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ORIGINAL ARTICLE *Infertility*

First PGT-A using human *in vivo* blastocysts recovered by uterine lavage: comparison with matched IVF embryo controls†

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STUDY QUESTION: After controlled ovarian stimulation (COS) and IUI, is it clinically feasible to recover *in vivo* conceived and matured human blastocysts by uterine lavage from fertile women for preimplantation genetic testing for aneuploidy (PGT-A) and compare their PGT-A and Gardner scale morphology scores with paired blastocysts from IVF control cycles?

SUMMARY ANSWER: In a consecutive series of 134 COS cycles using gonadotrophin stimulation followed by IUI, uterine lavage recovered 136 embryos in 42% (56/134) of study cycles, with comparable *in vivo* and *in vitro* euploidy rates but better morphology in *in vivo* embryos.

WHAT IS KNOWN ALREADY: *In vivo* developed embryos studied in animal models possess different characteristics compared to *in vitro* developed embryos of similar species. Such comparative studies between *in vivo* and *in vitro* human embryos have not been reported owing to lack of a reliable method to recover human embryos.

STUDY DESIGN, SIZE, DURATION: We performed a single-site, prospective controlled trial in women ($n = 81$) to evaluate the safety, efficacy and feasibility of a novel uterine lavage catheter and fluid recovery device. All lavages were performed in a private facility with a specialized fertility unit, from August 2017 to June 2018. Subjects were followed for 30 days post-lavage to monitor for clinical outcomes and delayed complications. In 20 lavage subjects, a single IVF cycle (control group) with the same ovarian stimulation protocol was performed for a comparison of *in vivo* to *in vitro* blastocysts.

PARTICIPANTS/MATERIALS, SETTINGS, METHODS: Women were stimulated with gonadotrophins for COS. The ovulation trigger was given when there were at least two dominant follicles ≥18 mm, followed by IUI of sperm. Uterine lavage occurred 4–6 days after the IUI. A subset of 20 women had a lavage cycle procedure followed by an IVF cycle (control IVF group). Recovered embryos were characterized morphologically, underwent trophectoderm (TE) biopsy, vitrified and stored in liquid nitrogen. Biopsies were analyzed using the next-generation sequencing technique. After lavage, GnRH antagonist injections were administered to induce menstruation.

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MAIN RESULTS AND THE ROLE OF CHANCE: A total of 134 lavage cycles were performed in 81 women. Uterine lavage recovered 136 embryos in 56 (42%) cycles. At the time of cryopreservation, there were 40 (30%) multi-cell embryos and 96 (70%) blastocysts. Blastocysts were of good quality, with 74% (70/95) being Gardener grade 3BB or higher grade. Lavage blastocysts had significantly higher morphology scores than the control IVF embryos as determined by chi-square analysis ($P < 0.05$). This is the first study to recover *in vivo* derived human blastocysts following ovarian stimulation for embryo genetic characterization. Recovered blastocysts showed rates of chromosome euploidy similar to the rates found in the control IVF embryos. In 11 cycles (8.2%), detectable levels of hCG were present 13 days after IUI, which regressed spontaneously in two cases and declined after an endometrial curettage in two cases. Persistent hCG levels were resolved after methotrexate in three cases and four cases received both curettage and methotrexate.

LIMITATIONS, REASON FOR CAUTION: The first objective was to evaluate the feasibility of uterine lavage following ovarian stimulation to recover blastocysts for analysis, and that goal was achieved. However, the uterine lavage system was not completely optimized in our earlier experience to levels that were achieved late in the clinical study and will be expected in clinical service. The frequency of chromosome abnormalities of *in vivo* and IVF control embryos was similar, but this was a small-size study. However, compared to larger historical datasets of *in vitro* embryos, the *in vivo* genetic results are within the range of high-quality *in vitro* embryos.

WIDER IMPLICATIONS OF THE FINDINGS: Uterine lavage offers a nonsurgical, minimally invasive strategy for recovery of embryos from fertile women who do not want or need IVF and who desire PGT, fertility preservation of embryos or reciprocal IVF for lesbian couples. From a research and potential clinical perspective, this technique provides a novel platform for the use of *in vivo* conceived human embryos as the ultimate benchmark standard for future and current ART methods.

STUDY FUNDING/COMPETING INTEREST(S): Previvo Genetics, Inc., is the sole sponsor for the Punta Mita, Mexico, clinical study. S.M. performs consulting for CooperGenomics. J.E.B. and S.A.C. are co-inventors on issued patents and patents owned by Previvo and ownshares of Previvo. S.N. is a co-author on a non-provisional patent application owned by Previvo and holds stock options in Previvo. S.T.N. and M.J.A. report consulting fees from Previvo. S.T.N., S.M., M.V.S., M.J.A., C.N. and J.E.B. are members of the Previvo Scientific Advisory Board (SAB) and hold stock options in Previvo. J.E.B and S. M are members of the Previvo Board of Directors. A.N. and K.C. are employees of Previvo Genetics. L.V.M, T.M.M, J.L.R and S. S have no conflicts to disclose.

TRIAL REGISTRATION NUMBER: Protocol Registration and Results System (PRS) Trial Registration Number and Name: Punta Mita Study TD-2104: Clinical Trials NCT03426007.

Key words: *in vivo* / uterine lavage / blastocyst / next-generation sequencing / IVF / morphology / preimplantation genetic testing for aneuploidy / reciprocal pregnancy for lesbian couples

Introduction

Early work performed by Buster and colleagues recovered a limited number of human embryos from the uteri of fertile ovum donors who underwent lavage in natural ovulatory cycles (Buster *et al*., 1985). Although full-term pregnancies were obtained in recipients following the donation and transfer of the retrieved *in vivo* embryos, chromosome testing of these embryos was not possible in the early 1980s. Preimplantation genetic testing for aneuploidy (PGT-A) was introduced later (Munné *et al*., 1993). Since then, the field of ART and PGT has significantly evolved. It has been shown that embryonic chromosome abnormalities are the major cause for the failure of implantation with advancing maternal age, with rates ranging from 40% in egg donors to 80% in women >42 years old (Ata *et al*., 2012), and that replacing euploid embryos reverses the decline in implantation since normal embryos implant equally well at any age (Harton *et al*., 2013). Indeed, most randomized clinical trials using PGT-A have shown a significant increase in implantation rates compared to control groups in sub-groups of patients (Yang *et al*., 2012; Forman *et al*., 2013, Scott *et al*., 2013; Rubio *et al*., 2017). However, maternal age is not the only factor contributing to chromosome abnormalities; performance of IVF by itself can induce aneuploidy or mosaicism (Munné *et al*., 1997; Munné and Alikani, 2011; Munné *et al*., 2017b). Indeed, there is great variability of aneuploidy rates *in vitro* by the IVF center, ranging from 17 to 60% abnormal blastocysts in egg donors (Munné *et al*., 2017a). Unfortunately, other than IVF, no other current method has been available to obtain human blastocysts for PGT. Uterine lavage, which does not involve IVF, provides a new alternative for recovery of blastocysts for the performance of genetic testing on *in vivo* conceived human embryos. *In vivo* embryos may offer an ideal control standard for comparison against current and new ART procedures.

In vivo developed embryos in animal models possess different characteristics (e.g. lower rates of chromosomal abnormalities, higher rates of blastocyst formation and reduced genetic aberrations) compared to *in vitro* developed embryos of similar species (Hyttel *et al*., 2000; Hamm *et al*., 2014). Recent studies performed using genetic stability measurements in bovine models have shown significantly higher chromosomal instability *in vitro* cultured rather than *in vivo* derived embryos (Tšuiko *et al*., 2017).

In this study, we demonstrate for the first time the feasibility of recovering multiple *in vivo* fertilized human blastocysts following controlled ovarian stimulation (COS) and IUI, without IVF, using a specialized uterine catheter and lavage system. We aim further to characterize the recovered *in vivo* blastocysts using PGT-A via next-generation sequencing (NGS) methods and report for the first time a comparative Gardner scale morphological analysis of *in vivo* versus *in vitro* conceived embryos.

Materials and Methods

Recruitment and ethical approval

Approval for the research protocol was obtained from the Ministry of Health of the State of Nayarit, Mexico (Servicios de Salud de Nayarit) and the Western Institutional Review Board, USA (Study no. 1176044). This research trial was registered with ClinicalTrials.gov (Identifier: NCT03426007). Subject recruitment, COS and all lavage procedures were conducted at Punta Mita Hospital, Punta de Mita, Mexico. Research study volunteers were recruited from the hospital's oocyte donor list during the study period of August 2017 to June 2018. All anonymous oocyte donors gave written informed consent before study inclusion. Each donor gave her consent for oocytes and embryos to be used for research or potential anonymous donation. Anonymous sperm donors were recruited via local advertisements. All oocyte and sperm donors were tested for sexually transmitted infectious diseases and had negative results for hepatitis B surface antigen, hepatitis C core antibody, human immunodeficiency virus, syphilis, gonorrhea and chlamydia. Donor sperm preparation and insemination followed the local standard of care protocols at Punta Mita Hospital. Sperm donors gave consent for sperm cryopreservation, quarantine and fertilization of donor oocytes for the above embryos. A small number of IUI procedures ($n = 28$) were performed with fresh anonymous donor sperm, with the remaining majority of IUI procedures performed with anonymous cryopreserved donor sperm ($n = 106$). Both oocyte and sperm donors received financial compensation for their participation. Both oocyte and sperm donors waived rights to their oocytes, sperm and embryos after gamete donation to the Punta Mita Hospital.

For inclusion, the oocyte donors were required to be of good general health with two ovaries and no known reproductive health issues affecting their fertility, by design. Each subject had her uterine cavity assessed by a hysterosalpingogram to document a normal uterine cavity and bilateral tubal patency. Subjects were excluded if they had a BMI >40 kg/m^2, history of a prior ovarian cystectomy or contraindications to the use of oral contraceptive pills (OCPs) or other study medications. Subjects gave their informed written consent that in the case of persistent hCG levels after uterine lavage they may be treated with uterine curettage and/or the administration of methotrexate. Subjects were grouped into specific research study cohorts for efficiency and protocol uniformity. Ten cohorts of study subjects were enrolled for COS followed by uterine lavage. Table I details the physical profiles of the subject for uterine lavage. The male sperm donors were of good general health and had normal semen parameters before and after semen cryopreservation.

Of the 134 lavages, 11/134 (8.2%) were infertile couples attempting to conceive for more than 1 year. These women fulfilled the same inclusion and exclusion criteria as the oocyte donors. The male partners of these women were of good general health and had normal semen parameters. The IUI procedures were performed with fresh sperm ($n = 11$). These treatment cycles were randomly interspersed among the research study cohorts.

A subset of the uterine lavage subjects who produced at least one blastocyst was eligible to undergo a single cycle of IVF at a later date (control IVF group). From July 2018 to August 2018, 20 women who had a prior *in vivo* conceived blastocyst biopsied for PGT-A underwent a single cycle of IVF. These subjects gave their informed consent for embryo donation to infertile couples. A single embryo biopsy was performed on all IVF blastocysts.

Table I Demographic profiles of women who underwent uterine lavage.

Number of uterine lavage procedures	134
Number of women	81
Average age (years ± SD)	26.3 ± 5.3
Average BMI (kg/m^2 ± SD)	25.6 ± 4.6
Parity	54.3% multiparous (44/81) 45.7% nulliparous (37/81)
Profile	86.4% oocyte donors (70/81) 13.6% infertile (11/81)

COS protocol

Prior to COS, subjects were started on OCPs for central suppression and synchronization of the study cohort with respect to ovarian folliculogenesis. OCPs were discontinued for 4 days prior to beginning gonadotrophin therapy. Initial doses of gonadotrophins were recombinant FSH (rFSH) 100 IU/day and human menopausal gonadotrophins (hMGs) 75 IU/day for 3 days. On the fourth stimulation day, women underwent a transvaginal ultrasound examination and a serum estradiol (E_2) measurement. Gonadotrophin doses were adjusted to achieve a minimum of two follicles ≥18 mm in diameter. Ovulation was triggered with 5000 IU hCG in the first cohort of subjects ($n = 13$, 10.4%). Subsequent subjects received leuprolide acetate 4 mg and 2500 IU of hCG ($n = 121$, 90.2%). The trigger medication was adjusted to minimize the side effect of ovarian hyperstimulation symptoms after the ovulatory trigger. Subjects returned 36 h later for IUI of donor or partner's sperm. After the IUI, a single uterine lavage procedure was scheduled 4 to 6 days after the IUI to coincide with the human window of implantation period.

For subjects participating in an IVF cycle, COS regimens were designed to match the gonadotrophin dosage and follicular response observed during the lavage cycle with blastocyst recovery. On the day of retrieval, an ultrasound-guided oocyte retrieval was performed under anesthesia using a 16-gauge aspiration needle (Cook Medical, Bloomington, IN, USA). Oocytes were retrieved into a warmed test tube (37°C) containing 1 mL HEPES-HTF (LifeGlobal, Guilford, CT, USA) with 5% human serum albumin (LifeGlobal, Guilford, CT, USA) and transferred to the embryology laboratory for oocyte isolation, fertilization and culture. Following retrieval, subjects were monitored for adverse events for up to 30 days after the retrieval.

Uterine lavage, blastocyst biopsy and genetic analysis

The uterine lavage system consists of a lavage catheter with a collection bottle and a reusable lavage controller (Fig. 1). The lavage catheter is a single-use, sterile handheld instrument containing a fluid supply port positioned at the distal end of the catheter, and collection ports along the longitudinal axis of the catheter tip. The fluid supply port delivers

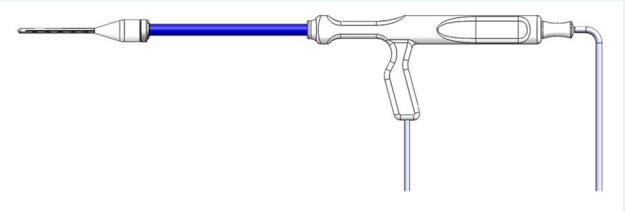

Figure 1 Previvo uterine lavage system. The uterine lavage system consists of a lavage catheter that drains into a collection bottle connected to a reusable lavage controller positioned on a mobile cart. Lavage fluid enters the catheter (on the right side of catheter). Collected fluid exits the lavage catheter (through the handle portion of the catheter).

lavage fluid into the uterine cavity followed by embryo recovery into the collection ports along the length of the catheter tip. The lavage catheter is connected to a fully automated programmable controller for accurate delivery of the lavage fluid to the catheter tip. Subjects were administered i.v. sedation and were positioned in the dorsal lithotomy position. A single-sided speculum was placed for visualization of the cervix. The vagina and cervix were rinsed with normal saline, and a tenaculum was placed on the cervix to ensure a snug contact between the cervix and the uterine lavage catheter. Prior to the beginning of the lavage procedure, the catheter was primed with ~30 mL of Global® Collect Media (LifeGlobal, Guilford, CT, USA) with human serum albumin (HSA) (LifeGlobal, Guilford, CT, USA). The lavage catheter was inserted through the cervical canal and the catheter tip positioned in the middle of the uterine cavity for equal expansion of the uterine cavity (Fig. 2). Approximately 115 mL of lavage fluid, comprising Global® Collect Media (LifeGlobal, Guilford, CT, USA) with a minimal concentration of HSA (LifeGlobal), was infused into the uterus over a 60–90-s period and the returning fluid drained into a collection bottle. The lavage fluid was immediately inspected for any cellular tissues. In this initial series of subjects, the same physician author (S.N.) performed all lavage procedures. All embryos were photographed, and blastocysts were biopsied for PGT-A. Cleavage-stage embryos that matured to blastocysts were cultured for further development. Following culture, any cleavage-stage embryos that made it to the blastocyst stage were included in the PGT-A analysis. The remaining multi-cell embryos were biopsied at their final maturation state but were not included in the current analysis. Vitrification was performed using Kitazato vitrification media kits with the Cryotop® open system (Kitazato, Tokyo, Japan) and a micromanipulator (Integra 3™, CooperSurgical, Inc., Trumbull, CT, USA), with ~3–5 cells removed per blastocyst.

After the uterine lavage procedure, an endometrial biopsy was performed, and a course of GnRH antagonist (GnRHant) injections was administered. For the first 69 lavages, subjects were given a 0.25-mg/day GnRHant i.m. injection (Merck, Naucalpan de Juarez, Mexico) after the lavage and self-administered the daily dose for an additional 2 days. Starting in January 2018, the remaining 65 lavages were given a higher i.m. dose of GnRHant, 0.75 mg, immediately after the endometrial biopsy. Subjects returned 13 days after IUI and had a serum sample drawn for hCG determination. Levels ≥2 mIU/mL were prospectively followed. Cycles where hCG levels were noted to rise were treated with uterine curettage and/or the administration of methotrexate. Subjects were followed for 30 days post-lavage to monitor clinical outcomes and to survey for any delayed complications.

Embryo biopsies were amplified at Punta Mita Hospital IVF laboratory in Mexico to facilitate transport to the PGT laboratory in the USA. Amplified DNA was analyzed by CooperGenomics (Livingston, NJ, USA) using the VeriSeq (Illumina, San Diego, CA, USA) NGS platform, while the cytogenetic analysis was performed with the BlueFuse Multi v3 software (Illumina, San Diego, CA, USA) as described previously (Munné *et al.*, 2017b). This platform can detect the presence of mosaicism with a resolution of 20–80% in a biopsied trophectoderm (TE) sample, as demonstrated previously by mixing ratios of normal to abnormal cell lines. The VeriSeq NGS platform can detect between one and four mosaic cells in a five-cell TE biopsy. Embryo biopsies were classified as a mosaic (from here onwards, these embryos are referred to as 'mosaic embryos') if the abnormality was in between normal and abnormal. Embryo biopsies with normal NGS results were classified as euploid, and embryo biopsies with chromosome abnormalities present in all the cells were classified as aneuploid. Mosaicism lower than 20% (<1/5 cells) or higher than 80% (>4/5 cells) was considered not differentiable due to technical limitations; therefore, <20% mosaics was classified as euploid and >80% was classified as aneuploid. Mosaic embryos with 20–40% abnormal cells were classified as low-impact mosaics, and embryos with >40 to 80% abnormal cells as high-impact mosaics. Irrespective of the percentage of abnormal cells, mosaic biopsies involving three or more chromosomes were classified as complex mosaics. Similarly, embryos with three or more chromosomes with full aneuploidy were classified as complex abnormal.

Blastocyst re-biopsy

PGT data indicated a higher than expected percentage of the complex abnormal blastocyst, which could be predicated on the DNA amplification being performed in the IVF laboratory and not following the CooperGenomics protocol. Therefore, it was decided that all blastocysts diagnosed as complex abnormal, blastocysts without results, as well as some euploid-classified blastocysts (to be used as controls for the PGT results that were euploid), would be re-biopsied with a larger biopsy taken, to re-confirm those diagnoses. The re-biopsy samples were amplified at CooperGenomics in contrast to the amplification performed at the Punta Mita clinical site in the original PGT testing. Chromosome abnormality rates for *in vivo* conceived embryos were compared to the IVF control group, as defined earlier. For purposes of comparison, the results obtained from the re-biopsy of *in vivo* conceived embryos took precedent over the prior PGT diagnosis since more cells were analyzed in the re-biopsy, and strict adherence to the CooperGenomics protocol was followed.

Results

Embryo recovery and safety results

A total of 81 women were enrolled in 134 stimulated cycles for 10 subject cohorts. A total of 1152 mature follicles ≥16 mm were counted. The current recovery efficiency of the catheter for oocytes and embryos was 53% (71/134) per cycle, and 18% (212/1152)

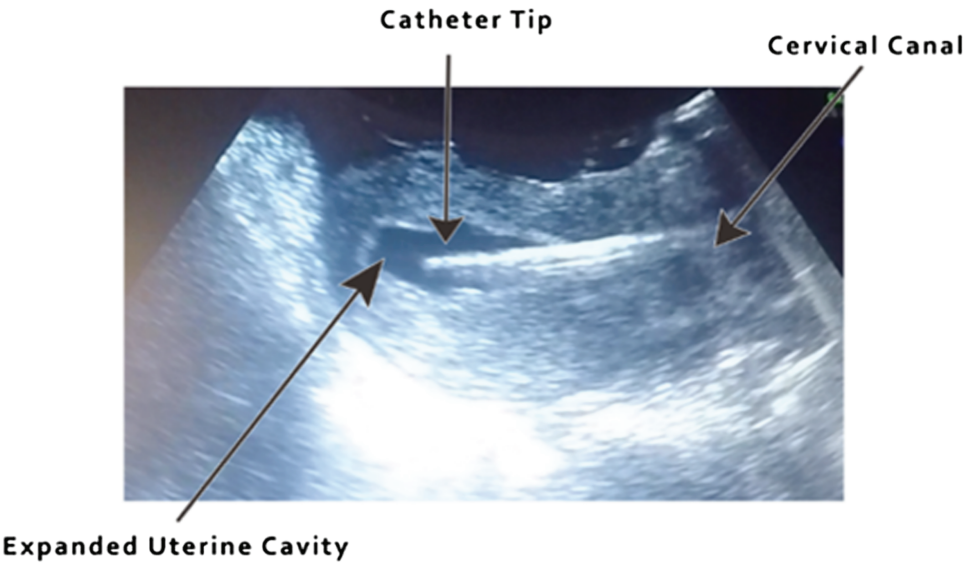

Figure 2 Placement of the uterine lavage catheter tip. The ultrasound image shows the uterine lavage catheter tip positioned in the middle of the woman's uterus with expansion of the uterine cavity during infusion of lavage fluid.

per mature follicle ≥16 mm for all cycles, and 31% (212/676) per follicle ≥16 mm for cycles in which an oocyte or embryo was recovered. When looking at embryo (blastocysts and cleavage stage) efficiency alone, the efficiency was 42% (56/134) and blastocyst-only recovery efficiency was 34% (46/134) (Fig. 3). The embryo recovery with and without oocytes by study cohort demonstrated variability over the study period. The earlier cohorts were associated with lower efficiency. With the optimization in technique and system optimization, the efficiency increased and maintained a range of 50 to 60% embryos per cycle over the last two cohorts. Proper catheter positioning, improved fluid dynamics and enhanced catheter echogeneity have been found to contribute significantly to recovery efficiency.

Uterine lavage was performed at an average of 120 h post IUI with a range of 96–144 h. A total of 136 embryos were collected. At the point of initial embryo recovery, 53% (72/136) of the recovered embryos were blastocysts. In total, 47% (64/136) of the recovered embryos were cleavage stage ranging from three-cell to morula stage. All cleavage-stage embryos were placed into *in vitro* culture for 2–3 days, and following culture, 38% (24/64) of the cultured multicell embryos became blastocysts. Within the group of cleavage stage embryos that made it to blastocyst, 63% (15/24) were at morula stage at the time of lavage recovery, and 37% (9/24) started as earlier-stage embryos, the earliest of which was a four-cell. At the time of biopsy and cryopreservation, 70% (96/136) of the recovered embryos were either blastocyst (53%, 72/136) or were cultured to blastocyst (17%, 24/136). A high percentage of lavages where an embryo (93%, 52/56 lavages) or specifically a blastocyst (93%, 43/46 lavages) was recovered occurred when the lavage was performed at 115–129 h post-insemination (Table II).

At the time of trigger administration, the mean ± SEM E_2 level was 2388 ± 135 pg/mL and the mean number of follicles ≥16 mm was 8.5 ± 0.4 (Table III). At the time of the lavage, the average serum E_2 was 1453 ± 99 pg/mL (range 58–5779 pg/mL) and the progesterone (P_4) was 40 ± 3.0 ng/mL (range 0.5–167 ng/mL) (Table III). Spontaneous menses occurred in women without detectable hCG levels on average 12.2 days after the hCG trigger (range 7–16). Eleven cycles (8.2%, 11/134) had detectable hCG levels (>2 mIU/mL) 13 days after the IUI, which regressed spontaneously in two cases and declined after curettage in two cases (Figs 4A and B). Of the 11 cycles with detectable hCG levels, seven occurred in Cohort 5. Detectable hCG levels (highest level 2652 mIU/mL) were treated with one dose of methotrexate (MTX) at 50 mg/m^2 in five cycles and two doses in two cycles. All subjects with persistent hCG levels were asymptomatic, and there was no evidence of a clinical ectopic pregnancy in the 11 lavage cycles. There were no significant side effects noted after the MTX administration. There were no signs of ovarian hyperstimulation syndrome (OHSS) after the lavage procedure and GnRHant administration in any of the treatment cycles. Other side effects noted were nausea and headache during post-lavage days 1–2 in six women (6/134, 4.4%) and one woman (1/134, 0.7%), respectively.

IVF control cycles

A single COS cycle was performed in 20 women who had previously had blastocysts recovered by uterine lavage. The COS stimulation protocol was similar, and the trigger injection was identical in the IVF control cycles. No ICSI was performed. At the time of trigger administration, the mean ± SEM E_2 level was 2628 ± 353 pg/mL and the mean number of follicles ≥16 mm was 9.7 ± 1.3 (Table IV). In the 20 women who had both an IVF and uterine lavage cycle, the COS cycles were similar with no significant difference on the day of trigger administration in the mean E_2 level or number of follicles ≥16 mm (Table V). Since six of the 20 women contributed two uterine lavages and one contributed three uterine lavages to the total number of lavages (n = 28), the E_2 level and the number of follicles ≥16 mm were averaged for a given individual and the mean level was used in

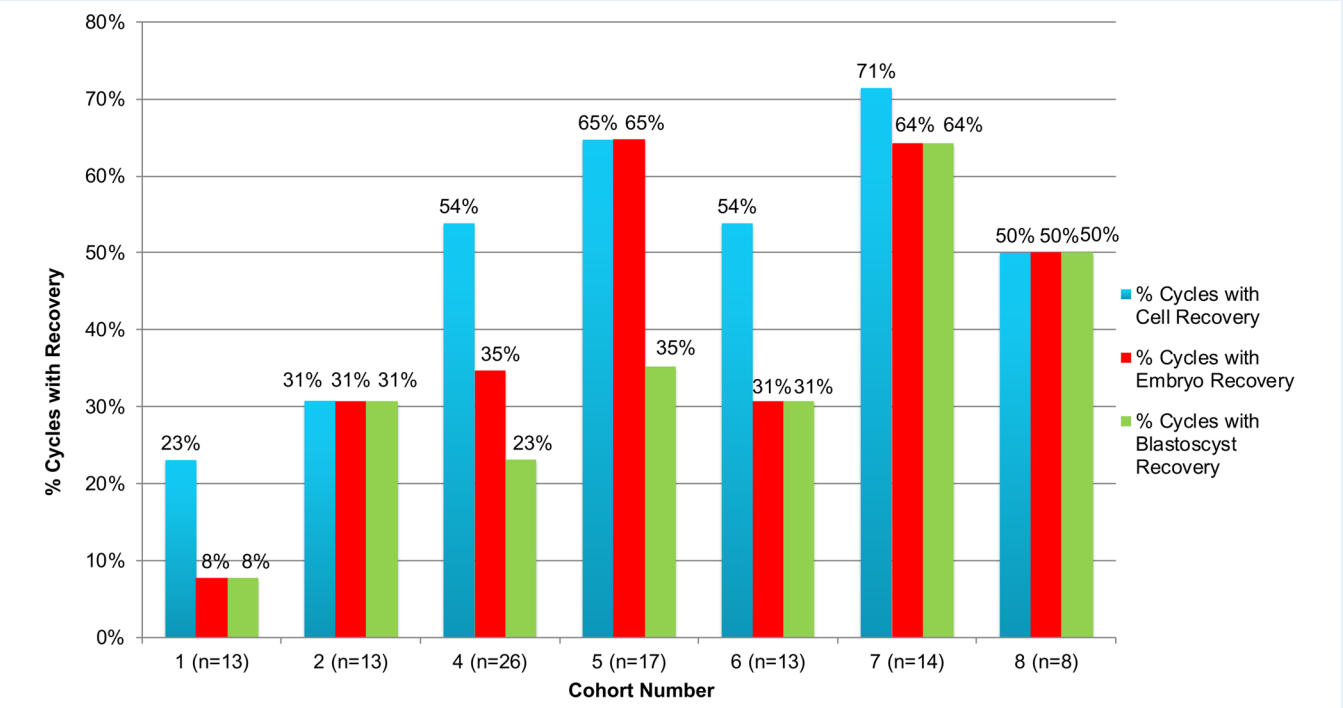

Figure 3 Results of embryo recovery after uterine lavage in women. The cellular recovery of oocytes and embryos (cells/cycle, blue graph), embryos (blastocysts and cleavage stage) (embryos/cycle, red graph) and blastocysts (blastocyst/cycle, green graph) by study cohort. Note: Cohort 3 was excluded from analysis as cycles were unstimulated.

Table II Time interval between IUI and uterine lavage.

	85–99 hours	100–114 hours	115–129 hours	130–145 hours
Distribution of the 134 lavages by time interval	2	7	121	4
Number of lavages with embryo recovery	0	1	52	3
Number of lavages with blastocyst recovery	0	1	43	2

Table III Uterine lavage cycle characteristics.

	Mean	Range	SEM	95% CI
E_2 at ovulation trigger	2388 pg/mL	352–8538 pg/mL	135 pg/mL	2132, 2642 pg/mL
Follicles ≥16 mm at trigger (*n*)	8.5	2–18	0.4	7.8, 9.3
E_2 at lavage	1453 pg/mL	58–5779 pg/mL	99 pg/mL	1287, 1640 pg/mL
P_4 at lavage	40 ng/mL	0.5–167 ng/mL	3.0 ng/mL	34, 46 ng/mL

E_2: estradiol, P_4: progesterone

the analysis. After oocyte retrieval and IVF with sperm, a total of 163 blastocysts were available for PGT-A analysis.

Aneuploidy testing

Supplementary Table SI summarizes the results of PGT-A and compares re-biopsy results of *in vivo* embryos with IVF embryos from the same patient cohorts. A total of 136 *in vivo* embryos were recovered by uterine lavage, which by the time of cryopreservation included 40 cleavage stage and 96 blastocysts. A detailed description of each *in vivo* embryo can be found in the Supplementary Table SI. Regarding blastocysts, three did not produce any result. The remaining 93 blastocysts were diagnosed, and the results are shown in Table VI. Considering euploidy and low-rate mosaics as a whole, the overall rate of chromosome abnormalities in *in vivo* conceived blastocysts (63%) was similar to that of IVF conceived blastocysts (64%). High-mosaicism

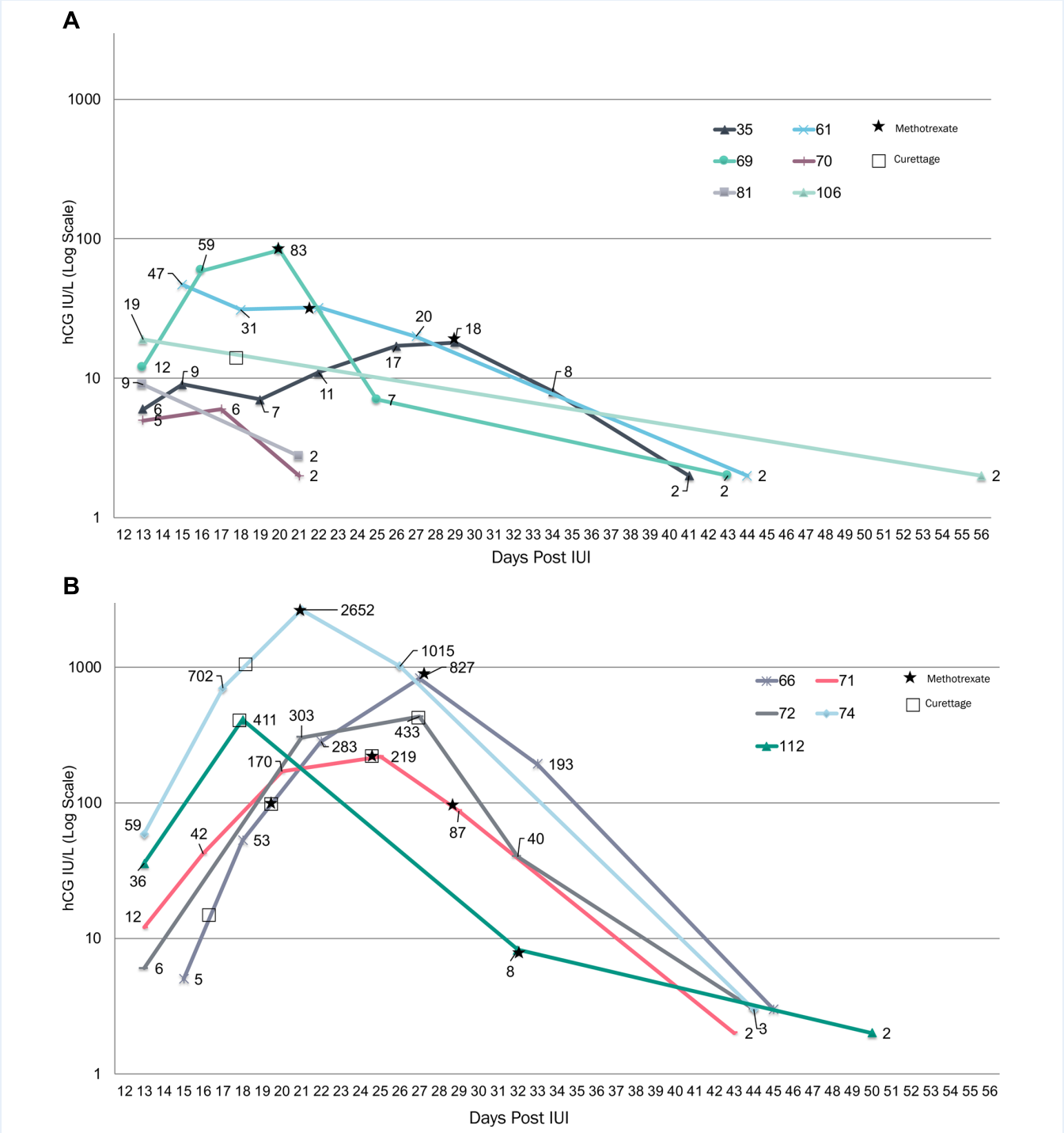

Figure 4 Detectable hCG levels in women after uterine lavage (*n* = 11). Eleven cycles had detectable hCG levels 13 days after the IUI. (**A**) hCG levels ≤100 mIU/mL (*n* = 6). The hCG levels regressed spontaneously in two cycles, declined after curettage in one cycle and resolved after methotrexate in three cycles. All hCG levels were followed to an undetectable level. (**B**) hCG levels >100 mIU/mL (*n* = 5). The hCG levels declined after curettage in one cycle and resolved after both curettage and methotrexate in four cycles. All hCG levels were followed to an undetectable level.

rate (11 versus 9%), complex abnormal rates (8 versus 10%) and aneuploidy rates (19 versus 17%) were also similar. We also examined the blastocyst results from the 20 women that participated in both a lavage and IVF cycle (Table VI). The blastocyst data from the same patients show similar rates of euploidy and aneuploidy for both *in vivo* and IVF blastocysts (control group). The multi-cell embryos recovered were cultured in the laboratory and biopsied, but their results on aneuploidy (seen in Supplementary Table SI) were not compared to control Day 3 PGT-A results since currently a Day 3 biopsy is considered unreliable. Thus, we had no comparison control group.

Table IV IVF cycle characteristics.

	Mean	Range	SEM	95% CI
E_2 at ovulation trigger	2628 pg/mL	339–5931 pg/mL	353 pg/mL	1962, 3293 pg/mL
Follicles ≥16 mm at trigger (*n*)	9.7	0–19	1.3	7.3, 12.1

Table V Uterine lavage and IVF cycles in the same 20 women.

	Uterine lavage Mean ± SEM	IVF Mean ± SEM	Unpaired Student's *t* test
E_2 at ovulation trigger	2521 ± 325	2628 ± 353	$p = 0.83$
Follicles ≥16 mm at trigger (*n*)	9.3 ± 0.9	9.7 ± 1.3	$p = 0.77$

Table VI Genetic analysis of *in vivo* and IVF (control group) blastocysts.

	In vivo blastocysts		IVF blastocysts	
	Data from all lavage cycles	Excluding lavage women with no IVF cycles		P-value
N (total cycles)	134	50	20	-
N (cycles with embryo recovery)	56	28	20	-
N (women)	81	20	20	-
N (embryos collected and analyzed)	93	65	163	
Euploid	50 (54%)	35 (54%)	83 (51%)	
Low-grade mosaic (≤40%)	8 (9%)	6 (9%)	22 (13%)	NS, $P > 0.05$
High-grade or complex mosaic (>40%)	10 (11%)	3 (5%)	14 (9%)	
Aneuploid	18 (19%)	13 (20%)	28 (17%)	
Complex abnormal, triploid	7 (8%)	8 (12%)	16 (10%)	

P = *p*-value comparing genetic results of *in vivo* and IVF blastocysts. No significant differences were found among the values of *in vivo* and IVF blastocysts. All statistical tests were performed using chi-square analysis. If a re-biopsy was performed on *in vivo* blastocysts, only the result of the second biopsy was utilized. (No re-biopsies were performed on the *in vitro* blastocysts.)

Embryo morphology

Of the 96 *in vivo* conceived blastocysts recovered, a rating was assigned to 95/96 (99%) blastocysts using the Gardner classification to assess and rank embryo quality (Gardner *et al*., 2000). There was one blastocyst which was not assigned a grading due to inability to visualize the inner cell mass (ICM). Blastocysts were placed into two groups: 'good morphology' if the blastocyst was ≥3BB, or 'poor morphology' if the blastocyst was <3BB or had a 'C' grading in its TE or ICM. Of the graded blastocysts, 74% (70/95) were assigned to the good morphology group, and 26% (25/95) were assigned to the poor morphology group (Table VII). A large proportion of the blastocysts graded were assigned 4AA (16%, 15/95) or 5AA (12%, 11/95), respectively. Blastocyst photos were re-evaluated by an independent embryologist using still images taken from the micromanipulator prior to cryopreservation. Of the blastocysts analyzed, 99% (95/96) were assigned grading. In this second evaluation, the finding of a significantly higher percentage of 'good morphology' *in vivo* blastocysts compared to the control IVF group was confirmed. The results of the ratings are shown in Tables VII and VIII. As a secondary analysis, uterine lavage subjects who did not participate in the IVF were excluded and compared. Similar to the previous analysis, a significant difference was found between the embryo morphology of the *in vivo* and IVF blastocysts.

Discussion

In this report, we describe the first large-scale clinical trial of uterine lavage procedures performed after COS and IUI in which we demonstrate the reliable recovery of *in vivo* conceived human embryos and the successful management of the occasionally retained embryo. Uterine lavage allows simplified access to *in vivo* conceived embryos, and data from these embryos will likely provide a benchmark for future comparisons to *in vitro* cultured embryos. For women with open fallopian tubes and the absence of male factor infertility, the recovery of *in vivo* blastocysts by uterine lavage for PGT, fertility preservation or reciprocal conception for lesbian couples may offer a significant

Table VII ***In vivo*** **versus IVF blastocyst morphology comparisons for all women.**

	IVL-PMH (*n*)	IVL-IHE (*n*)	IVF	P1*	P2*
Good morphology blastocysts (*n*)	70 (73.7%)	72 (75.8%)	77 (43.3%)		
Poor morphology blastocysts (*n*)	25 (26.3%)	23 (24.2%)	101 (56.7%)	$p < 0.05$	$p < 0.05$
Total	95**	95**	178		

Comparison of good morphology and poor morphology of the *in vivo* lavage (IVL) and IVF blastocysts using the entire IVL blastocyst population.
*P1 = *p*-value comparing the grades of the IVL blastocysts (as graded by Punta Mita Hospital: PMH) to the IVF blastocysts. P2 = *P* value comparing the grades of the IVL blastocysts (as graded by the in house embryologist: IHE) to the IVF blastocysts. All statistical tests performed using chi-square analysis.
**Total value excluded the non-graded blastocyst ($n = 1$)

Table VIII ***In vivo*** **versus IVF morphology comparison in the same woman.**

	IVL-PMH (*n*)	IVL-IHE (*n*)	IVF	P1*	P2*
Good morphology blastocysts (*n*)	45 (68.1%)	47 (71.2%)	77 (43.3%)		
Poor morphology blastocysts (*n*)	21 (31.9%)	19 (28.8%)	101 (56.7%)	$P < 0.05$	$P < 0.05$
Total	66	66	178		

Comparison of good morphology and poor morphology IVL and IVF blastocysts using IVL blastocyst data from women who also underwent a lavage cycle.
*P1 = *P* value comparing the grades of the IVL blastocysts, as graded by PMH, to the IVF blastocysts. P2 = *P* value comparing the grades of the IVL blastocysts, as graded by the in-house embryologist, to the IVF blastocysts

clinical advantage due to its simplicity compared to the traditional IVF procedure. Most women considering assisted reproduction to address inheritable single-gene mutations, and in other cases recurrent pregnancy loss, are fertile and can conceive without IVF.

Initial reports of uterine lavage described its use with donor oocytes at a time when researchers did not have access to methods for PGT and embryo vitrification, which would also have allowed genetic diagnosis (Buster *et al.*, 1985). In this first series of uterine lavages reported here, the catheter design used demonstrated the recovery of oocytes and embryos in 53% (71/134) of lavage procedures. In the initial four cohorts of lavage procedures, there was a steady increase in the number of cells (oocytes and embryos) and number of blastocysts recovered (Fig. 3). The total number of oocytes and embryos per cycle is likely an indicator of catheter efficiency, or the ability to retrieve any pertinent reproductive cell if present in the uterus. Similarly, the total number of blastocysts per cycle is an indicator of the relevant clinical efficiency of the uterine lavage procedure since collected oocytes 4–6 days after IUI would not be suitable for fertilization and thus clinically irrelevant. We attribute the initial increase in cellular recovery in the first four cohorts of subjects to careful positioning of the tip of the catheter into the middle of the uterine cavity. Great care was taken to avoid digging the catheter tip into the endometrium. Further positioning of the tip of the catheter 1 cm below the fundus of the uterus allowed for uniform expansion of the cavity, as noted by ultrasound. In Fig. 3, from subject cohorts #5 to #11 ($n = 82$), mean catheter efficiency was 2.0 cells/cycle, and the mean number of blastocysts recovered was 0.96 blastocysts/cycle. In comparison to IVF, however, uterine lavage with this current catheter design is less efficient than a standard oocyte retrieval and *in vitro* culture of embryos. In the 20 women who had both an IVF cycle and at least one uterine lavage, the cumulative number of blastocysts collected was 65 blastocysts in 28 lavage cycles versus 163 blastocysts in 20 IVF cycles, despite similar ovarian stimulation results at the time of trigger administration.

It is clear that multiple factors could impact negatively on the recovery of suitable embryos for analysis and for reproductive services. These include variable response in the COS regimen prescribed, semen parameters, subtle tubal disease, uterine configuration and variations in endometrial receptivity leading to the variable attachment of the blastocyst to the endometrium (Ruiz-Alonso *et al.*, 2013). In two subjects, the P_4 levels were noted to be 0.5 ng/mL at the time of lavage, strongly suggesting that the patient did not ovulate either due to faulty administration of the trigger medication or a failure to respond to the trigger. It is indeed difficult to determine the efficiency of each lavage procedure, due to the inability to verify the number of oocytes ovulated and number of embryos available for recovery. Although a hysterosalpingogram was performed prior to the uterine lavage, an occult tubal factor could be responsible for the absence of embryos and oocytes available for recovery. Furthermore, the exact hour of fertilization is unknown with conception following IUI, which may impact optimal timing for the lavage procedure. We performed lavage at 120 h post IUI as it was expected that the majority of embryos present in the uterus would be blastocysts.

Further examination of the lavage cycles that yielded blastocyst compared to those with no recovery revealed no difference in the mean E_2 or P_4 levels at the time of lavage. It is unclear whether elevated levels of E_2 and P_4 may be responsible for endocrine changes that might influence embryo implantation or the tubal transport of the embryo into the uterine cavity. Although these initial uterine lavages were performed with conscious sedation, further changes to the lavage procedure are planned to allow for lavages to be performed without sedation.

After uterine lavage, a course of GnRHant was administered to minimize the possibility of retained embryos. In this series, there were

11 cases in which hCG was detected after lavage for an incidence of 8.2% (11/134 lavages). In the initial 52 lavages, there was one subject with a detectable hCG level, for a 1.9% incidence of a retained embryo (1/52). After noting a marked increase in post-lavage detectable hCG (7/17) in cohort #5, the protocol was changed to prevent patient non-compliance in self-administering the two additional (0.25 mg) doses of GnRHant. In subsequent lavage cycles, a single dose of GnRHant (0.75 mg) was administered by a staff member immediately after the endometrial biopsy. After this change, the number of lavages with detectable hCG dropped to 4.6% (3/65). In managing post-lavage detectable hCG levels, a uterine curettage and/or the administration of MTX was performed more earlier than in routine clinical practice.

Genetic examination of the *in vivo* conceived embryo showed that the rate of euploid and low-mosaic blastocysts (classified as normal if analyzed by array comparative genomic hybridization: aCGH) is comparable to control IVF blastocyst from the same patient population. The rate of 'normal' embryos derived from uterine lavage is also within the wide range seen between ART centers, ranging from 40 to 80% for ART blastocysts analyzed by aCGH (Munné *et al*., 2017a). Also, one must consider that after the lavage procedure, residual hCG production was detected despite GnRHantag administration, suggesting that some good prognosis (euploid) embryos may not have been retrieved and included in the study. Overall, we conclude that the rate of chromosome abnormalities in *in vivo* conceived embryos is within the range of high quality donated *in vitro* embryos that would be selected for donation.

Although chromosome abnormalities of the *in vivo* conceived embryos seem to be in the range of *in vitro* produced embryos, the morphology demonstrated by the *in vivo* embryo is significantly better. The data suggests the rate of good morphology for *in vivo* blastocysts to be between 64 and 77% based on the gradings assigned by the respective embryologists. This is in contrast to the rate of good morphology IVF blastocysts which we have found to be 43%, a value within the range of data previously published by Rubio and colleagues, estimating the rate of good morphology blastocysts to be 36% (Rubio *et al*., 2014).

Our study has limitations. Multi-cell embryos obtained by uterine lavage between 85 and 145 h after IUI must be further cultured to the blastocyst stage prior to PGT-A testing. These embryos may represent either oocytes fertilized at a later time after the IUI or embryos developing at a slower rate than expected. It has been reported that sub-optimal biopsy, tubing or sample transport methods can produce complex abnormal embryos (three or more chromosomes involved in an abnormality), complex mosaic and no results (Witney *et al*., 2018). Owing to regulations that restrict the transport of biological samples, the original embryo biopsies were amplified *in situ* following a different protocol than that used at the reference PGT laboratory. Given this deviation, we chose to re-biopsy all complex abnormal, mosaics and aneuploidy embryos with results and no results as well as a fraction of euploid embryos, using the euploid ones as control. As shown in Supplementary Table SI, most euploid embryos (8/9) were confirmed as such, while 7/15 complex abnormal embryos were reclassified as euploid or low-risk mosaic, while others as aneuploid. Complex abnormal embryos in egg donor embryos should be a rare event, and therefore, we explain these PGT results as an artifact of the biopsy, tubing, transport to the USA or not using the correct amplification protocol, while trusting the euploid results of the PGT. Initial results of embryo transfers utilizing the euploid blastocysts into infertile recipients ($n = 5$) include two ongoing pregnancies (40%, 2/5) into the second trimester. The ongoing pregnancies are a single and twin intrauterine pregnancy, for an implantation rate of 33% (3/9).

Conclusion

This study demonstrates that uterine lavage is a potentially feasible method to recover multiple embryos in fertile patients who desire to preserve embryos, are planning for genetic testing of embryos or wish shared maternity. Uterine lavage may offer a nonsurgical minimally invasive alternative to IVF. Further research in this area will include potential modifications to the stimulation protocol and determining the optimal endocrine environment for uterine lavage. These protocol changes in addition to catheter and controller modifications should help to improve embryo recovery further thereby reducing the risk of retained pregnancy to a rare event.

Uterine lavage is not without its limitations. Patients should have patent fallopian tubes, normal ovarian reserve and no male factor. Uterine lavage relies on a normal *in vivo* fertilization process. In *in vivo* fertilization, there is no way to verify the total number of oocytes ovulated or whether fertilization occurred. Uterine lavage may have its best results in a fertile population for general screening of genetic abnormalities and may be less effective than IVF for infertile couples.

Although a nascent technology, uterine lavage has the potential to provide a new tool to acquire genetic information on *in vivo* conceived embryos. The lavage procedure provides a low-cost, minimally invasive, reproducible and effective way to acquire substantial numbers of *in vivo* conceived, and *in vivo* 'cultured', late-stage blastocysts that have had minimal exposure to laboratory media. In the early experience from the 1980s, *in vivo* blastocysts used for donation were producing acceptable implantation rates for the time (Buster *et al*., 1985). In this current report, the possible clinical superiority of *in vivo* over IVF embryos remains to be proven and will require a larger dataset. Uterine lavage has utility for patients who are at risk of having a pregnancy or child with genetically inherited illnesses. It may also be used to address infertility in cases where advanced maternal age or multiple miscarriages may be the primary cause of reproductive failure. Lastly, uterine lavage provides health care providers yet another ART option to address an increasingly diverse patient population. From a research perspective, this technique offers the possibility to use *in vivo* conceived embryos as the ultimate control to benchmark current and future ART methods and provide new scientific insights into human embryology by characterizing any difference between embryos conceived within or outside the fallopian tubes.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

S.M. and S.T.N. conceived and designed the study and wrote and edited the manuscript. S.T.N., J.L.R. and S.N. developed the clinical protocol and supervised the clinical study. M.V.S., M.J.A., C.N., S.A.C. and J.E.B. developed the protocol, advised the study and assisted in the editing of the manuscript. S.S. conducted the genetic sequencing and data analysis. L.V.M., A.N. and K.C. managed the clinical study and assisted with data analysis and editing of the manuscript, and T.M.M. conducted embryology procedures.

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Conflict of interest

S.M. performs consulting for CooperGenomics and own shares of Previvo. J.E.B. and S.A.C. are co-inventors on issued patents and patents owned by Previvo and own shares of Previvo. S.N. is a co-author on a non-provisional patent application owned by Previvo and holds stock options in Previvo. S.T.N. and M.J.A. report consulting fees from Previvo. S.T.N., S.M., M.V.S., M.J.A., C.N. and J.E.B. are members of the Previvo Scientific Advisory Board (SAB) and hold stock options in Previvo. J.E.B and S. M are members of the Previvo Board of Directors. A.N. and K.C. are employees of Previvo Genetics. L.V.M, T.M.M, J.L.R. and S.S. have no conflicts to disclose.

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