Accepted Manuscript

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PII: S0301-2115(18)31067-4
DOI: https://doi.org/10.1016/j.ejogrb.2018.10.053
Reference: EURO 10601

To appear in: *EURO*

Received date: 13 August 2018
Revised date: 26 October 2018
Accepted date: 28 October 2018

Please cite this article as: Ferrarini Zanetti B, de Almeida Ferreira Braga DP, Souza Setti A, de Cássia Sávio Figueira R, Iaconelli A, Borges E, Is perivitelline space morphology of the oocyte associated with pregnancy outcome in intracytoplasmic sperm injection cycles?, *European Journal of Obstetrics and Gynecology* (2018), https://doi.org/10.1016/j.ejogrb.2018.10.053

Title:Is perivitelline space morphology of the oocyte associated with pregnancy outcome in intracytoplasmic sperm injection cycles?

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Abstract

Objective(s): To investigate the effect of perivitelline space (PVS) abnormalities on the outcomes of intracytoplasmic sperm injection (ICSI) cycles in which the entire cohort was affected.

Study Design: Data from 9,752 oocytes obtained from 1,151 ICSI cycles performed from June/2010 to August/2016 in a private university-affiliated IVF centre. Cycles were divided into four groups according to the presence or absence of PVS abnormalities: PVS-L group (cycles with the entire oocyte cohort affected by large PVS, n=265), PVS-G group (cycles with the entire oocyte cohort affected by PVS granularity, n=280), PVS-L + PVS-G group (cycles with the entire oocyte cohort affected by PVS-L and PVS-G, n=204), and control group (cycles with the entire oocyte cohort free of PVS abnormalities, n=402). The effect of PVS abnormalities on ICSI outcomes was assessed by GLM adjusted for potential confounders.

Results: Groups with PVS abnormalities presented substantially higher FSH/follicle ($p<0.001$) and FSH/oocyte ($p<0.001$) ratios, and lower numbers of follicles ($p<0.001$), oocytes ($p<0.001$) and embryos ($p=0.002$) compared to the control group. PVS-L + PVS-G implantation ($p=0.044$) and pregnancy ($p=0.004$) rates were significantly lower than in cycles with isolated PVS abnormalities and controls.

Conclusion(s): Cycles in which the entire oocyte cohort is affected by both large PVS and PVS granularity have compromised implantation and pregnancy rates.

Keywords: large perivitelline space; perivitelline space granularity; oocyte morphology; oocyte dysmorphism; ICSI

Introduction

As a routine procedure in intracytoplasmic sperm injection (ICSI) cycles, denudation of retrieved oocytes allows the determination their maturation status and the assessment of morphological features of the cytoplasm, perivitelline space (PVS) and zona pellucida (1–3). Even though oocytes presenting extra and intracytoplasmic dysmorphisms have been related to impairment of developmental potential and implantation competence of derived embryos (4–6), different stimulation protocols and evaluation criteria may have biased the literature regarding the extent to which oocyte morphology correlates to ICSI outcomes (1,7,8).

The PVS abnormalities are amongst the most prevalent dysmorphisms of the extra cytoplasmic component. Large PVS (PVS-L) and the presence of PVS granularity (PVS-G) are repeatedly seen in retrieved oocytes of the same cohort and have already been used as parameters in mathematical models to predict embryo quality (9,10).

A previous meta-analysis showed that the probability of an oocyte becoming fertilised is significantly reduced by the presence of PVS-L, although the effects on implantation and pregnancy rates remain unclear (5). PVS-G has been negatively associated with implantation and pregnancy rates in cycles in which the entire oocyte cohort is affected, although the number of analysed cycles was limited (11).

The effects of extra cytoplasmic morphological deviations on embryo implantation potential are still controversial and the number of reports is scarce. In most cycles, morphologically abnormal oocytes are harvested together with oocytes free of morphological abnormalities, making it difficult to determine the origin and consequences of such characteristics. For a meaningful interpretation of pregnancy outcomes, the ideal study design should include only cycles with single embryo transfers, or those in which the entire oocyte cohort is affected by a given dysmorphism. To date, there has only been one study analysing the association between PVS abnormalities and ICSI outcomes; however, only 65 cycles were analysed (11). Therefore, the objective of this study was to evaluate the effect of PVS-L and

PVS-G, isolated and/or combined, on embryo quality and ICSI outcomes, in cycles in which the entire oocyte cohort was affected.

Materials and Methods

Experimental design, patients, and inclusion and exclusion criteria

This retrospective cross-sectional study included data from 9,752 oocytes obtained from 1,151 intracytoplasmic sperm injection (ICSI) cycles performed from June 2010 to August 2016, in a private university-affiliated *in vitro* fertilisation centre. The inclusion criteria were as follows: couples undergoing first or second ICSI cycles with fresh embryo transfer in which the entire oocyte cohort was affected by PVS-L, PVS-G or both. Cycles free of oocyte dysmorphisms were used as controls. Representative oocytes images from each group are shown in Figure 1.

The exclusion criteria were as follows: couples undergoing ICSI with vitrified/thawed or donated oocytes, surgical sperm retrieval, vitrified/thawed embryo transfer, or pre-implantation genetic diagnosis or screening.

Cycles were divided into four groups according to the presence or absence of PVS abnormalities as follows: PVS-L group (n= 265, cycles with the entire oocyte cohort affected by large PVS and free of PVS granularity), PVS-G group (n=280, cycles with the entire oocyte cohort affected by PVS granularity and free of large PVS), PVS-L + PVS-G group (n=204, cycles with the entire oocyte cohort affected by both large PVS and PVS granularity), and control group (n=402, cycles with the entire oocyte cohort free of PVS abnormalities).

The relation of PVS abnormalities with (i) the total dose of FSH administered, (ii) FSH per follicle rate, (iii) FSH per oocyte rate, (iv) oestradiol level on hCG trigger day, (v) the number of follicles, (vi) the number of retrieved oocytes; (vii) the mature oocyte rate, (viii) the fertilisation rate, (ix) the number of obtained embryos, (x) the embryo quality at cleavage-

stage days two and three, (xi) the blastocyst formation rate, (xii) the blastocyst quality rate, (xiii) the number of transferred embryos, (xiv) the endometrial thickness, (xv) the implantation rate, (xvi) the pregnancy rate, and (xvii) the miscarriage rate, were evaluated.

All patients signed a written informed consent form and the study was approved by the local institutional review board.

Controlled ovarian stimulation

Controlled ovarian stimulation was achieved by the administration of daily doses of follicle-stimulating hormone (rFSH, Gonal-F®, Merck KGaA, Geneva, Switzerland); beginning on day three of the cycle. Pituitary blockage was achieved by gonadotropin-releasing hormone (GnRH) antagonist (GnRHa, Cetrotide®; Merck KGaA, Darmstadt, Germany) beginning when at least one follicle ≥14 mm was visualised. Follicular growth was monitored by transvaginal ultrasound examination. When ≥3 follicles attained a mean diameter of ≥ 17 mm and adequate serum oestradiol levels (E2) were observed, recombinant human chorionic gonadotrophin (r-hCG, Ovidrel®, Merck KGaA, Geneva, Switzerland) was administered to trigger final follicular maturation. The oocytes were collected through transvaginal ultrasound ovum pick-up 35 hours later.

Oocyte preparation and morphology assessment

Retrieved oocytes were maintained in culture media (Global for fertilisation, LifeGlobal, Guilford, USA) supplemented with 10% protein (LGPS, LifeGlobal, Guilford, USA) and covered with paraffin oil (Paraffin oil P.G., LifeGlobal, Guilford, USA). After 2–3h, surrounding cumulus cells were removed after exposure to hyaluronidase (80 IU/ml, LifeGlobal, Guilford, USA) and the remaining cells were mechanically removed by gently pipetting with a hand-drawn Pasteur pipette (Humagen Fertility Diagnostics, Charlottesville, USA). Oocyte morphology was assessed using an inverted Nikon Diaphot microscope with a

Hoffmann modulation contrast system under 400x magnification (Eclipse TE 300 microscope, Nikon, Tokyo, Japan), just before sperm injection (5-7 hours after retrieval). The oocyte dysmorphisms individually recorded were the presence of intracytoplasmic granulation clusters, smooth endoplasmic reticulum clusters, dark cytoplasm, vacuoles, PVS-L, PVS-G, zona pellucida dysmorphisms, and polar body fragmentation.

Intracytoplasmic sperm injection

Intracytoplasmic sperm injection was performed according to Palermo et al. (1992). Only oocytes that had released the first polar body (Metaphase II -MII- oocytes) were considered mature and were used for ICSI. Sperm selection was performed at 400x magnification using an inverted Nikon Eclipse TE 300 microscope (Nikon, Tokyo, Japan). Fertilisation was confirmed by the presence of two pronuclei and the extrusion of the second polar body 16-18 hours after ICSI. Embryos were maintained in a 50-µL drop of culture medium (Global®, LifeGlobal, Guilford, USA) with 10% protein supplement and covered with paraffin oil in a humidified atmosphere under 6% CO_2 at 37°C for up to five days.

Embryo quality and embryo transfer

Embryos were morphologically evaluated on days two, three and five of development using an inverted Nikon Diaphot microscope with a Hoffmann modulation contrast system (Nikon, Tokyo, Japan) under 400x magnification. High-quality cleavage-stage embryos were defined as those with all of the following characteristics: 3−5 cells on day 2 and 8−10 cells on day 3, <15% fragmentation, symmetric blastomeres and absence of multinucleation.

The blastocyst rate was defined as the number of embryos that reached blastocyst stage at day five by the number of 2PN zygotes on day 1.

Blastocyst morphology on day five was evaluated according to Gardner and Schoolcraft (1999). The following parameters were recorded: degree of blastocoel expansion,

trophectoderm (TE) quality and cell number, and inner cell mass (ICM) quality and cell number. Blastocoel expansion was classified as 1- early blastocyst; 2- blastocyst; 3- full blastocyst; 4- expanded blastocyst; 5- hatching blastocyst; and 6- hatched blastocyst. TE quality was defined as A- many cells forming a cohesive epithelium; B- few cells forming a loose epithelium; and C- very few large cells. ICM cells were classified as A- tightly packed with many cells; B- loosely grouped with several cells; and C- very few cells and disorganised. Blastocyst quality rate was determined by dividing the number of top scoring blastocysts (≥ 3AA) by the total number of blastocysts.

Embryo transfer was performed on the third or fifth day of development using a soft catheter with transabdominal ultrasound guidance. One to three embryos were transferred per patient, depending on embryo quality and maternal age.

Clinical follow-up

A serum β-hCG pregnancy test was performed 10 to 12 days after embryo transfer. All women with a positive test (serum β-hCG >40 mIU/mL) received a transvaginal ultrasound scan after two weeks. Clinical pregnancy was achieved when a foetal heartbeat was detected. Pregnancy rates were calculated per embryo transfer. Implantation rate was calculated in each cycle of embryo transfer as the number of gestational sacs divided by the number of embryos transferred. Miscarriage was defined as a pregnancy loss before 20 weeks.

Data analysis and statistics

The previtelline abnormalities were analysed as the percentage of oocytes affected per cycle. The sample size calculation using G*Power 3.1.7 (Franz Faul, Universität Kiel, Germany) suggested that 1,095 subjects would be sufficient to demonstrate a 10% effect with an 80% power and 5% significance level (α).

The effects of PVS abnormalities on laboratorial and clinical outcomes were assessed using the Generalised Linear Model with Linear Distribution followed by Bonferroni Post hoc. The statistical analyses were performed using SPSS Statistics 21 (IBM, New York, New York, USA). Data are expressed as mean ± standard deviation for continuous variables, while percentages are used for categorical variables. All post hoc significances are annotated as different letters. Controlled ovarian stimulation (COS) outcomes were adjusted for maternal age, maternal BMI, total dose of FSH administered and oestradiol (E2) level on hCG trigger day; Laboratory outcomes were adjusted for maternal age and BMI, total dose of FSH administered, oestradiol level on hCG trigger day, and number of retrieved oocytes; clinical outcomes were adjusted for maternal age and BMI, total FSH dose, oestradiol level on hCG trigger day, number of retrieved oocytes, number of transferred embryos and endometrial thickness.

Results

There was no significant difference in total FSH dose used for COS; however, the FSH/follicle ($p<0.001$) and FSH/oocyte ($p<0.001$) ratios were substantially higher in the groups PVS-L, PVS-G and PVS-L + PVS-G compared to control group. Oestradiol level peak on hCG trigger day was lower in PVS-L group compared to others ($p<0.001$). Groups with PVS abnormalities (PVS-L, PVS-G and PVS-L + PVS-G) presented lower number of follicles and oocytes compared to control group ($p<0.001$) (Table 1).

The number of obtained embryos was higher in the control group compared to the others ($p=0.002$). Fertilisation, embryo quality on days two and three, blastocyst formation and quality rates were similar between the groups (Table 2).

The presence of isolated PVS dysmorphisms, that is, PVS-L or PVS-G, had no impact on clinical outcomes; however, in the PVS-L + PVS-G group, implantation ($p=0.044$) and pregnancy

(p=0.004) rates were lower than all the other groups, although miscarriage rates were similar (Table 3).

Discussion

Denudation of oocytes before fertilisation by ICSI offers a unique opportunity to evaluate oocyte morphology and analyse its correlation with embryo viability and development potential. Oocyte quality is a key factor that influences the successful outcome of assisted reproduction treatment cycles; nevertheless, there are no clear and well-defined criteria for oocyte morphology evaluation and the role of specific oocyte dysmorphisms on further embryo development is still a matter of controversy, since, in most cycles, normal and abnormal oocytes are harvested together, thus resulting in the transfer of embryos derived from oocytes with heterogeneous morphological features (5,13).

In our study, we selected cycles in which the entire oocyte cohort presented PVS abnormalities, and studied the cause/effect relationship between this specific morphological abnormalities and clinical outcomes. We found that fertilisation rate and embryo quality were not affected by these extracytoplasmic features; however, compared with a control group in which all embryos were derived from morphologically normal oocytes, significantly reduced implantation and pregnancy rates were observed in the group that presented both large and granular PVS.

It is known that oocyte quality is a result of serial events occurring following its meiotic initiation in the foetal ovary, follicle formation in the perinatal period, and naturally or induced follicle growth and oocyte maturation in the adult (14). The PVS size is associated with the maturational phase of the oocyte, while in the Germinal Vesicle (GV) stage, the expansion of PVS is minimal, there is an increase in PVS after re-assumption of meiosis (Metaphase I -MI- stage) and its full size is reached after the completion of maturation in the MII stage (15). The presence of granulation in the PVS has also been correlated to the oocyte maturation status,

since this dysmorphism was detected in 34.3%, 4% and 0% of MII, MI and GV stage oocytes, respectively, and was positively correlated to the administered gonadotrophin dose (16).

Interestingly, we noticed that higher doses of FSH were needed per recruited follicle and per obtained oocyte in cycles in which the entire cohort presented PVS-L, PVS-G or PVS-L+PVS-G, even though the total dose of FSH administered was similar to control cycles. In addition, the PVS-L group presented substantially lower serum oestradiol concentrations compared to the control group. In addition, cycles that presented PVS abnormalities resulted in lower numbers of follicles, oocytes and embryos compared to the control group. These results suggest that COS efficiency evaluation, in terms of FSH dose per follicle or per obtained oocyte, can be more informative than the total dose of FSH administered, which is conventionally used (11,17). It is important to highlight that patients were stimulated with recombinant FSH, and that oocyte dysmorphism patterns can be potentially influenced by the type of FSH administered (18).

Previous studies pointed out that single oocyte anomalies had no direct effect on fertilisation and embryo quality rates (1,7,11,18,19), which are in accordance with that which was observed in this study. The presence of isolated PVS dysmorphisms, that is, PVS-L or PVS-G, had no significant impact on clinical outcomes, which differs from the single study that analysed cycles in which all of the oocyte cohort was affected by PVS granularity (11). Nevertheless, we observed that the presence of both PVS abnormalities in the entire oocyte cohort highly compromised both implantation and pregnancy rates.

It has been suggested that extracytoplasmic dysmorphisms should be considered only as phenotypic deviations, given the heterogeneity of the retrieved oocytes after COS (6). However, our results suggest that the presence of PVS abnormalities in the entire cohort may impact the outcomes of ICSI cycles. Despite the consistency of the results, it is important to emphasise that this phenomena was observed in a specific population that presented oocyte cohorts which were completely affected by the same dysmorphisms, indicating that these

patients might have had inherently compromised oocyte development, which could be related to the lower FSH efficacy.

In conclusion, the findings of this study suggest that PVS abnormalities are associated with lower FSH efficacy per follicle and oocyte, implantation and pregnancy rates in ICSI cycles. The morphological evaluation of the oocyte should be coupled with a detailed evaluation of the resulting embryo to allow the selection of the best embryo for transfer, thus improving the chances of implantation and pregnancy.

Condensation

ICSI cycles in which the entire oocyte cohort is affected by large perivitelline space and perivitelline space granularity results in compromised implantation and pregnancy outcomes.

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Figure 1- Representative images for the groups PVS-L (oocyte affected by large perivitelline space (PVS) and free of PVS granularity), PVS-G (oocyte affected by PVS granularity and free of large PVS), PVS-L + PVS-G group (oocyte affected by both large PVS and PVS granularity), and control (oocyte free of dysmorphism).

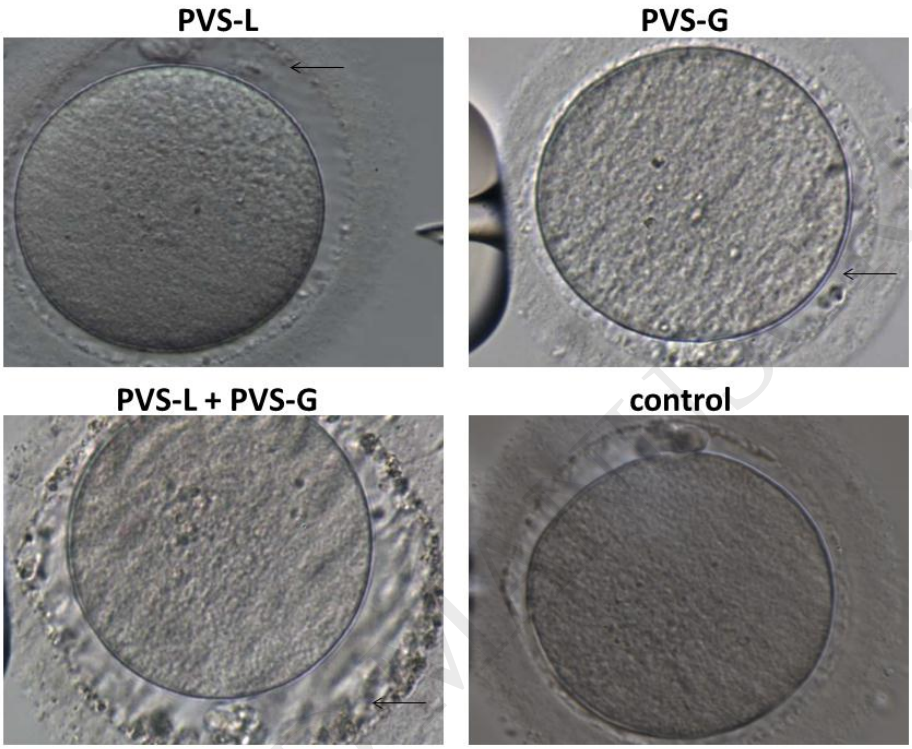

Table 1- Descriptive statistics of COS outcomes.

	PVS-L	PVS-G	PVS-L + PVS-G	control	*p*
Cycles (n)	265	280	204	402	
Total dose FSH (IU)	2609.08 ± 164.58	2419.69 ± 62.01	2609.12 ± 71.21	2475.61 ± 51.46	0.198
FSH/follicle	590.94 ± 36.26 [b]	446.27 ± 21.77 [c]	604.55 ± 33.29 [b]	158.49 ± 6.88 [a]	**<0.001**
FSH/oocyte	1062.26 ± 63.43 [c]	912.58 ± 44.63 [c]	1169.43 ± 64.02 [b]	228.77 ± 9.90 [a]	**<0.001**
Oestradiol level (pg/ml)	1038.85 ± 247.17 [a]	2260.42 ± 159.08 [b]	1953.35 ± 189.73 [b]	2371.55 ± 129.44 [b]	**<0.001**
Follicles (n)	9.47 ± 4.53 [b]	13.93 ± 1.00 [b]	11.57 ± 1.13 [b]	17.89 ± 0.98 [a]	**<0.001**
Oocytes (n)	4.97 ± 2.71 [b]	7.87 ± 0.63 [b]	6.52 ± 0.71 [b]	12.19 ± 0.74 [a]	**<0.001**
Mature oocytes rate (%)	73.51 ± 15.45	76.36 ± 2.49	76.38 ± 3.39	72.86 ± 1.90	0.657

Note: a≠ b ≠ c (GzLM Bonferroni Post hoc p<0.05). BMI: body-mass index, COS: controlled ovarian stimulation, FSH: follicle stimulating hormone. Data was adjusted for maternal age, maternal BMI, FSH dose and oestradiol level on hCG trigger day.

Table 2- Descriptive statistics of laboratory outcomes.

	PVS-L	PVS-G	PVS-L + PVS-G	control	*p*
Fertilization rate	84.65 ± 4.60	82.90 ± 2.22	76.34 ± 1.90	81.79 ± 2.69	0.077
Embryos obtained	4.18 ± 0.63[b]	6.57 ± 2.53[b]	4.96 ± 0.72[b]	9.74 ± 0.41[a]	**0.002**
Embryos transferred	1.23 ± 0.17	1.48 ± 0.08	1.25 ± 0.10	1.57 ± 0.07	0.059
High-quality embryo at day two	27.26 ± 10.75	35.21 ± 2.73	33.32 ± 1.80	42.64 ± 4.75	0.275
High-quality embryo at day three	44.10 ± 5.96	49.10 ± 2.88	41.65 ± 2.47	47.53 ± 3.47	0.225
Blastocyst rate	36.66 ± 8.65	43.98 ± 4.26	42.08 ± 4.94	46.52 ± 3.42	0.199
Blastocyst quality	31.35 ± 6.67	31.57 ± 6.49	41.10 ± 7.21	42.54 ± 3.99	0.322

Note: a≠ b (GzLM Bonferroni Post hoc p<0.05). Data was adjusted for maternal age, maternal BMI, FSH dose, oestradiol level on hCG trigger day, and number of oocytes.

Table 3- Descriptive statistics of clinical outcomes.

	PVS-L	**PVS-G**	**PVS-L + PVS-G**	**control**	***p***
Endometrial thickness	10.87 ± 1.92	10.73 ± 0.23	10.23 ± 0.32	10.68 ± 0.18	0.632
Implantation rate	17.62 ± 2.33 [b]	17.24 ± 3.83 [b]	15.61 ± 2.33 [a]	20.81 ± 2.94 [b]	**0.044**
Pregnancy rate	27.9% [b]	30.6% [b]	18.1% [a]	33.7% [b]	**0.004**
Miscarriage rate	9.1%	7.8%	10.2%	7.3%	0.962

Note: a≠ b (GzLM Bonferroni Post hoc p<0.05). Data was adjusted for maternal age, maternal BMI, FSH dose, oestradiol level on hCG trigger day, number of oocytes, number of transferred embryos, and endometrial thickness.