes that couples continue to return for transfers.*

B. Response: Great point! Indeed, potentially similar cumulative live birth rates are based on the premise that couples in the cleavage-stage group are willing to continue embryo transfer. We have added this point to the discussion.

C. Changes made: We added **"**The cumulative live birth rate might **in the end** be the same in both treatment groups, ~~if women continue to transfer more cleavage stage embryos~~ **assuming that women in the cleavage-stage group would continue to return for embryo transfers."**.

D. Location of changes: Please refer to the **Discussion**, page 9, line 236-238 in the revised manuscript with highlighted track changes.

Reviewer #3:

Remarks to the Author:

Reviewer 3, comment 1

A. Reviewer: *My main concerns with this paper remain and which deal with the design of the study. I think it is well known (Cochrane 2022, 15 studies, 2219 women) that blastocyst transfer is superior to cleavage stage transfer and that this is an effect of embryo selection. It is then quite obvious that also three blastocyst transfers should be superior to three cleavage stage transfers which is the result and the main conclusion from this trial. The message that blastocyst transfer is superior to cleavage stage transfer in terms of live birth rate is thus not new or unique.*

B. Response: We thank the reviewer very much for these comments. We are actually not sure if we agree with the reviewer's statement. Glujovsky Cochrane 2022 reports on live birth following fresh transfer (analysis 1.1) and on cumulative pregnancy rate (analysis 2.1). The reviewer's reference (15 studies, 2219 women) is to analysis 1.1 (live birth following fresh transfer), which is non-surprisingly better after blastocysts transfer (RR 1.27 [1.06, 1.51]).[1] The real important comparison however is in our opinion the cumulative pregnancy rate (analysis 2.1) which involves five studies, 632 patients and reports a RR of 0.89 [0.64, 1.22]. There is actually a lack of studies on cumulative live birth rate-the most important outcome.[1] We respectfully think in that context that our finding of cumulative live birth rate after three blastocyst transfers being superior to cleavage transfer (RR 1.13 [1.04-1.22]) and all transfers within the study period (RR1.10 [1.02-1.19]) is not that obvious, but rather an important asset to the literature.

We want to stress that in the 2022 Cochrane review, even data after one fresh blastocyst transfer was classified as "low-quality evidence for live birth".[1] In addition, there is a scarcity of high-quality data on cumulative live birth rates after multiple embryo transfers from a single oocyte retrieval cycle, which is the most important patient-centered outcome.[11] Many studies had a much shorter follow-up than our study, which is a concern as highlighted by reviewer 2, in comment 3.

Our trial provides high-quality evidence of cumulative live birth rates and obstetrical-perinatal outcomes that the Cochrane review and the European IVF Monitoring Consortium for the European Society of Human Reproduction and Embryology have been advocating for.[1,2] The rational for our trial have been clearly described in the Introduction page 4, line 90-102, and Discussion page 8, line 190-208.

We understand that, despite the lack of high-quality evidence, it can be hypothesized that the estimate of cumulative live birth rates after three single blastocyst transfers may be higher than that after three cleavage-stage transfers, based on previous reports of higher live birth rates after one single blastocyst transfer in fresh cycles.

C. Changes made: We added **"Our trial shows that the cumulative live birth rate after three single blastocyst transfers is higher than that after three cleavage-stage transfers, which might be hypothesized based on previous reports of higher live birth rates after one fresh blastocyst transfer.[11] Since depletion of embryos by blastocyst culture leads to a reduction in the number of embryos,** ~~Therefore, until now it is uncertain~~ **data are needed to confirm** whether blastocyst transfer really improves the cumulative outcomes in couples undergoing IVF**."** in the revised Discussion.

D. Location of changes: Please refer to the **Discussion**, page 8, line 203-208 in the revised manuscript with highlighted track changes.

Reviewer 3, comment 2

A. Reviewer: *The authors have done a secondary, post hoc analysis, including all transfers until July 2023 and by then delivery rates do not differ any longer between the groups. However, this analysis can not be basis for conclusion since not included in the protocol and a mix of transfers has taken place.*

B. Response: We appreciate the reviewer's comment. We agree that we cannot draw the conclusions based on the results of this long-term follow-ups, because the transfers did not follow our pre-specified protocol. Our main conclusions were based on the primary analysis. Our secondary analysis was to ensure that our main findings were robust after further scrutiny.

C. Changes made: We added **"**We conducted **a secondary, post-hoc analysis of** the long-term follow-ups from randomization day to July 28th, 2023, and found similar cumulative live birth rate between the two group.**" "Therefore, this analysis cannot be used as a basis for conclusions."** in the revised Discussion.

D. Location of changes: Please refer to the **Discussion**, page 9, line 227-228; line 232 in the revised manuscript with highlighted track changes.

Reviewer 3, comment 3

A. Reviewer: *There is another thing that confuses me. You state that the primary outcome was "cumulative live-birth rate following first 3 single embryo transfers from one oocyte retrieval and all embryo transfers within 1 year of randomization".*

I don't think this is quite correct and it does not fit. First it is obvious from Suppl Table that a considerable portion of the patients, not achieving live birth after first ET, never go for a second transfer. The same pattern in both groups and the same pattern for third transfer. This is not strange. That is how it works, all patients do not get embryos in the freezer and thus don't get further transfers but then the design should not say cumulative livebirth after three transfers but cumulative livebirth after a maximum of three transfers.

In summary I think the design should have been one of the following:

1.Cumulative livebirth after maximum of three transfers

2. All transfers within a year after randomization

I can't understand the design otherwise.

B. Response: The primary outcome of this trial was the cumulative live birth rate for maximum of the first three embryo transfers resulting from one oocyte retrieval cycle, as far as these transfers occurred within 1 year of randomization (and in times of COVID-19 restrictions 1 years and 3 months). This definition of the primary outcome is consistent across the manuscript, the protocol and online registration (NCT03152643). Referring to the comment of two options mentioned by the reviewer, our primary outcome was option 1. Cumulative livebirth after maximum of three transfers, as long as these transfers happened in the first year after randomization. The reason to limit ourselves to the first three single embryo transfers is that after these three single embryo transfers, many couples and their doctors are unable to continue single embryo transfers without treatment to increase the chances of pregnancy, eg. double embryo transfers. We have explained the reasons for this design as detailed in our previous responses, and in the discussion section of the manuscript, page 12, line 307-313.

According to the protocol, we calculated the cumulative live birth rates from the maximum of the first three embryo transfers within 1 year of randomization as the primary outcome, and the live birth rates from all embryo transfers within 1 year of randomization as the secondary outcome. We revised the entire manuscript to make the description clearer.

C. Changes made: To make this as clear as possible, we revise the description of the primary outcome throughout the revised manuscript.

> **Abstract**
>
> "The primary outcome was cumulative live-birth rate following **a maximum of** first 3 single embryo transfers from one oocyte retrieval. All embryo transfers within 1 year of randomization were followed up for the occurrence of live birth until 2 years after randomization **as the secondary outcome**."
>
> **Discussion**
>
> **"**However, considering that the first three SETs may achieve most pregnancies, as well as the feasibility and applicability of the trial to the real-world clinical practice, we ~~used~~ **studied** the live births from **a maximum of** first three SETs as the primary outcome, **which happened in the first year after randomization**, ensuring equal number of embryos transferred in both groups, to reveal the efficacy and safety of the two strategies.**"**
>
> **Methods**
>
> **Outcomes**
>
> **"**The primary outcome was cumulative live birth rate for **a maximum of** ~~**up to**~~ the first three embryo transfers resulting from one oocyte retrieval cycle, **as long as these transfers happened in the first** ~~**within 1**~~ **year after** ~~**of**~~ randomization (or 1 year and 3 months in case of delays ~~**from**~~ **due to** COVID-19).**"**
>
> **"Outcomes from all embryo transfers within 1 year of randomization were followed up for the occurrence of live birth until two years after randomization as the secondary outcome."**

D. Location of changes: Please refer to the **Abstract**, page 3, line 62-64; **Discussion**, page 12, line 309-313; **Methods**, page 29, line 700-702; line 710-712 in the revised manuscript with highlighted track changes. We revised the entire manuscript to make the description of the outcome clearer.

Reviewer 3, comment 4

A. Reviewer: *I can't understand either how it can be both three embryo transfers and within one year after randomization. How was it planned to be handled if one woman did her third ET one month after this year and that resulted in a livebirth? was only the first two ET (both within a year) included for that patient?*

B. Response: Please see our response on the previous comment (Reviewer 3, comment 3). Based on the protocol, in details, if one woman underwent her third embryo transfer after 1 year of randomization, this transfer fell outside of our follow-up period and was therefore not counted for calculation of the primary outcome. If a patient did her fourth embryo transfer within 1 year, this was also not counted for the primary outcome, but it was included as a secondary outcome - all transfers in 1 year after randomization, as is presented in Supplementary Table 9.

C. Changes made: Not applicable.

D. Location of changes: Not applicable.

Reviewer 3, comment 5

A. Reviewer: *Further, were there other reasons (than no embryos frozen or no embryos surviving freezer) for not getting ET 2 and ET 3?*

B. Response: In response to the reviewer's comments, we performed follow-ups on the reasons for women who did not undergo a second and third embryo transfer (**Response Table 1**). The main reasons for not continuing the transfers were no plans for embryo transfers and family issues, in addition to loss-to-follow up. We would like to stress that this reflects the reality of clinical practice. While the reviewer is correct that when all embryos are used, the day 3 and day 5 strategies would have equal cumulative live birth rates, the reality is that not all patients use all embryos, with patients dropping out after the first cycle.

Response Table 1. Reasons for not performing ET 2 and 3 (in addition to not having frozen embryos)

Reasons	**Number (%)** **N=49**
Natural conception	5 (10.2)
Divorce or other social family reasons	9 (18.4)
No plans for embryo transfers recently	12 (24.5)
Unable to continue transfers due to physical reasons-Uterine rupture	1 (2.0)
Unable to continue transfers due to physical reasons- Gastric tumor	1 (2.0)
Unable to continue transfers due to physical reasons-Uterine adhesion	1 (2.0)
Lost to follow-up	16 (32.7)
Received treatment at other hospitals	4 (8.2)

C. Changes made: Not applicable.

D. Location of changes: Not applicable.

References:

1. Glujovsky, D.*, et al.* Cleavage-stage versus blastocyst-stage embryo transfer in assisted reproductive technology. *Cochrane Database Syst Rev* **5**, CD002118 (2022).
2. European IVF Monitoring Consortium (EIM), for the European Society of Human Reproduction and Embryology (ESHRE), et al. ART in Europe, 2018: results generated from European registries by ESHRE. Hum Reprod Open 2022, hoac022 (2022).
3. Centers for Disease Control and Prevention. *2021 Assisted Reproductive Technology Fertility Clinic and National Summary Report.* US Dept of Health and Human Services; 2023. https://www.cdc.gov/art/reports/2021/summary.html
4. Xiaodong Zhang, Yuan Shen, Juanzi Shi, et al. Annual report on assisted reproductive technology of Chinese Society of Reproductive Medicine in 2019. The Journal of Reproductive Medicine, 2022(008):031.

5. G.D Adamson. Global ART data: what do they tell us about our world? 2022 https://www.icmartivf.org/wp-content/uploads/ASPIRE-Bruno-Lunenfeld-Lecture-v10-presented-pdf-format-20220407-2.pdf
6. Yan, J., et al. Live Birth with or without Preimplantation Genetic Testing for Aneuploidy. N Engl J Med 385, 2047-2058 (2021).
7. Practice Committee of the Society for Reproductive Endocrinology and Infertility, Quality Assurance Committee of the Society for Assisted Reproductive Technology, and the Practice Committee of the American Society for Reproductive Medicine. Multiple gestation associated with infertility therapy: a committee opinion. *Fertil Steril* **117**, 498-511 (2022).
8. G.D Adamson and others, O-151 ICMART preliminary world report 2018, Human Reproduction, Volume 37, Issue Supplement_1, July 2022, deac105.057, https://www.icmartivf.org/wp-content/uploads/ICMART-ESHRE-WR2018-Preliminary-Report.pdf
9. Bai, F., et al. Assisted reproductive technology service availability, efficacy and safety in mainland China: 2016. Hum Reprod 35, 446-452 (2020).
10. De Geyter, C., et al. 20 years of the European IVF-monitoring Consortium registry: what have we learned? A comparison with registries from two other regions. Hum Reprod 35, 2832-2849 (2020).
11. Harbin Consensus Conference Workshop, G. Improving the Reporting of Clinical Trials of Infertility Treatments (IMPRINT): modifying the CONSORT statement. Fertil Steril 102, 952-959 e915 (2014).

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

I once again appreciate the very detailed responses of the authors and feel that they in principle addressed all of my concerns. I was to a degree surprised to see how young their study population was and just wasn't to, again, emphasize the importance of making it clear to often only superficial readers what kind of patients the study was made up with. I, therefore, also appreciate the title change because what this study reports as outcomes can really only be applied to very young good-prognosis patients.

Reviewer #2 (Remarks to the Author):

I am happy with the responses to my comments. I would prefer a statement in the abstract reflecting the fact that blastocyst transfer provides a much greater increase in RR of pre-term birth (>3) compared to a small increased RR of live birth (1.13). I would also add that the increased number of frozen embryos in store with cleavage cycles will reduce this RR of 1.13 even further (albeit outside the trial design). This will allow clinicians and patients to more easily assess the risk:benefit ratio of blastocyst transfer versus cleavage transfer.

Reviewer #3 (Remarks to the Author):

My main concerns with this paper remain and which deal with the design of the study. I think it is well known (Cochrane 2022, 15 studies, 2219 women) that blastocyst transfer is superior to cleavage stage transfer and that this is an effect of embryo selection. It is then quite obvious that also three blastocyst transfers should be superior to three cleavage stage transfers which is the result and the main conclusion from this trial. This is also obvious from Tables presenting live birth per first, second and third transfer. The message that blastocyst transfer is superior to cleavage stage transfer in terms of live birth rate is thus not new or unique .

This trial does not calculate cumulative live birth rate, according to international definition (Zeger-Hochschild 2017), which would have been very interesting and unique, but only livebirth after a max of three embryo transfers. When adding transfers on surplus embryos (more embryos available in the cleavage stage group) it is obvious that cumulative live birth rates do not longer differ. However, this is outside the study and can correctly not be used for conclusion.

Response to Reviewers' Comments:

We want to thank all reviewers for their helpful comments and criticisms on this and previous versions of our paper.

Reviewer #1

A. Reviewer:

I once again appreciate the very detailed responses of the authors and feel that they in principle addressed all of my concerns. I was to a degree surprised to see how young their study population was and just wasn't to, again, emphasize the importance of making it clear to often only superficial readers what kind of patients the study was made up with. I, therefore, also appreciate the title change because what this study reports as outcomes can really only be applied to very young good-prognosis patients.

B: Response: We are grateful to reviewer 1 for these encouraging comments as well as for the comments in previous rounds!

C. Changes made: None.

D. Location of changes: Not applicable.

Reviewer #2:

A. Reviewer*: I am happy with the responses to my comments. I would prefer a statement in the abstract reflecting the fact that blastocyst transfer provides a much greater increase in RR of pre-term birth (>3) compared to a small increased RR of live birth (1.13). I would also add that the increased number of frozen embryos in store with cleavage cycles will reduce this RR of 1.13 even further (albeit outside the trial design). This will allow clinicians and patients to more easily assess the risk: benefit ratio of blastocyst transfer versus cleavage transfer.*

B. Response: We appreciate reviewer 2's comments. Our study shows that blastocyst transfer results in a higher live birth rate (RR 1.13, absolute rates 74.8% versus 66.3%) for a higher rate of spontaneous preterm birth (RR 2.29, absolute rates 4.6% versus 2.0%) and preterm premature rupture of membranes (RR 3.11, absolute rate 5.0% versus 1.6%). As more couples in the cleavage stage group without a live birth have embryos left, this difference might become smaller. We have added this statement in the abstract.

C. Changes made: The abstract now contains information on cumulative live birth rates and preterm birth rates: "Live-birth rates were 74.8% in blastocyst group versus 66.3% in cleavage-stage group (relative risk 1.13, 95%CI:1.04-1.22; $P_{non\text{-}inferiority}<0.001$, $P_{superiority}=0.003$) (1-year cumulative live birth rates of 76.8% versus 68.5%). Blastocyst transfer increased the risk of preterm birth (4.6% vs 2.0%; *P*=0.02)."

D. Location of changes: Please refer to the **Abstract**, page 4, line 63-66 in the clean version of manuscript.

Reviewer #3:

A. Reviewer: *My main concerns with this paper remain and which deal with the design of the study. I think it is well known (Cochrane 2022, 15 studies, 2219 women) that blastocyst transfer is superior to cleavage stage transfer and that this is an effect of embryo selection. It is then quite obvious that also three blastocyst transfers should be superior to three cleavage stage transfers which is the result and the main conclusion from this trial. This is also obvious from Tables presenting live birth per first, second and third transfer. The message that blastocyst transfer is superior to cleavage stage transfer in terms of live birth rate is thus not new or unique.*

This trial does not calculate cumulative live birth rate, according to international definition (Zeger-Hochschild 2017), which would have been very interesting and unique, but only livebirth after a max of three embryo transfers. When adding transfers on surplus embryos (more embryos available in the cleavage stage group) it is obvious that cumulative live birth rates do not longer differ. However, this is outside the study and can correctly not be used for conclusion.

B. Response: We appreciate reviewer 3's comments on cumulative live birth. As we previously stated, there is a lack of evidence on the strategy of blastocyst transfer versus cleavage-stage transfer on the cumulative live birth rate (CLBR), as advocated by the latest Cochrane review and the European IVF Monitoring Consortium for the European Society of Human Reproduction and Embryology. We respectfully consider our findings as an important asset to the existing evidence.

As we previously responded, we agree that cumulative live birth rates are the most relevant outcome. Indeed, CLBRs from all embryo transfers within 1 year of randomization (a secondary outcome) are almost similar to the CLBR from 3 single embryo transfers (SETs) (our primary outcome). As we have previously indicated, it is almost impossible to conduct a clinical trial without a fixed time frame. For pragmatic reasons, we therefore limited the follow-up period to 1 year after randomization, which reflects the reality of clinical practice. Patients cannot stick to the assigned group if pregnancy does not occur within 1 year (Please see Supplementary Table 13) and 44 women gave up continuing embryo transfers after ET 1 (Please see Table 1 in previous response NCOMMS-23-33214B1).

We want to stress however that the choice for cleavage or blastocyst stage is not so much made at the moment of transfer, but – apart from the first fresh transfer - rather at the moment of freezing. Among 497 women assigned to the blastocyst group, only four women (0.8%) had frozen cleavage-stage embryos, two of them also frozen blastocysts. Five women (1.0%) did not have blastocyst-stage embryos after extended culture. Of 727 embryo transfers in the blastocyst group, there were only 8 (1.1%) undergoing cleavage-stage transfer.

Among 495 women assigned to the cleavage-stage group, nine women (1.8%) had frozen blastocysts only; 130 women (26.3%) frozen both cleavage-stage and blastocyst-stage embryos, with 114 women (87.7%) freezing $\geqslant$3 cleavage-stage embryos, which stored enough cleavage-stage embryos for transfers within the study period. Of 875 embryo transfers in the cleavage-stage group, there were 42 (4.8%) undergoing blastocyst transfer.

In our opinion, these protocol deviations are limited. We designed the trial to compare the strategies of cleavage-stage and blastocyst-stage embryo transfers within the period of 1 year (reasonable period in clinical practice), not only on three embryo transfers. Thus, while the reviewer is correct that 3 blastocyst transfers are superior to 3 cleavage stage transfers, we want to stress that we compare a strategy of blastocyst versus cleavage stage transfer.

We have added the above on page 7, line 143-152 of the revised manuscript.

In terms of results, the long-term difference stays in the advantage of blastocyst transfer. As the number of couples without a baby and without embryos left is 9.3% versus 4.4%, this 4.9% difference is smaller than the 8.6% difference in CLBR (74.8% versus 66.3%). For follow-up of all embryo transfers within 1 year of randomization, the 3.6% difference for women with no livebirths and without embryos (9.9% versus 6.3%) is still lower than 6.8% increase in CLBRs (75.7% vs 68.9%). Even at follow-up after the end of the study (Please see Table 5 main text), the 1.0% difference in women without a frozen embryo and without a livebirth (11.7% versus 10.7%) do not make up for the 3.3% difference higher CLBRs (80.9% versus 77.6%). Therefore, our RCT shows that CLBRs are unlike to be equal for the two strategies in real practice.

We have also emphasized this point in our previously revised manuscript, as follows:

Discussion (Page 11, line 240-245)

"Our trial shows that the cumulative live birth rate after three single blastocyst transfers is higher than that after three cleavage-stage transfers, which might be hypothesized based on previous reports of higher live birth rates after one fresh blastocyst transfer.[11] Since depletion of embryos by blastocyst culture leads to a reduction in the number of embryos, data are needed to confirm whether blastocyst transfer really improves the cumulative outcomes in couples undergoing IVF."

Discussion-limitation (line 344-350)

"Finally, we calculated a maximum of the first three SETs as the primary outcome, and all embryo transfers within the study period as the secondary outcome. Ideally, the "true" cumulative live birth rate would be obtained after all embryo have been transferred. However, considering that the first three SETs may achieve most pregnancies, as well as the feasibility and applicability of the trial to the real-world clinical practice, we studied the live births from a maximum of first three SETs as the primary outcome, which happened in the first year after randomization, ensuring equal number of embryos transferred in both groups, to reveal the efficacy and safety of the two strategies."

Discussion (line 316-326)

"To our knowledge, this is the largest randomized controlled trial to date and the first to provide robust data on cumulative live birth and obstetrical-perinatal outcomes of the two embryo transfer strategies. The strengths of this study include its large sample size, the low loss-to-follow-up rate, randomized allocation in multiple cycles over the course of a year, the multicenter and pragmatic design that improves generalizability of our results, and strict adherence to SETs in both groups, that ensures the comparable number of embryos between groups. In addition, our study informs the discussion on blastocyst versus cleavage-stage transfer and the design of such studies. We use both absolute and relative terms in expressing success rates and risks, which strongly contributes the clinical message conveyed to clinicians and patients. Furthermore, our study had for pragmatic reasons a follow-up period of 1 year after randomization. While this might favour blastocyst transfer, as the cleavage stage group has more unused embryos left, we also think that a 1-year follow-up reflects the reality of clinical practice."

C. Changes made: None.

D. Location of changes: Not applicable.