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James. M Kemper , Yanhe Liu , Masoud Afnan , Ben W.J. Mol , Dean E. Morbeck

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What happens to abnormally fertilised embryos? A scoping review

James. M Kemper[1,2]

Yanhe Liu[3,4,5,6]

Masoud Afnan[7]

Ben W. J. Mol[1,2,8]

Dean E. Morbeck[2,9,10]

[1]Monash Women's, Monash Health, Clayton, Australia

[2]Department of Obstetrics & Gynaecology, Monash University, Clayton, Australia

[3]Fertility North, Joondalup, Australia

[4]School of Human Sciences, University of Western Australia, Crawly, Australia

[5]School of Medical and Health Sciences, Edith Cowan University, Joondalup, Australia

[6]School of Health Sciences and Medicine, Bond University, Robina, Queensland, Australia

[7]Qingdao United Family Hospital, Qingdao, China

[8]Aberdeen Centre for Women's Health Research, School of Medicine, Medical Sciences and Nutrition, University of Aberdeen, Aberdeen, United Kingdom

[9]Fertility Associates, Auckland, New Zealand

[10]Department of Obstetrics & Gynaecology, University of Auckland, New Zealand

Corresponding Author

James M. Kemper

Monash Women's, Monash Health

246 Clayton Road, Clayton, 3168

Australia

dr.james.kemper@gmail.com

+ 61 3 9594 6666

ORCID: 0000-0002-9483-6639

Abstract

A dearth of evidence exists surrounding embryos derived from oocytes without two pronuclei (2PN) or 'normal fertilisation'; that is, embryos arising from non-pronuclear oocytes (0PN), mono-pronuclear oocytes (1PN) and tri-pronuclear oocytes (3PN). We searched the published literature regarding non-2PN oocytes and their clinical outcomes using a two-part collection strategy of relevant articles. A total of 33 articles were deemed eligible for the scoping review. There is a significant difference between potential development of oocytes with an abnormal number of pronuclei and those with 2PN in most studies; they occur rarely and there is significant attrition between day 1 and day 6, with corresponding reduction in chromosome integrity and clinical utility. Most recent studies describe outcomes of blastocysts derived from non-2PN oocytes, rather than cleavage stage embryo transfers. Compared to 2PN oocytes, blastocyst rates are lower in 1PN oocytes (68.3 vs. 32.2%), with larger 1PN oocytes having better developmental potential compared to their smaller counterparts. Blastocysts from 1PN oocytes appear to have a slightly reduced implantation potential compared to those from 2PN blastocysts (33.3% vs. 35.9%), with a reduced ongoing pregnancy rate (27.3% vs. 28.1%). Live birth rates were only reported in 13 of the included studies. The comparators varied between studies, with live birth rates provided ranging from 0-59.0%, with two case reports (100%); this is a clear indication of the variability in practices and the significant heterogeneity of the studies. A distinct lack of evidence exists surrounding non-2PN oocytes; however, it appears that most abnormally fertilised oocytes that are non-viable will developmentally arrest in culture, and those that are viable can form viable pregnancies. Concerns continue to exist regarding the outcome of pregnancies arising from the use of abnormally fertilised oocytes. Coupled with appropriate outcome measures, abnormally fertilised oocytes hold the potential to increase the pool of embryos eligible for transfer.

Key Words

Fertilisation; pronuclear; oocyte; embryo.

Key Message

Live births have been reported from transfer of embryos obtained from non-2PN oocytes. Further studies are needed to better understand the origin and potential of these embryos so patients can be properly advised.

Introduction

The drive to improve success rates within in vitro fertilisation (IVF) has focussed on producing top-quality embryos, ranking the available embryos, and transferring those with the highest chances of sustained development before transferring others with less developmental potential. We recently discussed the lack of evidence in predicting the success rates of low-grade blastocysts (commonly classed as <3BB), thereby expressing the concern of neglecting these embryos in couples who lack good-quality embryos, and noting that once implantation is achieved, the course of pregnancy is usually uncomplicated, without an increase in adverse perinatal or pregnancy outcomes (Hammond *et al.*, 2021; Kemper *et al.*, 2021).

'Normal fertilisation' is defined as the presence of two pronuclei and two polar bodies in an oocyte. A dearth of evidence exists surrounding embryos derived from oocytes without such normal fertilisation; that is, embryos arising from non-pronuclear oocytes (0PN), mono-pronuclear oocytes (1PN) and tri-pronuclear oocytes (3PN). This is not a new concept; Levron et al. (Levron *et al.*, 1995) and Sultan et al. (Sultan *et al.*, 1995) were the first do describe the potential fertilisation of 1PN IVF oocytes; 26 years ago, Manor et al. published a paper titled 'Undocumented embryos: do not trash them, FISH them', discussing that undocumented embryos should be analysed, not discarded (Manor *et al.*, 1996). Data on clinical prognosis of embryos from non-2PN oocytes are generally limited as most clinics do not offer the option to use them. However, assessment for fertilisation can be seen as a diagnostic test, with both false positives and false negatives, particularly with a predominately static snapshot observation where true fertilisation status may not be fully revealed (Alpha Scientists in Reproductive Medicine *et al.*, 2011). Even with improved visualisation of pronuclei with the assistance of time-lapse videography, the prognosis of such "abnormally" fertilised embryos is still poorly understood. As with low-grade

blastocysts, embryos from non-2PN oocytes may be the only options available for some patients; thus, clinicians should be able to accurately advise patients about the relative chances of viability of embryos from non-2PN oocytes.

In this scoping review, we examine the available published literature regarding the outcomes for embryos from non-2PN oocytes and highlight the need for dedicated studies examining these important clinical questions.

Methods

We searched the published literature regarding non-2PN oocytes and their clinical outcomes using a two-part collection strategy of relevant articles. Two different PubMed searches were conducted; given some results were only included in one of the searches, both were included. The first search was conducted utilising the following terms: (((blastocyst) AND (("abnormal pronuclear" OR "abnormal fertilisation" OR "abnormal fertilization" OR "monopronucl*" OR "micropronucl*" OR "nonpronuclear")) OR ("1PN" OR "3PN" OR "0PN"))) AND ("ploidy" OR "aneuploid" OR "euploid"). The second search utilised: (blastocyst[Title]) AND ((((((abnor*[Title]) OR (pronuc*[Title])) OR (fertil*[Title])) OR ((multi* nuc*) OR (monopron*) OR (nonpronuc*))) OR ((1PN) OR (3PN) OR (0PN))) OR ((*ploid*[Title]))), and was limited to 1990-present, human species, full-text, and English language. Finally, we utilised personal knowledge of the topic to collate a list of articles, some of which overlapped with the formal search strategy.

Results

The first search found 18 results, of which 8 were deemed to be applicable to the study question. The second search identified 122 results, of which 11 results were included; 5 of these overlapped with the first search. Expert input added a further 19 articles.

A total of 33 articles were deemed eligible for the scoping review (Balakier *et al.*, 1993; Levron *et al.*, 1995; Palermo *et al.*, 1995; Sultan *et al.*, 1995; Staessen and Steirteghem, 1997; Gras and Trounson, 1999; Petignat *et al.*, 2001; Dasig *et al.*, 2004; Otsu *et al.*, 2004; Noyes *et al.*, 2008; Liao *et al.*, 2009; Reichman *et al.*, 2010; Wang *et al.*, 2012; Mateo *et al.*, 2013, 2020; Itoi *et al.*, 2015; Li *et al.*, 2015, 2016, 2020, 2021; Liu *et al.*, 2016; Yin *et al.*, 2016; Bradley *et al.*, 2017; Capalbo *et al.*, 2017; Dai *et al.*, 2017; Araki *et al.*, 2018; Destouni *et al.*, 2018; Kai *et al.*, 2018; Xie *et al.*, 2018; Fabozzi *et al.*, 2019; Si *et al.*, 2019; Tan *et al.*, 2019; Paz *et al.*, 2020). No randomised controlled trials were identified; the included studies feature thirteen retrospective studies, eight experimental/point-in-time analyses, seven prospective studies, four case reports, and one case-control trial.

Despite the relative lack of evidence on the outcomes of non-2PN oocytes, there are some key findings obtainable from the published literature. Most recent studies describe outcomes of blastocysts derived from non-2PN oocytes, rather than cleavage stage embryo transfers. This is largely a function of the increase in the practice of blastocyst culture as well as a trend to keeping all oocytes, regardless of fertilisation status, in culture with minimal interruption through day five and beyond.

There is limited evidence on the incidence of useable blastocysts from non-2PN oocytes, particularly for presumed 0PN oocytes. While most clinics report 2PN rates of 65-80% per mature oocyte, the incidence of 1PN oocytes is 1-8% and 3PN oocytes is 1-7% (Papale *et al.*, 2012; ESHRE Special Interest Group of Embryology *et al.*, 2017; Li *et al.*, 2020). A recent report of 6466 oocytes reported that 11.2% and 14.8% of 0PN and 1PN embryos formed good-quality blastocysts, with blastulation rates higher following IVF compared to ICSI (Chen et al., 2020).

Compared to 2PN oocytes, blastocyst rates are lower in 1PN oocytes (68.3% vs. 32.2%, N=557 vs. N=47, $p<0.01$; 'good quality' blastocysts 38.5% vs. 17%, $p<0.01$) (Araki

et al., 2018), with 1PN oocytes with a larger pronucleus having better developmental potential compared to their smaller counterparts (56.1% for an area of pronucleus ≥710μm vs. 17.9% ≤509μm) (Araki *et al.*, 2018). The use of either conventional IVF (cIVF) or intracytoplasmic sperm injection (ICSI) has an impact on the number of blastocysts formed from 1PN oocytes (cIVF 2PN 55.7% vs. 1PN 21.4%; ICSI 2PN 46.4% vs. 1PN 10.7%) and the implantation rate (cIVF 2PN 39.0% vs. 1PN 33.3%; ICSI 2PN 46.7% vs. 1PN 0%) (Itoi *et al.*, 2015). Data on developmental potential of presumed 0PN oocytes is not as robust as most studies only provide blastocyst transfer results or genetic analyses.

Blastocysts from 1PN oocytes appear to have a marginally reduced implantation potential compared to those from 2PN blastocysts following cIVF (33.3% vs. 39%), with a reduced ongoing pregnancy rate (adequate for 1PN blastocysts from IVF but not from ICSI) (Itoi *et al.*, 2015). Presumed 0PN oocytes have a similar or reduced implantation potential compared to those from 2PN blastocysts (Itoi *et al.*, 2015); evidence for relative developmental potential of presumed 0PN compared to 1PN blastocysts is lacking.

Live birth rates were only reported in 13 of the included studies, as seen in Table 1 (Gras and Trounson, 1999; Dasig *et al.*, 2004; Reichman *et al.*, 2010; Itoi *et al.*, 2015; Liu *et al.*, 2016; Bradley *et al.*, 2017; Capalbo *et al.*, 2017; Destouni *et al.*, 2018; Xie *et al.*, 2018; Si *et al.*, 2019; Li *et al.*, 2020, 2021; Paz *et al.*, 2020). The comparators varied between studies, with rates provided ranging from 0-59%, with two case reports. This is a clear indication of the variability in practices and the significant heterogeneity of the studies.

Limitations of the Current Literature

Naturally, embryos from 2PN zygotes are deemed ideal and are used preferentially; as such, significant evidence exists regarding their outcomes.

Despite the limited evidence available for the outcomes of blastocysts from abnormally fertilised embryos, a small proportion of blastocysts from presumed 0PN and 1PN oocytes have the ability to form viable pregnancies, as most of the abnormally fertilised oocytes will naturally become nonviable and developmentally arrest in culture.

One of the important limitations of the current literature relates to the timing of the fertilisation check. As recommended by ESHRE (ESHRE Guideline Group on Good Practice in IVF Labs *et al.*, 2016), "all inseminated or injected oocytes should be examined for the presence of pronuclei and polar bodies at 16-18 hours post insemination". It is notable that both amongst conference presentations and in formal trials, some centres still examine fertilisation at up to 20 hours post insemination (Yin *et al.*, 2016). It is theoretically possible, and the rates thereof are currently unknown, that the brief appearance of one or both pronuclei may be missed by static observation; time-lapse imaging would conclusively show these oocytes as being potentially 2PN. In a study with >50,000 embryos, as many as 13% had faded pronuclei by 20 hours post-insemination, representing 3000 2PN that would have been missed without time-lapse monitoring; indeed, up to 300 blastocysts which resulted in live births would have been categorised as unfertilised (Barrie *et al.*, 2021). With ICSI, more 2PN zygotes will be missed when analysed >18 hours post insemination. This is supported by time-lapse studies showing different timings of pronuclear fading between zygotes created via IVF and ICSI. Relative to insemination, ICSI-originated zygotes were shown to have pronuclear fading 1.4 hours earlier than their IVF counterparts (Cruz *et al.*, 2013), a difference that is made inconsequential for time-lapse algorithms by using pronuclear fading as a reference starting point rather than insemination (Liu *et al.*, 2015, 2016). This indicates a significant 'delay' in sperm entry in IVF which requires additional steps than ICSI, including interactions between sperm and cumulus cells as well as zona pellucida; sperm injected via ICSI would have skipped these procedures, potentially leading to the observed earlier

pronuclear fading; thus this 1.4 hour advancement in oocyte activation in ICSI cycles results in an important shift in pronuclear fading in relation to fertilization assessment. There is no guideline proposing earlier assessment for ICSI cycles to minimise the risk of missing pronuclei when using static observations. Even with time-lapse imaging, the full developmental footage of pronuclei in zygotes created via IVF is difficult to capture because oocytes are generally co-incubated with sperm overnight before fertilisation check. As such, observation of initial pronuclear formation (a few hours after sperm entry) following sperm entry is not possible due to the layers of cumulus cells surrounding the oocytes (Mio and Maeda, 2008). However, short insemination protocols, whereby cumulus cells are removed 2-3 hours following insemination, combined with time-lapse imaging offers a better chance to observe pronuclei following IVF (Menezo and Barak, 2000; Kattera and Chen, 2003).

Recent publications, as we do here, have called into question the 'sacrosanct' nature of the fertilisation check (Doody, 2020), noting the significant time taken and the additional complexity. Doody notes that, amongst other indications, there are two reasons to identify abnormally fertilised eggs: they will either not produce viable embryos or will result in high-risk pregnancies (Doody, 2020). The study by Li et al. (Li *et al.*, 2021), included in our dataset, detected reasonable implantation and live birth rates. They conclude that rather than discarding zygotes after the fertilisation check, culture should continue to the blastocyst stage, at which point development can be reassessed. The findings of this review indicate that blastocysts that lack documented fertilisation have developmental potential and should be considered for use.

A recent report following embryos in time-lapse incubators suggests viable blastocysts from presumed 0PN oocytes are simply 2PN that faded early, rather than blastocysts that never formed visible pronuclei (Kobayashi *et al.*, 2021). While non-time-lapse, single point observations yielded 8% blastocysts from presumed 0PN oocytes, no

blastocysts were obtained from oocytes in which pronuclei were not observed throughout the first 20 hours of culture. Though this study was small, in all likelihood, time-lapse imaging in either ICSI cycles or IVF cycles with short insemination would detect PN formation and fading. Whilst time-lapse imaging is not universally utilised and analysed, it is important that studies investigating non-2PN oocytes ensure appropriate timing of the fertilisation check at 16-18 hours post insemination.

Another limitation of particular importance to clinicians and patients is the lack of evidence surrounding the pregnancy and perinatal outcomes arising from successful implantation of non-2PN oocytes. Chen et al. examined 6466 embryos that underwent extended culture to day 5 or day 6, derived from 0PN or 1PN oocytes, and low-quality day 3 embryos; blastulation occurred in 17.3%, with good-quality blastocyst formation present in 9.5% (Chen *et al.*, 2020). Of the 243 resulting cycles utilising blastocysts arising from non-2PN embryos, clinical pregnancies occurred in 44.9% (Chen *et al.*, 2020). Limited evidence has shown that the use of these oocytes may result in live births (Liu *et al.*, 2016), but it is currently unknown whether these pregnancies are more likely to be complicated antenatally or perinatally.

Of particular concern is the possibility that abnormally fertilised oocytes may result in molar pregnancies (Petignat *et al.*, 2001). We found only one case report of a molar pregnancy from a 1PN in a thorough review on incidence and genetics of 1PN oocytes (Rosenbusch, 2014), and in contrast, most published studies on 1PN oocytes do not report findings of this adverse outcome. Genetic studies have determined that most 1PN blastocysts are diploid, though haploid an uniparental disomy are possible (Bradley *et al.*, 2017; Kai *et al.*, 2018). Thus, further research is needed to determine the relative risk of abnormal pregnancies to allow proper counselling regarding risks.

The evidence is so scant that very few national/international guidelines exist regarding the use these abnormally fertilised embryos. The European Society of Human Reproduction and Embryology (ESHRE)'s Revised Guidelines in 2016 note that "embryos derived from ≥3PN oocytes should never be transferred or cryopreserved" (ESHRE Guideline Group on Good Practice in IVF Labs *et al.*, 2016). They go on to state that "even if no transferable embryos derived from 2PN oocytes are available, the use of embryos derived from 1PN oocytes or oocytes showing no PN is not recommended" (ESHRE Guideline Group on Good Practice in IVF Labs *et al.*, 2016), a guideline that merits review.

Finally, this scoping review encountered several methodological difficulties. As can be seen from the article collection process, over 50% of the included studies were not found via two PubMed searches. The authors struggled to identify terms that would capture the majority of studies included in a single search; this unfortunately makes a formal systematic review difficult to conduct. Additionally, the heterogenicity of study designs, investigations, and outcomes hinders efforts at conducting a meta-analysis. Finally, the lack of standardised terminology in this area complicates efforts further.

Future Directions

This review highlights the need for future research to accurately assess and resolve some of the key issues surrounding blastocysts arising from oocytes with no evidence or atypical fertilisation. The use of time-lapse imaging may allow for significantly enhanced understanding of embryo development, and of the impact of the timing of pronuclear development and disappearance on development. Transfer of blastocysts derived from 0PN or 1PN oocytes can result in normal live births (Fu *et al.*, 2020). Some examples include more extensive assessment of the impact of variations in pronuclei size (Otsuki *et al.*, 2019)

as well as the impact of micro nuclei associated with two normal pronuclei (Capalbo *et al.*, 2017).

In addition to new information, time-lapse imaging allows for more timely and accurate fertilisation checks, to aid in detecting those 'missed' pronuclear bodies that present in between static assessment checks. It also allows improved categorisation of pronuclear development; for example, pronuclear oocytes that present with unequal sized nuclei, nuclei displaced to the periphery of the cell, or nuclei that fail to join by 16-18 hours have been characterised to have lower developmental potential (Sadowy *et al.*, Scott, 2003). Polarisation of nucleolus precursor bodies predicts implantation as well as embryo morphology assessed on day three (Nagy *et al.*, 2003). In addition, time-lapse monitoring enables differentiation between sperm- and oocyte-originated pronucleus by its proximity to the 2nd polar body, with different morphokinetic and morphometric features reported between the two pronuclei (Orevich *et al.*, 2022). A more recent study has proposed the use of migration speed of precursor bodies within the male (sperm-originated) pronucleus as a novel predictor of live birth (Inoue *et al.*, 2021). Furthermore, studies show that existing markers are better assessed by TLI, including the cytoplasmic wave (Payne *et al.*, 1997) and the kinetics of pronuclear chromatin polarisation (Coticchio *et al.*, 2017).

Consensus on the fate of oocytes within 3PN, that they are definitively abnormal, is being challenged by genetic analyses that enable further assessment of these typically discarded embryos. One study reported a diploidy rate of 23% amongst 30 3PN oocytes following FISH (Grau *et al.*, 2015). The authors went on and hypothesised potential autocorrection mechanisms such as direct cleavage into 3 even daughter cells provided maternal haplotype duplication is absent. It's important to note that further studies are required to confirm whether diploid embryos from 3PN zygotes contain uniform biparental composition. In contrast to typical 3PN, where all nuclei are of similar size, oocytes with two

normal sized and one small pronucleus (2.1 or 2+1 PN), or a micronucleus, are also observed. Capalbo et al. (Capalbo *et al.*, 2017) used next-generation sequencing to demonstrate that 12 of 14 (86%) of blastocysts from 2.1 PN oocytes were in fact diploid (Capalbo *et al.*, 2017). The mechanism or significance of a micronucleus coupled with two normal PN is unknown; this small dataset indicates blastocysts derived from these oocytes are mostly normal and merit consideration for transfer. Of note, since NGS alone does not exclude uniparental disomy, future genetic studies should apply NGS with karyomapping functionality. Nonetheless, these findings highlight that guidelines stating that only zygotes with 2 pronuclei are diploid are imperfect and merit further attention. While the examples provided show that fertilisation check is prone to false-negatives, false positives also occur, as illustrated by the 0.5% rate of triploidy in blastocyst biopsied for preimplantation genetic testing for aneuploidy (Marin *et al.*, 2017). The addition of artificial intelligence algorithms may also enable improved accuracy of fertilisation status, with the caveat that any assessor (human or computer) must be able to appropriately visualise the developing embryonic components. For example, the rapid advancement of computer vision offers further insight in automation of pronuclear detection, with the potential for more accurate measurement of any new parameters (Fukunaga *et al.*, 2020). With the increasing computing power, deep learning algorithms may be developed to facilitate identification of unknown features at the pronuclear stage, by utilising enormous amounts of high-resolution imaging data (Riegler *et al.*, 2021).

Authorship

Please see the attached 'Authorship Form'.

JMK, YL, MA, DEM and BWJM conceived of the study. JMK completed the data collection and wrote the first manuscript. All authors interpreted the data, edited the manuscript, provided responses to the reviewers/editors, and agreed upon the final version.

Conflicts of Interest

Please see the attached 'Authorship Form'.

JM Kemper reports no conflict of interest.

Y Liu reports no conflict of interest.

M Afnan reports no conflict of interest.

DE Morbeck reports consultancy for Cook Medical, CooperSurgical and Fujifilm Irvine Scientific.

BWJ Mol is supported by a NHMRC Practitioner Fellowship (GNT1082548) and reports consultancy for ObsEva, Merck Merck KGaA and Guerbet.

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Data Availability

All raw data is available by contacting the corresponding author.

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Dr. James M. Kemper – Biography

James M. Kemper is a Registrar at Monash Women's, Monash Health, and an Affiliate Researcher with the Department of Obstetrics & Gynaecology, Monash University. His

publications have focused on embryo quality, preimplantation testing, and research quality.

Table 1

Article	Investigation	Live Birth Rate % (N)
Gras et al. 1999	1PN	100% (1/1)
Dasig et al. 2004	1PN	100% (1/1)
Reichman et al. 2010	1PN 3PN	1.3% (1/78) 0% (0/9)
Itoi et al. 2015	1PN IVF 1PN ICSI	27.2% (9/33) 0% (0/4)
Liu et al. 2016	0PN	4.7% (13/285)
Bradley et al. 2017	1PN	30.8% (8/26)
Capalbo et al. 2017	1PN	0.5% (3/719)
Destouni et al. 2018	0PN	23.1% (3/13)
Xie et al. 2018	1PN	66.7% (2/3)
Si et al. 2019	1PN	14.0% (26/186)
Li et al. 2020	1PN IVF Fresh 1PN ICSI Fresh 1PN IVF Frozen 1PN ICSI Frozen	7.6% (9/113) 0% (0/70) 32.1% (63/196) 15.3% (9/59)
Li et al. 2021	0PN 1PN	35.6% (155/435) 27.4% (77/281)
Paz et al. 2021	0PN	48.1% (13/27)