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ORIGINAL RESEARCH

Metabolic profile of *in vitro* derived human embryos is not affected by the mode of fertilization

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ABSTRACT: The pattern of metabolism by early embryos *in vitro* has been linked to a range of phenotypes, including viability. However, the extent to which metabolic function of embryos is modified by specific methods used during ART has yet to be fully described. This study has sought to determine if the mode of fertilization used to create embryos affects subsequent embryo metabolism of substrates. A metabolic profile, including consumption of key substrates and the endogenous triglyceride content of individual IVF and ICSI supernumerary embryos, was assessed and compared. Embryo development and quality was also recorded. All embryos were donated at a single clinical IVF center, on Day 5, from 36 patients aged 18–38 years, The data revealed that consumption of glucose and pyruvate, and production of lactate, did not differ between embryos created by IVF or ICSI. Similarly, the mode of insemination did not impact on the triglyceride content of embryos. However, ICSI-derived embryos displayed a more active turnover of amino acids ($P = 0.023$), compared to IVF embryos. The specific amino acids produced in higher quantities from ICSI compared to IVF embryos were aspartate ($P = 0.016$), asparagine ($P = 0.04$), histidine ($P = 0.021$) and threonine ($P = 0.009$) while leucine consumption was significantly lower ($P = 0.04$). However, importantly neither individual nor collective differences in amino acid metabolism were apparent for sibling oocytes subjected to either mode of fertilization. Embryo morphology (the number of top grade embryos) and development (proportion reaching the blastocyst stage) were comparable in patients undergoing IVF and ICSI. In conclusion, the microinjection of spermatozoa into oocytes does not appear to have an impact on subsequent metabolism and viability**.** Observed differences in amino acid metabolism may be attributed to male factor infertility of the patients rather than the ICSI procedure *per se*.

Key words: blastocyst / embryo / human / metabolism / ICSI / viability

Introduction

Since the first live birth following IVF in 1978, over 8 million children have been conceived using ART (De Geyter, 2018) The introduction of ICSI in 1992 (Palermo *et al.*, 1992) has in part contributed to this rise, since it revolutionized the treatment of male factor infertility. Worldwide, approximately two-thirds of all ART procedures performed involved ICSI and use has risen in recent years (Dieke *et al.*, 2018), regardless of the etiology of infertility (Nyboe Andersen *et al.*, 2008; Dieke *et al.*, 2018). It has been proposed that ICSI may be beneficial even in cases when no male factor is involved in order to avoid unanticipated failed fertilization, which occurs in an estimated 10% of IVF cycles (Staessen *et al.*, 1999; Komsky-Elbaz *et al.*, 2013).

Higher fertilization rates have been reported for ICSI compared to IVF when comparing sibling oocytes and normozoospermic samples (Komsky-Elbaz *et al.*, 2013) and one study has reported that the proportion of ICSI embryos reaching the 4-cell stage at 42 hours post insemination is significantly higher than that from IVF (Staessen *et al.*, 1999). However, studies have failed to identify any significant impact of either mode of insemination on embryo quality, as assessed using conventional morphological, cleavage stage grading techniques (Calderon *et al.*, 1995; Yang *et al.*, 1996; Ruiz *et al.*, 1997; Komsky-Elbaz *et al.*, 2013). Despite this, questions remain about the safety of the ICSI technique since microinjection and the manipulations involved make ICSI significantly more invasive than conventional IVF. Such *in vitro* handling techniques reportedly increase the possibility of detrimental epimutations (Morgan and Whitelaw, 2008; Rivera *et al.*, 2008).

The ICSI procedure carries several identified risks; there is the possibility of damage incurred during the injection process, for example, the risk of disturbing the spindle during the introduction of the pipette (Blake *et al.*, 2000; Dumoulin *et al.*, 2001) and the risk of injecting foreign contaminants. Moreover, ICSI bypasses the normal spermatozoon–oocyte interaction and fusion process since the zona pellucida and oolema are selective for binding sperm with normal

morphology (Garrett *et al.*, 1997), whereas in ICSI any oocyte-led selection of the spermatozoa is bypassed entirely. In addition, in the pig, sperm nuclear decondensation has been shown to be delayed in ICSI compared to IVF (Katayama *et al.*, 2002). One explanation for this may be the retention of the acrosome, which may physically disturb sperm chromatin decondensation, resulting in asynchronized decondensation of sperm chromosomes (Terada *et al.*, 2000). Furthermore, there are reported differences in the pattern of calcium-induced transients (Tesarik *et al.*, 2004), replication of the genome and positioning of chromosomes (Terada *et al.*, 2000) between ICSI- and IVF-derived zygotes.

A number of studies have reported that children born as a result of ART have increased frequencies of a number of disorders of epigenetic etiology (DeBaun *et al.*, 2003; Maher *et al.*, 2003; Moll *et al.*, 2003; Halliday *et al.*, 2004). Studies specifically comparing malformation rates in IVF- and ICSI-conceived children have thus far been inconclusive, although recent evidence hints that males born from ICSI might have depressed sperm counts (Belva *et al.*, 2016). Some reports have suggested that ICSI births are more likely to be associated with imprinting errors leading to disorders such as Beckwith–Wiedemann syndrome and Angelmann syndrome (Aytoz *et al.*, 1998; Cox *et al.*, 2002). However, meta-analyses comparing malformation rates in infants conceived naturally and through IVF or ICSI have failed to reach a satisfactory consensus. The lack of an appropriate control group means that it has not been possible to determine if the risks are due to the influence of infertility *per se* or the specific interventions used in ART (Rimm *et al.*, 2004).

Studies on individual supernumerary embryos generated from IVF and ICSI patients have also informed the debate surrounding the safety of these techniques. Thus, Munne *et al.* (1998) reported no differences between rates of chromosome abnormalities from IVF and ICSI embryos. Likewise, Santos *et al.* (2010) concluded that ICSI does not lead to an increased incidence of epigenetic errors. However, in a murine model using sibling oocytes fertilized by IVF or ICSI, Bridges *et al.* (2011) observed significant differences in the expression of a number of genes that regulate metabolic pathways, including cholesterol and lipid metabolism/catabolism.

Embryo metabolism may provide an objective biomarker of human embryo physiology, and techniques are available to examine the depletion of glucose and pyruvate and appearance of lactate, the turnover of amino acids and the total content of triglyceride within single human embryos. Compelling evidence has indicated that the consumption and release of metabolites into embryo culture medium can serve as a quantifiable means of assessing embryo quality (Houghton *et al.*, 2002; Brison *et al.*, 2004; Sturmey *et al.*, 2010; Gardner *et al.*, 2011). Collectively, these data indicate that during each development stage there are upper and lower values of metabolic normality, outside which embryos show a decline in viability (Gardner *et al.*, 2011; Guerif *et al.*, 2013), a concept described as the 'Goldilocks Zone' by Leese *et al.* (2016). Comparable data from animal studies (Rivera *et al.*, 2008; Sturmey *et al.*, 2010; Van Hoeck *et al.*, 2011; Guerif *et al.*, 2013) indicate that early embryo metabolism impacts on the physiology and health of the early embryo, in a way which might persist into adulthood, and it is therefore vital to determine the effect in human embryos. However, to date, the impact of IVF and ICSI techniques on embryo metabolism in humans has not been evaluated directly. Uppangala *et al.* (2016) described differences in amino acid metabolism between embryos derived from parents with infertility ascribed to male factor, compared to embryos from cases of tubal factor infertility. However, the potentially critical role of insemination technique on embryo metabolism, which impacts on the success of pregnancy and health of the offspring, has yet to be explored.

In this study we have used a range of quantitative metabolic assessments to compare the phenotype and viability of individual human embryos produced by IVF with those derived through ICSI. Importantly, we have analyzed data from couples undergoing a split IVF/ICSI treatment cycle. Such approaches provide the opportunity to determine if embryo metabolism is linked to the mode of insemination or relates to the severity of male factor infertility of the patients treated.

Materials and Methods

Patient selection and embryo source

All research was carried out according to licence conditions of the Human Fertilisation and Embryology Authority (Licence R0067), with full ethical approval (09/HI304/44). All patients indicating a willingness to be approached about research were given the opportunity to participate in the study. Twenty-nine consecutive patients presenting for ART at the Hull IVF Unit donated a total of 150 embryos with full informed consent. In this cohort there were 91 IVF and 59 ICSI embryos (study group A). During the course of further study, a small number of patients who had borderline 'normal' semen evaluations had elected to undergo split IVF/ICSI treatment and all such patients willing to participate in research were included in the study. This led to an additional seven patients who donated 26 embryos that had been generated from IVF and 24 produced by ICSI after sibling oocytes had been randomly assignment to a treatment group (study group B).

Patient data available from routine clinical investigation included a description of the cause of infertility, hormonal profile and antral follicle counts as well as details of the treatment, including the number and quality of eggs retrieved, rates of fertilization and embryo development, pregnancy and miscarriage.

Ovarian stimulation and oocyte collection were performed as described in Dickerson *et al.* (2010). Oocytes were cultured at 37°C in 6%CO_2/5%O_2, in Sage Fertilization Medium (Cooper Surgical, Trumball, CT, USA). Oocytes were fertilized by conventional IVF or ICSI on the basis of semen quality in accordance with the World Health Organization (WHO) guidelines (World Health Organization, 2010). In cases of a 'borderline' normal semen result, sibling oocytes were assigned randomly to IVF or ICSI treatment; both procedures were performed simultaneously within 5 hours after retrieval. A semen result was classified as 'borderline', if at least one of the individual parameters was below the WHO reference value, but within the calculated uncertainty of measurement for the laboratory, as per ISO15189 sample reporting requirements. Embryos were cultured in groups until Day 3 in 1 ml droplets of Sage Cleavage medium and in Sage Blastocyst medium (Cooper Surgical) until Day 5. One embryo was transferred to the patient on Day 5.

Research embryo culture and assessment

Surplus embryos that on the afternoon of Day 5 were not suitable for transfer or cryopreservation were donated to research, with full

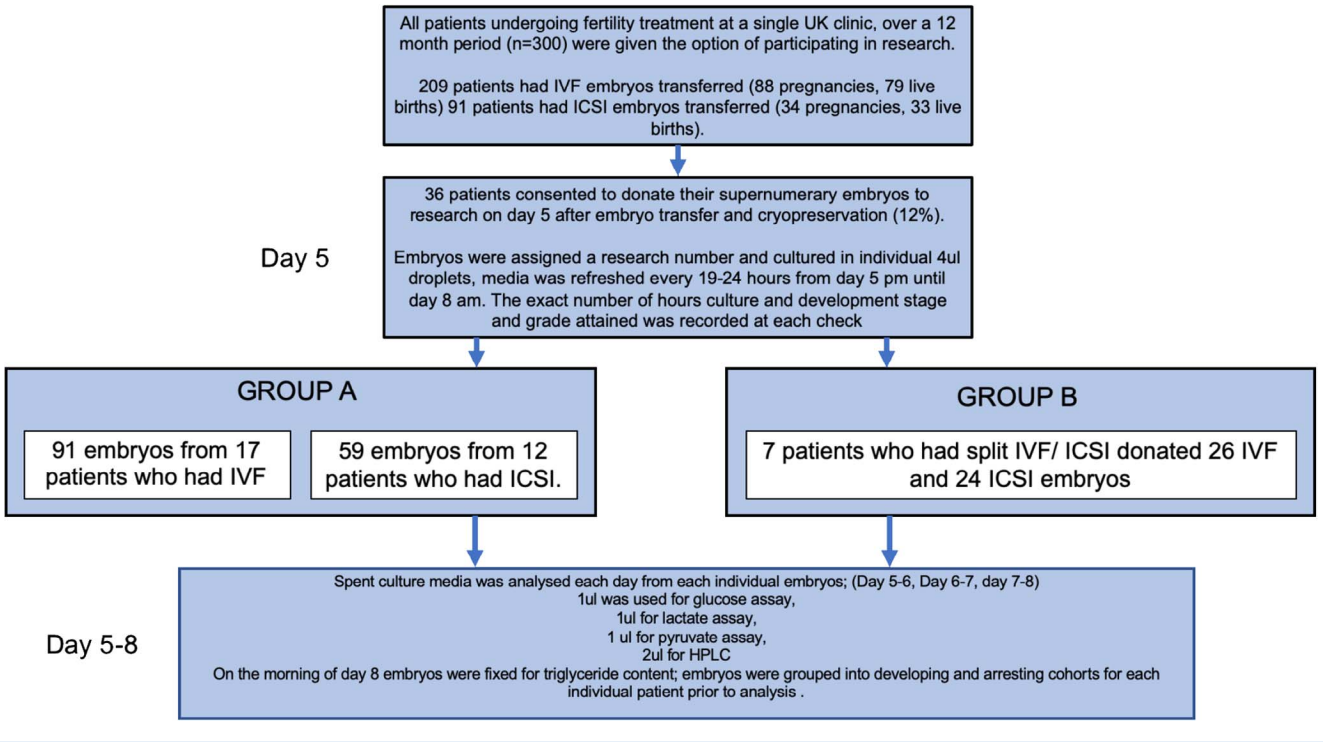


Figure 1 Overview of study design depicting patient, embryo and sample utilization. HPLC: high performance liquid chromatography.

patient consent. All reagents were sourced from Sigma Aldrich (Poole, UK), unless stated otherwise. Stage of development was recorded and embryos placed individually into 5 µl droplets of culture medium specifically validated for use with metabolic assays: an Earl's balanced salt solution, supplemented with 0.5%(v/v) human serum albumin, 1 mM glucose, 0.47 mM pyruvate, 5 mM Lactate and a physiological mixture of amino acids (Houghton *et al.*, 2002). Embryos were cultured under oil at 37°C in 6%CO_2/5%O_2, for 19–24 hours alongside embryo-free control drops. Culture media was refreshed every 24 hours and stage of embryo development and quality was recorded. As observations were taken across a period of 24 h, the day that observation started was used to describe embryos. Embryos that had failed to form a blastocyst, but continued to develop and undergo cell division were classified as slow developing, whereas those that reached the blastocyst stage were classified according to their degree of expansion, according to European consensus (Balaban *et al.*, 2011). An embryo that failed to change in development stage after 48 hours in culture was classified as arrested. Observations were continued until Day 8, or developmental arrest, to permit data capture from slower developing embryos. After each period of 19–24 hours incubation, the spent culture medium was frozen immediately at −80°C for subsequent metabolic analysis (Fig. 1).

Metabolic profiling

Metabolic profiles were determined by measuring the depletion and appearance of glucose, pyruvate, lactate and 18 amino acids, according to established techniques that may be applied to individual oocytes and embryos (Guerif *et al.*, 2013) and provide quantitative markers of embryo health.

Depletion of glucose and pyruvate consumption and accumulation of lactate were measured using ultramicrofluorometric assays, as described in Leese and Barton (1984) and modified by Guerif *et al.* (2013). Some of the sample medium (1 µl) was added to 10 µl of assay mixture. Uptake or production measurements were determined in triplicate for standards and control droplets; however, single measurements were taken from test droplets to maximize the number of assays that could be performed simultaneously.

A coupled colorimetric assay, as described in Sturmey and Leese (2003), was used to measure the triglyceride content of groups of 2–5 embryos that had reached equivalent development stages for each patient at the end of the culture period. Analysis of pooled embryos was necessary to overcome the limits of sensitivity of the assay, and thus data points for individual embryos are not shown for these assays.

Amino acid concentrations in spent culture droplets were determined using reverse-phase high performance liquid chromatography (HPLC), as described in Sturmey *et al.* (2009), using an Agilent 1100 HPLC system (Agilent Technologies, Stockport, UK). Briefly, 2 µl of sample was diluted 1:12:5 in HPLC grade water. Amino acid concentrations were determined by analyzing area under the curve of individual peaks, which were then normalized to a non-metabolizable internal standard. Mean sums of amino acid production and depletion were expressed in pmol per embryo per hour for Days 5 to 8 of culture. Results were recorded according to stage reached at the end of the period of culture.

Table I Patient demographics for each treatment group are comparable, with the exception of the semen characteristics, which are significantly poorer for the ICSI group.

Group A	IVF mean (SEM) n=17 patients	ICSI mean (SEM) n=12 patients	* *p* value
Female age years	34.67 (0.99)	32.91 (1.44)	0.31
Female BMI m/kg^2	22.89 (0.99)	23.65 (0.94)	0.6
Anti-Mullerian hormone pmol/l	20.66 (4.32)	20.89 (3.75)	0.97
no. oocytes	9.6 (0.98)	9.91 (0.83)	0.82
Male age years	36.33 (1.26)	38.73 (2.12)	0.31
Male BMI m/kg^2	26.77 (0.86)	24.42 (0.86)	0.07
Sperm concentration M/ml	77.04 (14.93)	18.42 (5.11)	<0.01
Sperm motility %	67.0 (2.97)	40.91 (4.37)	<0.001
Sperm morphology %	7.20 (0.34)	3.73 (0.45)	<0.001
no. fertilized oocytes	7.0 (0.94)	6.0 (0.38)	0.39
no. top grade embryos	3.73 (0.71)	3.0 (0.56)	0.73
no. blastocysts	1.67 (0.37)	1.64 (0.39)	0.96
Clinical pregnancy rate %	10/17 58.8	3/12 25	0.13
Live birth rate %	10/17 58.8	3/12 25	0.13
Gestation (weeks)	39.6 (0.57)	40.5 (0.5)	0.59
Birthweight (g)	3244.7 (231.5)	3620.0 (220.0)	0.46

*Student's t-test, and Fishers exact test.

All values for substrate depletion/appearance are expressed as pmol per embryo per hour. This was calculated by subtracting the amount of a given substrate in the embryo droplet from that of the undisturbed medium controls. Where the concentration of a substrate was lower in the embryo droplet than the control, a negative value was recorded and this was assumed to represent depletion; where the concentration in the embryo droplet was greater than that of the control, a positive value was indicated and this was assumed to represent release from the embryo into the medium. The difference from control values was then divided by the precise duration of culture in hours i.e. (i) afternoon of Day 5–6 (i.e. Day 5) ~19 hours; Day 6–7 (i.e. Day 6) and Day 7 and (ii) morning of Day 8 (i.e. Day 7); each ~24 hours.

Statistical analysis

The data from the metabolic profiling of spare embryos was compared between IVF and ICSI embryos and correlated retrospectively to embryo developmental progress. Data are expressed as pmol per embryo per hour (pmol/e/h). Analyses were performed using GraphPad Prism 8 (GraphPad, San Diego, CA, USA) for macOS and IBM SPSS (V5; IBM Porstmouth, UK). Power calculations were performed using the Birkett and Day (1994) method and studies were designed to achieve 80% power, unless stated otherwise. Based on previous validation studies, the expected blastocyst development rate for supernumerary research embryos is 30% (±12) and therefore a minimum of 56 embryos would be required in each group for 0.8 power to be achieved. Levene's test for normality was performed and parametric/non-parametric methodology was used as indicated. A *P*-value of <0.05 was considered significant.

Univariate regression analysis was used to compare continuous data, while independent sample Student's t-test or Mann Whitney U test were used to compare grouped two-sample data. Intra- and inter-patient variability within the embryo cohort were assessed by ANOVA in combination with multiple log-linear regression analysis, with Holms test (if $F < 0.05$) to assess the predictive accuracy of metabolic profile on blastocyst development rate. Kruskal–Wallis H test was used when data exhibited significant variance in homogeneity and was used to compare metabolic profile differences between IVF and ICSI embryos, with *post hoc* testing (Duncan's test) if the necessary level of statistical significance was achieved. Principal component analysis (PCA) was used to reduce the dimensionality of the individual 18 amino acid measurements and adjust for multiple testing.

Results

Study group A—exclusive IVF or ICSI treatment

In study group A, 58.6% of patients had embryos fertilized by gamete co-incubation ($n = 17$, 91 embryos) and 41.4% had ICSI treatment ($n = 12$, 59 embryos). There were no significant differences between the patient age, BMI, ovarian response, embryo development and implantation. Semen parameters were, however, significantly poorer for the ICSI patient group (Table I; $P < 0.01$). There was no significant difference in blastocyst formation between the groups: 42.9% by Day 7 in the IVF group compared to 32.2% from the ICSI group ($P = 0.23$).

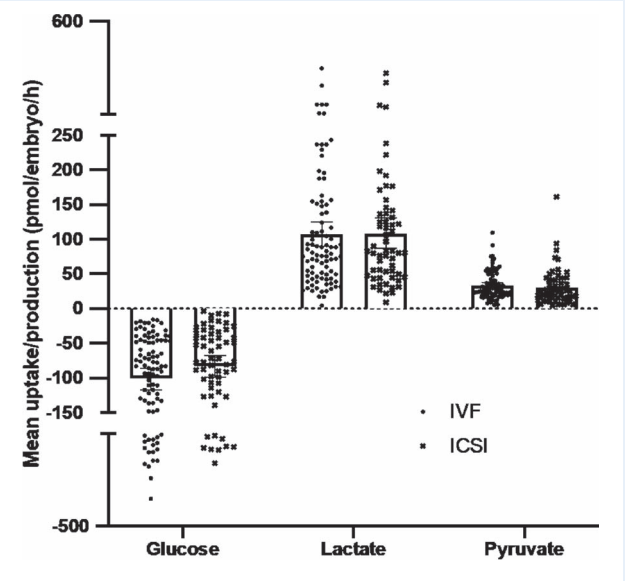


Figure 2 The depletion and release of glucose, pyruvate and lactate by IVF ($n = 91$) and ICSI-derived ($n = 59$) embryos between Days 5 and 7 of culture. Data are expressed as pmol/embryo/hr.

Glucose, lactate and pyruvate

In total, 150 embryos were assayed for glucose and pyruvate metabolism and lactate production. Figure 2 shows that uptake of glucose and pyruvate did not differ according to the method of insemination. Glucose consumption was 101.6 and 83.1, and pyruvate consumption was 33.6 and 31.2 pmol/e/h for IVF and ICSI embryos, respectively. There were also no differences in lactate production (107.8 versus 108.8 pmol/e/h for IVF and ICSI embryos, respectively).

Mean glucose consumption of all embryos fell from 115.8 pmol/e/h on Day 5 to 76.05 pmol/e/h on Day 7; lactate production fell from 114.2 to 90.97 pmol/e/h and pyruvate consumption remained relatively stable, at between 30.05 and 30.42 pmol/e/h, during the period of culture analysis. However, embryos reached the blastocyst stage on different days in culture and the corresponding results displayed a wide range in values. Importantly, when values were evaluated according to stage of development the variation in glucose consumption was reduced dramatically (Table II); embryos at corresponding stages of development had comparable metabolic profiles in the IVF and ICSI cohorts (ANOVA $P > 0.05$). Furthermore, this was independent of grade and the day of development (as determined by one-sample, two-tailed Kolmogorov–Smimov test $P > 0.05$).

There were no significant differences in the kinetics of IVF and ICSI embryos; during the 5–7 days of extended culture 43% (39/91) of IVF embryos continued developing to reach the blastocyst stage, compared to 32% (19/59) of ICSI embryos ($P = 0.23$). The mean (±SEM) glucose consumption for developing blastocysts was 103.5 (±9.9) and 106.9 (±15.4) for IVF and ICSI embryos, respectively ($P = 0.85$). The values for IVF and ICSI embryos, respectively, for pyruvate consumption were 33.8 (±3.3) pmol/e/h and 30.9 (±4.8) ($P = 0.63$) pmol/e/h, and for lactate production were 104.6 (±12.1) pmol/e/h and 105.3 ± (15.9) pmol/e/h ($P = 0.97$). The glycolytic rate was comparable for blastocysts derived from IVF and ICSI embryos.

Multiple logistic regression was used to evaluate the statistical significance of each independent variable to predict blastocyst development and account for possible inter- and intra-patient variability. The mode of fertilization did not influence the probability of continued blastocyst development (odds ratio 1.77 (0.85–3.4)). Glucose uptake was a significant predictor variable (odds ratio 1.02 (1.01–1.02)). Embryos consuming higher quantities of glucose were more likely to form full blastocysts.

Amino acids

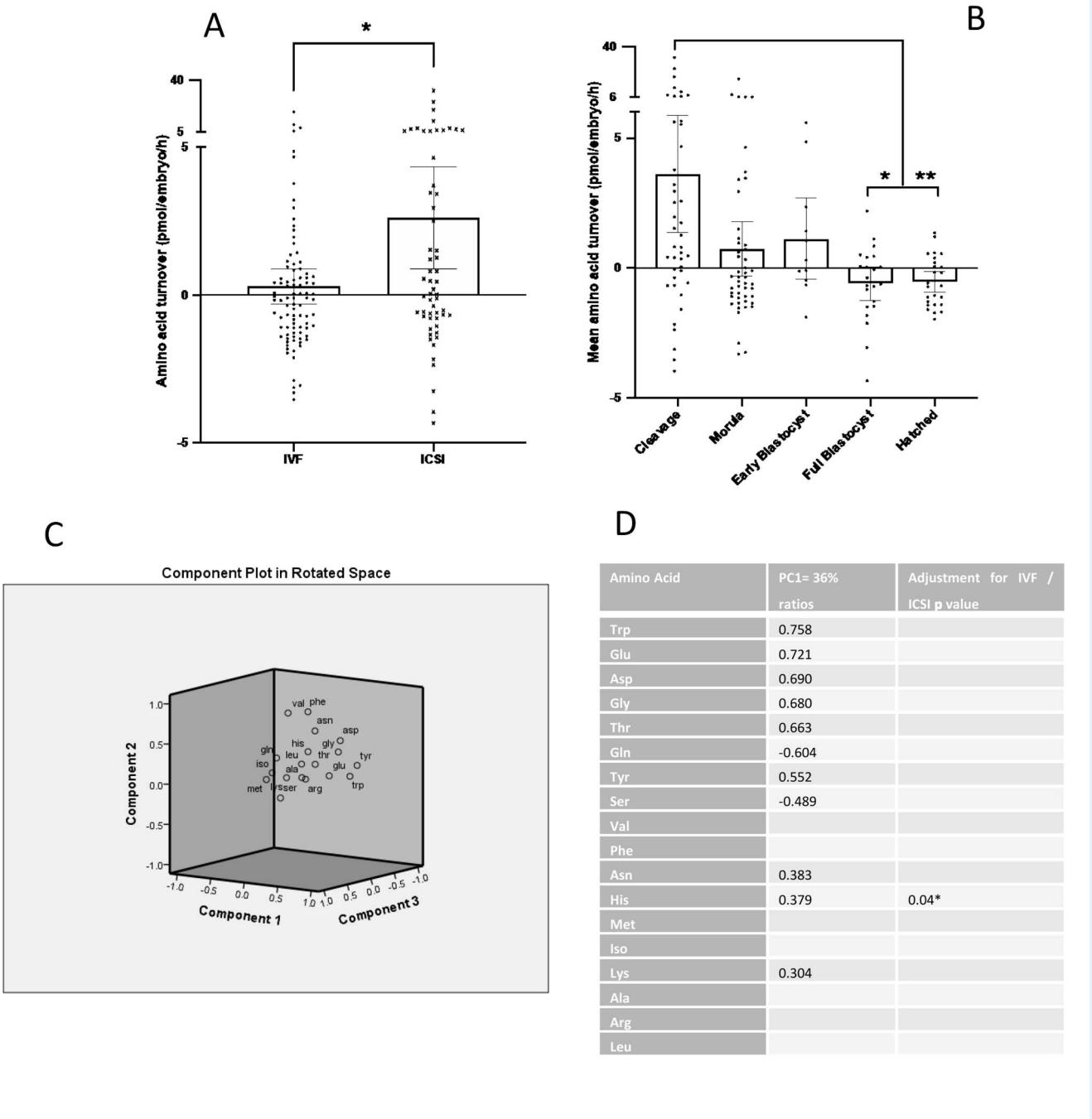
We next examined the amino acid profile of ICSI and IVF embryos and found that ICSI embryos produced significantly more amino acids than IVF-generated embryos ($P = 0.023$; Fig. 3A). Multivariate analysis was used to determine the specific amino acids produced in higher quantities by ICSI embryos, which were aspartate (0.29 for ICSI and 0.17 pmol/e/h for IVF; $P = 0.016$), asparagine (0.23 and 0.04 pmol/e/h, respectively; $P = 0.04$), histidine (0.55 and 0.22 pmol/e/h; $P = 0.021$) and threonine (0.41 and 0.17 pmol/e/h; $P = 0.009$). The consumption of leucine was significantly lower (0.01 for ICSI and 0.23 pmol/e/h for IVF; $P = 0.04$) in ICSI than IVF embryos.

Given the observed differences in amino acid profiles between embryos from patients receiving ICSI versus the IVF counterparts, we further examined the net rates of depletion or appearance of amino acids dependent on the stage of development attained using multiple log-linear regression (Fig. 3B). Despite there being no significant differences in blastocyst development rates between IVF- and ICSI-derived embryos, there was a trend for higher rates of early arrest

Table II Mean metabolite consumption/release according to development stage attained for 91 IVF and 59 ICSI embryos.

	IVF mean (SEM)*				ICSI mean (SEM)*			
	n	Glucose	Lactate	Pyruvate	n	Glucose	Lactate	Pyruvate
Cleavage	19	87.1 (13.9)	96.3 (21.6)	33.8 (4.6)	24	65.5 (11.8)	130.1 (22.5)	31.3 (6.6)
Morula	33	107.8 (16.7)	117.7 (15.5)	33.2 (3.4)	16	81.3 (12.3)	82.5 (10.8)	31.3 (5.9)
Early	9	61.4 (7.7)	73.8 (16.1)	21.0 (7.8)	2	100.9 (13.3)	92.3 (49.1)	12.8 (7.2)
Full	15	125.1 (19.4)	115.6 (20.3)	39.5 (6.9)	7	76.5 (16.2)	96.0 (26.0)	24.0 (7.0)
Hatched	15	107.1 (13.6)	112.1 (22.1)	35.7 (4.2)	10	129.4 (25.5)	114.4 (24.1)	38.3 (7.3)

*all presented as pmol/embryo/h. There were no significant differences between embryos derived from IVF or ICSI at corresponding stages of development (ANOVA $P > 0.05$).



Amino Acid	PC1= 36% ratios	Adjustment for IVF / ICSI p value
Trp	0.758	
Glu	0.721	
Asp	0.690	
Gly	0.680	
Thr	0.663	
Gln	-0.604	
Tyr	0.552	
Ser	-0.489	
Val		
Phe		
Asn	0.383	
His	0.379	0.04*
Met		
Iso		
Lys	0.304	
Ala		
Arg		
Leu		

Figure 3 The effect of mode of fertilization on amino acid metabolism by single human embryos. (**A**) The sum of mean amino acid production and depletion, expressed in pmol/embryo/hour for IVF- and ICSI-derived embryos between Days 5 to 7 of culture. Results are recorded according to IVF ($n = 91$) and ICSI ($n = 59$). Significantly lower production and depletion of amino acids was evident for IVF embryos, $P < 0.05$ (Mann Whitney U test. Error bars represent 95% CI. (**B**) Significant differences in amino acid turnover were evident between those failing to form blastocysts and those blastocysts that were able to complete ($n = 43$ cleavage stage, 48 morula, 11 early, 22 full and 25 hatched). Error bars represent 95% CI, $^*P < 0.05$. (**C**) PC used for all 18 amino acids per embryo for developing blastocysts to identify outlying amino acids and determine differences in IVF ($n = 39$) and ICSI ($n = 19$) populations. Principal component (PC) plot in rotated space rotation converged in 11 iterations (PC1; trp, glu, asp, gly, thr, gln,tyr, ser, asn, his, lys). (**D**) Only histidine is significantly different when PC1 components are separated into IVF and ICSI cohorts. The suitability of the data for this type of analysis was confirmed; standards met = Kiaser–Meyer–Olkin measure of sample adequacy = 0.77, Bartlett's test 0.000).

in the ICSI population. Nineteen of the 91 IVF embryos arrested at the cleavage stage, compared to 24 of the 59 ICSI embryos, and the mean amino acid turnover for these embryos was 1.32 (±0.7) pmol/e/h and 5.45 (±1.8) pmol/e/h, respectively, ($P = 0.07$). Comparatively, 30 IVF embryos reached full or hatched blastocyst stage and 17 ICSI embryos, with mean amino acid turnover rates of −0.48 (±0.2) pmol/e/h

and −0.70 (±0.3) pmol/e/h, respectively ($P = 0.5$). At each individual development stage there were no significant differences in the turnover of amino acids for IVF- versus ICSI-derived embryos.

Overall, embryos that arrested at the earlier stages of development had a higher turnover of amino acids; specifically, asparagine (0.19 compared to −0.01 pmol/e/h; $P = 0.002$), aspartate (0.25 and 0.16 pmol/e/h; $P = 0.01$) and glutamate (0.44 and 0.26 pmol/e/h; $P = 0.001$) were consumed in significantly higher quantities by those arresting, and alanine production was greater (1.35 and 1.25 pmol/e/h; $P = 0.02$) compared to those that formed blastocysts. Those embryos that developed beyond the unexpanded blastocyst stage produced significantly lower quantities of amino acids, but consumption of methionine was significantly higher in developing blastocysts (0.18 and 0.06 pmol/e/h; $P = 0.001$), as was arginine (0.79 and 0.68 pmol/e/h; $P = 0.003$), and histidine production was high in expanded and hatched blastocysts (0.38 and 0.28 pmol/e/h, respectively; $P = 0.01$).

The data from the 18 amino acids were transformed by PCA, as it is likely that differences in the consumption and release of individual amino acids may be related. Therefore, prior to further multivariate testing, the variance for each of the 18 individual amino acids was used to create a smaller set of linear combinations. The data were transformed with five eigenvectors, accounting for 69% of the total variance. A three-component solution explains 54.9% of the data and is presented in Fig. 3C. Component 1 (PC1) accounted for 36% of the variance and the majority of the amino acids included in PC1 are neutral and polar and appear to belong to system N transporters. After the PC adjustment for covariation, a permutated-based adjustment of P values was undertaken—only histidine production significantly differed ($P < 0.05$) between developing blastocysts generated through IVF and ICSI.

Triglyceride

Reassuringly, the mode of insemination did not impact on the triglyceride content of embryos (Fig. 4A). However, by Day 7 of development, striking differences were observed in the triglyceride content of embryos reaching the blastocyst stage compared to those undergoing early cleavage stage arrest (6.7 ng versus 11.32 ng, respectively; $P < 0.001$). Blastocysts generated through both ICSI and IVF contained significantly less triglyceride than their cleavage-stage-arrested counterparts (Fig. 4B).

Study group B; IVF/ICSI sibling embryos

To understand more about the possible impact that the injection procedure could have on subsequent embryo metabolism, 50 sibling embryos derived by IVF or ICSI were compared. Blastocyst development rates were comparable ($P = 0.11$), as were rates of uptake and production of glucose ($P = 0.47$), lactate ($P = 0.75$) and pyruvate ($P = 0.93$) for IVF and ICSI embryos (Fig. 5A). Similarly, there were no significant differences in the triglyceride content of ICSI and IVF sibling embryos (Fig. 5C).

Amino acids

Individual and collective differences in amino acids were not apparent for sibling embryos created by IVF or ICSI (Fig. 5B). Interestingly, amino acid turnover was higher for IVF embryos in group B patients who had borderline suboptimal semen parameters, compared to embryos generated via IVF in group A patients, who had normal semen parameters (0.30 versus 0.83 pmol/mbryo/h for groups A and B, respectively) and, conversely, amino acid turnover was lower for ICSI embryos in group B (borderline suboptimal semen evaluation) compared to group A patients (abnormal semen evaluation).

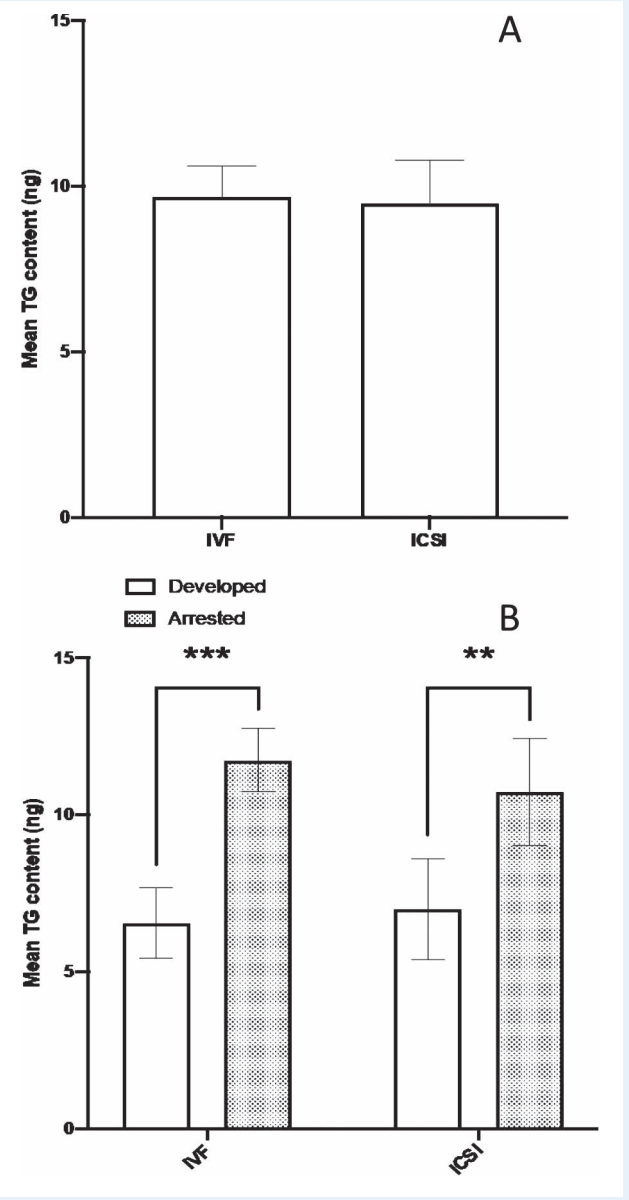


Figure 4 Mode of fertilization does not affect mean triglyceride content of human embryos. (**A**) Mean triglyceride (TG) content of embryos fertilized by IVF ($n = 86$) or ICSI on Day 7 ($n = 54$), expressed as ng/embryo. (**B**) TG content of embryos according to development stage attained for ICSI- and IVF-generated embryos $n = 34$ developed IVF and 52 arrested; $n = 18$ developed ICSI and 36 arrested embryos on Day 7. Error bars represent 95% CI, $^{**}P < 0.01$ $^{***}P < 0.001$, Mann Whitney U test. No sample results possible for 10 embryos, as samples perished during this analysis.

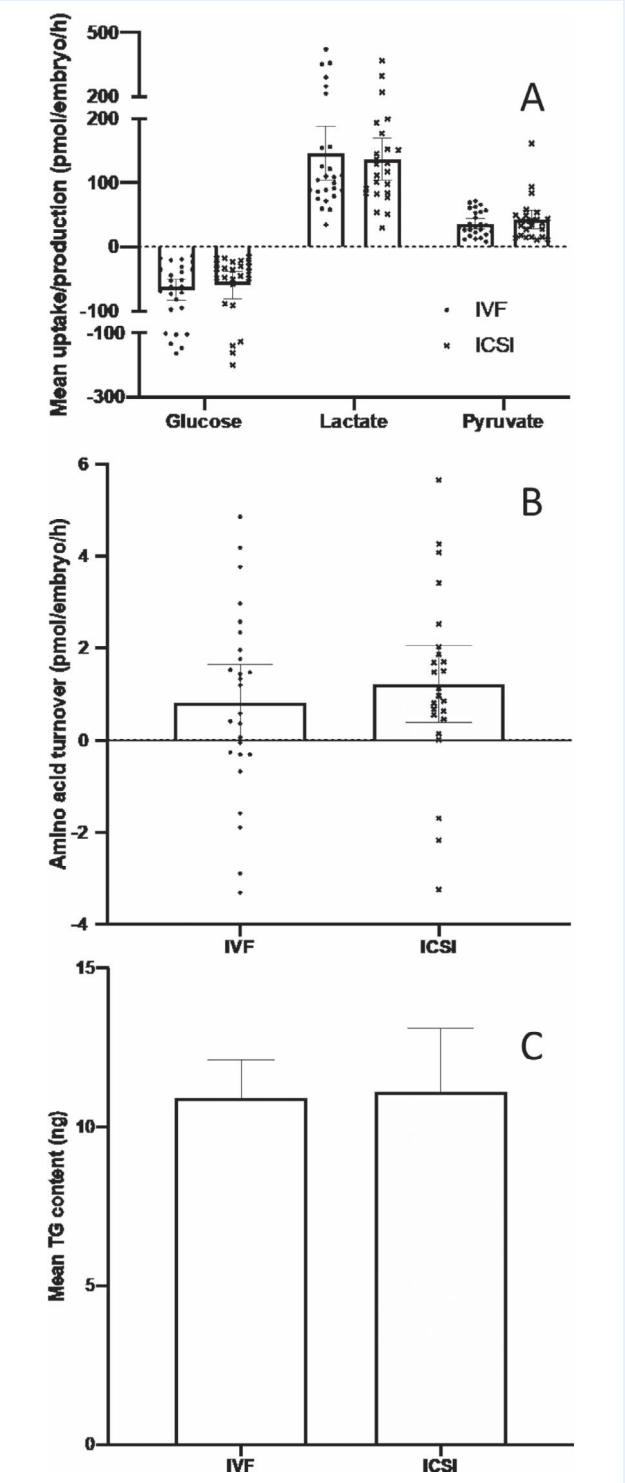


Figure 5 Metabolic profile of sibling embryos does not appear to be influenced by mode of fertilization. (**A**) Mean glucose and pyruvate uptake and lactate production between Days 5 and 7 of culture, expressed as pmol/embryo/h for sibling embryos derived by IVF ($n = 26$) or ICSI ($n = 24$). (**B**) Mean sums of amino acid production and depletion, Days 5–7; comparable for sibling embryos ($P = 0.39$). (**C**) TG content on Day 7 for sibling embryos derived by IVF ($n = 21$) or ICSI ($n = 22$). No sample results possible for TG analysis for $n = 5$ IVF and $n = 2$ ICSI.

Discussion

A number of studies have now been published identifying a higher incidence of rare imprinting disorders in children born following ICSI treatment (Vermeiden and Bernardus, 2013; Lazaraviciute *et al.*, 2014; Uk *et al.*, 2018). In addition, animal studies have shown significant differences in the expression of genes that regulate metabolic pathways, including cholesterol and lipid metabolism/catabolism in embryos derived from IVF and ICSI (Sanchez-Calabuig *et al.*, 2014). We therefore set out to discover if the early stages of development, from cleavage divisions to blastocyst formation, and the corresponding processes of embryo metabolism are perturbed in embryos produced by ICSI. In this study, we have shown that embryo morphology (the number of top grade embryos) and development (proportion reaching the blastocyst stage) were comparable in patients undergoing IVF and ICSI. This is consistent with the previously reported findings of Plachot *et al.* (2002) and Fishel *et al.* (2000), but contrary to the findings of Griffiths *et al.* (2000) who reported that blastocyst development rates were compromised by the ICSI procedure. Such studies have given inconsistent results and the subjective nature of embryo grading schemes challenges the validity of the conclusions that can be reached. By contrast, the use of metabolic biomarkers enables quantitative comparisons of embryo physiology and viability to be made.

The metabolism of substrates is indicative of embryo developmental progress, with embryos developing to blastocysts having higher glycolytic activity and a lower triglyceride content than those arresting at the early stages of development (Gardner *et al.*, 2011; Leary *et al.*, 2015). The data presented in this analysis illustrate an altered amino acid metabolism at each developmental stage. Reassuringly, the data presented show that the method of fertilization had only a limited impact on the developmental stage specific utilization of substrates.

Our data revealed that glucose and pyruvate depletion and lactate release into the medium did not differ according to the mode of fertilization. Moreover, levels of endogenous triglyceride were not influenced by mode of fertilization. However, when we compared embryos from patients undergoing IVF with those having an ICSI cycle, we observed significant differences in overall amino acid profile. Embryos from couples undergoing ICSI had a higher overall amino acid metabolism, despite there being no significant differences in development stage attained for embryos in each group. Increased amino acid turnover by embryos has previously been associated with sub-optimal embryo viability (Houghton *et al.*, 2002; Brison *et al.*, 2004; Picton *et al.*, 2010). Indeed, our own data here support the notion that amino acid profile differs significantly between embryos that develop and those which arrest. Notably, once results had been corrected to control for confounding variables and adjusted for multiple testing, histidine was produced in higher quantities from ICSI-generated embryos. Histidine is transported by a system N mechanism of amino acid transport, a Na^+-dependent channel that is mostly expressed in the central nervous system. The significance of this finding is not clear, especially since histidine handling has not previously been reported to differ between viable and non-viable human embryos (Houghton *et al.*, 2002; Brison *et al.*, 2004; Picton *et al.*, 2010), although it does differ between *in vivo* derived cattle embryos and those created by IVF (Sturmey *et al.*, 2010).

As we were unable to conclude from this data set whether it was the mode of fertilization that caused a difference in metabolism, or whether the origin of these differences was a feature of the cause of

infertility, we compared the impact of the mode of fertilization on the metabolism of sibling oocytes using semen from men with mild factor male infertility (study group B). In this analysis, there were no significant differences in the metabolic utilization and fate of glucose, lactate and pyruvate. Triglyceride levels were comparable in stage-equivalent embryos. Importantly, the observed differences in amino acid turnover were no longer apparent and likewise there were no significant differences in any of the 18 individual amino acids. Amino acid turnover was marginally higher for IVF-derived embryos created in group B than IVF embryos from group A; the difference being that in group B patients semen parameters were in the borderline normal/abnormal range. One plausible explanation for the general rise in amino acid turnover for embryos generated from poorer quality semen samples may be related to higher levels of molecular damage. A higher incidence of numerical and structural chromosome aberrations is associated with suboptimal semen parameters (Sakkas *et al.*, 2002). Metabolic activity has been correlated with molecular damage; embryos with lower levels of damage have characteristically low metabolic activity (Sturmey *et al.*, 2009). This finding would seem to suggest that the differences identified in group A between IVF and ICSI embryos could be attributed to the underlying andrological infertility. It is thus plausible that the underlying male infertility and any sperm DNA damage is promutagenic and may introduce mutations that become fixed in the germline (Cox *et al.*, 2002).

The main limitation of this study is that adverse effects of fertilization route may not manifest at cleavage and blastocyst stage. Implantation is a test of embryo viability; however, the metabolic studies were performed on poorer quality sibling embryos that were not suitable for transfer or cryopreservation. In addition, it may well be that any metabolic differences between IVF- and ICSI-conceived populations do not become apparent until organ morphogenesis is complete. Some degree of assurance is granted by the finding that rates of spontaneous abortions are comparable for IVF and ICSI conceptions (Bettio *et al.*, 2008). In addition, studies comparing ICSI children with the general population have reported no differences in development, growth, weight, height, head circumference and pubertal staging (Belva *et al.*, 2008, 2011; Wennerholm *et al.*, 2009). Likewise, there are no reported differences in cognitive and motor development between IVF- and ICSI-conceived children (Rimm *et al.*, 2004; Bonduelle *et al.*, 2005). The caveat of these reports is that many of the studies have a high incidence of patients lost to follow-up.

In conclusion, the data presented provide assurances about the ICSI procedure; however, the aim of any reproductive intervention should be to use the simplest, least expensive procedure, with the greatest chance of producing healthy children (Barker *et al.*, 1993). It would therefore seem reasonable to suggest that ICSI should be reserved for male factor cases of infertility. The microinjection of spermatozoa into oocytes does not significantly impact on the metabolism and viability embryos. However, andrological infertility of the patients rather than the ICSI procedure *per se* may lead to minor alterations of embryo metabolism post fertilization.

Authors' roles

RGS and CL conceived the study, CL conducted the analysis, RGS and CL analyzed the results, RGS and CL wrote the manuscript.

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Conflict of interest

The authors declare that there are no conflict of interests relating to this work.

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