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Offspring physiology following the use of IVM, IVF and ICSI: a systematic review and meta-analysis of animal studies

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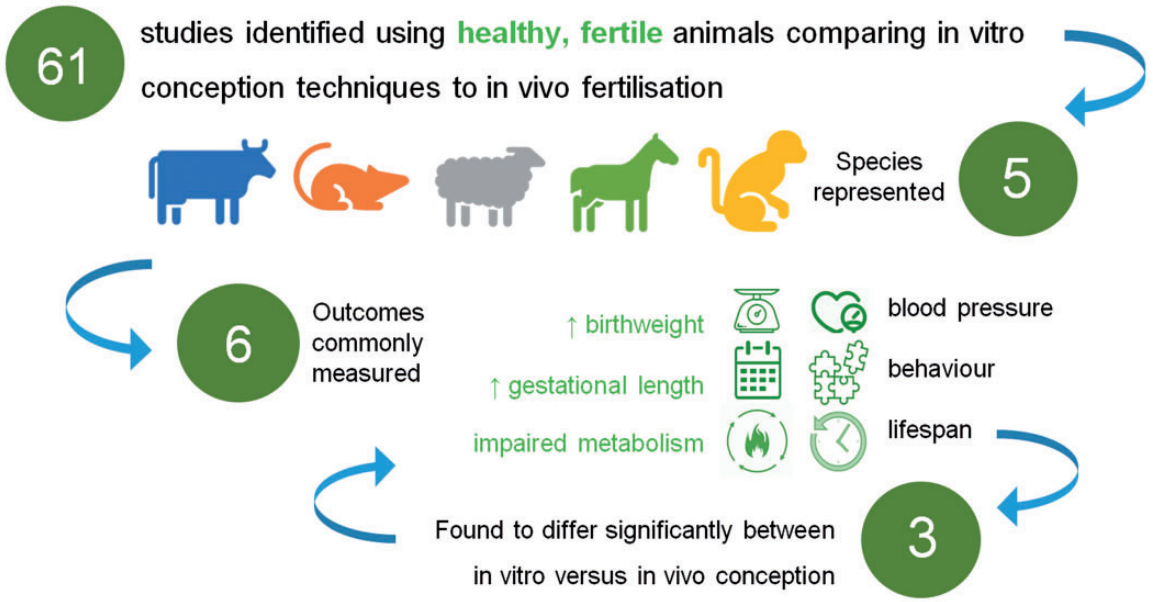

Offspring produced through IVM/IVF/ICSI can have different physiology to their *in vivo* conceived counterparts.

BACKGROUND: Since the birth of the first baby using IVF technology in 1978, over 10 million children have been conceived via ART. Although most aspects of ARTs were developed in animal models, the introduction of these technologies into clinical practice was performed without comprehensive assessment of their long-term safety. The monitoring of these technologies over time has revealed differences in the physiology of babies produced using ARTs, yet due to the pathology of those presenting for treatment, it is challenging to separate the cause of infertility from the effect of treatments offered. The use of systematic review and meta-analysis to investigate the impacts of the predominant ART interventions used clinically in human populations on animals produced in healthy fertile populations offers an alternative approach to understanding the long-term safety of reproductive technologies.

OBJECTIVE AND RATIONALE: This systematic review and meta-analysis aimed to examine the evidence available from animal studies on physiological outcomes in the offspring conceived after IVF, IVM or ICSI, compared to *in vivo* fertilization, and to provide an overview on the landscape of research in this area.

SEARCH METHODS: PubMed, Embase and Commonwealth Agricultural Bureaux (CAB) Abstracts were searched for relevant studies published until 27 August 2021. Search terms relating to assisted reproductive technology, postnatal outcomes and mammalian animal models were used. Studies that compared postnatal outcomes between *in vitro*-conceived (IVF, ICSI or IVM) and *in vivo*-conceived mammalian animal models were included. *In vivo* conception included mating, artificial insemination, or either of these followed by embryo transfer to a recipient animal with or without *in vitro* culture. Outcomes included birth weight, gestation length, cardiovascular, metabolic and behavioural characteristics and lifespan.

OUTCOMES: A total of 61 studies in five different species (bovine, equine, murine, ovine and non-human primate) met the inclusion criteria. The bovine model was the most frequently used in IVM studies (32/40), while the murine model was mostly used in IVF (17/20) and ICSI (6/8) investigations. Despite considerable heterogeneity, these studies suggest that the use of IVF or maturation results in offspring with higher birthweights and a longer length of gestation, with most of this evidence coming from studies in cattle. These techniques

may also impair glucose and lipid metabolism in male mice. The findings on cardiovascular outcomes and behaviour outcomes were inconsistent across studies.

WIDER IMPLICATIONS: Conception via *in vitro* or *in vivo* means appears to have an influence on measurable outcomes of offspring physiology, manifesting differently across the species studied. Importantly, it can be noted that these measurable differences are noticeable in healthy, fertile animal populations. Thus, common ART interventions may have long-term consequences for those conceived through these techniques, regardless of the pathology underpinning diagnosed infertility. However, due to heterogeneous methods, results and measured outcomes, highlighted in this review, it is difficult to draw firm conclusions. Optimizing animal and human studies that investigate the safety of new reproductive technologies will provide insight into safeguarding the introduction of novel interventions into the clinical setting. Cautiously prescribing the use of ARTs clinically may also be considered to reduce the chance of promoting adverse outcomes in children conceived before long-term safety is confidently documented.

Key words: ICSI / IVM / IVF / ART / assisted reproduction / postnatal outcomes / animal model / systematic review

Introduction

Since its inception, the use of reproductive technologies has resulted in the birth of over 10 million children (ESHRE, 2022). Initially developed to overcome the physical obstacle of blocked fallopian tubes, the application of IVF has now expanded to be used for idiopathic infertility, age-related infertility and male factor infertility as well as fertility preservation. The development of ICSI to further address severe male-factor infertility was introduced clinically in 1992 (Palermo *et al.*, 1992) and widely adopted into practice with little investigation into any potential long-term health effects on offspring. Further, while established for animal application, IVM is increasingly emerging as a technique for clinical use in human infertility, albeit with some variation in protocol compared to animal models where the oocyte is often hormonally 'primed' (treated with exogenous hormones prior to oocyte collection) *in vivo* prior to IVM (Krisher, 2022). Currently, IVF and ICSI are regarded as a set of relatively safe clinical and laboratory procedures that are standardized and available in most countries, with IVM becoming more accessible through practising clinics globally.

The short- and long-term health outcomes of children conceived using IVF and ICSI continue to stimulate research (Painter and Roseboom, 2007; Hart and Norman, 2013; Kamphuis *et al.*, 2014; Pinborg, 2019). This is in part because the Developmental Origins of Health and Diseases (DOHaD) paradigm indicates that there are critical periods of development, including the periconception period where epigenetic reprograming is taking place, that when perturbed, predispose an adult to cardiovascular and metabolic disorders (Wadhwa *et al.*, 2009). A greater body of evidence is now emerging that suggests altered conditions during periconception can not only influence gamete maturation and preimplantation development but also affect foetal and postnatal growth, as well as adult glucose metabolism, fat deposition and vascular function (Feuer and Rinaudo, 2016).

Existing epidemiological studies, although limited, have shown that children born to infertile parents conceived through ART may be associated with a lower birth weight, higher blood pressure and higher fasting glucose when compared to children conceived spontaneously (Ceelen *et al.*, 2008; Hart and Norman, 2013; Berntsen *et al.*, 2019; Cui *et al.*, 2020). Birth defects and other perinatal outcomes are reported to be more common in ART versus non-ART-conceived children (Pandey *et al.*, 2012; Hansen *et al.*, 2013). Although evidence on cognitive development following ART is subject to methodological limitations, it has been shown that ART, especially ICSI, may be a determinant of child developmental outcomes (Rumbold *et al.*, 2017). In addition, evidence has been presented to indicate that the incidence of autism spectrum disorder is increased in children conceived by ART (Liu *et al.*, 2017).

These clinical epidemiological studies on outcomes following ART have a major challenge in differentiating 'the chicken and the egg'; it is difficult to attribute the effects on the outcomes to ART or infertility *per se* (Berntsen *et al.*, 2019). Such a challenge cannot be solved by the clinical 'gold standard' method, a randomized controlled trial, as it is impossible to randomize couples with infertility to the natural conception (NC) group due to biological limitations, or to randomize couples without infertility to the ART group due to ethical considerations. Therefore, animal studies play a unique role in this dilemma and animal models are widely used to investigate the impact of specific ART processes on embryo development and postnatal physiology. ART has been used extensively in a wide range of species, not only for research purposes but also for the management of genetics in commercially important agricultural species, and for wildlife conservation. The major advantage of studying ART in animal models is that, unlike in human clinical studies, animals with presumably normal fertility are used, removing the confounding factor of sub-fertility in the population utilizing ART. Furthermore, animal populations are more genetically homogeneous compared to human sub-populations, which may be of benefit in recognizing the true magnitude of variability in treatment outcomes.

Systematic reviews are commonly performed in clinical reproductive medicine, but less common in animal research despite their value to inform pre-clinical, translational and clinical research and practice (Ritskes-Hoitinga *et al.*, 2014). This systematic review aimed to assess the scientific literature reporting animal studies that measured postnatal physiological outcomes following *in vitro* conception (IVF, IVM or ICSI) compared to *in vivo* fertilization and to provide an overview on the landscape of research in this area.

Methods

This review was registered in Prospero: CRD42020191346

Search strategy

Literature reporting on the impact of the mode of conception on offspring health was searched, only to include animal models. We identified studies by searching the PubMed, Embase and *Commonwealth Agricultural Bureaux* (CAB) Abstracts databases from their inception

until 27 August 2021. We used a broad combination of search terms relating to assisted reproductive technology, mammalian animal models and postnatal outcomes (Supplementary Table SI). Only full-text studies in English were included.

Eligibility criteria

To be eligible, studies had to meet all of the following inclusion criteria: (i) conducted in a mammalian animal model; (ii) included animals conceived using an ART intervention, i.e. *in vitro* conception via IVF or ICSI with or without IVM; (iii) included animals conceived following an control group involving an *in vivo* fertilization process: mating/NC, artificial insemination (AI), or NC or AI followed by embryo transfer with or without *in vitro* culture (*in vivo*-ET); and (iv) reported on at least one outcome of interest.

The schematic diagram on the definitions of the ART interventions and *in vivo* controls is presented in Supplementary Fig. S1. Studies reporting on transgenic animals and studies that quantified purely genomic or proteomic measures from organ and tissue samples were excluded.

Experimental ART protocols

Experimental protocols used in the creation of an *in vitro* embryo combine a multitude of steps that vary greatly from the source of the gametes used, to the composition of the culture media chosen, to the micromanipulations applied by the operator. However, all introduce an exposure to IVF, ICSI or IVM. In Supplementary Fig. S1, a comparative summary of the protocols compared in this review is presented.

Outcomes

The outcomes of interest were birth weight, length of gestation, cardiovascular (primarily blood pressure), metabolic (fasting glucose, fasting insulin, area under the curve of glucose and insulin and other metabolites such as lipids) and behavioural measures, and lifespan.

Study selection, data extraction and risk of bias assessment

Potentially eligible articles were first identified by screening titles and abstracts of the search results, followed by a full-text review in Covidence, with both processes conducted independently by at least two authors (K.H.B., E.K., I.M.v.M., J.G.T. and R.L.R.). Differing assessments were resolved by involving another author (K.H.B. or R.W.).

For eligible studies, the following information was extracted by two authors (I.M.v.M., E.K., J.G.T., B.W.J.M. and R.W.) independently: name of the first author, year of publication, country, species, sex, sample size and details on the interventions, controls and outcomes. For all outcome measures, information on method, unit and age of measurements were all extracted. For continuous variables, means and SDs were extracted. If SEs were reported, they were converted to SDs according to a standard approach (Higgins *et al.*, 2022). If the outcomes were only displayed graphically, they were extracted in an online tool, WebPlotDigitizer (version 4.5) (Rohatgi, 2021).

The risk of bias was assessed using a modified SYRCLE risk of bias tool for animal studies (Hooijmans *et al.*, 2014). The following eight domains were evaluated: baseline characteristics, random housing, blinding (performance bias), random outcome assessment, blinding (detection bias), incomplete outcome data, selective outcome reporting and other sources of bias. We did not assess sequence generation and allocation concealment as randomization may not always be applicable for studies evaluating offspring outcomes.

Two authors (from K.H.B., E.K., T.J.R. and R.W.) independently assessed each included article and discrepancies were solved by consulting another author (K.H.B. or R.W.). The results were graphically displayed using the *robvis* tool (McGuinness and Higgins, 2021).

Data synthesis

To provide an overview on the landscape of research in this area, we plotted an alluvial diagram of included studies by visually linking three categories (year of publication, intervention of interest and species investigated). For continuous outcomes, the summary effects were expressed as mean differences with 95% CIs. Meta-analyses were performed using a random-effects model with the restricted maximum likelihood method. Heterogeneity between studies was quantified using the I^2 statistic. Forest plots were presented for all meta-analyses and different *in vitro* interventions (IVM, IVF and ICSI) were analysed separately when sufficient numbers of studies are available. We explored the source of heterogeneity by stratifying data in subgroup analyses according to different types of controls. We also used a funnel plot to evaluate small-study effects. As *in vivo* fertilization followed by embryo transfer is not practiced in human clinical practice, we performed a sensitivity analysis by limiting the studies to those with an NC or AI control group.

When meta-analyses were impossible due to heterogeneity, we presented forest plots of individual studies without synthesizing the overall estimates. When meta-analyses were impossible due to the missing reporting of variances (SE or SD) in multiple studies, we summarized the means of included studies in Box-and-whisker plots as suggested by the Cochrane handbook (McKenzie and Brennan, 2022). For other circumstances where meta-analyses were impossible, we tabulated the findings of included studies. Results were stratified by the sex of the offspring when possible.

The alluvial diagram was produced in RAWGraphs (Mauri *et al.*, 2017) and data analysed were performed using 'meta' suite in Stata (version 16.1 StataCorpLP, TX, USA).

Results

Search results

The database search returned 5192 articles, of which 61 primary studies met the inclusion criteria after full-text review (Behboodi *et al.*, 1995; Sinclair *et al.*, 1995; Thompson *et al.*, 1995; Holm *et al.*, 1996; Otoi *et al.*, 1996; Kruip and den Daas, 1997; Agca *et al.*, 1998; McEvoy *et al.*, 1998; van Wagtendonk-de Leeuw *et al.*, 1998, 2000; Ptak *et al.*, 1999; Jacobsen *et al.*, 2000a,b, 2002, 2003; Numabe *et al.*, 2000, 2001; Sangild *et al.*, 2000; Yang *et al.*, 2001; Bertolini *et al.*, 2002; Lazzari *et al.*, 2002; Martinez *et al.*, 2002; Ptak *et al.*, 2002; Sakaguchi *et al.*, 2002; Bertolini *et al.*, 2004; Breukelman *et al.*, 2004, 2005; Quaresma *et al.*, 2004; Wolf *et al.*, 2004; Walmsley *et al.*, 2004; Park *et al.*, 2005; Rerat *et al.*, 2005; Givens *et al.*, 2006; Sackett *et al.*, 2006; Hashimoto *et al.*, 2007; Liang *et al.*, 2007; Fernández-Gonzalez *et al.*, 2008; Camargo *et al.*, 2010; Scott *et al.*, 2010; Li *et al.*, 2011;

Kohda *et al.*, 2011; Pimenta-Oliveira *et al.*, 2011; Rexhaj *et al.*, 2013; Wang *et al.*, 2013; Chen *et al.*, 2014a,b; Donjacour *et al.*, 2014; Bonilla *et al.*, 2014; Feuer *et al.*, 2014; Kannampuzha-Francis *et al.*, 2015; Strata *et al.*, 2015; Rexhaj *et al.*, 2015; Cerny *et al.*, 2017; Siqueira *et al.*, 2017; Wang *et al.*, 2017; Valenzuela *et al.*, 2018; Le *et al.*, 2019; Aljahdali *et al.*, 2020; Lewon *et al.*, 2020; Narapareddy *et al.*, 2021; Qin *et al.*, 2021). The PRISMA flow diagram illustrating the process of study screening and selection is shown in Fig. 1.

Study characteristics

The characteristics of the 61 included studies are detailed in Table I and Supplementary Table SII. Briefly, the 61 studies reported on 5 different species (bovine, equine, murine, ovine and non-human primate) published between 1995 and 2021. The research landscape of the animal studies that report on the postnatal outcomes of offspring following the use of ART is presented in Fig. 2. The bovine model was the most frequently used in IVM studies (32/40), while the murine model was mostly chosen for studies that used IVF (17/20) and ICSI (6/8) interventions. There was a shift in the technology studied from IVM in the 1990s to ICSI and IVF from the early 2010s, with the bulk of studies also moving away from bovine and towards murine as a study animal (Fig. 2).

Risk of bias

The complete risk of bias assessment for each included study is presented in Supplementary Fig. S2. While the risk of selection bias was low in 26/61 studies, it was unclear in the remaining studies where there were baseline differences between the ART and the control group including source of the gametes, breed of the animal models and the timing of the intervention. The animals were housed under the same conditions in 37/61 studies resulting in low risk of bias for that domain, although six studies housed their animals in multiple locations resulting in high risk of bias (Sinclair *et al.*, 1995; Kruip and den Daas, 1997; van Wagtendonk-de Leeuw *et al.*, 1998, 2000; Camargo *et al.*, 2010; Valenzuela *et al.*, 2018). The remaining 18 studies did not report housing conditions. Blinding of caregivers and/or investigators was only reported in 1/61 studies (Siqueira *et al.*, 2017). There was low risk of detection bias amongst the studies due to the objective nature of most of the included outcomes (61/61). The risk of reporting bias was low for most studies (60/61), although it was unclear whether all of one study's prespecified outcomes were reported due to the vague nature of its aim (Otoi *et al.*, 1996). For attrition bias, there was low risk in all but twelve studies (50/61); the risk of bias was unclear in eleven studies as it was not clear if all animals were included in the analysis, and it was high in one study which did not report the reason for attrition (Hashimoto *et al.* 2007).

Birthweight

Overall, there were 49 studies that reported birthweight (bovine, n = 32; murine, n = 9; ovine, n = 5; equine, n = 1; primate, n = 2). Forest plots depicting our meta-analysis of birthweight between

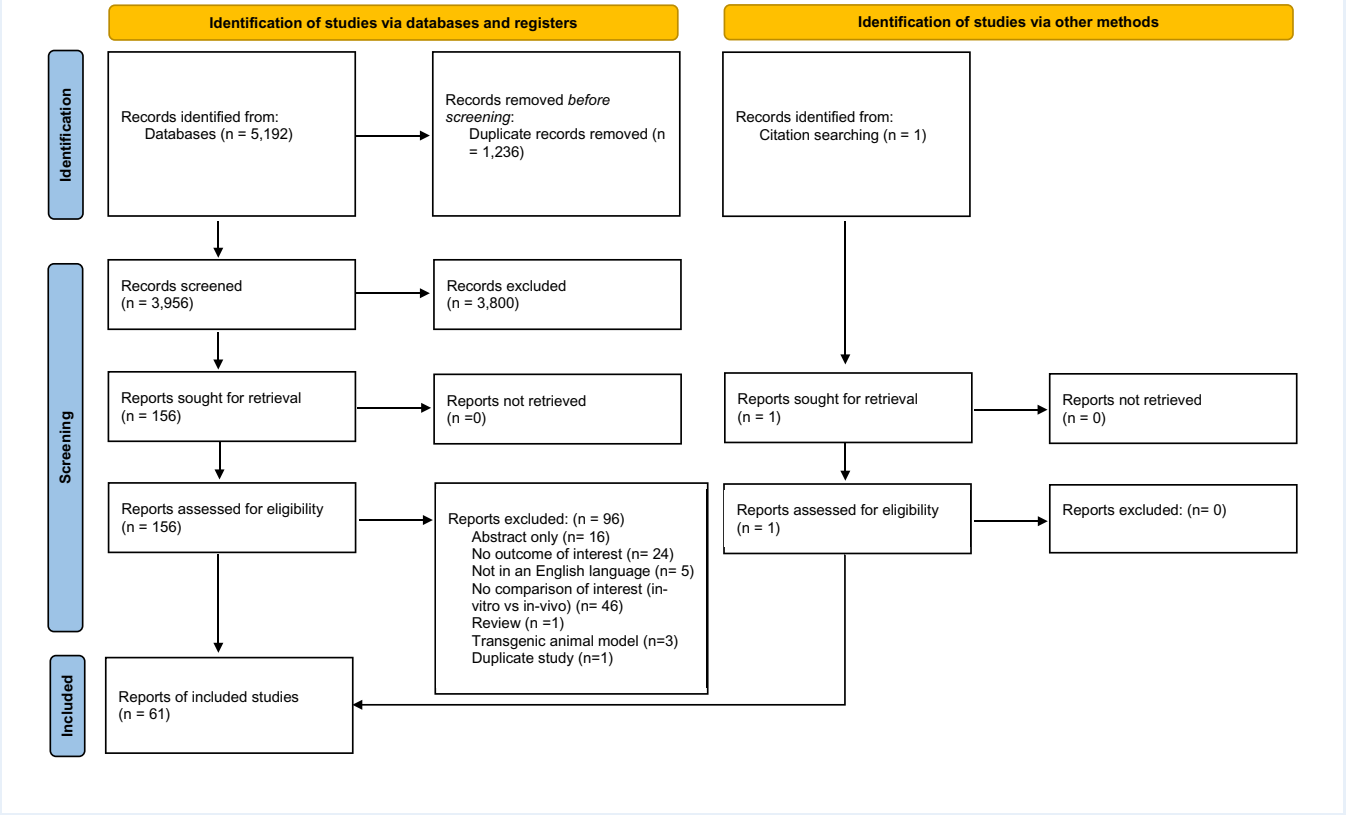

Figure 1. PRISMA flowchart.

Table I Characteristics of included studies.

Study	Country	Species	Intervention	Control
Agca (1998)	USA	Bovine	IVM	AI
Aljahdali (2020)	UK	Murine	IVF	Natural; *in vivo*-ET
Behboodi (1995)	USA	Bovine	IVM	AI
Bertolini (2002)	USA	Bovine	IVM	*In vivo*-ET
Bertolini (2004)	USA	Bovine	IVM	*In vivo*-ET
Bonilla (2014)	USA	Bovine	IVM	AI
Breukelman (2004)	Netherlands	Bovine	IVM	*In vivo*-ET
Breukelman (2005)	Netherlands	Bovine	IVM	*In vivo*-ET
Camargo (2010)	Brazil	Bovine	IVM	AI/natural
Cerny (2017)	Switzerland	Murine	IVF	Natural
Chen (2014a)	Australia	Murine	IVF	*In vivo*-ET
Chen (2014b)	Australia	Murine	IVF	*In vivo*-ET
Donjacour (2014)	USA	Murine	IVF	Natural
Fernández-Gonzalez (2008)	Spain	Murine	ICSI	*In vivo*-ET
Feuer (2014)	USA	Murine	IVF	*In vivo*-ET
Givens (2006)	USA	Bovine	IVM	Natural
Hashimoto (2007)	Japan	Murine	IVF	Natural
Holm (1996)	Australia	Ovine	IVM	*In vivo*-ET
Jacobsen (2000a)	Denmark	Bovine	IVM	AI
Jacobsen (2000b)	Denmark	Bovine	IVM	AI
Jacobsen (2002)	Denmark	Bovine	IVM	AI
Jacobsen (2003)	Denmark	Bovine	IVM	AI
Kannampuzha-Francis (2015)	USA	Bovine	IVM	AI
Kohda (2011)	Japan	Murine	IVF; ICSI	*In vivo*-ET
Kruip (1997)	Netherlands	Bovine	IVM	AI
Lazzari (2002)	Italy	Bovine	IVM	AI; *in vivo*-ET
Le (2019)	China	Murine	IVM; IVF; ICSI	*In vivo*-ET
Lewon (2020)	USA	Murine	ICSI	Natural
Li (2011)	China	Murine	IVF	Natural
Liang (2007)	China	Bovine	IVM	AI
Martinez (2002)	Argentina	Bovine	IVM	*In vivo*-ET
McEvoy (1998)	UK	Bovine	IVM	*In vivo*-ET
Narapareddy (2021)	USA	Murine	IVF	Natural
Numabe (2000)	Japan	Bovine	IVM	*In vivo*-ET
Numabe (2001)	Japan	Bovine	IVM	*In vivo*-ET
Otoi (1996)	Japan	Bovine	IVM	AI
Park (2005)	South Korea	Bovine	IVM	*In vivo*-ET
Pimenta-Oliveira (2011)	Brazil	Bovine	IVM	AI
Ptak (1999)	Italy	Ovine	IVM	*In vivo*-ET
Ptak (2002)	Italy	Ovine	IVM	Natural; AI
Qin (2021)	China	Murine	IVF	Natural
Quaresma (2004)	Portugal	Bovine	IVM	AI
Rerat (2005)	Switzerland	Bovine	IVM	AI
Rexhaj (2013)	Switzerland	Murine	IVF	AI; natural
Rexhaj (2015)	Switzerland	Murine	IVF	Natural
Sackett (2006)	USA	Primate	IVF; ICSI	AI; natural
Sakaguchi (2002)	Japan	Bovine	IVM	AI
Sangild (2000)	UK	Bovine	IVM	AI

(continued)

Table I Continued

Study	Country	Species	Intervention	Control
Scott (2010)	USA	Murine	IVF; ICSI	Natural
Sinclair (1995)	UK	Bovine	IVM	*In vivo*-ET
Siqueira (2017)	USA	Bovine	IVF	AI; *in vivo*-ET
Strata (2015)	USA	Murine	IVF	Natural
Thompson (1995)	New Zealand	Ovine	IVM	Natural
Valenzuela (2018)	USA	Equine	IVM	Natural; *in vivo*-ET
van Wagtendonk-de Leeuw (1998)	Netherlands	Bovine	IVM	AI
van Wagtendonk-de Leeuw (2000)	Netherlands	Bovine	IVM	AI; *in vivo*-ET
Walmsley (2004)	Canada	Ovine	IVM	Natural
Wang (2013)	China	Murine	IVF; ICSI	Natural
Wang (2017)	China	Murine	IVM	Natural
Wolf (2004)	USA	Primate	IVF; ICSI	Natural
Yang (2001)	South Korea	Bovine	IVM	AI; *in vivo*-ET

AI, artificial insemination; ET, embryo transfer.

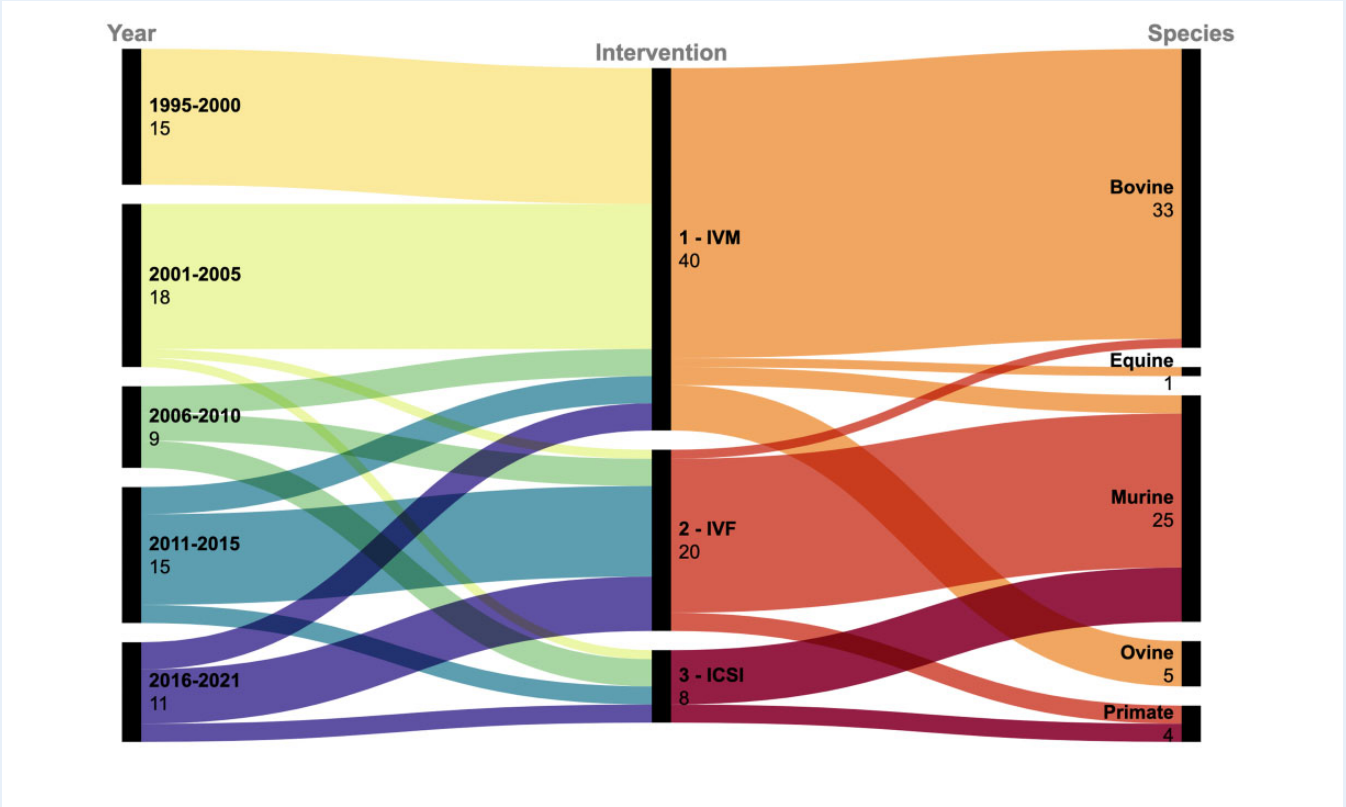

Figure 2. Alluvial diagram of included studies on the research landscape. The alluvial diagram illustrates animal research on ARTs and postnatal outcomes by visually linking three categories (year of publication, intervention of interest and species investigated). The height of the black rectangle is proportional to the number of studies in each subgroup under each category and the width of curved coloured lines are proportional to the number of studies linking two categories. Note that studies on two or more interventions are considered as separate studies in the diagram and therefore the total number here is 68 instead of 61 (five studies included two interventions and one study included three interventions).

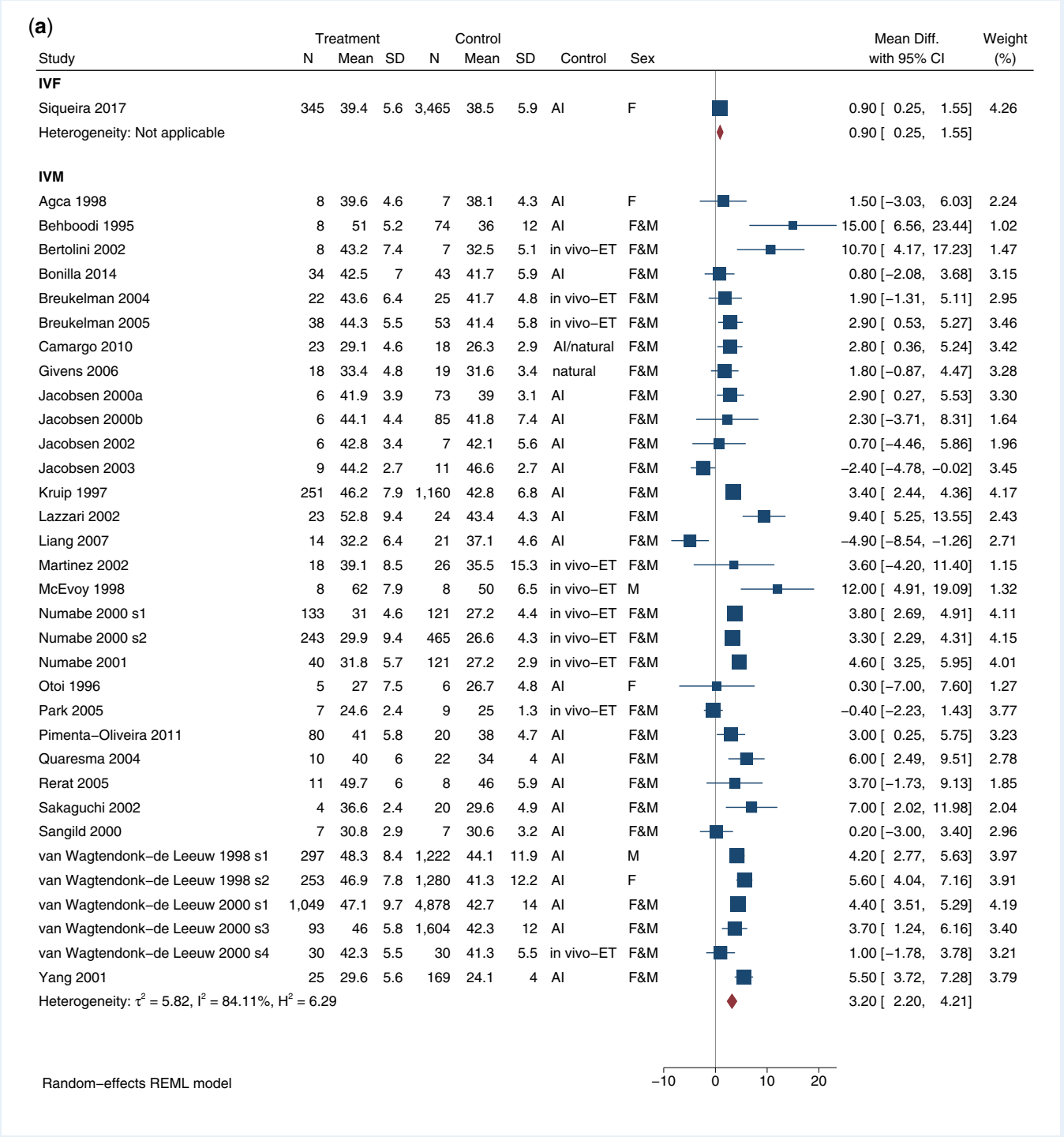

Figure 3. Forest plots for birth weight. (**a**) Bovine studies. (**b**) Murine studies. (**c**) Primate studies. Continued

different species are presented in Fig. 3a–c and subgroup analyses based on the different types of controls are presented in Supplementary Fig. S3. A funnel plot for small study effects is presented in Supplementary Fig. S4.

The meta-analysis of birthweight in bovine models included 30 studies and showed that birthweights in the IVF and IVM group were both higher compared to *in vivo* controls (IVF: 1 study, 3810 cattle, mean difference (MD) 0.9 kg, 95% CI 0.25 to 1.55; IVM: 29 studies, 18240 cattle, MD 3.2 kg, 95% CI 2.21 to 4.21, $I^2 = 84\%$; Fig. 3a). The heterogeneity between studies was high but the direction of effect was consistent across studies as shown in Fig. 3a. Subgroup analysis based on types of controls did not reveal differences between

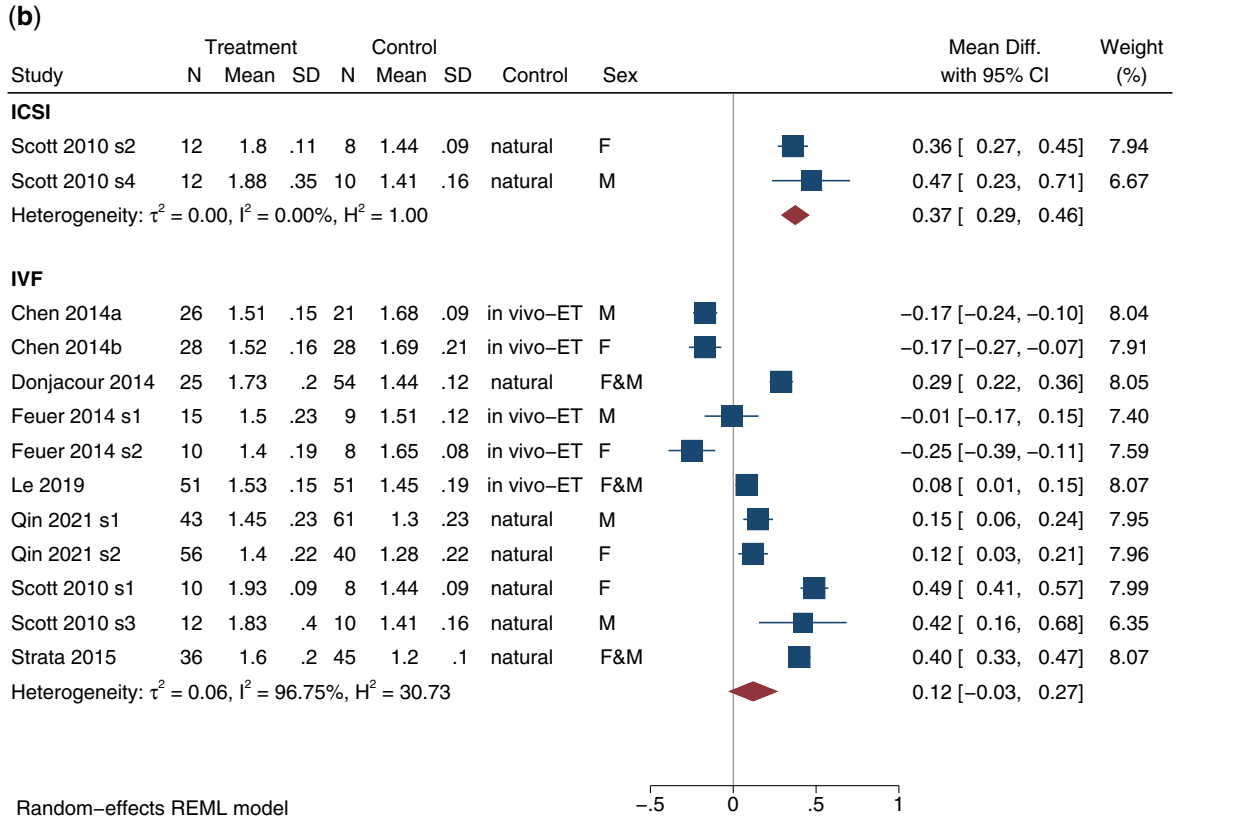

Figure 3. Continued.

subgroups (Supplementary Fig. S3a). The funnel plot was symmetrical overall, indicating no evidence of small-study effects (*P* for Egger's test = 0.16, Supplementary Fig. S4a). Sensitivity analysis limiting to NC or AI controls showed similar findings as the main analysis (IVF: 1 study, 3810 cattle, MD 0.9 kg, 95% CI 0.25 to 1.55; IVM: 21 studies, 13018 cattle, MD 3.1 kg, 95% CI 1.8 to 4.4,

$I^2 = 84\%$). Two bovine studies (Sinclair *et al.*, 1995; Kannampuzha-Francis *et al.*, 2015) were not included in our meta-analyses as detailed data were not extractable. They both reported a non-significant difference in birthweight.

Nine studies were included in the meta-analysis of birthweight in murine models. Compared to the control group, ICSI was associated with a small increase in birth weight (1 study, 42 mice, MD 0.37 g, 95% CI 0.29 to 0.46), while the evidence on the difference between IVF and the control group was inconclusive (8 studies, 689 mice, MD 0.12 g, 95% CI −0.03 to 0.27, $I^2 = 96\%$; Fig. 3b). Subgroup analysis showed that IVF was associated with a small or no decrease in birth weight compared to the *in vivo* group (MD −0.10 g, 95% CI −0.22 to 0.02, $I^2 = 87\%$), while IVF was associated with an increase in birth weight compared to the natural group (MD 0.30 g, 95% CI 0.18 to 0.43, $I^2 = 91\%$; P for interaction < 0.001; Supplementary Fig. S3b). Although the heterogeneity between studies in the natural subgroup was still high, the direction of effect was consistent across studies. Therefore, the overall high heterogeneity could be partly explained by the choice of controls. When limiting to the NC controls in a sensitivity analysis, both ICSI and IVF were associated with a small increase in birth weight (ICSI: 1 study, 42 mice, MD 0.37 g, 95% CI 0.29 to 0.46; IVF: 4 studies, 400 mice, MD 0.30 g, 95% CI −0.18 to 0.43, $I^2 = 91\%$). In addition, one study (Wang *et al.* 2017), which was not included in meta-analyses because data on variance were not extractable, reported comparable birthweights between IVM and control (natural and *in vivo*-ET).

Two studies reported birth weight in primate models (Wolf *et al.*, 2004; Sackett *et al.*, 2006). Meta-analyses did not show significant differences for ICSI or IVF compared to the control group (2 studies, 109 primates, MD −10.27 g, 95% CI −43.61 to 23.08, $I^2 = 0$; Fig. 3c). Subgroup analysis based on types of controls did not reveal significant differences between subgroups (Supplementary Fig. S3c). As all studies had a natural or AI control group, no sensitivity analysis was performed.

Five studies reported birth weight using an ovine model (Thompson *et al.*, 1995; Holm *et al.*, 1996; Ptak *et al.*, 1999, 2002; Walmsley *et al.*, 2004), and one in an equine model (Valenzuela *et al.*, 2018), with all studies comparing IVM and a control group. Meta-analyses were not performed due to limited extractable data. Four ovine studies reported no significant differences in the birthweight of lambs produced after the use of ART (Thompson *et al.*, 1995; Holm *et al.*, 1996; Ptak *et al.*, 1999, 2002) and one study reported more 'abnormally large lambs' (defined as birth weight more than 99th percentile) in the IVM group, compared to the natural conceived control (Walmsley *et al.*, 2004). No significant differences in birth weight were noted by Valenzuela *et al.* in horses (Valenzuela *et al.*, 2018).

Length of gestation

Overall, 32 studies reported gestational length as an outcome (bovine, n = 27; murine, n = 1; ovine, n = 1; equine, n = 1; primate, n = 2). A forest plot depicting the meta-analysis is presented in Supplementary Fig. S5 and subgroup analyses based on different types of controls are presented in Supplementary Fig. S6. A funnel plot for small study effects is presented in Supplementary Fig. S4b.

There were 27 studies included in a meta-analysis of bovine data (Supplementary Fig. S5). Overall, our meta-analysis showed no difference in gestational length in the IVF group versus controls (1 study, 3810 cattle, MD 0.20 days, 95% CI −0.47 to 0.84) and a longer gestational length in the IVM group compared to controls (26 studies, 18876 bovine, MD 2.17 days, 95% CI 0.87 to 3.46, $I^2 = 87\%$; Supplementary Fig. S5). Subgroup analysis showed that IVM was associated with an increase in gestational length compared to the AI group (MD 2.71 days, 95% CI 1.27 to 4.16, $I^2 = 83\%$) but was inconclusive about IVM compared to the *in vivo*-ET group (MD 2.31 days, 95% CI −0.03 to 4.66, $I^2 = 93\%$) or the NC group (MD −2.00 days, 95% CI −5.09 to 1.09; Supplementary Fig. S6b). Heterogeneity within subgroups remained high but the direction of effect was consistent in the IVM versus AI subgroup. Therefore, the overall high heterogeneity could be partly explained by the choice of controls. Sensitivity analysis limiting to NC or AI controls showed similar findings as the main analysis (IVF: 1 study, 3810 cattle, MD 0.20 days, 95% CI −0.47 to 0.84; IVM: 19 studies, 13669 bovine, MD 2.33 days, 95% CI 0.93 to 3.72, $I^2 = 84\%$). The funnel plot was symmetrical overall, indicating no evidence of small-study effects (P for Egger's test = 0.95, Supplementary Fig. S4b).

Two studies reported length of gestation in a primate model (Wolf *et al.*, 2004; Sackett *et al.*, 2006), one used an equine model (Valenzuela *et al.*, 2018), one in a murine model (Le *et al.*, 2019) and another used an ovine model (Holm *et al.*, 1996). Meta-analysis of studies in the primate model showed that both IVF and ICSI were associated with a slightly shorter gestational length compared to the AI group (MD −4.28 days, 95% CI −5.63 to −2.93; MD −3.00 days, 95% CI −5.15 to −0.85) but the differences were not observed when compared to the NC group (Supplementary Fig. S6b). There was no difference observed between the length of gestation in equine, murine or ovine studies following the use of an ART intervention.

Blood pressure

There were seven included studies that used a mouse model to measure blood pressure as an indicator of cardiovascular health in animals conceived following IVF (including ICSI) and culture and a control group (Table II). Four studies reported the systolic/diastolic and mean blood pressure of offspring at a fixed time point between 9 and 52 weeks of age (Fernández-Gonzalez *et al.*, 2008; Rexhaj *et al.*, 2013; Donjacour *et al.*, 2014), with varied results, as shown in Table II. Two studies recorded arterial blood pressure in 12–14-week-old male mice both at a fixed time point and continuously over a 48-h period using telemetry, and the mean fixed and continuous arterial pressures were significantly higher in mice conceived by IVF (Rexhaj *et al.*, 2013, 2015). One study reported blood pressure in 1.5-year-old mice, with an increased systolic blood pressure in female mice conceived through IVM (Le *et al.*, 2019), but not in male mice or those conceived through IVF or ICSI.

Metabolic outcomes

The outcomes of glucose and lipid metabolism reported in included studies are summarized in Supplementary Table SIII. Glucose metabolism was reported in 16 studies (bovine n = 3; murine, n = 13) and lipid metabolism was reported in four studies using mouse models.

Table II Studies reporting blood pressure in mouse models.

Study	Intervention	Age	Method of measurement	Outcome (blood pressure)	Findings
Aljahdali (2020)	IVF	9/15/21 weeks	Tail-cuff method	Systolic	↑
Donjacour (2014)	IVF	38 weeks	Tail-cuff method	Systolic	↓
				Diastolic	→
				Mean	→
Fernández-Gonzalez (2008)	ICSI	1 year	Tail-cuff method	Systolic	→
Le (2019)	IVF; ICSI; IVM	1.5 years	Tail-cuff method	Systolic	↑ (IVM, female) → (ICSI; IVF)
				Diastolic	→
				Mean	→
Narapareddy (2021)	IVF	39 weeks	Tail-cuff method	Systolic	→
				Diastolic	→
				Mean	→
Rexhaj (2013)	IVF	12–14 weeks	Telemetry	Mean	↑
Rexhaj (2015)	IVF	12–14 weeks	Telemetry	Mean	↑

All studies used mouse models. ↑ Intervention higher than control; ↓ Intervention lower than control; → no significant differences between groups.

Of the three bovine studies, two measured serum glucose levels within 24 h after birth (Sangild *et al.*, 2000; Bertolini *et al.*, 2002) and one measured glucose and insulin levels on Day 2 after birth (Jacobsen *et al.*, 2000a), all comparing IVM and a control group (Supplementary Table SIII). None of the studies reported significant differences on these outcomes between groups. Among 13 studies reporting outcomes on glucose metabolism in a mouse model, all 13 reported serum fasting glucose levels, 8 reported fasting insulin levels, 11 reported glucose area under the curve (AUC) during a glucose tolerance test and 5 reported insulin AUC. Most studies reporting fasting glucose and insulin did not report information on variance (SD or SE), therefore meta-analysis could not be performed. Instead, a box-and-whisker plot was used to visualize the distribution of effect estimates across studies (Fig. 4a and b). The mean difference on fasting glucose levels between the ART and the control groups varied from −0.7 to 2.4 mmol/l (10 studies, median 0.4) in females and from −0.6 to 2.2 mmol/l (10 studies, median 0.8) in males, with more studies in males showing a higher fasting glucose level in the ART group (Fig. 4a). The mean difference on fasting insulin levels between the ART and the control groups varied from −0.35 to 0.2 ng/ml (median 0.01) in females and from −0.92 to 0.15 ng/ml/l (median −0.04) in males, with more studies in males showing a lower fasting insulin level in the ART group (Fig. 4b).

For glucose and insulin AUC, meta-analyses were not performed due to the variation of age at which the outcomes were measured (8–78 weeks; Supplementary Table SIII). Instead, forest plots without summary estimates were used to visualize the difference in glucose AUC and insulin AUC between groups (Fig. 5a and b).

Eight studies (13 comparisons) reported glucose AUC in female mice, and 8 studies (10 comparisons) reported this outcome in male mice (Fig. 5a). The mean difference for glucose AUC ranged from −200 to 630 mmol/l*2 h in studies on females (95% CI of individual studies: −381 to 856) and from −353 to 930 mmol/l*2 h in studies on males (95% CI of individual studies: −912 to 1067). More studies in males showed a higher glucose AUC in the ART group than those in females. Three studies reported data on the insulin AUC following an insulin tolerance test in female mice, and four did the same using male mice (Fig. 5b). The mean difference for insulin AUC ranged from −2.96 to−0.41 ng/ml*2 h in studies on females (95% CI of individual studies: −4.27 to 0.49) and from −2.28 to 3.24 ng/ml in studies on males (95% CI of individual studies: −3.44 to 4.49). Studies in female mice showed a lower insulin AUC in the ART group, while studies in male mice showed inconsistent findings.

Four studies reported serum lipid metabolites in mice. Three reported cholesterol and triglycerides levels (Le *et al.*, 2019; Narapareddy *et al.*, 2021; Qin *et al.*, 2021). Meta-analysis was performed in two studies with outcomes measured at 12 and 20 weeks. It showed that IVF did not increase cholesterol levels (MD 0.08, 95% CI −0.24 to 0.41, $I^2 = 60\%$) compared to naturally conceived group and subgroup analysis based on sex showed that the difference mainly occurred in females (males: MD −0.07, 95% CI −0.36 to 0.22, I2 = 0; females: MD 0.30, 95% CI 0.10 to 0.50; *P* for interaction 0.04; Supplementary Fig. S7a). There was insufficient evidence between IVF and naturally conceived group on triglycerides levels (MD 19.21, 95% CI −0.90 to 39.32, $I^2 = 66\%$), and subgroup analysis based on sex showed that the difference mainly occurred in males (males: MD 28.60, 95% CI 15.16 to 42.04, $I^2 = 0$; females: MD −2.30, 95% CI −24.30 to 19.70; *P* for interaction 0.02; Supplementary Fig. S7b). The other study reported data on fatty acid composition in the liver and adipose tissue and found that these outcomes were also altered in male IVF/ICSI mice (Wang *et al.*, 2013).

Behaviour outcomes

Nine studies assessed behavioural characteristics in animals conceived using ART (bovine, n = 2; murine, n = 6; primate, n = 1) (van Wagtendonk-de Leeuw *et al.*, 1998; Bertolini *et al.*, 2002; Sackett *et al.*, 2006; Fernández-Gonzalez *et al.*, 2008; Kohda *et al.*, 2011; Li *et al.*, 2011; Strata *et al.*, 2015; Wang *et al.*, 2017; Lewon *et al.*, 2020).

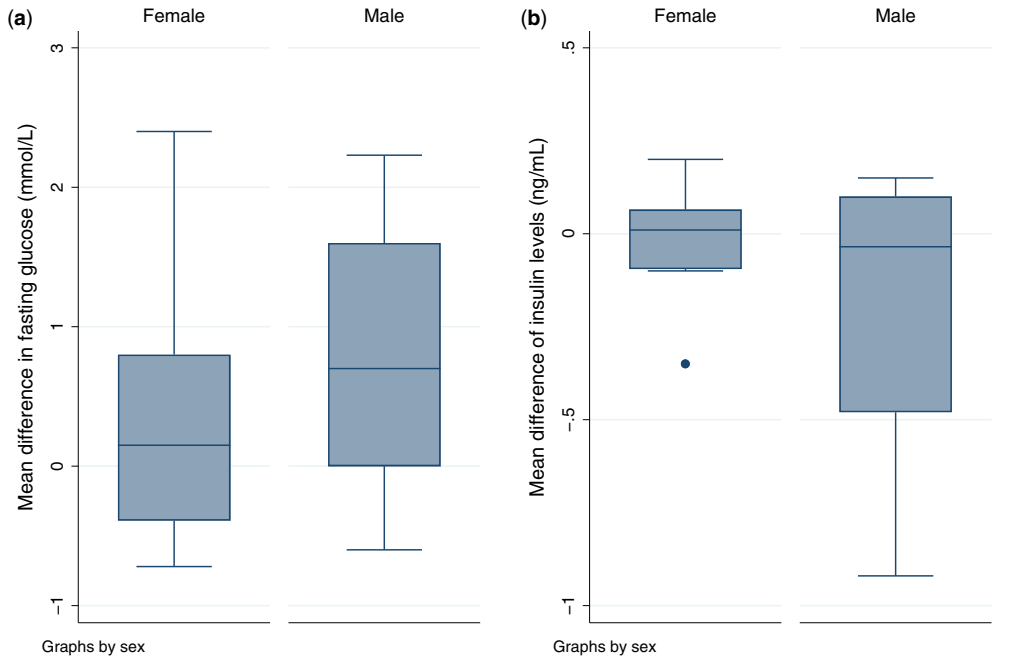

Figure 4. Box-and-whisker plots for fasting glucose and insulin in mice studies. (**a**) Box-and-whisker plot for fasting glucose levels (mean difference between the intervention and the control, mmol/l). The upper and lower limits of the box, represent the 75th and 25th percentiles, respectively. The line within the box represents the 50th percentile (median), and the whiskers represent the ranges. (**b**) Box-and-whisker plot for fasting insulin levels (mean difference between the intervention and the control, ng/ml).

There was no difference in the overall cognitive development as observed in rhesus macaque neonates and infants after conception by ICSI, IVF, AI, or NC (Sackett *et al.*, 2006). No difference in spatial learning memory ability between IVF and naturally conceived mice was found at 6–7 weeks of age (Li *et al.*, 2011). Yet, in a complete behavioural analysis of mice, female mice born via *in vivo* conception performed better during acquisition learning than ICSI females, a difference not observed in males, and did not demonstrate the same deficit in implicit memory as ICSI-conceived individuals (Fernández-Gonzalez *et al.*, 2008). In a switching discrimination task and delayed non-matching-to-position memory task, *in vivo* derived males performed better at learning and memory compared to their ICSI produced counterparts, and this was not observed in females (Lewon *et al.*, 2020). While Stata *et al.* (Strata *et al.*, 2015) found that there was no difference in anxiety-like behaviour in mice at ages 10–28 weeks, when additional prenatal and postnatal metabolic stress was added through diet, IVF mice show less anxiety-like behaviour. No difference in newborn behavioural traits such as standing time, suckling time and respiratory distress was observed in IVM compared to *in vivo*-conceived calves (Bertolini *et al.*, 2002). However, the need of a breathing stimulus at birth was significantly increased for calves produced after the use of IVM compared to *in vivo*-conceived animals ($P < 0.05$), especially after a Caesarean delivery. Importantly, this effect only occurred in calves conceived by IVM and using a co-culture cell embryo culture system and not in calves conceived by IVM without this culture system (van Wagtendonk-de Leeuw *et al.*, 2000).

Lifespan

The lifespan of mice conceived after IVF was shorter compared to those conceived naturally, but the difference only found to be statistically significant when they were fed a high fat diet (Rexhaj *et al.*, 2013). Under these conditions, median survival was 582 days (n = 42) vs 787 days (n = 53) in IVF and *in vivo*-conceived mice, respectively.

Discussion

Summary of key findings

This systematic review and meta-analysis were conducted to identify whether postnatal outcomes in offspring born following *in vitro* conception differed from that of offspring born after *in vivo* conception, as evidenced by animal studies. We identified 61 primary studies that met the inclusion criteria of our search, regarding five species (bovine, murine, ovine, equine and non-human primate). Despite considerable heterogeneity, combined analysis of these studies suggests that the use of ART results in offspring with higher birthweights, and a longer length of gestation, with most of this evidence coming from studies in cattle. Reports on blood pressure were inconsistent, but two of the five studies that measured mean arterial blood pressure demonstrated an increase in Blood pressure (BP) following the use of *in vitro* conception in mice. Studies that measured glucose and insulin sensitivity lacked standardization in both the timing of measurement (age) and the

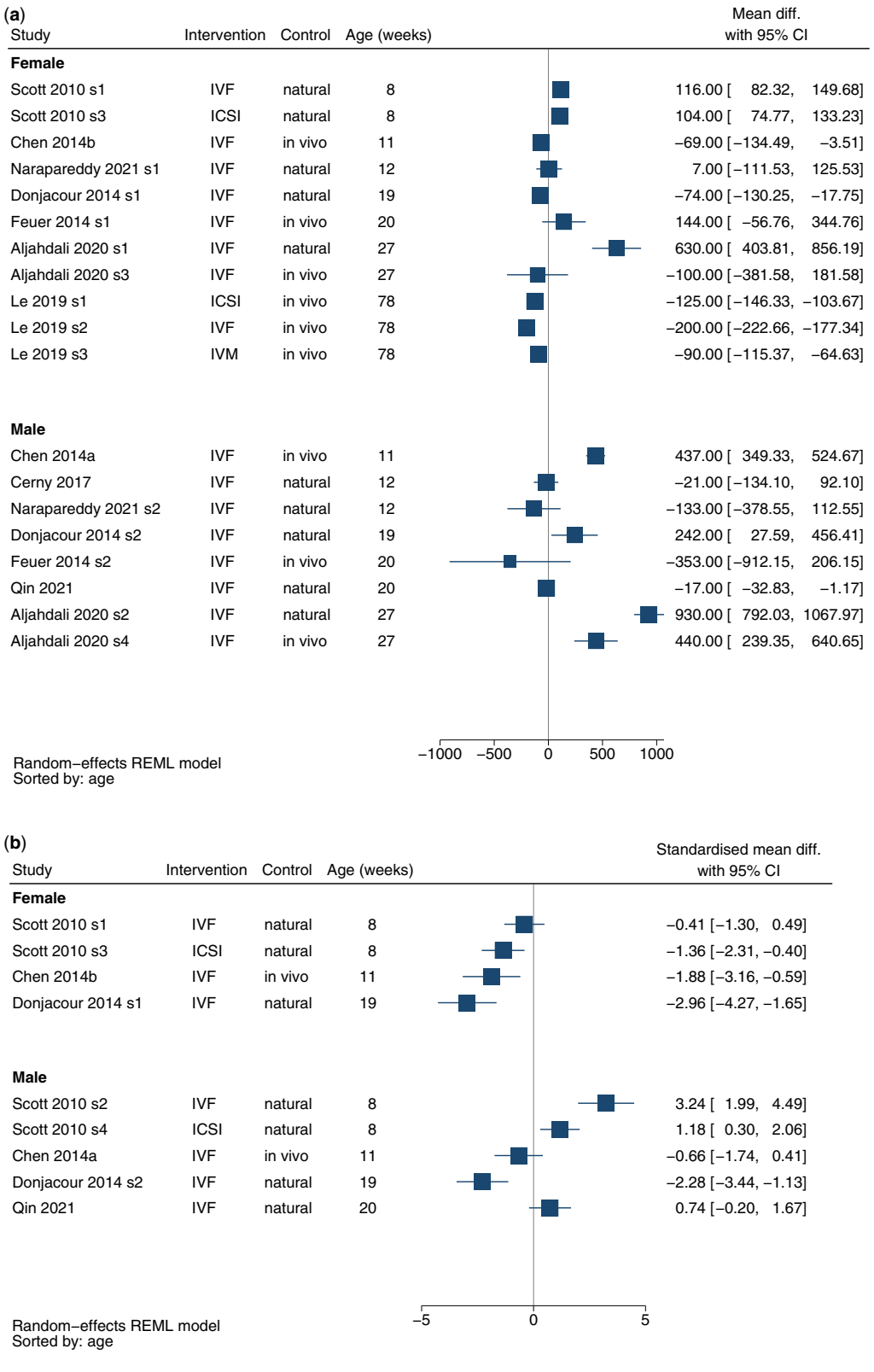

Figure 5. Forest plots for metabolic outcomes in mice studies. (**a**) Glucose area under the curve during glucose tolerance test (mmol/l*2 h). (**b**) Insulin area under the curve during insulin tolerance test (ng/ml*2 h).

methods used, yet indicate sex-specific differences in systemic metabolism after the use of an ART intervention. Serum lipids were also reported to be altered in animals conceived *in vitro*, again in a sex-dependant manner. Aspects of animal behaviour were found to be affected in offspring produced using ART interventions, more specifically ICSI, yet these observations have yet to be replicated between test methods or species. Some of the behavioural characteristics observed also appear to be sex specific. Only one study considered the effect of ART on lifespan, demonstrating this to be shorter for mice conceived after the use of IVF when fed a high-fat diet, compared to spontaneously conceived mice on a high-fat diet.

Interpretation

Assisted reproduction comprises of numerous parallel and in series variations in protocols for clinical and animal systems. ART interventions in our review were limited to IVF, IVM and ICSI and did not include *in vivo* produced embryos followed by embryo transfer with or without *in vitro* culture. This choice was made to mimic technologies commonly used in human clinical practice. Although from a technological perspective, *in vivo* embryos recovered by uterine lavage is completely possible in humans and its use in preimplantation genetic testing - aneuploidy (PGT-A) has been recently reported in a research context (Munné *et al.*, 2020), the underlying ethical challenges are enormously controversial (Lambalk *et al.*, 2020; Oron, 2020; Pennings, 2020).

To make broad comparisons on impact we can at best take these together as a sum of interactions. As shown in both human and animal studies, there appears to be an impact, at least under some experimental designs and conditions, of ART on offspring produced. Our review demonstrates that this impact is taking place regardless of the study cohort/population and can be attributed to the intervention itself. Yet caution must be taken in extrapolating specific results, and here we quite importantly address some of the nuances between clinical and animal studies, areas of caution regarding interpretation, and explanatory mechanisms that may help in understanding the observations made.

The ART used in animal models is overall quite different from clinical approaches in humans. For example, donors and recipients are not the same animal, while in humans, the woman usually plays both roles. Differences in embryo collection methodologies vary greatly for mice, cattle and human populations, as do the hormonal stimulation protocols and culture media additives used, such as growth factors like Epidermal growth factor (EGF) that routinely feature in animal culture systems but are largely unapproved for use in human clinical settings. Similarly, IVM protocols differ between animal and human methodologies where the use of hormonal priming may be used in the case of the latter, making the data hard to extrapolate. The age of animals used should also be highlighted, especially in mouse models where pre-pubertal animals are often used at the time of ovarian stimulation to generate *in vivo* matured oocytes or *in vivo*-derived embryos. In contrast, the use of assisted reproduction in human populations is largely concentrated on older individuals. In mouse studies it is important to control for litter size, as this influences birth weight. Most studies controlled for litter size by matching the number of embryos transferred to pseudopregnant recipients, some considering the *in vivo*-conceived control group with embryo transfer as more suitable than a naturally conceived group, as the former takes superovulation, litter size and embryo transfer procedure into account. One study removed an oviduct from mice in the control group before mating with a male (Scott *et al.*, 2010), but still litter size ranged from 2 to 10 in naturally conceived mice compared with IVF or ICSI-conceived mice that had litter sizes ranging from 1 to 4. In cattle and sheep studies, many of those reported here used serum or co-culture during *in vitro* culture which itself has been associated with alterations in birth weight (Walker *et al.*, 1992; van Wagtendonk-de Leeuw *et al.*, 1998; Hasler, 2000). Yet despite these differences, animal models allow for a focus on specific mechanisms, including epigenetic regulation, to be more closely investigated, and for outcomes with broad variability, such as behaviour to be more easily documented.

Our meta-analysis supports the observation of higher calf birthweight and longer length of gestation when embryos are *in vitro* produced. This phenomenon is in part attributed to the co-culture of these embryos with epithelial cells or serum as a protein source in media, as indicated in a recent review (Duranthon and Chavatte-Palmer, 2018). This practice, while increasing the *in vitro* embryonic yield, alters embryo quality resulting in overgrowth syndrome, as recently reviewed in bovine and ovine studies (Li *et al.*, 2019). A protein source provides important support to *in vitro* culture systems as they prevent the sticking of cells to glass and plastic surfaces which cause mechanical damage, yet even well-defined protein sources such as Bovine serum albumin (BSA) have been shown to influence birthweight (Lazzari *et al.* 2002), demonstrating a complex scenario regarding balancing all physiological needs. To that point, the issue of difference in oxygen concentration between protocols (5% or 20%) need also be mentioned as an important and common variable, although one not focused on in this review.

Altered fetoplacental development in cattle that are *in vitro* conceived has been linked to placental compensation from impaired vascular development in the early stages of pregnancy, and not directly attributed to length of gestation (Miles *et al.*, 2005). The mechanisms of this observation still require elucidation, however, disruption to the epigenetic processes that occur during embryogenesis appear to contribute to impaired trophectoderm differentiation and placentation, leading to disease-related outcomes. This is not just the case as seen in animal studies, but also using human data sets that consider epigenetic changes in candidate genes and genome-wide epigenetic changes in cord blood/placenta and early pregnancy tissues (Mani *et al.*, 2020). Here, the authors examined the link between commonly used ART interventions, changes in DNA methylation and the development of short- and long-term adverse outcomes, and much like the current study concluded that inconsistencies between experimental design and endpoints require further investigation to delineate causal associations.

Foetal growth and birthweight are also changed in murine offspring following the use of ART interventions (Vrooman and Bartolomei, 2017), but not with the same consistent trend as seen in cattle, as supported by our analysis. Rather, there appears to be an intersectional effect of culture system, most commonly in association with culture media composition, but also inbred or outbred animal strains and/or stage at embryo transfer, and this has also been seen to have a sex-specific effect (Bloise *et al.*, 2014; Donjacour *et al.*, 2014; Feuer *et al.*, 2014; Ozil *et al.*, 2017). This acts as a reminder regarding the complexity of the preimplantation environment, including any ARTs

used, and the impact of epigenetic modifications during the sensitive pre- and post-implantation process.

Few studies in cattle measured glucose metabolism (n = 3) and none found differences between control and treatment groups. A more extensive body of literature exists for glucose metabolism in mice (n = 15), however, the studies varied in outcome, with a sex-specific effect inconsistently identified. Most notably, male mice produced using ART tended to have higher fasting glucose and lower fasting insulin, and a higher glucose AUC than control animals. Also observed more notably in male offspring was an increase in triglyceride level in ART mice compared to a naturally conceived control group, as shown by meta-analysis in the current study. To identify the molecular mechanisms driving these observed differences, a recent study found 21 genes were differentially regulated in *in vitro* produced mouse embryos, along with a noticeably altered epigenetic signature when compared to their *in vivo*-conceived counterparts. Yet the analysis of male and female embryos separately found minimal differences in epigenetic signature and no gene expression changes (Ruggeri *et al.*, 2020). Given the sexually dimorphic nature of disease prevalence (Ober *et al.*, 2008), the genetic mechanisms that are altered via the use of *in vitro* technologies may manifest differently in male and female phenotypes and may be observable at different times across an animal's lifespan. While the triglyceride levels measured in *in vitro* and *in vivo*-conceived mice differ, the DNA methylation signature in the liver of adult mouse offspring also changes, resulting in hypomethylation of genes involved in metabolism and gene transcription regulation in mice produced via IVF, but no difference between male and female animals (Lira-Albarran *et al.*, 2022). Finally, evidence of the use of media additives, such as melatonin, have been shown to reduce the impact of ART on the glucose metabolism of embryos, demonstrating once again the complex impact of the *in vitro* culture environment (Jia *et al.*, 2022).

Strengths and limitations

The strengths of our systematic review include comprehensive literature search, rigorous data extraction (including data extraction from figures) and a variety of data visualization approaches. There are also some limitations. First, we excluded abstract-only publications due to limited extractable information and five non-English publications. Second, highlighted by the SYRCLE tool used, the use of investigator blinding was rarely mentioned and differences in baseline characteristics were only reported in approximately half of the included studies. These might have contributed to performance and selection bias. Third, studies that compared *in vivo* fertilization (NC or AI) followed by *in vitro* culture without IVF versus NC or AI were not included, even if referred to as ART in an animal context. The included interventions in our review (IVF, ICSI and IVF) reflect the use of technologies in humans, where offspring created are done so in combination of IVF and culture. Finally, the use of *in vivo* controls was not commonly reported in studies that observed offspring outcomes following cryopreservation and as such this was not a focus of our review.

Conclusions

There are noticeable differences in some physiological measures made on offspring born after the use of ART in healthy animal populations. ART use in human populations continues to increase in cycle numbers each year, globally. Improved efficacy and access of treatment has no doubt played a role in its increased application, and healthy children continue to be born as a result. However, given a growing body of evidence, in animal and human studies that offspring born following the use of ART can display differences in physiology compared to those who are spontaneously conceived, circumspect application and further scientific enquiry are required. Greater consideration of the methods and controls used to specifically investigate aspects of *in vitro* culture and fertilization are needed, especially to aid clearer decision-making regarding the clinical application of these technologies.

Supplementary data

Supplementary data are available at *Human Reproduction Update* online.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Authors' roles

B.W.J.M. and J.G.T. conceived the study. K.H.B., E.K., T.J.R., I.M.v.M., J.G.T., R.J.N., R.L.R., B.W.J.M. and R.W. contributed to the study design and protocol development. I.M.v.M. and E.K. performed the search. I.M.v.M., E.K., B.W.J.M., J.G.T. and R.W. extracted the data. I.M.v.M. and R.W. performed the analysis. E.K., T.J.R., R.W. and K.H.B. performed the quality assessment. I.M.v.M., J.G.T., B.W.J.M., K.H.B. and R.W. drafted the manuscript. K.H.B., E.K., T.J.R., I.M.v.M., J.G.T., R.J.N., R.L.R., B.W.J.M. and R.W. interpreted the data, critically revised the article, and approved the final version.

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Conflict of interest

B.W.J.M. reports consultancy for ObsEva, Merck KGaA, Guerbet, iGeonomix & Merck and travel support from Merck. The other authors declare no conflicts of interest.

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