The duration of embryo culture after mouse IVF differentially affects cardiovascular and metabolic health in male offspring

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Running title: Mouse embryo culture duration and long-term health

Abstract

STUDY QUESTION: Do the long-term health outcomes following IVF differ dependent upon the duration of embryo culture before transfer?

SUMMARY ANSWER: Using a mouse model, we demonstrate that in male but not female offspring adverse cardiovascular health was more likely with prolonged culture to the blastocyst stage, but metabolic dysfunction was more likely if embryo transfer (ET) occurred in early cleavage.

WHAT IS KNOWN ALREADY: Assisted reproductive treatments (ART) associate with increased risk of adverse cardiovascular and metabolic health in children, findings confirmed in animal models in the absence of parental infertility issues. It is unclear which specific ART treatments may cause these risks. There is increasing use of blastocyst versus cleavage-stage transfer in clinical ART which does not appear to impair perinatal health of children born, but the longer-term health implications are unknown.

STUDY DESIGN, SIZE, DURATION: Five mouse groups were generated comprising: (a) NM - naturally mated, non-superovulated and undisturbed gestation; (b) IV-ET-2Cell - in vivo derived 2-cell embryos collected from superovulated mothers, with immediate ET to recipients; (c) IVF-ET-2Cell – IVF generated embryos with oocytes from superovulated mothers cultured to 2-cell stage before ET to recipients; (d) IV-ET-BL – in vivo derived blastocysts collected from superovulated mothers, with immediate ET to recipients; (e) IVF-ET-BL - IVF generated embryos with oocytes from superovulated mothers cultured to blastocyst stage before ET to recipients. Both male and female offspring were analysed for growth, cardiovascular and metabolic

markers of health. 8-13 litters were generated for each group for analyses; postnatal data were analysed by multilevel random effects regression to take account of between-mother and within-mother variation and litter size.

PARTICIPANTS/MATERIALS, SETTINGS, METHODS: C57/BL6 female mice (3-4 weeks old) were used for oocyte production; CBA males for sperm with HTF medium used for IVF. Embryos were transferred (ET) to MF1 pseudo-pregnant recipients at the 2-cell stage or cultured in KSOM medium to blastocyst stage before ET. Control in vivo embryos from C57BL6 x CBA matings were collected and immediately ET at 2-cell or blastocyst stages. Postnatal assays included growth rate up to 27 weeks; systolic blood pressure (SBP) at 9, 15 and 21 weeks; lung and serum angiotensin converting enzyme (ACE) activity at time of cull (27 weeks); glucose tolerance test (GTT; 27 weeks); basal glucose and insulin levels (27 weeks); lipid accumulation in liver cryosections using Oil Red O imaging (27 weeks).

MAIN RESULTS AND THE ROLE OF CHANCE: Blastocysts formed by IVF developed at a slower rate and comprised fewer cells that in vivo generated blastocysts without culture ($P<0.05$). Postnatal growth rate was increased in all 4 experimental treatments compared with NM group ($P<0.05$). SBP, serum and lung ACE, and heart/body weight were higher in IVF-ET-BL versus IVF-ET-2Cell males ($P<0.05$) and higher than in other treatment groups, with SBP and lung ACE positively correlated ($P<0.05$). Glucose handling (GTT AUC) was poorer and basal insulin levels higher in IVF-ET-2Cell males than IVF-ET-BL ($P<0.05$) with glucose:insulin ratio more negatively correlated with body weight in IVF-ET-2Cell males than in other groups. Liver/body weight and liver lipid droplet diameter and density in IVF-ET-2Cell males were higher

than in IVF-ET-BL males (P<0.05). IVF groups had poorer health characteristics than their in vivo control groups indicating outcomes were not caused specifically by background techniques (superovulation, ET). No consistent health effects from duration of culture were identified in female offspring.

LIMITATIONS, REASONS FOR CAUTION: Results from experimental animal models cannot be extrapolated to humans. Nevertheless, they are valuable to develop conceptual models in this case in the absence of confounding parental infertility in assessing the safety of ART manipulations.

WIDER IMPLICATIONS OF THE FINDINGS: The study indicates that longer duration of embryo culture after IVF up to blastocyst before ET leads to increased dysfunction of cardiovascular health in males compared with IVF and shorter cleavage-stage ET. However, the metabolic health of male offspring is poorer after shorter versus longer culture duration. This distinction indicates that the origin of cardiovascular and metabolic health phenotypes after ART may be different. The poorer metabolic health of males after cleavage-stage ET coincides with embryonic genome activation occurring at ET.

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Introduction

Infertility is thought to affect an estimated 186 million people globally (Inhorn and Patrizio 2015). The development of assisted reproductive treatments (ART) has provided a partial clinical resolution to infertility with over 8 million children born to date, representing some 2-6% births in developed countries (Crawford and Ledger 2019; Berntsen, et al. 2019). Although most IVF children appear healthy according to numerous systematic reviews, ART has been linked with a small increased risk of adverse obstetric and perinatal outcomes and birth defects compared with naturally conceived children (Berntsen et al. 2019; Pinborg, et al. 2013; Qin, et al. 2017). In addition, longer-term health concerns of ART offspring have been associated mainly with altered birthweight and growth (Ceelen, et al. 2009; Kleijkers, et al. 2016; Kleijkers, et al. 2014); increased risk of cardiovascular (CV) dysfunction comprising CV remodelling during pregnancy with vascular impairment and raised blood pressure evident in children through to at least adolescence (Ceelen et al. 2009; Ceelen, et al. 2008; Guo, et al. 2017; Meister, et al. 2018; Sakka, et al. 2010; Scherrer, et al. 2012; Valenzuela-Alcaraz, et al. 2013; von Arx, et al. 2015; Zhou, et al. 2014); and susceptibility to metabolic dysfunction including poorer glucose handling, insulin resistance and increased triglycerides (Chen, et al. 2014; Gkourogianni, et al. 2014;

Guo et al. 2017; Pontesilli, et al. 2015; Sakka et al. 2010). In a minority of studies, impairment to neurological and cognitive health have also been reported (Goldsmith, et al. 2018; Liu, et al. 2017; Sandin, et al. 2013).

These sustained health effects have been linked to the 'Developmental Origins of Health and Disease' (DOHaD) concept suggesting environmental factors during development, especially the peri-conceptional period, may alter subsequent growth and morphogenesis through epigenetic, cellular and physiological processes (Feuer and Rinaudo 2016; Fleming, et al. 2018). However, evaluation of ART childrens' health is complex and confounded by the actual technologies and precise protocols applied in clinics, the gradual refinement in practice over time, and appropriateness of controls and comparator groups to distinguish between consequences mediated through parental infertility and ART practice (Berntsen et al. 2019).

With these considerations in mind, animal models have been invaluable to assess effects of ART-associated technologies on long-term offspring health, removing confounders such as parental infertility, treatment variability and including suitable controls. These indicate ART treatments do indeed affect long-term health. Thus, IVF and/or mouse embryo culture and transfer result in offspring with altered growth trajectory, cardiovascular abnormalities and glucose/insulin dysfunction (Cerny, et al. 2017; Chen et al. 2014; Donjacour, et al. 2014; Feuer, et al. 2014; Le, et al. 2013; Ramirez-Perez, et al. 2014; Rexhaj, et al. 2013; Schenewerk, et al. 2014; Scott, et al. 2010; Wang, et al. 2018; Watkins, et al. 2007).

In the last decade, there has been a gradual switch in ART practice from cleavage stage embryo transfer (ET) to blastocyst stage ET to facilitate embryo selection and improve synchronicity with the uterine environment although at the potential risk of increased embryo environmental perturbation. Whilst fresh blastocyst ET may marginally improve live birth rate (Glujovsky, et al. 2016) without significantly affecting birthweight (De Vos, et al. 2018) or risk of adverse perinatal outcomes (Shi, et al. 2019), it is unknown whether extended culture negatively impacts on later health status. In the current study we have used a mouse model to assess the effect of cleavage or blastocyst ET on offspring health across a range of growth, cardiovascular and metabolic criteria.

Materials and Methods

Animals

Animal treatments were conducted in accordance with the UK Home Office Animal (Scientific procedure) Act 1986 and local ethics committee at the University of Southampton. CBA male and C57/BL6 female mice (source of embryos) and MF1 females (pseudo-pregnant recipients) were bred in-house (University of Southampton, Biomedical Research Facility) on a 07:00-19:00 light cycle, 24°C, fed *ad libitum* from weaning on a standard chow diet (Special Diet Service, Ltd, Witham, Essex, UK) and water.

Embryo production and treatment

159 Virgin female C57/BL6 mice (3-4 weeks old) were superovulated by i.p. injection of 5
160 IU pregnant mare's serum gonadotropin (PMSG, Intervet, Cambridge, UK) and 46 h
161 later, 5 IU human chorionic gonadotropin (hCG, Intervet, Cambridge, UK). For *in vivo*
162 produced embryos, females were housed overnight with CBA males. Plug positive
163 females at embryonic day 0.5 (E0.5) (i.e. midday of plug detection day) were housed
164 individually and, at E1.5 and E3.5, females were killed by cervical dislocation and 2-
165 cell embryos and blastocysts flushed from dissected oviducts and uteri, respectively,
166 into prewarmed H6 medium supplemented with 4 mg/ml bovine serum albumin
167 (BSA, A3311, Sigma, UK) (Nasr-Esfahani, et al. 1990). Some females were also
168 naturally-mated without superovulation.

169 For IVF embryo production, sperm was retrieved from the cauda epididymis
170 of CBA males (8 weeks old) and placed into 90 µl sperm pre-incubation medium TYH-
171 MBCD (Takeo and Nakagata 2011) made in-house and equilibrated for 1 h at 37^{o}C in
172 5% CO_2 in air. C57/BL6 females were superovulated as above and cumulus masses
173 collected from the oviduct ampulla 13 h post hCG injection were placed directly into
174 200 µl fertilisation drop containing Human Tubal Fluid (HTF) medium made in-house
175 with 1.0 mM reduced glutathione (GSH, Sigma: G4251). Sperm (3-5 µl from pre-
176 equilibrated TYH-MBCD drop) were added to the fertilisation drop and incubated for
177 3-4 h to allow fertilisation to occur (Ishizuka, et al. 2013). Presumptive zygotes were
178 washed through four drops HTF medium without GSH and then cultured in the
179 fourth drop under oil at 37^{o}C and 5% CO_2 in air to the next day (E1.5) before
180 calculating the fertilisation rate. IVF embryos (2-cell stage) were then divided into
181 two groups, the first was washed in pre-warmed M2 medium (Sigma; Cat No.

M7167) before transfer to E0.5 MF1 pseudo-pregnant mothers. The second group was cultured in potassium simplex optimised medium with amino acids and BSA (KSOM; Sigma-Aldrich) (Biggers, et al. 2005) at 37^{o}C in 5% CO_2 in air to the blastocyst stage before washing in M2 medium and transfer to E2.5 MF1 pseudo-pregnant mothers.

In vivo and IVF generated blastocyst TE and ICM cell numbers were determined by differential nuclear staining as described (Handyside and Hunter 1984) with modifications (Velazquez et al. 2018).

Embryo transfer

Embryo transfer (ET) was performed by flank laparotomy in pseudo-pregnant MF1 recipients (7–8.5 weeks) obtained by mating with vasectomized MF1 males. 2-cell embryos and blastocysts, were washed three times in M2 medium prior to embryo transfer into oviduct and uteri, respectively, in minimal medium, as previously described (Velazquez, et al. 2018). Recipients were anaesthetized by a single intraperitoneal injection of Ketamine (50 mg/kg, Ketaset, Pfizer, UK) and Xylazine (10 mg/kg, Rompun, Bayer, UK). Embryos were transferred (19.7 ± 6.05 per recipient) in equal numbers into both maternal tracts with separate recipients used for different treatments, as below. After transfer, exposed tracts were placed back into the abdominal cavity, the peritoneum sutured, and the skin closed with wound clips. Recipients were then kept individually in a clean cage in a warm room (28-30°C) to recover from anaesthesia. Females were then housed in a quiet room for the rest of their pregnancy and lactation. Litter size was adjusted to up to 8 per dam at birth with similar number of male and female.

205 **Animal treatment groups**

206 Eight to thirteen litters were generated from each of five treatments with groups
207 termed as follows: (a) **NM** - Naturally mated, non-superovulated and undisturbed
208 gestation; (b) **IV-ET-2Cell** - in vivo derived 2-cell embryos collected from
209 superovulated mothers, with immediate ET to recipients; (c) **IVF-ET-2Cell** – IVF
210 generated embryos with oocytes from superovulated mothers cultured to 2-cell
211 stage before ET to recipients; (d) **IV-ET-BL** – in vivo derived blastocysts collected from
212 superovulated mothers , with immediate ET to recipients; (e) **IVF-ET-BL** - IVF
213 generated embryos with oocytes from superovulated mothers cultured to blastocyst
214 stage before ET to recipients. These treatment groups are shown in **Figure 1**.

215 **Offspring analysis**

216 All offspring from the five treatment groups were weaned at 3 weeks and males and
217 females caged separately per litter. **Offspring body weight** was recorded weekly for
218 27 weeks. **Systolic blood pressure** (SBP) was measured at post-natal weeks 9, 15 and
219 21 by tail-cuff plethysmography with Non-Invasive Blood Pressure Monitor (NIBP-8,
220 Columbus Instruments, Columbus, OH, USA) in a pre-warmed room (28-30°C) to
221 which mice were acclimatized for 90 mins, as described previously (Velazquez et al.
222 2018). Five SBP readings with good waveforms and good overall quality were taken
223 per mouse, and the mean value of the three middle readings was calculated and
224 recorded. Heart rate was monitored as an indicator of stress, and if reaching >500
225 beats per minute, SPB readings were delayed until heart rate reduced. **Glucose**
226 **tolerance test** (GTT) was conducted at post-natal week 27 in unrestrained conscious
227 mice after 15 h overnight fast, with access to water. A standard protocol for GTT

using a blood glucose meter (Accu-Chek Aviva, Roche Diagnostics GmbH, Germany) to measure blood glucose in small drops collected by tail tipping was employed. Topical anaesthetic cream (Lidocaine 5%, Teva, UK) was applied to the tail 20 min before starting the GTT. After recording of fasting glucose level (0 min), a glucose (G8270, Sigma) solution (20%, in sterile distilled water) was i.p. injected at a dose of 2 g/kg. Blood glucose levels were measured at 15, 30, 60 and 120 min after glucose administration. Area under the curve (AUC) values were calculated by the trapezoidal rule (Matthews, et al. 1990). **Organ Allometry** was determined two days after GTT: mice were sacrificed by cervical dislocation, blood collected by heart puncture and organs (i.e. liver, heart, left and right kidneys, lung and spleen) weighed, snap frozen in liquid nitrogen and stored at -80°C. Blood samples were centrifuged at 4°C for serum collection and storage at -80°C.

Angiotensin Converting Enzyme (ACE) activity

The method was used as previously (Watkins, et al. 2006; Watkins et al. 2007) to measure serum and lung ACE activities, the classical enzyme regulator of the renin-angiotensin system converting Angiotensin I to the vasopressor Angiotensin II (Li, et al. 2017). The assay is based on the colorimetric determination of hippurate with cyanuric chloride/dioxan reagent. Briefly, for serum ACE activity, samples were incubated in hippuryl-L-histidyl-L-leucine (HHL; Sigma) solution in H_3PO_3 buffer at 37°C, the reaction terminated with HCl (Sigma) followed by addition of cyanuric chloride (Sigma) in 1,4-dioxan (Sigma) for yellow coloration to develop. Four replicates per sample were analyzed using a plate reader (Varioskan Flash Multimode Reader; Thermo Scientific) at 380 nm. Negative controls comprised

addition of HCl before HHL. A Hippurate standard curve (20 µM to 100 µM) was prepared from 112 mg Hippuric acid (Sigma) solution in 250 mL 20 mmol/L NaOH, treated as samples except the addition of HHL. Each of the 4 replicates per sample were analyzed in duplicate, and the average of these eight readings taken. For lung ACE activity, lung samples of 50 +/- 1 mg were homogenised in 300 µl ice-cold boric buffer (H_3BO_3, 2M NaCl, pH 8.3; Sigma) with a PowerGen homogeniser, centrifuged at 16,400 rpm for 10 min at 4^{o}C, and the supernatant removed and stored at -80^{o}C. Pellets were homogenised in 300 µl buffer, centrifuged and supernatant removed and stored. Duplicate analysis of four replicate supernatants per sample were analyzed as described for serum ACE activity. Total protein content of samples was measured using a BioRad kit. Serum ACE activity was expressed as amount (in µM) of hippurate formed per ml of serum per minute; lung ACE activity was expressed as amount (in nM) of hippurate formed per mg of protein per minute. Serum and lung samples were selected from the same offspring at the middle weight across litters from the five treatment groups (one male and one female from each of 7-9 mothers per treatment) and frozen stored at 27 weeks. These same offspring were used for serum glucose and insulin assays and for the liver lipid metabolism assay.

Serum glucose and insulin analysis

Glucose concentration in offspring serum was measured using the glucometer as described in the GTT procedure. Serum insulin concentration was determined using an ELISA kit (Mercodia, Sweden, Mouse: 10-1247-01) based on the manufacturer's instructions. Briefly, 10 µl of each calibrator 0, 1, 2, 3, 4, and 5 and serum samples were incubated in coated microplate wells with 100 µl enzyme conjugate solution on a plate shaker at room temperature at 750 rpm for 2 hours before washing in 350 µl

of wash buffer repeated five times before addition of 200 µl TMB substrate and incubation for 15 minutes before addition of 50 µl stop solution. Absorbance was measured at 450 nm using a Varioskan Flash Multimode Reader (Thermo Scientific). Standard deviation and coefficient of variance were calculated for each sample run in duplicate in three plates and mean insulin values calculated. The glucose/insulin ratio (G:I) ratio was calculated to assess insulin resistance (McAuley, et al. 2001). A total of 6-8 samples from each treatment, both male and female and each from a separate mother, were used for combined glucose and insulin analyses.

Liver morphometrics and metabolism

Frozen-stored adult offspring median lobe liver samples were embedded in OCT-compound and cryosections at 7µm were generated and stained with Oil Red O to visualise lipid accumulation and Mayer's Haematoxylin as counterstain before mounting in aqueous medium and applying coverslips. Images of sections were analysed and photographed using an Olympus dotSlide Virtual Microscopy System with an Olympus BX61 Microscope Frame at 10x magnification. Images (3 per liver sample) were quantified using Fiji software for red-stained lipid accumulation with the Watershed tool applied to separate grouped lipid droplets. A total of 6-9 offspring from each treatment, both male and female and each from a separate mother, were used for liver analyses.

Statistics

Statistical analyses were performed with the IBM SPSS Statistics software, version 21 (IBM Corporation) and significance was taken as $P \leq 0.05$. If a P value of between 0.1 and 0.05 was observed, a trend was assumed to exist. Blastocyst cell number, rates of blastocyst development and ET outcome (i.e. pregnancy rate, ET efficiency and

299 litter size) were analyzed using a one-way ANOVA followed by a pairwise t-test with
300 Bonferroni correction analysis. Percentage data were arcsine transformed before
301 ANOVA analysis. Postnatal data comprising offspring weights, SBP, GTT, organ
302 weights and ratios, post-culling serum glucose and insulin, serum and lung ACE
303 activities and liver lipid accumulation data were analyzed using multilevel random
304 effects regression models to compare treatment groups (Kwong et al. 2004) and to
305 analyse relationships between different readouts (i.e. correlations) within each
306 treatment group (Velazquez et al. 2018). All postnatal data were converted to Z-
307 scores before being analysed with the regression models which took into account
308 between-mother and within-mother variation and litter size (Kwong et al. 2004;
309 Watkins, et al. 2008).

310

311 ## Results:

312 **IVF and embryo culture delay blastocyst development and reduce cell proliferation**
313 Routine analysis of IVF embryo development was conducted throughout the study,
314 with eggs (n = 1,720) collected from 40 superovulated dams, used in 14 separate IVF
315 experiments, leading to an overall mean success rate of 2-cell embryo formation of
316 92%, and from these allocated to culture, 81% formed morulae and 72% developed
317 to blastocysts. The developmental rate of IVF embryos was compared with *in vivo*
318 embryos (superovulated; naturally mated; develop *in vivo*; collected at E3.5). IVF
319 embryos developed slower and only reached the morula stage at E3.5 whilst *in vivo*
320 embryos had become expanding blastocysts (**Table 1**). IVF embryos became
321 expanding blastocysts by E4.5 (**Figure 2A,B**; **Table 1**). Some IVF and *in vivo* mid-

322 expanded blastocysts at E4.5 and E3.5 days, respectively, were subjected to
323 differential cell staining which showed increased TE, ICM and total cell numbers in *in*
324 *vivo* versus IVF embryos ($P<0.05$) although the ICM:TE ratio did not differ between
325 the two groups (**Figure 2C,D**). IVF and prolonged culture therefore delayed blastocyst
326 formation and reduced associated proliferation of both cell lineages compared with
327 *in vivo* development.

328 **Postnatal offspring from ART treatments display increased body weight**

329 To study the effect of ART and embryo culture duration on postnatal development,
330 we generated the five treatment groups as shown in **Figure 1** with offspring
331 production criteria shown in **Table 2**. The ET pregnancy rate (% dams giving birth)
332 was significantly higher in the IV-ET-BL group compared with IVF-ET-BL, otherwise no
333 differences were found between groups (**Table 2**). ET efficiency (pups generated per
334 numbers of embryos transferred) was lower in IVF-ET-BL than other groups. Litter
335 size in the ET groups IV-ET-2Cell, IV-ET-BL and IVF-ET-BL was lower than the NM
336 group. The IVF-ET-BL litter size was also lower than the IVF-ET-2Cell group. Male:
337 female ratio was not different between any of the treatment groups (**Table 2**).

338 Male and female offspring body weight differences between groups were
339 analysed from weaning through to week 27, taking into consideration litter size and
340 individual maternal origin. All four ET groups were significantly heavier compared
341 with the NM control group, evident from week 5 (males) and 4 (females) through to
342 week 27 (**Figure 3A,C**). Z-score plots confirmed increased body weight for all ET
343 groups compared with the NM group up to week 27 (**Figure 3B,D**). Generally, weight
344 differences between different ET groups were minimal and are itemised in **Figure 3**

345 legend. Notably, IVF-ET-BL female mean weight was heavier than other ET groups
346 throughout the 27 week period (**Figure 3C,D**). Thus, the combined techniques of ART
347 (superovulation, IVF, culture, transfer, recipient gestation) in our model, or just some
348 of them (minimally superovulation, transfer, recipient gestation), resulted in
349 sustained increase in postnatal weight in both sexes compared with natural,
350 unstimulated reproduction.

351 **Male offspring from IVF and prolonged culture before ET develop cardiovascular**
352 **dysfunction**

353 Systolic blood pressure (SBP) was determined at 9, 15 and 21 weeks and the mean of
354 these also recorded as LIFE (**Figure 4**). In males, mean SBP for all time points was
355 consistently highest in IVF-ET-BL, then reducing in IVF-ET-2Cell, the two IV-ET control
356 groups, and the NM group (**Figure 4A**). IVF-ET-BL male SBP was increased at weeks
357 15, 21 and LIFE compared with IVF-ET-2Cell (P=0.032, 0.034 and 0.017, respectively)
358 and with IV-ET-BL (P= 0.003, 0.014 and 0.001, respectively) (**Figure 4A**). In females,
359 although a similar SBP pattern existed across treatment groups, differences were not
360 significant between ET groups (**Figure 4B**). However, NM females showed significant
361 lower SBP than females in IV-ET and IVF-ET groups at weeks 15, 21 and LIFE (P<0.05).

362 Serum and lung angiotensin converting enzyme (ACE) activity, known to
363 associate with increased SBP (Li et al. 2017), was further measured in offspring. Male
364 IVF-ET-BL offspring recorded the highest serum and lung ACE activity, both higher
365 (P<0.05) than the IVF-ET-2Cell males (**Figure 4C,D**). IVF-ET-BL lung ACE in males was
366 also higher than the control IV-ET-BL males (P<0.05) (**Figure 4D**). However, ACE
367 activities were not different across groups in female offspring (**Figure 4C,D**).

Correlation analysis of SBP and ACE activity revealed a significant positive correlation between both SBP 21 weeks and SBP LIFE with Lung ACE activity in male IVF-ET-BL offspring but not in females or in any other treatment group (**Table 3**).

The combined techniques of ART (superovulation, IVF, culture, ET, recipient gestation) therefore contribute to adverse postnatal CV health compared with natural unstimulated reproduction but with prolonged versus short embryo culture exacerbating these effects in male offspring.

Male offspring from IVF and short culture before ET develop impaired glucose and insulin metabolism

Glucose metabolism of offspring was assessed by glucose tolerance test (GTT) at postnatal week 27. Male offspring fasting glucose level (i.e. 0 min) and after 15 min, 30 min, 1 hr and 2 hrs of i.p glucose injection showed all treatment groups to have significantly slower recovery and larger area under the curve (AUC) than the NM control group (**Figure 5A,B**). Glucose recovery and AUC for IVF-ET-2Cell was poorer compared with both IV-ET-2Cell (P= 0.05 - 0.004) and IVF-ET-BL males (P= 0.03 - 0.003). In female offspring, fasting glucose level, glucose recovery and AUC also appeared poorer in treatment groups compared with the NM control although not always significantly. No significant differences were detected between the four treatment groups in females (**Figure 5C,D**).

Serum samples collected at 27 weeks during animal culling were used to measure insulin and glucose levels and the glucose: insulin ratio (G:I), a measure of insulin effectiveness in glucose homeostasis. In male offspring, glucose levels were similar across treatments with IV-ET-BL higher than IV-ET-2Cell and NM ($P<0.05$;

391 **Figure 6A**). In contrast, insulin levels differed substantially across treatments with
392 IVF-ET-2Cell males significantly higher than all other groups (P<0.05; **Figure 6B**). The
393 lowest insulin level was in NM males which led to the highest G:I ratio in NM males
394 and significantly higher than in IV-ET-2Cell, IVF-ET-2Cell and IV-ET-BL groups
395 (P=0.005, P=0.001 and P=0.038 respectively; **Figure 6C**). Female serum glucose was
396 unchanged across treatments (**Figure 6A**) while insulin was lowest in NM and
397 significantly raised in IVF-ET-2Cell females (P<0.05; **Figure 6B**), resulting in G:I ratio
398 highest in NM females, as in males, and significantly above IV-ET-BL and IVF-ET-2Cell
399 females (P<0.05; **Figure 6C**).

400 Metabolic outcomes were analysed for possible associations with other
401 phenotypes; the G:I ratio in particular was found to be significantly negatively
402 correlated both with body weight throughout postnatal life and with AUC from GTT
403 in the IVF-ET-2Cell male but not female offspring (**Table 3**). Other groups with ET
404 treatment also showed a weaker association between these parameters but not the
405 NM group (**Table 3**).

406 The combined techniques of ART (superovulation, IVF, culture, ET, recipient
407 gestation) therefore contribute to adverse postnatal metabolic health as measured
408 by glucose homeostasis compared with natural unstimulated reproduction. Here,
409 evidence of insulin resistance was most pronounced after short embryo culture
410 particularly in male offspring.

411 **Offspring from IVF and short culture before ET develop increased lipid**
412 **accumulation in liver**

Metabolic health of offspring was also assessed by analysis of lipid accumulation in liver cryosections stained with Oil Red O using organs stored at 27 weeks at culling. Representative images of lipid accumulation in male liver sections are shown in **Figure 7A**. Lipid droplet size was increased in IVF-ET-2Cell offspring relative to other groups and especially in males. IVF-ET-2Cell lipid size was increased compared with IVF-ET-BL (P=0.015) and with control IV-ET-2Cell (P=0.015) males (**Figure 7B**). Moreover, the relative percentage area of lipid accumulation was increased in IVF-ET-2Cell versus IVF-ET-BL at trend level (t=0.065) and versus control IV-ET-2Cell (P=0.003) in males (**Figure 7C**). Thus, IVF and transfer after short rather than long culture contributes to adverse liver lipid accumulation as well as impaired glucose-insulin metabolism, especially in males.

Postnatal offspring from ART treatments display altered organ allometry

Offspring were sacrificed at postnatal week 27 and organ /body weight ratios determined before organ freeze storage. Male offspring organ weight was generally proportional to body weight but with exceptions (see **Figure 8** for details). Notably, IVF-ET-2Cell males had relatively smaller lung, heart and right kidney and larger livers compared with NM males, whilst IVF-ET-BL males also had larger liver and spleen compared with NM males (**Figure 8A**). IVF-ET-BL males had larger heart and smaller liver than IVF-ET-2Cell males. The IV-ET-2Cell and IV-ET-BL control groups had few organ size differences from NM males. In contrast, female offspring from ART treatments generally had smaller proportioned organ sizes, especially lungs and heart, compared with NM females but differences between the two IVF groups were not apparent (**Figure 8B**). The combined techniques of ART (superovulation, IVF,

culture, transfer) therefore contribute to altered organ allometry in both male and female offspring compared with natural unstimulated reproduction.

Discussion

We have used an animal model to address the safety for long-term offspring health of specific ART techniques in common practice in clinics and in the absence of confounding parental infertility. Given the past record of adverse offspring health risk mediated through embryo culture (Cagnone and Sirard 2016; Fleming et al. 2018; Sunde, et al. 2016), the model was designed to distinguish specifically between short and long culture duration either up to cleavage-stage (2-cell) or blastocyst transfer, respectively. Both groups (IVF-ET-2Cell; IVF-ET-BL) were supported by direct in vivo controls for transfer at these two stages (IV-ET-2Cell; IV-ET-BL) which included the background ART techniques (superovulation; ET) but in the absence of the tested techniques (IVF; short or long culture). These four groups were also compared with a natural mating (NM) group where no ART techniques were applied. Thus, the model is suitable for direct comparison of the health consequences for offspring arising from IVF and culture duration independent of other techniques, but also permits evaluation of the background techniques and the collective of all ART techniques. However, our design required the use of atmospheric oxygen rather than 5% for culture, although the former is reported to still be practised in some 40% IVF cycles worldwide (van Montfoort et al. 2020). This choice was necessary to maintain consistency between the two IVF groups and their two IV controls where embryo incubation was kept to an absolute minimum, essentially the time to complete ET in

459 the surgery room, and could not be accomplished at 5% O_2 for practicalities. Lastly,
460 the statistical approach of random effects regression analysis on the dataset permits
461 outcomes to be evaluated in the entire offspring generated in each treatment rather
462 than just on litter means, thereby integrating variability both within- and between-
463 mothers and independent of the effect of litter size (Kwong et al. 2004), as used in
464 our previous periconceptional DOHaD models (Watkins et al. 2008; Velazquez et al.
465 2016, 2018).

466 One enduring feature of the dataset was the distinction between offspring
467 phenotype arising from all four manipulated groups with that of the NM group. Thus,
468 compared with the NM group, offspring from manipulated groups exhibited
469 increased postnatal growth and poorer CV and metabolic health across the spectrum
470 of assays undertaken, commonly in both male and female offspring at significant
471 levels. This broad and unequivocal phenotypic consequence at one level
472 demonstrates the collective effect of the ART techniques applied over the lifespan
473 but is likely to be exaggerated because of the use of MF1 recipients for gestation and
474 lactation. For example, it is established that the maternal uterine genotype of mouse
475 recipients can influence offspring phenotype such as postnatal growth rate (Cowley
476 et al. 1989). Whilst we used inbred C57BL6/CBA embryos for genomic stability and
477 capacity to overcome the '2-cell block' in culture, outbred MF1 recipients were
478 necessary to enhance pregnancy efficiency, a combination we have used successfully
479 previously for DOHaD-related mouse studies (Velazquez, et al. 2016). Thus, growth
480 rate of offspring from the manipulated groups here broadly matched that as
481 previously reported (Velazquez et al. 2016) and is similar to MF1 offspring from

natural pregnancies (Watkins et al. 2008) or slightly below that following MF1 embryo manipulations and transfer to MF1 recipients (Velazquez et al. 2018).

In the critical group comparison of culture duration after IVF with all other ART techniques normalised, we found a curious dichotomy between IVF-ET-2Cell and IVF-ET-BL offspring, and particular males, in that CV outcomes (SBP; ACE activity; larger heart/body mass) were poorer in IVF-ET-BL treatments but conversely, metabolic outcomes (glucose response; raised basal insulin; increased liver/body mass; increased liver lipid accumulation) were poorer in the IVF-ET-2Cell group. Both CV phenotype in IVF-ET-BL and metabolic phenotype in IVF-ET-2Cell males were poorer than their respective controls (IV-ET-BL; IV-ET-2Cell) indicating outcomes were predominantly derived from IVF and culture duration, perhaps in combination with the timing of ET (discussed later), rather than by in vitro manipulations and ET *per se*. To assess the basis for this dichotomy in health outcomes in IVF-ET-BL and IVF-ET-2Cell offspring, we first need to consider the direct effects of in vitro culture on the early embryo.

Our study showed that in vitro culture, although permissive for blastocyst formation, was suboptimal, slowing development and reducing proliferation of TE and ICM cells, as previously reported in other mouse ART models (Chen, et al. 2019; Watkins et al. 2007). Culture conditions can interfere with two critical aspects of preimplantation development, namely embryo metabolism and the epigenetic regulation of the new embryonic genome. Embryo metabolism matures progressively from a low rate during fertilisation and early cleavage dependent upon mitochondrial oxidative phosphorylation for energy production which increases

505 substantially at the blastocyst stage (Houghton, et al. 1996; Leese 2012). This
506 progression is accompanied by upregulated glycolysis in late cleavage, further
507 enhancing energy availability for blastocyst morphogenesis, especially epithelial
508 transport activity and increased protein synthesis for growth (Houghton et al. 1996;
509 Leese 2012). Mitochondrial morphology also matures during cleavage with normal
510 transverse cristae formation coinciding with the increased efficiency of ATP
511 production at morula and blastocyst stages (Harvey 2019). The unnatural metabolite
512 milieu experienced in embryo culture can induce oxidative stress through increased
513 production of reactive oxygen species (ROS) alongside ATP in the mitochondrial
514 electron transport chain (Cagnone and Sirard 2016; Takahashi 2012). Whilst natural
515 protective mechanisms exist through antioxidant enzymes to maintain the redox
516 balance, culture conditions can perturb this balance leading to impaired
517 development affecting growth, gene expression and survival (Cagnone and Sirard
518 2016; Leese 2012; Takahashi 2012). Indeed, direct manipulation of energy
519 substrates, mitochondrial activity and redox potential in mouse zygotes leads to
520 altered postnatal growth rates (Banrezes, et al. 2011). Further, a range of
521 environmental factors including maternal over-nutrition and obesity have also been
522 shown to disturb mitochondrial functioning, localisation and mtDNA copy number in
523 oocytes and early cleavage embryos with enduring effects on fetal and postnatal
524 growth and metabolism (Grindler and Moley 2013; Igosheva, et al. 2010; Wu, et al.
525 2015).

526 The second consequence of adverse culture environment is to interfere with
527 the epigenetic reprogramming of the new embryonic genome (Cagnone and Sirard
528 2016; Chason, et al. 2011; Sunde et al. 2016). Global demethylation of the genome

during cleavage is followed by a gradual, lineage-specific pattern of de novo methylation initiated in the blastocyst to coordinate development (Seisenberger, et al. 2013). Thus, culture environment may alter the expression and methylation level of imprinted genes within the embryo persisting into later developmental stages (de Waal, et al. 2014; Doherty, et al. 2000). Non-imprinted genes are also vulnerable to culture conditions with the global pattern of gene expression (Feuer, et al. 2016) and DNA methylation distinct from that of in vivo embryos (Canovas, et al. 2017; Salilew-Wondim, et al. 2015; Wright, et al. 2011). Epigenetic disturbance may at least partially derive from mitochondrial dysfunction since mitochondria supply intermediates in DNA methylation and histone acetylation through the 1-carbon metabolism pathway (Cagnone and Sirard 2016; Ducker and Rabinowitz 2017; Xu and Sinclair 2015).

The poorer CV outcomes identified in IVF-ET-BL males after long culture versus both control IV-ET-BL and short culture IVF-ET-2Cell groups likely reflects the progressive negative effects of in vitro culture on embryo metabolism and epigenetic stability. Indeed, we show progressive increase in SBP in male offspring based upon the duration of culture from IV controls through to IVF-ET-BL offspring. Cardiac and associated vasculature form very early during development, from E8.5 in mouse, and is a complex morphogenetic process essential for embryo survival with recent research identifying significant epigenetic regulation (Kathiriya, et al. 2015). Adult CV dysfunction occurs in response to a wide range of peri-conceptional environments, indicating its sensitivity (Fleming et al. 2018). Moreover, an epigenetic basis for adverse CV health including arterial hypertension has been reported in a mouse ART model, mediated through altered DNA methylation of the endothelial eNOS gene in

553 the aorta leading to reduced eNOS expression and disturbed NO signalling (Rexhaj et
554 al. 2013). Notably, the CV phenotype and associated epigenetic alteration in the
555 eNOS gene can be prevented by inclusion of the epigenetic regulator, melatonin, in
556 embryo culture medium (Rexhaj et al. 2015). Indeed, the significant positive
557 correlation identified between SBP and lung ACE level in the IVF-ET-BL males, but not
558 other groups, suggests that ACE expression, known to be epigenetically regulated
559 (Mudersbach et al. 2019) and sensitive to peri-conceptional environment (Watkins et
560 al. 2006, 2007), may contribute an epigenetic pathway to affect later CV health.

561 In contrast to the clear link between extended culture and offspring CV
562 dysfunction, it is the IVF-ET-2Cell group with shorter culture duration that lead to the
563 poorer metabolic phenotype in offspring. Collectively, male offspring from this
564 treatment demonstrated poorer glucose handling which correlated negatively with
565 body mass, increased basal insulin levels, increased relative liver sizing and liver lipid
566 accumulation than either the direct control group (IV-ET-2Cell) or the IVF-ET-BL
567 group. Increased birth weight and poorer glucose and insulin regulation was
568 previously reported in mouse IVF offspring following ET at the 2-cell stage but
569 predominantly in females (Scott et al. 2010). Similar poorer glucose handling mainly
570 in female offspring following IVF was found after mouse blastocyst ET and coincided
571 with metabolic dysfunction across several tissues including liver, evidenced by
572 microarray analysis (Feuer et al. 2014). Further, liver metabolic dysfunction including
573 accumulation of monounsaturated fatty acids has been reported following mouse
574 IVF and ET at the 2-cell stage (Wang, et al. 2013). Mouse IVF also leads to increased
575 phospholipid accumulation in fetal liver (Li, et al. 2016), indicating prenatal origin of
576 ART-mediated metabolic impairment. Given the increased accumulation of lipid in

577 the male IVF-ET-2Cell liver, it would be interesting in future studies to determine
578 serum lipid levels and adipose tissue composition for broader understanding of lipid
579 dysregulation in this group. The co-occurrence of markers of metabolic disease risk
580 in several studies, as well as in our current study, confirm the link between ART and
581 adult metabolic health.

582 This distinction in outcomes between IVF-ET-2Cell and IVF-ET-BL groups
583 suggests different mechanisms and biological pathways may be at work for
584 metabolic and CV outcomes. Apart from the shorter culture duration, the IVF-ET-
585 2Cell group experienced ET during the 2-cell stage when the mouse embryonic
586 genome is predominantly activated (EGA) (Flach, et al. 1982). The period of EGA at
587 the transition from maternal to embryonic control of development is recognised as
588 one of particular sensitivity to culture conditions across mammalian species,
589 affecting embryo potential (Lonergan, et al. 2003; Zander, et al. 2006) and discussed
590 in detail elsewhere (Brison, et al. 2014). A convincing argument suggests that
591 stressful manipulations during EGA (such as ET here) may be accentuated by the
592 absence of gap junction communication between blastomeres to coordinate
593 homogeneity and protection in intercellular maturation (Brison et al. 2014). EGA in
594 the human occurs slightly later in cleavage, at the 4- to 8-cell transition (Braude, et
595 al. 1988; Vassena, et al. 2011), but cleavage ET in human ART normally coincides
596 with this cellular stage.

597 A further characteristic of our study has been the clear disparity in outcomes
598 based upon offspring sex with males far more sensitive that females. Sexual
599 dimorphism has been commonly found in periconceptional DOHaD programming

600 studies in response to diverse challenges including ART-based models and evident in
601 small and large mammals and humans (Fleming et al. 2018; Hansen, et al. 2016). In
602 mouse studies of embryo culture effects on offspring cardiometabolic health, males
603 commonly show increased sensitivity, as here (Donjacour et al. 2014; Velazquez et al.
604 2018) but female vulnerability has been shown elsewhere (Feuer et al. 2014)
605 indicating strain differences may be contributory. This also likely reflects different
606 susceptibilities to CV disease based on sex which arise in utero (Schalekamp-
607 Timmermans, et al. 2016). Environmental conditions such as nutrient and metabolite
608 levels both in vivo and in vitro can differentially influence embryo response in terms
609 of signalling activity, gene expression and morphogenesis in a sex-specific manner
610 that can persist through gestation and postnatal life (Hansen et al. 2016).

611

612 ## Conclusion

613 We have shown that IVF and embryo culture in a mouse model specifically associate
614 with adverse CV and metabolic outcomes particularly in male offspring independent
615 of background superovulation and ET techniques. Our study shows a clear effect of
616 culture duration after IVF with long culture to the blastocyst stage before ET leading
617 to a poorer CV phenotype while shorter culture to the 2-cell stage before transfer
618 resulting in a poorer metabolic health phenotype. We consider this distinction in
619 outcome likely reflects different pathways leading to these health conditions
620 initiated from preimplantation environment and the interaction between culture
621 duration and the timing of ET in relation to EGA. These findings further pinpoint the
622 risks of preimplantation manipulations in the programming of long-term health

outcomes. From a clinical perspective, whilst our data do not identify a safer strategy for IVF and culture duration, they do show the biological and health implications that derive from the ART culture protocol.

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Authors' roles

A.A. performed experiments, analysed data, wrote and edited the manuscript. R.K.R.I.A, B.S. and K.W. performed experiments. M.A.V. provided technical support, analysed data and edited the paper. A.J.W. and J.J.E. analysed data and edited the paper. C.O. provided statistical expertise. N.R.S. performed experiments, analysed data and edited the paper. T.P.F. conceived and designed the study, wrote and edited the manuscript.

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Conflict of interest

The authors have no conflicts of interest to declare.

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910

911 Figure legends

912 **Figure 1.** Experimental design showing the five treatment groups used.

913

914 **Figure 2.** Effect of IVF and prolonged embryo culture on blastocyst development cell
915 number. IVF embryos at E3.5 comprise morulae (A) and at E4.5 comprise blastocysts
916 (B); bar = 100 µm. (C) IVF embryos (n= 50) have fewer cells than in vivo embryos (n=
917 87) at the blastocyst stage. Mean (±SEM) blastocyst cell number for IVF compared
918 with in vivo embryos ($P<0.05$). (D) Mean (±SEM) ICM/TE ratio of blastocysts. *
919 $P<0.05$.

920

921 **Figure 3.** Effect of ART techniques on growth of offspring. Body weight and Z-score
922 analysis in male (A and B) and female (C and D) offspring. Multilevel random effects
923 regression analysis. *Indicates a significant difference ($P < 0.05$) between NM and
924 other treatments; ⏀ denotes NM vs. (IV-ET-2Cell, IVF-ET-2Cell and IVF-ET-BL) at week
925 3 and NM vs. IV-ET-BL at week 4 ($P \leqslant 0.05$), Δ indicates IV-ET-2-Cell vs. IV-ET-BL and
926 ○ indicates IVF-ET-BL vs. IVF-ET-2Cell ($P \leq 0.05$). Mean (±SEM) body weight from 3 to
927 27 weeks (from 8-13 litters); n of mothers or foster mothers ♀, n of offspring.

928

Figure 4. Effect of ART techniques on cardiovascular function in offspring. Postnatal SBP at indicated weeks of age and mean of these for individual offspring (LIFE), in male (A) and female (B) offspring; mean (±SEM) from 8-13 litters. Serum ACE activity (C) and lung ACE activity (D) in male and female offspring; mean (±SEM) from 7-9 litters per treatment. Multilevel random effects regression analysis. *Indicates a significant difference ($P < 0.05$) between NM and selected groups; ◊ indicates IV-ET-BL vs. IVF-ET-BL, and ○ indicates IVF-ET-BL vs. IVF-ET-2Cell differences ($P<0.05$). n of mothers or foster mothers ♀, n of offspring.

Figure 5. Effect of ART techniques on glucose metabolism in offspring. Intraperitoneal GTT at 0, 15, 30, 60 and 120 minutes and AUC in male (A and B) and female (C and D) offspring; mean (±SEM) in 8-13 litters per treatment. Multilevel random effects regression analysis. *Indicates a significant difference ($P < 0.05$) between NM and selected groups; Δ indicates IV-ET-2-Cell vs. IV-ET-BL, # indicates IV-ET-2Cell vs. IVF-ET-2Cell, and ○ indicates IVF-ET-BL vs. IVF-ET-2Cell differences ($P\leq0.05$). n of mothers or foster mothers ♀, n of offspring.

Figure 6. Effect of ART techniques on glucose and insulin levels in offspring. Serum glucose (A), serum insulin (B) and G:I ratio (C) in male and female offspring; mean (±SEM) from 6–8 litters per treatment. Multilevel random effects regression analysis. *Indicates a significant difference ($P < 0.05$) between NM and selected groups; Δ indicates IV-ET-2-Cell vs. IV-ET-BL, # indicates IV-ET-2Cell vs. IVF-ET-2Cell, and ○

951 indicates IVF-ET-BL vs. IVF-ET-2Cell differences (P≤0.05). n of mothers or foster
952 mothers ♀, n of offspring.

953

954 **Figure 7.** Effect of ART techniques on lipid accumulation in offspring liver.
955 Representative images of liver cryosections from male offspring stained with Oil Red
956 O from each treatment group (A). Average lipid droplet diameter (B) and percentage
957 lipid-stained area (C) in male and female offspring; mean (±SEM) from 6-9 litters
958 per treatment. *Indicates a significant difference (P < 0.05) between NM and
959 selected groups; ○ indicates IVF-ET-BL vs. IVF-ET-2Cell (B, P=0.015; C, t=0.065), and
960 # indicates IV-ET-2Cell vs. IVF-ET-2Cell differences (P<0.05). n of mothers or foster
961 mothers ♀, n of offspring.

962

963 **Figure 8.** Effect of ART techniques on organ/body weight in adult offspring. Organ
964 allometry variables in male (A) and female (B); mean (±SEM) organ: body weight
965 ratio from 8–13 litters per treatment. Multilevel random effects regression analysis.
966 *Indicates a significant difference (P < 0.05) between NM and selected groups, Δ
967 indicates IV-ET-2-Cell vs. IV-ET-BL, # indicates IV-ET-2Cell vs. IVF-ET-2Cell, and ○
968 indicates IVF-ET-BL vs. IVF-ET-2Cell differences (P<0.05). n mothers or foster mothers
969 ♀, n offspring.

Table 1. Developmental rate of in vivo and IVF embryos at E3.5 and E4.5.

Group	Number (%) Mean (SD ± SEM) from each dam						
	Dam number	**Embryo number**	**Morula**	**Early blastocyst**	**Mid blastocyst**	**Late blastocyst**	**Arrested (early cleavage)**
in vivo E3.5	22	469	15 (3.2) 0.71 (0.72±0.15)	40 (8.5) 1.9 (0.89±0.19)	114 (24.3) 5.43 (1.29±0.27)	295 (62.9) 14.05 (3.31±0.7)	5 (1.1) 0.24(0.44±0.09)
IVF E3.5	40	1076[1]	876 (81.4) 62.57 (38.14±6.03)	0	0	0	200 (18.6) 14.28 (5.69±0.9)
IVF E4.5			109 (12.4)[2] 7.78 (5.1±0.81)	51 (5.8)[2] 3.64 (1.98±0.31)	83 (9.5)[2] 5.92 (3.19±0.5)	633 (72.3)[2] 45.21 (28.12±4.45)	0

Early blastocyst: has a blastocoel volume less than half of the total embryo volume.
Mid blastocyst: has a blastocoel volume equal to or larger than the total embryo volume.
Late blastocyst: blastocoel fully expanded within the embryo whilst the zona pellucida (ZP) is thinning.
[1]Number of 2-cell embryos cultured after IVF
[2]% of morulae at E3.5

Table 2. Offspring production criteria for the five treatment groups as shown in Figure 1. Data were analysed using ANOVA (mean± SEM).

Treatment Group	ET pregnancy rate[1] % (dam numbers)	ET efficiency[2] % (pups/ embryos transferred)	Birth litter size[3] Mean (SD ± SEM) [litter number]	Offspring number	No. male/female pups	Ratio Male: Female
NM	N/A	N/A	8 (1.33±0.42)[a] [10]	80	40/40	1
IV-ET-2Cell	88.9 (8/9)	31.7 (57/180)[a]	7.12(4.36±1.54)[b] [8]	57	32/25	1.3
IVF-ET-2Cell	73.3 (11/15)	16.7 (75/450)[a]	8.33 (3.74±1.25)[a1] [9]	75	42/33	1.2
IV-ET-BL	81.8 (9/11)[a]	30.5 (47/154)[a]	5.88 (1.73±0.61)[b] [8]	47	22/25	0.9
IVF-ET-BL	48.3 (14/29)[b]	9.1 (42/464)[b]	3.23 (1.79±0.5)[b,b1] [13]	42	26/16	1.6

[1] Dams that gave birth/total number of ETs performed.
[2] Total number of pups at birth (before litter size correction)/total embryos transferred (7-15 per side).
[3] Calculated on dams with live pups at birth (before litter size correction).
[a,b; a1,b1] Within a column, values with different letters are significantly different (P<0.05).

Table 3. Phenotypic correlations between different offspring outcomes across treatments.

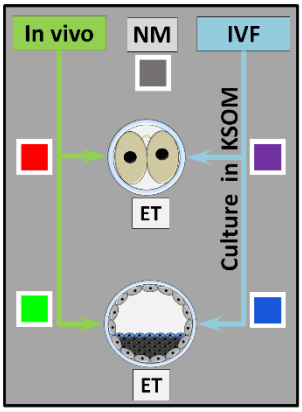
	Natural mating		IV-ET-2cell		IV-ET-BL		IVF-ET-2cell		IVF-ET-BL	
	Male (n=8)	Female (n=8)	Male (n=6–8)	Female (n=7–8)	Male (n=6–8)	Female (n=6–7)	Male (n=8–9)	Female (n=7–9)	Male (n=7–9)	Female (n=7–9)
Cardiovascular phenotype										
SBP wk21 − Lung ACE	−0.190	0.415	0.556	0.370	0.184	0.130	−0.026	0.223	0.902*	−0.119
SBP LIFE − Lung ACE	0.118	0.107	0.415	0.353	0.693$	0.328	0.101	0.195	0.714*	−0.177
G:I and body weight										
G:I ratio − BW3	−0.305	−0.351	−0.211	−0.566	−0.403	0.080	−0.224	0.160	−0.676$	−0.268
G:I ratio − BW9	0.111	0.169	−0.621	−0.649	0.936*	0.789$	−0.806*	−0.073	−0.664	−0.013
G:I ratio − BW15	0.128	−0.303	−0.761$	−0.735$	−0.904*	0.749$	−0.807*	−0.309	−0.700$	−0.053
G:I ratio − BW21	0.287	−0.184	−0.848*	−0.710$	−0.871*	0.664	−0.812*	0.008	−0.748$	0.122
G:I ratio − BW27	0.001	−0.448	−0.832*	−0.883*	−0.768$	0.910*	−0.935*	−0.026	−0.806*	−0.741$
G:I ratio − FG	−0.523	−0.183	−0.040	−0.431	−0.651	0.471	−0.640$	−0.090	0.437	0.198
G:I ratio − GTT 15 min	−0.010	−0.021	−0.082	−0.282	−0.383	0.679	−0.806*	0.137	−0.767*	0.234
G:I ratio − GTT 120 min	0.298	−0.350	−0.753$	−0.547	−0.432	0.927*	−0.648$	0.043	−0.707$	−0.201
Insulin – AUC	0.023	−0.111	−0.013	0.208	0.252	−0.912*	0.860*	−0.315	0.659	0.420
G:I ratio − AUC	−0.195	−0.107	−0.263	−0.371	−0.540	0.960*	−0.862*	0.116	−0.819*	−0.530

SBP LIFE=Average (SBP9, SBP15 and SBP21); ACE= Angiotensin Converting Enzyme; G:I G:I ratio=Serum glucose: Serum insulin ratio. BW=body weight as specific week; AUC=area under the curve for GTT test; Insulin=Serum insulin; FG fasting glucose

$ $P < 0.1$ * $P < 0.05$

Figure 1.

Group	Superovulation	IVF	Embryo transfer
Normal Mating (Undisturbed development) (NM)	✗	✗	✗
In vivo 2-cell, immediate transfer (IV-ET-2CELL)	✓	✗	✓
In vivo blastocyst, immediate Transfer (IV-ET-BL)	✓	✗	✓
IVF-ET 2-cell (Short culture) (IVF-ET-2CELL)	✓	✓	✓
IVF-ET-blastocyst (Long culture) (IVF-ET-BL)	✓	✓	✓



Groups:
NM – Naturally mated, no superovulation (SO) and undisturbed gestation
IV-ET-2Cell – In vivo derived 2-cell embryos from SO mothers, with immediate ET to recipients
IVF-ET-2Cell – IVF embryos with eggs from SO mothers cultured to 2-cell stage before ET
IV-ET-BL – In vivo derived blastocysts from SO mothers, with immediate ET
IVF-ET-BL – IVF embryos with eggs from SO mothers cultured to blastocyst stage before ET

Figure 2.

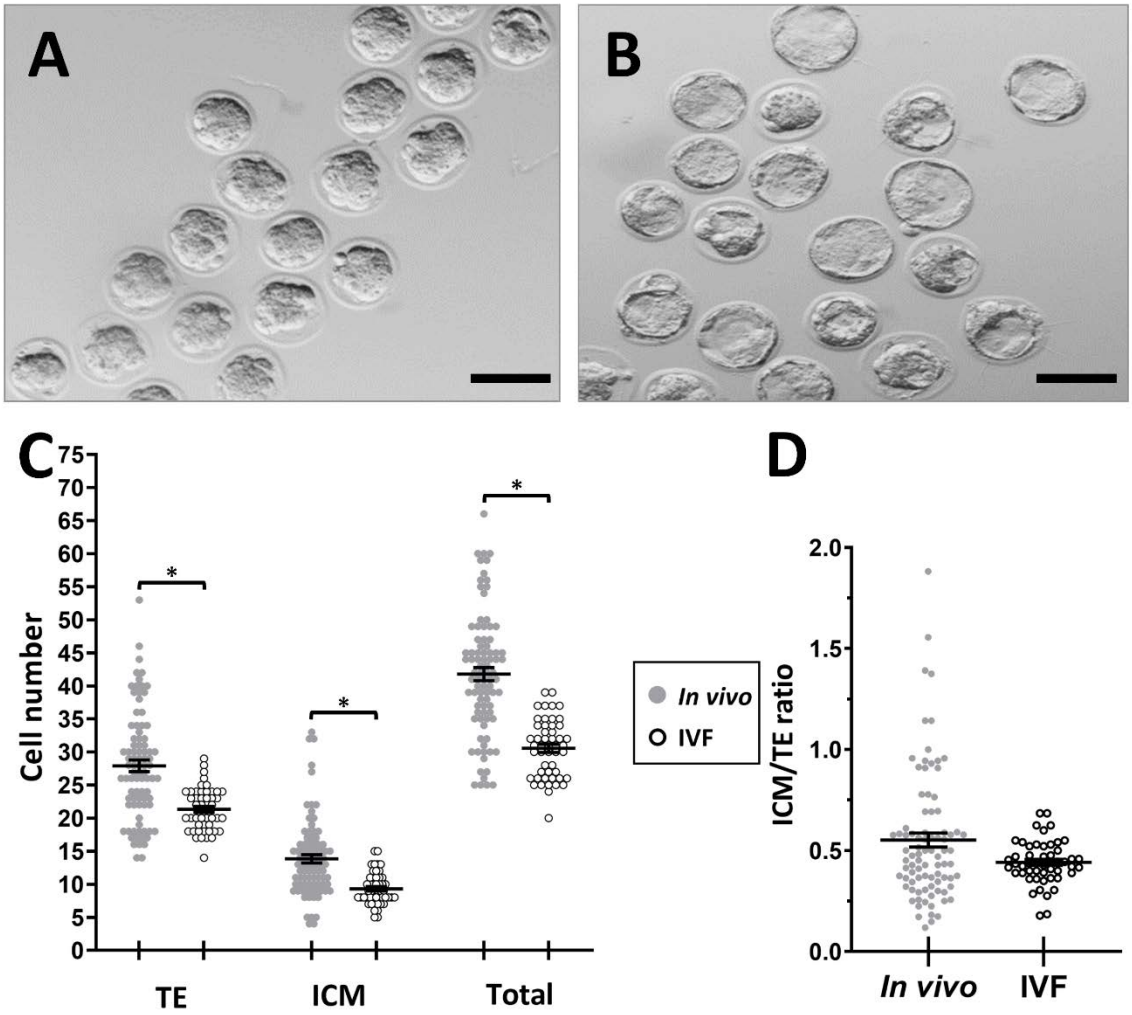

A
B
C
Cell number
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
*
*
*
TE
ICM
Total
In vivo
IVF
D
ICM/TE ratio
2.0
1.5
1.0
0.5
0.0
In vivo
IVF

Figure 3.

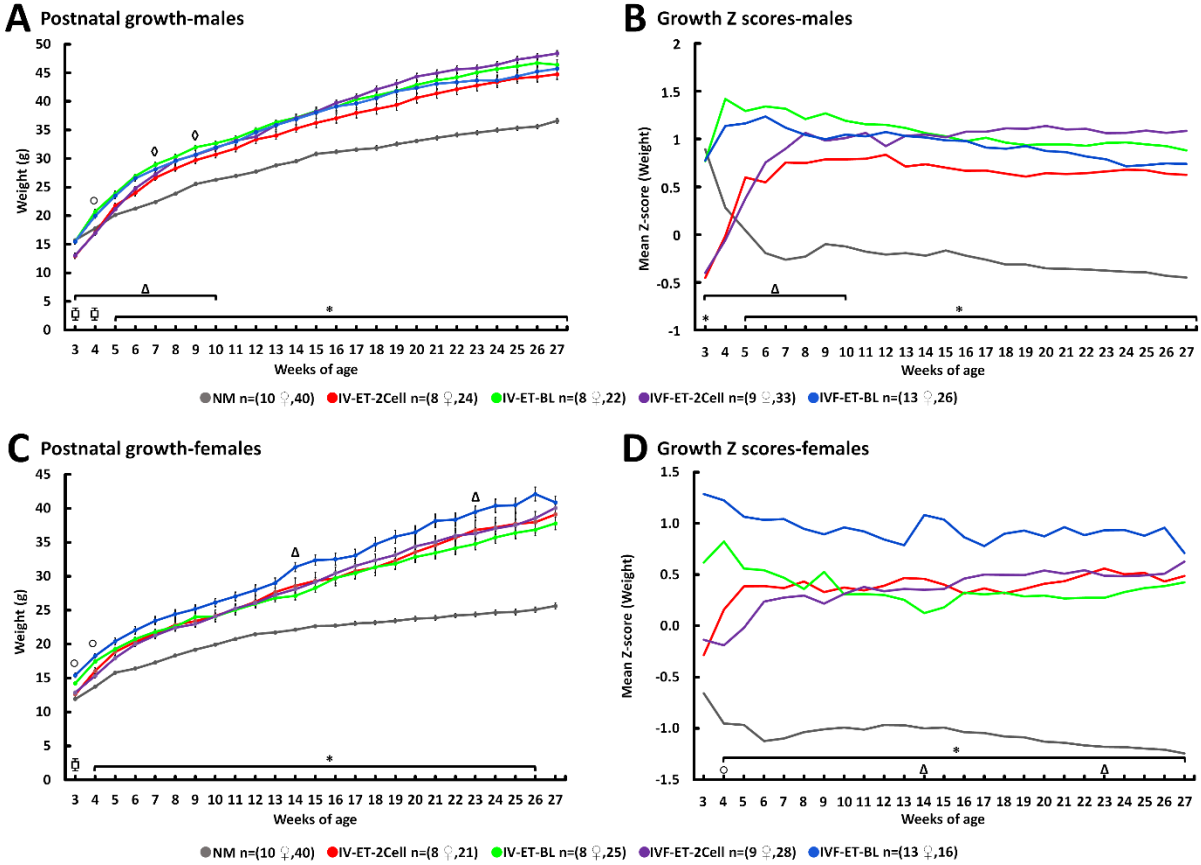

A Postnatal growth-males
Weight (g)
Weeks of age
B Growth Z scores-males
Mean Z-score (Weight)
Weeks of age
NM n=(10 ♀,40) IV-ET-2Cell n=(8 ♀,24) IV-ET-BL n=(8 ♀,22) IVF-ET-2Cell n=(9 ♀,33) IVF-ET-BL n=(13 ♀,26)
C Postnatal growth-females
Weight (g)
Weeks of age
D Growth Z scores-females
Mean Z-score (Weight)
Weeks of age
NM n=(10 ♀,40) IV-ET-2Cell n=(8 ♀,21) IV-ET-BL n=(8 ♀,25) IVF-ET-2Cell n=(9 ♀,28) IVF-ET-BL n=(13 ♀,16)

Figure 4.

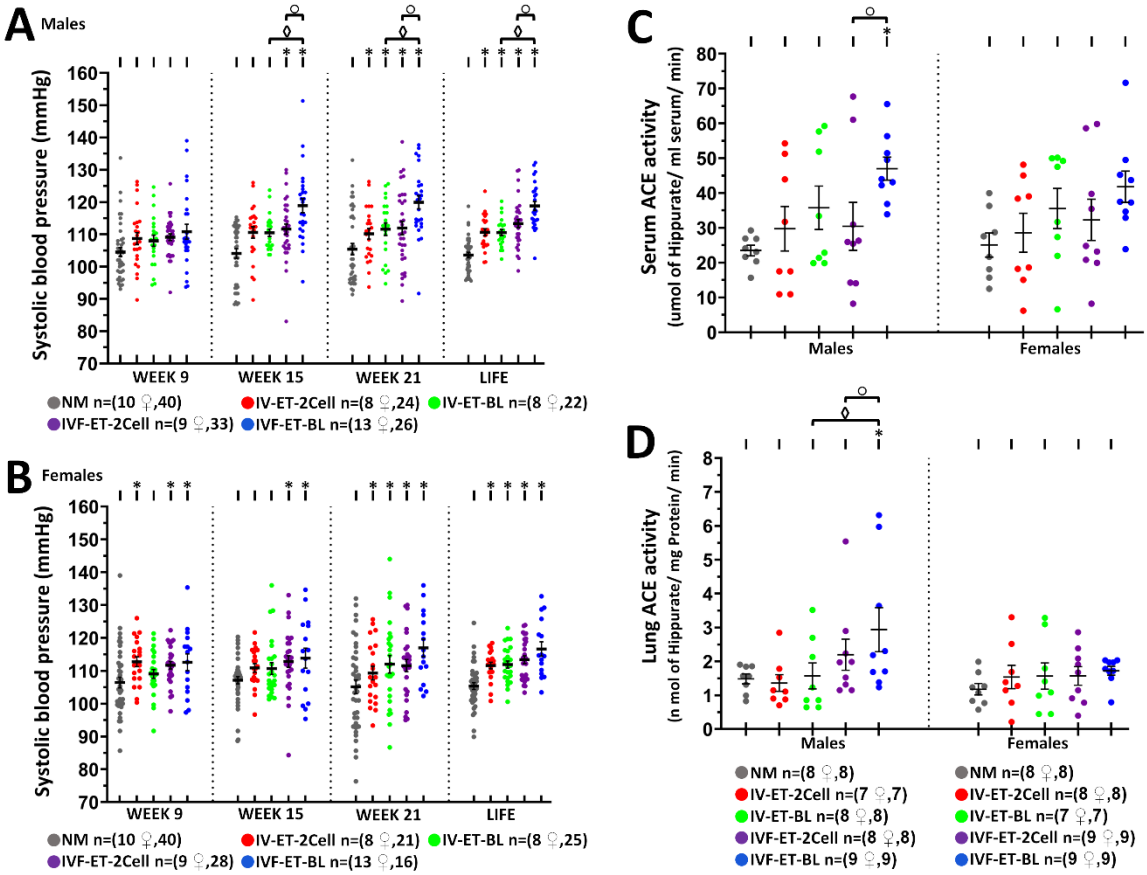

A
Males
Systolic blood pressure (mmHg)
WEEK 9
WEEK 15
WEEK 21
LIFE
NM n=(10 ♀,40)
IV-ET-2Cell n=(8 ♀,24)
IV-ET-BL n=(8 ♀,22)
IVF-ET-2Cell n=(9 ♀,33)
IVF-ET-BL n=(13 ♀,26)
B
Females
Systolic blood pressure (mmHg)
WEEK 9
WEEK 15
WEEK 21
LIFE
NM n=(10 ♀,40)
IV-ET-2Cell n=(8 ♀,21)
IV-ET-BL n=(8 ♀,25)
IVF-ET-2Cell n=(9 ♀,28)
IVF-ET-BL n=(13 ♀,16)
C
Serum ACE activity
(umol of Hippurate/ ml serum/ min)
Males
Females
D
Lung ACE activity
(n mol of Hippurate/ mg Protein/ min)
Males
Females
NM n=(8 ♀,8)
IV-ET-2Cell n=(7 ♀,7)
IV-ET-BL n=(8 ♀,8)
IVF-ET-2Cell n=(8 ♀,8)
IVF-ET-BL n=(9 ♀,9)
NM n=(8 ♀,8)
IV-ET-2Cell n=(8 ♀,8)
IV-ET-BL n=(7 ♀,7)
IVF-ET-2Cell n=(9 ♀,9)
IVF-ET-BL n=(9 ♀,9)

Figure 5.

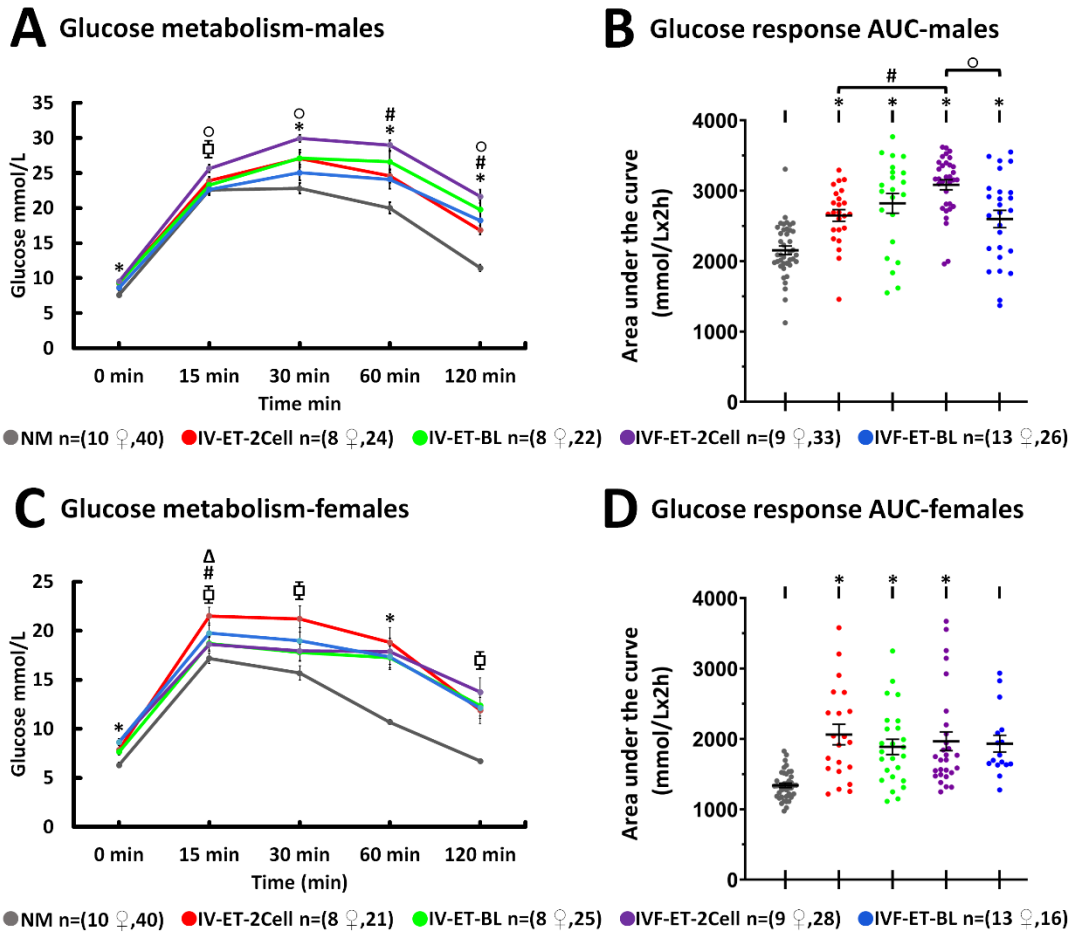

A Glucose metabolism-males
Glucose mmol/L
Time min
0 min
15 min
30 min
60 min
120 min
B Glucose response AUC-males
Area under the curve (mmol/Lx2h)
NM n=(10 ♀,40)
IV-ET-2Cell n=(8 ♀,24)
IV-ET-BL n=(8 ♀,22)
IVF-ET-2Cell n=(9 ♀,33)
IVF-ET-BL n=(13 ♀,26)
C Glucose metabolism-females
Glucose mmol/L
Time (min)
D Glucose response AUC-females
Area under the curve (mmol/Lx2h)
NM n=(10 ♀,40)
IV-ET-2Cell n=(8 ♀,21)
IV-ET-BL n=(8 ♀,25)
IVF-ET-2Cell n=(9 ♀,28)
IVF-ET-BL n=(13 ♀,16)

Figure 6.

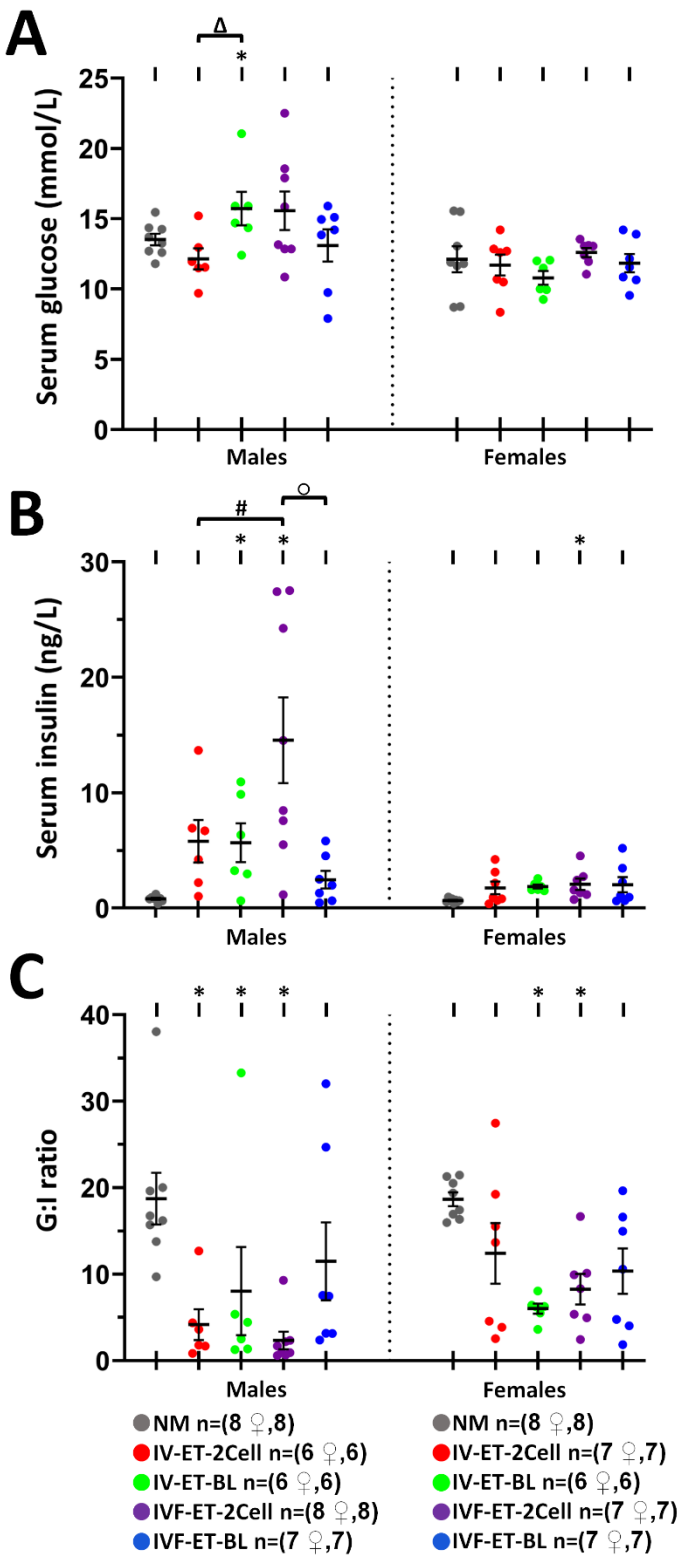

A
Δ
*
Serum glucose (mmol/L)
25
20
15
10
5
0
Males
Females
B
#
*
*
○
*
Serum insulin (ng/L)
30
20
10
0
Males
Females
C
*
*
*
*
*
G:I ratio
40
30
20
10
0
Males
Females
NM n=(8 ♀,8)
IV-ET-2Cell n=(6 ♀,6)
IV-ET-BL n=(6 ♀,6)
IVF-ET-2Cell n=(8 ♀,8)
IVF-ET-BL n=(7 ♀,7)
NM n=(8 ♀,8)
IV-ET-2Cell n=(7 ♀,7)
IV-ET-BL n=(6 ♀,6)
IVF-ET-2Cell n=(7 ♀,7)
IVF-ET-BL n=(7 ♀,7)

Figure 7.

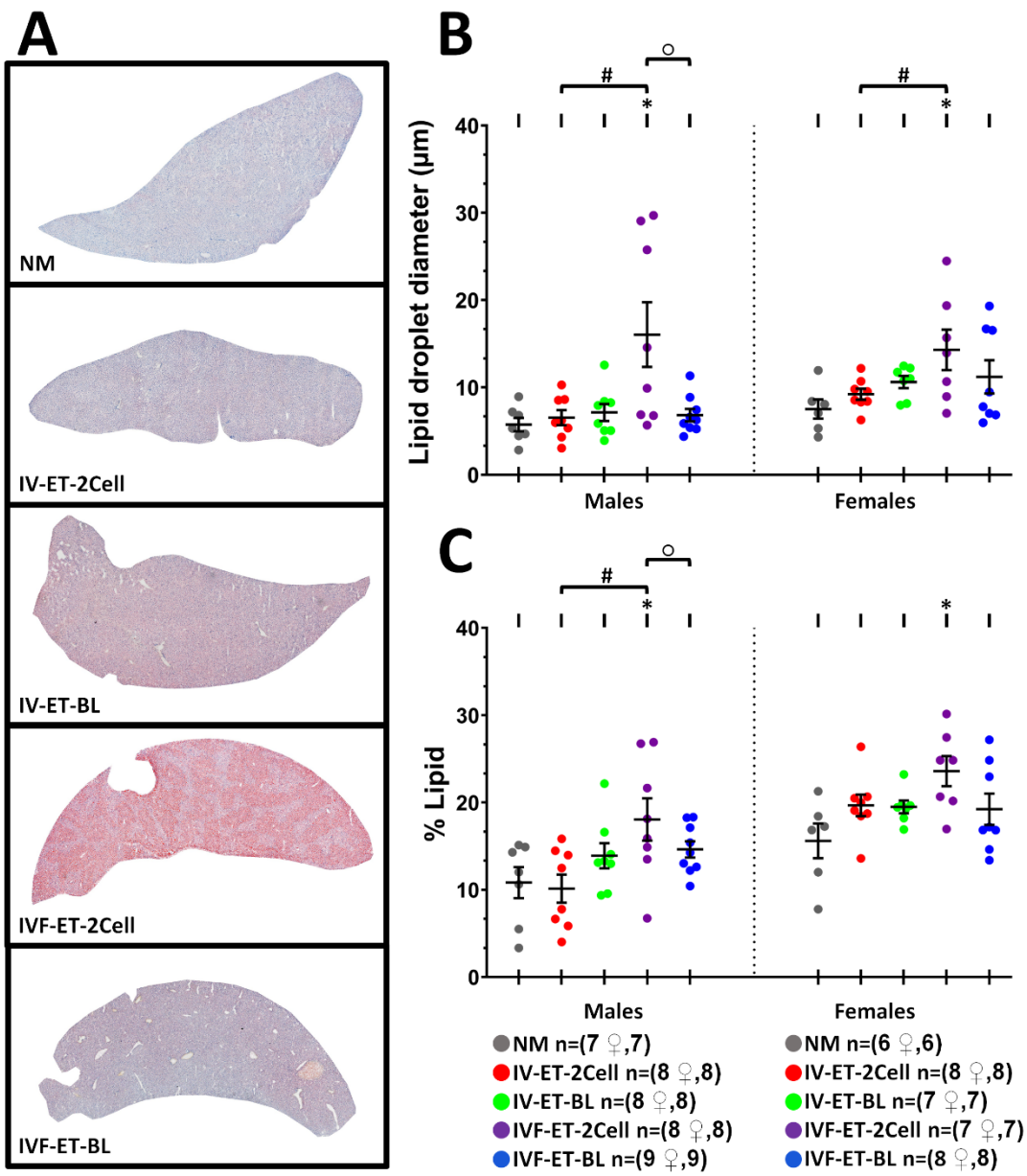
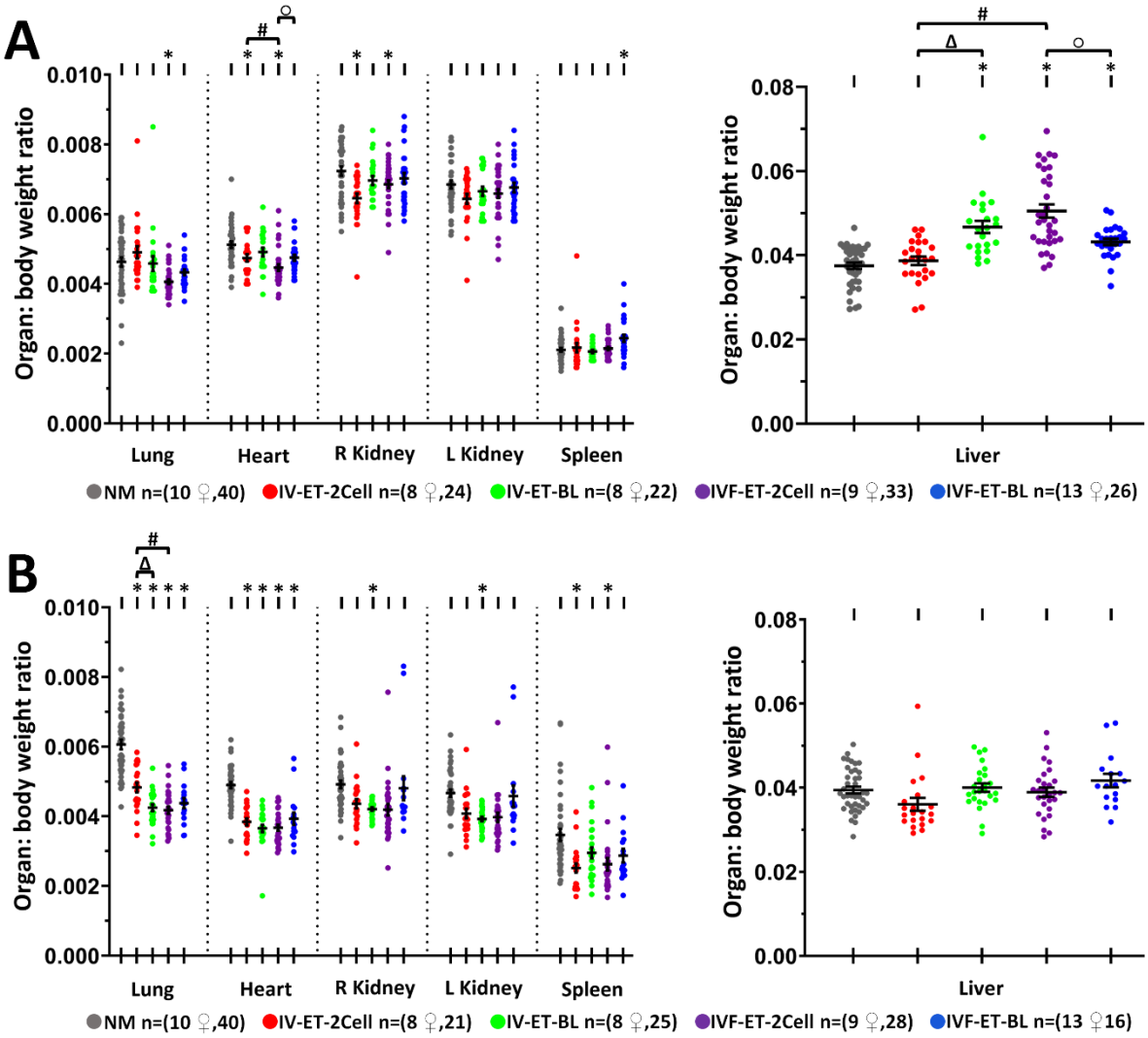

A
NM
IV-ET-2Cell
IV-ET-BL
IVF-ET-2Cell
IVF-ET-BL
B
Lipid droplet diameter (µm)
0
10
20
30
40
#
*
○
Males
Females
C
% Lipid
0
10
20
30
40
#
*
○
Males
Females
NM n=(7 ♀,7)
IV-ET-2Cell n=(8 ♀,8)
IV-ET-BL n=(8 ♀,8)
IVF-ET-2Cell n=(8 ♀,8)
IVF-ET-BL n=(9 ♀,9)
NM n=(6 ♀,6)
IV-ET-2Cell n=(8 ♀,8)
IV-ET-BL n=(7 ♀,7)
IVF-ET-2Cell n=(7 ♀,7)
IVF-ET-BL n=(8 ♀,8)

Figure 8.


A
B
Organ: body weight ratio
0.010
0.008
0.006
0.004
0.002
0.000
0.08
0.06
0.04
0.02
0.00
Lung
Heart
R Kidney
L Kidney
Spleen
Liver
NM n=(10 ♀,40)
IV-ET-2Cell n=(8 ♀,24)
IV-ET-BL n=(8 ♀,22)
IVF-ET-2Cell n=(9 ♀,33)
IVF-ET-BL n=(13 ♀,26)
NM n=(10 ♀,40)
IV-ET-2Cell n=(8 ♀,21)
IV-ET-BL n=(8 ♀,25)
IVF-ET-2Cell n=(9 ♀,28)
IVF-ET-BL n=(13 ♀16)