Zygote

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Detailed analysis of cytoplasmic strings in human blastocysts: new insights

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Research Article

Cite this article: Eastick J *et al*. (2023) Detailed analysis of cytoplasmic strings in human blastocysts: new insights. *Zygote*. **31**: 78–84. doi: 10.1017/S0967199422000570

Received: 23 September 2022
Accepted: 14 October 2022
First published online: 17 November 2022

Keywords:
Blastocyst; Cytoplasmic string; Embryo development; Time-lapse; Ultrastructure

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Summary

The aim of this study was to determine if there was an association between the presence of cytoplasmic strings (CS) and their characteristics, with blastocyst quality, development and clinical outcome in human blastocysts. This two-centre cohort study was performed between July 2017 and September 2018 and involved a total of 1152 blastocysts from 225 patients undergoing *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI). All embryos were cultured in Embryoscope+ and were assessed for CS using time-lapse images. A single assessor examined all blastocysts and reviewed videos using the EmbyroViewer® Software. Blastocyst quality was assessed on day 5 of embryo development. The number of CS, location and duration of their activity was recorded on days 5/6. A positive association between the presence of CS in human blastocysts with blastocyst quality was identified. Blastocysts with a higher number of CS present, were of higher quality and were in the more advanced stages of development. Top quality blastocysts had CS activity present for longer, as well as having a higher number of vesicles present travelling along the CS. Blastocysts that had CS present, had a significantly higher live birth rate. This study has confirmed that a higher number of CS and vesicles in human blastocysts is associated with top quality blastocysts and is not a negative predictor of development. They had a higher number of CS present that appeared earlier in development and, although ceased activity sooner, had a longer duration of activity. Blastocysts with CS had a significant increase in live birth rate.

Introduction

The selection of the most viable embryo for a single blastocyst transfer remains to be the dilemma that embryologists are challenged with on a daily basis. The introduction of time-lapse monitoring systems (TLM) into the *in vitro* fertilization (IVF) laboratory enables the continuous visualization of the patient's developing embryos. In conjunction with traditional grading systems (Gardner and Schoolcraft, 1999), TLM allows for further assessments on the cohort of embryos by application of morphokinetic algorithms (Kirkegaard *et al*., 2013; Basile *et al*., 2015; Petersen *et al*., 2016; Storr *et al*., 2018) and the most recently artificial intelligence systems (Khosravi *et al*., 2019; Tran *et al*., 2019; Hassan *et al*., 2020; VerMilyea *et al*., 2020). Currently, embryologists are presented with vast amounts of information to select a single embryo for transfer that will most probably result in a clinical pregnancy.

TLM has allowed the identification of morphokinetic and morphological events in embryo development that previously were unobserved. One such event is the formation and activity of cytoplasmic strings (CS). These ultrastructures are present during blastulation, and currently their function and role in human blastocysts are of unknown significance. Little information exists in the published literature (Ducibella *et al*., 1975; Salas-Vidal and Lomelí, 2004; Fierro-González *et al*., 2013), however recently a few studies have identified CS as a potential biomarker that may aid in the selection of blastocysts that have higher implantation potential for transfer (Eastick *et al*., 2021; Ma *et al*., 2021). In addition, it has been reported that CS did not have a negative effect on implantation (Ebner *et al*., 2020), contrary to a previous study (Scott, 2000). Another study found that blastocysts that contained a high number of CS and have vesicles travelling along the CS are associated with a positive clinical pregnancy with a fetal heart (Eastick *et al*., 2021). It has been suggested that the CS and their vesicles may act as a communication mechanism between the inner cell mass (ICM) and trophectoderm (TE) cells as the blastocoel cavity expands. One study identified two receptors, fibroblast growth factor 2 (FGF2) receptor and Erb-B2 receptor tyrosine kinase 3 (ErbB3), on CS that played important roles in embryonic development, in particular the proliferation of mural TE (mTE) cells and signal transduction activity (Salas-Vidal and Lomelí, 2004).

In mouse embryos, CS have been identified during the 8-cell to 16-cell stage and are associated with the compaction process (Fierro-González *et al*., 2013). Once compaction is complete, CS activity ceases. In human embryos, CS cannot be seen during the cleavage stages of embryo development with the current TLM software available in the IVF setting. The CS

re-appear as projections during the early stages of blastulation, it is at this stage that these projections can be identified on TLM systems.

It is these projections that have been linked to blastocysts that are of higher quality and are associated with a clinical pregnancy with a fetal heart (Eastick *et al.*, 2021). However, the study only included high quality blastocysts that were considered suitable for embryo transfer. It still remains to be seen if CS are associated with blastocyst quality, including those that are cryopreserved or discarded. The aim of this study was to determine if there was an association between the presence of CS and their characteristics, with blastocyst quality, development and live birth rate in human blastocysts.

Materials and Methods

Study design

This two-centre cohort study was performed between July 2017 and September 2018 and is an extension of the results published by Eastick *et al.* (2021). Approval for this study was obtained from the IVFAustralia Ethics Committee (2017/104). The study involved 225 patients and all embryos were cultured in EmbryoScope+. In total, 1152 blastocysts were assessed for CS using the time-lapse videos generated from EmbryoScope+ for every blastocyst generated.

Ovarian stimulation, oocyte retrieval, semen preparation and insemination

Ovarian stimulation, oocyte retrieval, semen preparation and insemination were performed as described previously (Eastick *et al.*, 2017). Briefly, stimulation was achieved using gonadotrophin releasing hormone (GnRH). Transvaginal ultrasounds and serum estradiol levels were performed regularly to monitor follicular growth. Human chorionic gonadotropin (hCG) was used to trigger oocyte maturation and retrieval was performed 36 h after the injection.

Semen samples were prepared using PureSperm density gradients (Nidacon, Sweden) and washed in G-MOPS (Vitrolife, Sweden) and held at room temperature until insemination. Oocytes were cultured in pre-equilibrated four-well Nunc dishes (ThermoFisher Scientific, USA) at 37°C, in 5% O_2 and 6% CO_2. Each well contained G-IVF and Ovoil mineral oil overlay (Vitrolife, Sweden). Insemination occurred between 39–41 h post trigger injection.

Embryo culture and blastocyst assessment

The EmbryoSlide® (Vitrolife, Sweden) was filled with prewarmed G-TL and Ovoil. The slide was loaded into an EmbryoScope+ at 37°C, in 5% O_2, 6% CO_2, balance N_2, immediately on completion of ICSI (day 0) or following the IVF fertilization check (day 1).

Embryo development was recorded using the image software EmbyroViewer® (EmbryoScope™, Vitrolife, Denmark). Videos of embryos that reached the blastocyst stage were reviewed to assess morphology using the Gardner criteria (Gardner *et al.*, 2000) between 118–120 hpi. Blastocysts were classified as top quality if ICM and TE were graded: AA or BA, medium quality: BB or AB; and poor quality was considered anything containing a C grade.

Cytoplasmic string assessment

The detailed methodology of the CS assessment is available in Eastick *et al.* (2021) however, in summary, a single assessor examined all blastocysts for the presence and absence of CS as seen in the time-lapse videos generated from the EmbryoViewer® software.

The time points and stage of embryo development in which the CS first appeared and the time their activity ceased were recorded. The number of CS present and the location of the CS within the developing blastocyst were also noted. The location of CS formation was based on the blastocyst polarity regions as defined by Gardner (1997) and a detailed explanation can be found in Eastick *et al.* (2021). Regions were classified as mural, polar–mural junction (p-mj) and both (mural and polar–mural junction). CS were further assessed to determine if they contained the presence of vesicle-like bulges. In addition, vesicle presence and direction were recorded in three categories: vesicles originating from the TE cells and travelling towards the ICM, originating from ICM and travelling towards the TE cells, or bidirectionally (back and forth trajectory between the ICM and TE cells). For each blastocyst containing the CS with vesicles, the number and direction of vesicle movement were recorded.

Statistical analysis

All statistical analyses were performed with STATA (v.14.2, StataCorp, USA) and statistical significance was set at $P \leq 0.05$. Logistic regression models using the generalized estimating equations framework were used to estimate the odds ratio (OR) with a 95% confidence interval (CI) and *P*-values. Adjustment for potential confounders was performed.

Results

Embryological characteristics

The current study contained 225 patients. The mean female age was 34.6 years (23.7–51.8) with a mean Anti-Mullerian Hormone (AMH) of 25.4 (0.1–150). The mean male age was 36.4 years (22.8–58.9). The majority of patients presented with primary infertility (93.6%). The indication for treatment fell into the categories: maternal (23.6%), paternal (16.8%), a combination (11.2%), social (17.3%) and other reasons (31.1%). The mean starting Follicle-Stimulating Hormone (FSH) dose was 216.7 IU (75–600) and 95% of patients had an antagonist cycle. The mean total FSH dose was 2232 IU (375–7800). The mean duration of stimulation was 10.3 days (3–22). Blastocyst stage of development was assessed for each blastocyst seen in the patients cohort (149 early blastocyst, 240 full blastocysts, 487 expanded blastocysts, 271 hatching blastocysts and five hatched blastocysts) ($P < 0.001$). Blastocyst quality was also assessed for those blastocysts at the full blastocyst stage and greater with no significant difference detected ($P = 0.938$). From these blastocysts, 209 were suitable for embryo transfer, 558 were cryopreserved and 385 were discarded.

Prevalence and characteristics of cytoplasmic strings and their vesicles

The prevalence of CS in 1152 blastocysts was examined (Table 1) and a significant difference was seen in the number of blastocysts with CS (77.0%) compared with those that did not (23.0%) ($P < 0.001$, CI 95% 74.5–79.4%). When the CS were examined for the presence of vesicles, there was a significant difference in

https://doi.org/10.1017/S0967199422000570 Published online by Cambridge University Press

Table 1. Prevalence and characteristics of cytoplasmic strings and their vesicles in the 1152 blastocysts examined

Parameter	Population
CS presence, *n* % (95% CI)	887, 77.0% (74.5–79.4%)
Vesicle presence, *n* % (95% CI)	797, 90.8% (87.7–91.8%)
CS appearance time in hours post insemination, mean (95% CI)	108.7 (108.0–109.4)
CS end time in hrs post insemination, mean (95% CI)	119.3 (118.6–119.9)
Duration of CS presence in hours, mean (95% CI)	10.6 (10.1–11.1)
Developmental stage when CS first appear *n* (%)	
EBL	523 (59.0)
BL	334 (37.7)
XBL	19 (2.1)
HGBL	11 (1.2)
HBL	0
Developmental stage when CS cease activity *n* (%)	
EBL	53 (6.0)
BL	246 (27.7)
XBL	435 (49.0)
HGBL	153 (17.2)
HBL	0
CS formation – region of blastocyst, *n* (%)	
P-mj	33 (3.7)
Mural	415 (46.8)
Both	439 (49.5)
Direction vesicles travelled, *n* (%)	
Bidirectional	168 (21.1)
TE–ICM	569 (71.4)
ICM–TE	60 (7.5)

the number of blastocysts with vesicles (90.8%), compared with those that did not (10.2%) ($P < 0.001$, CI 95% 87.7–91.8%).

The mean start time CS were first identified in the developing blastocysts was 108.7 hpi (CI 95% 108.0–109.4) and the mean time CS were no longer identified in blastocysts was 119.3 hpi (CI 95% 118.6–119.9), with a mean duration of CS activity being 10.6 h (CI 95% 10.1–11.1).

Blastocysts had CS first appear mostly at the early blastocysts stage (59.0%) and blastocyst stage (37.7%), whereas CS activity ceased mostly during the expanded stage (49.0%). The region in which the CS activity occurred and the direction the vesicles were seen travelling was also assessed for each of the blastocysts. The majority of the blastocysts had CS detected at both the mural region and polar–mural junction 49.5%, followed by 46.8% at the mural region only, and 3.7% at the polar–mural junction. The direction in which the vesicles were seen travelling along the CS was also assessed. The majority of the blastocysts had vesicles travelling from the TE cells to the ICM (71.4%), followed by 21.1% having bidirectional movement and 7.5% having vesicles travelling from the ICM to the TE cells.

Table 2. Descriptive characteristics and morphological features of blastocysts that presented with at least one cytoplasmic string compared with those that did not (*n* =1152)

Parameter	Blastocysts with CS (*n* = 887)	Blastocysts without CS (*n* = 265)	*P*-value
Insemination method *n* (%)	IVF: 620 (69.9)	IVF: 173 (65.3)	0.155[a]
	ICSI: 267 (30.1)	ICSI: 92 (34.7)	
D5 quality excluding EBL *n* (%)	Top: 321 (37.4)	Top: 12 (8.3)	<0.001[a]
	Fair: 300 (34.9)	Fair: 39 (27.1)	
	Poor: 238 (27.7)	Poor: 93 (64.6)	
Blastocyst stage of development assessed between 119–120 hpi *n* (%)	EBL: 28 (3.2)	EBL: 121 (45.7)	<0.001[a]
	BL: 159 (17.9)	BL: 81 (30.6)	
	XBL: 437 (49.3)	XBL: 50 (18.8)	
	HGBL: 258 (29.1)	HGBL: 13 (4.9)	
	HBL: 5 (0.5)	HBL: 0	
Fate, *n* (%)			
Transferred	170 (19.2)	39 (14.7)	<0.001[a]
Cryopreserved	487 (54.9)	71 (26.8)	
Discarded	230 (25.9)	155 (58.5)	
Live birth % (*n*) for single transferred blastocysts	34.8% (55/158)	17.1% (6/35)	0.042[a]

[a]Chi-squared test.

Descriptive characteristics and morphological features of blastocysts with and without CS present

The descriptive characteristics and morphological features for blastocysts that had CS present and those that did not were assessed (Table 2).

When evaluating blastocysts with CS they tended to originate from cycles with a lower FSH dose (2123.9 IU vs 2393.8 IU, $P = 0.005$) and there was no significant difference seen in the duration of stimulation ($P = 0.666$) or the type of trigger injection used ($P = 0.081$). When the insemination method used was evaluated between blastocysts with CS and those without CS, no significant difference was observed ($P = 0.155$).

Blastocysts with CS were seen to be of higher quality on day 5 (37.4% top quality) compared with those blastocysts without CS (8.3% top quality) ($P < 0.001$). When the two groups were assessed for the stage of blastocyst development between 119–120 hpi, a significant difference was observed with blastocysts that had CS being in the more advanced stages of development, i.e. expanded blastocyst (49.3%) compared with those that did not have CS present that were at the early blastocyst stage (45.7%) ($P < 0.001$). In addition, there was a significant difference in embryo fate, with more blastocysts containing CS being either suitable for transfer on day 5 (19.2%) or cryopreserved on days 5 or 6 (54.9%) compared with those that did not have CS present (14.7% suitable for transfer and 26.8% cryopreserved) ($P < 0.001$).

There was a significant difference seen in the live birth rate ($P = 0.042$) with blastocysts that had CS resulting in a live birth compared with those blastocysts that did not.

https://doi.org/10.1017/S0967199422000570 Published online by Cambridge University Press

Characteristics of CS and their vesicles according to blastocyst quality

The characteristics of CS and their vesicles according to blastocyst quality was assessed. Top quality blastocysts had a higher number of CS present (5.7 CS per blastocyst), compared with those that were of poor quality (3.4 CS per blastocyst) ($P = 0.000$). CS appeared 10 h earlier in top quality blastocysts (104.5 hpi; $P < 0.001$) and activity ceased approximately 7 h earlier ($P < 0.001$) compared with that of their poorer quality counterparts. The overall duration of the CS activity was assessed, with activity occurring the longest in top quality blastocysts (11.9 h), compared with that of the fair quality (10.3 h) and poor quality blastocysts (9.2 h) ($P < 0.001$).

The presence of vesicles seen travelling along the CS was recorded for each blastocyst quality. Top quality blastocysts had a higher percentage of CS with vesicles (96.3%) compared with those that were fair (92.7%) and poor quality (79.4%) ($P < 0.001$). Blastocysts that were either fair ($P < 0.001$, OR: 0.354, CI 95% 0.215–0.583) or poor quality ($P < 0.001$, OR: 0.103, CI 95% 0.065–0.164) had a 70% and 90% reduction, respectively, in vesicles being detected compared with their top quality blastocysts.

A significant difference was detected in the number of vesicles seen travelling along the CS, with a higher number observed in top quality blastocysts (3.7 vesicles), compared with their poorer quality counterparts (1.8 vesicles) ($P < 0.001$). There was no significant difference detected in the direction in which the vesicles were seen travelling between the three groups.

There was no significant difference seen when the stage of blastocyst development CS were first identified was assessed in the three groups, however the majority of blastocysts has CS first appear during the early blastocyst stage. There was a significant difference when CS activity ceased, with majority of the top quality blastocysts having CS activity cease around the later stages of development, compared with their poorer quality counterparts ($P < 0.001$). A significant difference was seen in the region of the blastocyst that CS activity was observed across the three groups ($P = 0.012$). There was a significant difference seen in the live birth rate ($P = 0.003$), with top quality blastocysts having a higher rate compared with their poorer quality counterparts.

Bivariate and multivariable logit regression analysis

A bivariate regression analysis was performed taking into consideration the results observed in Tables 2 and 3. Top quality blastocysts had a significantly higher chance of having CS present, with fair quality blastocysts having 0.39 the odds of having CS present ($P = 0.009$, OR: 0.399, CI 95% 0.201–0.795) and poor quality blastocysts having 0.13 the odds of having CS present ($P < 0.001$, OR: 0.133, CI 95% 0.070–0.254) compared with top quality blastocysts. Similarly, when assessing the blastocyst stage of development, the more advanced stages had a significantly higher chance of having CS present compared with the earlier stages. Hatching blastocysts were 2.13 times the odds of having CS present compared with expanded blastocysts ($P = 0.002$, OR: 2.133, CI 95% 1.113–4.088), whereas blastocysts had an 80% reduction in CS presence compared with expanded blastocysts ($P = 0.000$, OR: 0.262, CI 95% 0.172–0.399).

A multivariable regression analysis was performed to determine the predictive role of CS for the achievement of a clinical pregnancy with a fetal heart while controlling for the confounders, developmental stage, blastocyst quality, female age and insemination method (Table 4). After controlling for these, the presence of CS in blastocysts was not significantly associated with a clinical pregnancy with a fetal heart ($P = 0.627$, OR: 1.537, CI 95% 0.271–8.708). Blastocyst quality was significantly associated with a clinical pregnancy with a fetal heart with fair quality blastocysts having a 57% reduction ($P = 0.026$, OR: 0.435, CI 95% 0.210–0.904) and poor quality blastocysts ($P = 0.017$, OR: 0.141, CI 95% 0.028–0.704) having a 86% reduction in the odds, compared with top quality blastocysts.

Discussion

This study has identified a positive association between the presence of CS in human blastocysts with blastocyst quality. Blastocysts with a higher number of CS present were of higher quality and were in the more advanced stages of development. They had CS activity present for longer, as well as having a higher number of vesicles present travelling along the CS. Blastocysts that had CS present had a higher live birth rate. However, while controlling for confounders, no statistical significance was detected when assessing the predictive role of CS in the achievement of a clinical pregnancy with a fetal heart. Although the study was not designed to assess this as a primary outcome, given that the effect size was 1.54, this may indicate that a larger population size is required to detect differences and reach strong conclusions. This therefore opens the possibility of further studies to confirm the potential additional predictive value of cytoplasmic string presence in human blastocysts that could be used as an ultrastructure marker in the selection of embryos with higher implantation potential. This study has presented a more detailed analysis of the presence and characteristics of CS.

A previous study suggested that CS in mouse blastocysts could be identified in 40% of *in vivo* produced blastocysts, and suggested that CS would be present in all most all *in vitro* cultured blastocysts (Salas-Vidal and Lomelí, 2004). The current study identified 77% of *in vitro* cultured human blastocysts as having CS present and 90.8% having vesicle activity. Another study reported the incidence of CS at 43.9%, and vesicle activity in approximately 64.1% of the blastocysts studied (Ebner *et al.*, 2020). This reduction in incidence may be attributed to differences in the time-lapse equipment used, sample size or the fact that the current study looked at blastocysts of all qualities and not just those suitable for embryo transfer or cryopreservation. Intriguingly, another recent study reported identifying CS in *in vivo* produced blastocysts that were flushed from the uteri of volunteers (Munné *et al.*, 2020).

In the current study, CS were located in the mural and the polar–mural junctions of blastocysts. Furthermore, blastocysts that were more advanced and of higher quality had more CS and vesicles present. These finding may further support the theory that CS act as a communication mechanism. It is known that, as the blastocysts develop, the ICM induces a high rate of proliferation in the polar TE cells, resulting in a shift in cells from the polar to mural region. The cells in the mural TE are the furthest dispersed from the ICM, therefore exactly how these cells receive signals to proliferate remains unknown (Chai *et al.*, 1998), however the CS traversing the blastocoel cavity connecting the ICM and TE cells may facilitate these signals.

Interestingly, the current study found that the vesicles mostly travelled from the TE cells to the ICM and bidirectionally, but rarely solely from the ICM to TE cells. Another study also noted their bidirectional movement, however this study was in mouse blastocysts and frequencies were not reported

https://doi.org/10.1017/S0967199422000570 Published online by Cambridge University Press

Table 3. Characteristics of cytoplasmic strings and their vesicles in human blastocysts (excluding early blastocysts) according to blastocyst quality as graded by the Gardner criteria ($n = 887$)

Parameter	Top quality blastocysts	Fair quality blastocysts	Poor quality blastocysts	*P*-value
Mean number of CS/blastocyst mean (range)	5.7 (1–14)	4.6 (1–14)	3.4 (1–11)	0.000[b]
CS appearance time hours post insemination mean (95% CI)	104.5 (103.5–105.5)	107.7 (106.8–108.6)	114.5 (113.3–115.6)	<0.001[b]
CS end time hours post insemination mean (95% CI)	116.1 (115.7–117.5)	118.0 (117.2–118.9)	123.7 (122.5–124.8)	0.001[b]
Duration of CS presence hours mean (95% CI)	11.9 (11.3–12.8)	10.3 (9.7–10.9)	9.2 (8.5–9.9)	0.035[b]
Vesicle presence % (*n*)	96.3% (309/321)	92.7% (278/300)	79.4% (189/238)	<0.001[a] odds ratio (95% CI) Top: Reference; Fair: 0.354 (0.215–0.583) <0.001; Poor: 0.103 (0.065–0.165) <0.001
Mean number of vesicles travelling on a single CS mean (range)	3.7 (0–12)	2.9 (0–12)	1.8 (0–13)	<0.001[b]
Developmental stage when CS first appear, *n* (%)				
EBL	185 (57.6)	178 (59.3)	134 (56.3)	0.871[a]
BL	125 (38.9)	110 (36.7)	97 (40.7)	
XBL	6 (1.9)	9 (3)	4 (1.7)	
HGBL	5 (1.6)	3 (1)	3 (1.3)	
HBL	0	0	0	
Developmental stage when CS cease activity, *n* (%)				
EBL	3 (0.9)	8 (2.6)	18 (7.6)	<0.001[a]
BL	50 (15.6)	93 (31)	100 (42.0)	
XBL	183 (57.0)	152 (50.7)	99 (41.6)	
HGBL	85 (26.5)	47 (15.7)	21 (8.8)	
HBL	0	0	0	
CS formation – region of blastocyst, *n* (%)				
P-mj	9 (2.8)	9 (3)	11 (4.6)	0.012[a]
Mural	141 (43.9)	126 (42)	130 (54.6)	
Both	171 (53.3)	165 (55)	97 (40.8)	
Direction vesicles travelled, *n* (%)				
Bidirectional	78 (25.2)	58 (20.8)	31 (16.4)	0.166[a]
TE–ICM	210 (68)	197 (70.9)	146 (77.2)	
ICM–TE	21 (6.8)	23 (8.3)	12 (6.4)	
Fate, *n* (%)				
Transferred	90 (28.0)	59 (19.7)	16 (6.7)	<0.001[a]
Cryopreserved	224 (69.8)	232 (77.3)	24 (10.1)	
Discard	7 (2.2)	9 (3)	198 (83.2)	
Live birth % (*n*) for single transferred blastocysts	42.5% (43/90)	30.5% (18/59)	6.3% (1/16)	0.003[a]

[a]Chi-squared test.
[b]One-way analysis of variance (ANOVA).

(Salas-Vidal and Lomelí, 2004). From immunofluorescent analysis studies performed in mouse blastocysts, ErbB3 (known to be involved in the proliferation of the mTE cells) was found to be expressed in a speckled distribution along the long CS, leading to the suggestion that this may indicate active vesicular transport in the form of the visible vesicles (Salas-Vidal and Lomelí, 2004). This selective communication may allow for a more direct delivery of information to specific cells that may be too far from the ICM and may assist in explaining the positive association observed with CS presence and clinical outcomes.

https://doi.org/10.1017/S0967199422000570 Published online by Cambridge University Press

Table 4. Multivariable regression analysis of the predictive role of cytoplasmic strings for the achievement of clinical pregnancy with a fetal heart while controlling for confounders

Parameter	Adjusted odds ratio (95% CI)	*P*-value
CS presence	1.537 (0.271–8.708)	0.627
Female age	0.937 (0.871–1.009)	0.087
Insemination method		
ICSI, IVF	Reference, 0.455 (0.219– 0.949)	–, 0.036
Blastocyst quality		
Top	Reference	–
Fair	0.435 (0.210–0.904)	0.026
Poor	0.141 (0.028–0.704)	0.017
Blastocyst stage of development		
BL	0.829 (0.304–2.262)	0.714
XBL	Reference	–
HGBL	1.377 (0.590–3.213)	0.459

Previously in the published literature, it has been suggested that CS are an indication of a defect with *in vitro* culture (Hardarson *et al*., 2012) and their presence has been suggested to have a negative effect on embryo development, in particular when the CS persist beyond the expansion stage (Scott, 2000). The current study identified CS activity ceasing mostly during the expansion stage of development (49.1%), and in a small percentage of blastocysts in which activity ceased during the hatching stage (17.2%). These observations did not translate to a reduction in embryo fate of the live birth rate. Other researchers have found the presence of CS to have a positive effect (Eastick *et al*., 2021; Ma *et al*., 2021) or at the least no effect (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011; Ciray *et al*., 2014; Ebner *et al*., 2020) on blastocyst viability.

Cytoplasmic strings were detected as early as 90.3 hpi that was similar to that reported by Ebner *et al.* (2020) at 90.8 hpi. Interestingly, the authors of the study noted that CS were never seen in the early blastocyst stage, contrary to our report. Additionally, 59.0% of blastocysts had CS first appear during early blastulation and cease activity during the expansion stage of blastulation (49.1%). Two other studies have reported the presence of CS to be related to the grade of blastocoel expansion (Ebner *et al*., 2020; Ma *et al*., 2021). Similarly, the current study also indicated that the stage of expansion was related to CS presence, with a bivariate regression analysis suggesting that the chances of detecting CS in hatching blastocysts were 2.13 times more probable compared with expanded blastocysts. These findings could be attributed to differences in the time-lapse equipment used or the argument that, as the blastocoel cavity expands, visibility of the CS become more apparent. It has been suggested that the CS may be an artefact associated with blastocoelic collapses (Ebner *et al*., 2020).

A strength of the current study is that it is the largest study to date to assess CS presence in fresh cultured *in vitro* human blastocysts. Other studies that exist are limited to top quality blastocysts that are suitable for transfer or cryopreservation or blastocysts that have been frozen and thawed. Another strength of the study is that the entire cohort was included in the study, including blastocysts that were not suitable for transfer or cryopreservation, therefore allowing for a greater understanding of the role they play in blastocysts of differing quality. Similarly, Ebner *et al.* (2002) reported a large percentage (74.7%) of blastocysts that were considered high quality and had CS present (Ebner *et al*., 2020). Another strength of the study is that CS assessment was performed by a single assessor on all blastocysts, therefore eliminating the possibility of intra-observer variation.

The current study has some weaknesses that should be addressed. The fact that CS were more predominantly found in blastocysts that were expanded and top quality could be attributed to the fact that the blastocoel is clear and therefore more easily seen. However, contrary to this, blastocysts that are poor in quality may have less cells that form the TE, therefore also making it clearer to detect the CS. In addition, the interpretation of CS may be complicated due to reports of two types of CS being present at the same time: those that connect the ICM to the TE cells and those that appear as artefacts from blastocoelic collapses. This warrants further exploration in future studies.

In conclusion, this study has confirmed a positive association of the presence of CS in human blastocysts with blastocyst quality and is not a negative predictor of outcome. Furthermore, it has shown that blastocysts with a higher number of CS and vesicles present, were at the more advanced stages of development, and resulted in a higher live birth rate. The possibility of CS being used as an ultrastructure marker in the identification of higher quality blastocysts for embryo transfer requires further studies to confirm these findings and to gain more insight into their value in the modern IVF laboratory.

Data availability. The data underlying this article are available in UNSWorks at: http://hdl.handle.net/1959.4/100660

Financial support. Not applicable.

Conflict of interest. The authors declare none.

Ethics approval. This is an observational study. The IVFAustralia Research Ethics Committee has confirmed that no ethical approval is required.

Key message. Higher numbers of CS and vesicles in human blastocysts are associated with top quality blastocysts and are not a negative predictor of development. Blastocysts with larger numbers of CS and vesicles had a significant increase in live birth rate.

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https://doi.org/10.1017/S0967199422000570 Published online by Cambridge University Press