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Lessons from human umbilical cord: gender differences in stem cells from Wharton's jelly

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ABSTRACT

Objective: To study the molecular features of mesenchymal stem cells from Wharton Jelly (WJ-MSCs) of umbilical cord to predict their differentiation capacity.
Design: Comparison of gene expression from mesenchymal stem cells of male and female umbilical cord
Setting: University hospital
Patient (s): umbilical cords (n = 12, 6 males and 6 females) retrieved from spontaneous full-term vaginal delivery of healthy women
Intervention: we analyzed the expression of the stemness related genes C-MYC, OCT4, SOX2 and NANOG and of the epigenetic modulating gene DNA-methyltransferase 1 (DNMT1).
Mean outcome measure: WJ-MSCs were isolated by standard procedures and immunophenotypically characterized. Gene expression analysis of stemness related genes and the epigenetic modulating gene DNMT1 were performed by real-time PCR
Results: expression of the OCT4 and DNMT1 genes was significantly higher in WJ- MSCs isolated from male subjects, as compared to MSCs isolated from female-derived WJ. The resulting higher expression of OCT4 and DNMT1 in WJ-MSCs from males as compared with female WJ-MSCs for the first time identifies a specific relationship between stemness genes, an epigenetic modulator, and gender differences.
Conclusion: our findings disclose novel biomedical implications in WJ-MSCs related to the sex of the donor, thus providing additional cues to exploit their regenerative potential in allogenic transplantation.

Introduction

Cell therapy represents at the moment a crucial area in regenerative medicine. Aging of population prompts the need for innovative therapeutic modalities implementing the stem cell field of application based upon induced pluripotent stem cells (iPS), or mesenchymal stem cells (MSCs), obtained from sources such as the umbilical cord, Sertoli cells, dental pulp, or the adipose tissue [1–4]. Stem cells are undifferentiated elements capable to assume a specific phenotype when exposed to a conditioned milieu [5].

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They exhibit different levels of plasticity and differentiation potential according to the source tissue, and the age, as well as the sex of the donor. In this regard, other authors have observed that stem cells taken from the muscles of female mice afforded a better tissue regeneration than those harvested from male mice. The sex-related differences seemed to come from the different behavior of cells, that following transplantation, differently reacted to oxidative stress in the inflamed recipient tissue. In particular, mesenchymal stem cells (MSCs) derived from males exhibited a higher differentiation rate, as compared to female cells, thus leading to a reduced number of undifferentiated elements, with exhaustion of the tissue-resident pool of stem cells available to rescue the damaged tissue [6–7].

MSCs derived from amniotic fluid and Wharton Jelly (WJ), among all the different tissues sources of stem cells, represent a promising cell population due to their high pluripotency, and WJ-MSCs have also attracted interest for their banking and transplantation capacities [2,8]. The umbilical cord contains two arteries, a vein, Wharton's jelly enveloped in amniotic epithelium or, at the fetal end, a Malpighian keratinized epithelium [7]. Wharton's jelly is a gelatinous tissue within the umbilical cord that contains myofibroblast-like stromal cells. WJ is the primitive connective tissue of the human umbilical cord (UC), described for the first time by Thomas Wharton in 1656 [9]. Subsequently, research efforts have attempted at optimizing the isolation and differentiation of these cells from WJ [10–12]. The WJ cell population expresses the characteristic phenotype of MSCs, exhibiting plastic adhesion and the expression of CD90, CD73 and CD105 [13]. Differences between male and female embryos start very early during development, prior to the onset of hormonal influence, suggesting the presence of genetically driven sexual dimorphisms in humans [14]. The determination of sex is a two-step process for content of sex chromosomes and production of hormones. In the first phase, the sexual chromosomal content guides the differentiation of the bipotential gonadal ridges in testis or ovary [14]. Afterwards, sex hormones produced by the gonads drive the establishment of a range of anatomical and physiological characteristics known as phenotypic sex [15]. Within this scenario, a strong regulation of pluripotency arises as an important aspect of fetal male germ cell development. In human pluripotent stem cell lines, X inactivation frequently occurs in the undifferentiated state, resulting in an identical content of X chromosomes in males and females [16]. Within this context, female fetuses had smaller cord areas and less Wharton's jelly, as compared to the corresponding areas of male fetuses. This is consistent with previous studies showing that male and female fetuses develop their length of the cord and relative placental weight differently from each other under the influence of umbilical ring constriction. Stem cell pluripotency is a key feature orchestrated by a series of stemness regulating genes, among which SOX2, OCT4, NANOG and c-MYC have been largely enrolled also *in vitro* for the generation of iPS [17–18]. OCT4, SOX2, and NANOG form a transcription factor triad that is key to maintaining embryonic stem cell (ESC) identity by activating genes of the self-renewal program and repressing lineage commitment genes. These pluripotency transcription factors bind to and regulate thousands of loci in the genome to maintain pluripotency. These genes are described to be gradually downregulated during aging and stem cell senescence [19–21], thus affecting the capability of stem cells to assume a specific cellular phenotype. Moreover, in ESCs a 50% increase or decrease in OCT4 expression is able to promote differentiation into extraembryonic endoderm and mesoderm, or trophectoderm, respectively, suggesting that the threshold level of OCT4 required to maintain self-renewal and pluripotency is tightly regulated [22]. OCT4 was discovered and characterized 30 years ago [19–23], it is a key stemness marker that is involved in lineage specification and reprogramming of somatic cells in vitro [17]. OCT4, is a transcription factor encoded by the POU5f1 gene (located on chromosome 6 in human and 17 in mouse) and belongs to the POU family of DNA binding-proteins. These proteins regulate the expression of target genes by binding to the octamer motif ATGCAAAT within their promoter or enhancer regions [23]. OCT4, together with SOX2 and NANOG, comprises a core transcriptional network that regulates self-renewal and pluripotency by regulating their own expression, as well as the expression of other self-renewal and lineage-specific genes [24–25]. It is well known that the physical association between SOX2 and OCT4 when bound to DNA is likely to be critical for the induction of pluripotency [17–18]. Epigenetic features are also involved in stemness gene regulation during early male germ cell differentiation. In fact, male-specific methylation occurs in key functional elements of the NANOG and SOX2 promoters, leading to suppression of these genes [17–18]. These observations suggest a substantial difference in the expression of pluripotency genes among male and female fetuses, but there are not studies on the differences in expression between males and females in stem cells drawn from Warthon jelly. In the present study, we aimed at evaluating the molecular differences between male and female WJ-MSCs, by analyzing specific gene expression of c-MYC, OCT4, SOX2, NANOG and DNA-methyltransferase 1 (DNMT1), a major epigenetic remodeler. Our results highlight different features in stem cell morphology and stemness related gene expression between males and females. These findings could be critical in defining which kind or gender of stem cells may be used in regenerative medicine approaches attempting to restore the lost cellular functions among tissues.

Materials and methods

The study included umbilical cords (n = 12, 6 males and 6 females) retrieved from spontaneous full-term vaginal delivery of healthy women. Donors aged between 25 and 35 years, the recruitment criteria were spontaneous birth, donors free from drugs, smoking and diseases.

WJ-MSCs isolation and culture

Fresh human umbilical cords (n = 12) from both sexes were collected after birth by Natural childbirth section in the Gynecologic and Obstetric Clinic, at University of Sassari. The patients gave written informed consent according to the approval of this study by the Ethics Committee (Ethical Committee, CNR September 21, 2017, prot. n°. 0059862). The umbilical cords were collected in phosphate buffer saline (PBS) supplemented with 200 U/ml penicillin (Euroclone Italy), 100 μg/ml streptomycin (Euroclone Italy) and 2,5 μg/ml amphotericin B (Gibco Life Technologies) prior to storage at 4 °C for further WJ-MSCs isolation. Tissues were dissected into small pieces and then washed with an equal volume of PBS (200 U/ml penicillin, 100 μg/ml streptomycin and 2,5 μg/ml amphotericin B). The suspension was centrifuged at 300 x g, 4 °C for 10 min and supernatant was discarded. The precipitate (mesenchymal tissue) was digested with collagenase type I (2 mg/ml) Sigma at 37 °C for 16–18 h with agitation. After neutralization of the enzyme with 10% foetal bovine serum (FBS) (Life Technologies, Grand Island, NY, USA) and filtering (70 μm cell strainer) (Euroclone, Milano, Italy), samples were centrifuged at 600 × *g* for 10 min to separate distinct cell fractions. The MSCs from WJ were immunomagnetically sorted for c/kit using a monoclonal anti-c/kit (CD117) antibody (Miltenyi Biotech, Minneapolis, MN, USA) directly conjugated to microBeads (Miltenyi Biotech) and then expanded in subconfluent conditions in a basic medium (BM), Dulbecco's modified Eagle's Medium (DMEM) (Life Technologies Grand Island, NY, USA) supplemented with 10% foetal bovine serum (FBS) (Life Technologies, Grand Island, NY, USA), 200 mM l-

glutamine (Euroclone, Italy), and 200 U/mL penicillin—0.1 mg/mL streptomycin (Euroclone, Milano, Italy) and plated in 12 cm^2 flasks with this medium. The flasks were placed in the culture incubator at 37 °C with 5% CO2 and saturated humidity for 10–14 days. [26]. After 48 h of incubation, the cultures were washed with DPBS and kept in the fresh medium. The culture medium was changed every 3 days. When the cells reached 80–90% confluence, they were harvested using 0.25% Trypsin EDTA (Euroclone, Milano, Italy), counted and passaged into new flasks. The WJ-MSCs used in this study were stained positive for CD90, CD105, CD44, and CD29, typical of mesenchymal stem cells, and negative for CD34, CD133, and CD45. Cells were prepared for the flow-citometry analysis according to previously described method (26), using a flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA, USA) by collecting 10,000 events and the data analyzed using the Cell Quest Software (Becton Dickinson).

RNA Extraction and Quantitative Polymerase Chain Reaction

RNA was isolated from stem cells at confluence in the presence of the previously described conditions. The isolated component was used for quantitative polymerase chain reaction. RNA was collected by means of the Charge Switch total RNA Cell Kits (Life Technologies, Grand Island, NY, USA). Approximately 1 μg of total RNA was reverse-transcribed into cDNA using the Superscript cDNA kit (Applied Biosystems), according to the manufacturer's protocol. Quantitative polymerase chain reaction was performed using a CFX Thermal Cycler (Bio-Rad) in triplicate (Applied Biosystems), and then incubated under standard qRT-PCR conditions (50 °C for 2 min, 95 °C for 2 min, and then cycled at 95 °C for 15 s, 55–59 °C for 30 s and 60 °C for 1 min, for 40 cycles), according to the qRT-PCR protocol specified in the Quantitative PCRMaster Mix whit Power SYBR® Green, 0.1 μM of each primer, and 3 μL cDNA generated from 1 μg of the total RNA template were mixed in 25 μL volumes and added to each reaction [27]. Target Ct values were normalized to HPRT1 [26,28–29], considered as a reference gene, while the gene levels of stem cells were expressed as fold of change ($2^{-\Delta\Delta Ct}$) relative to the gene levels observed when stem cells reached 80% confluence. Each experiment included a distilled water control. The qRT-PCR analysis was performed for the following set of genes: OCT4, SOX2, NANOG, C-MYC and DNMT1. All primers used (Invitrogen) all of which are described in Table 1.

Statistical Analysis

The Kruskal-Wallis test was applied to compare the groups (male and female) in each target. The statistical analysis was performed with the SPSS software version 17.0. Reverse transcription followed by polymerase chain reaction (RT-PCR) is the most suitable method for the detection and quantification of mRNA. It provides high sensitivity, good reproducibility, and a wide-range quantification. Several mathematical algorithms have been developed to calculate a ratio of expression based on real-time PCR efficiency and the crossing point deviation of an unknown sample against a control. Then, a software tool named REST© (relative expression software) [30] was used, which compares two o more groups and different reference and target genes. The mathematical model used is based on the correction for exact PCR efficiencies and the mean crossing point deviation between sample group(s) and control group(s). Subsequently, the expression ratio results of the investigated transcripts are tested for significance by a Pair Wise Fixed Reallocation Randomisation Test © and plotted using standard error (SE) estimation via a complex Taylor algorithm. Expression variation for each gene is visualized in a whisker-box plot. In this study, the relative expression of the mRNAs was analyzed using the software REST. The non-parametric bootstrapping test was used to evaluate the different expression of gene between male and female.

Results

The results of the analysis performed with the Normfinder software have suggested HPRT1 as the best normalizer compared to GAPDH. Statistical analysis showed that data for all targets were not normally distributed. The Kruskal-Wallis test revealed no significant differences between the male and female groups for the SOX2, NANOG, C-MYC genes, confirming the results of the normoscope software, due to the greater stability of HPRT1 between the two normalizers. We analyzed five genes: SOX2, OCT4, NANOG, C-MYC, DNMT1 identified by a number from 1 to 5 in Fig. 1 and Table 2, using HPRT1 (number 6) as a normalizer (Table 2). This gene is not present in the graphs but only in the tables (Table 2).

We analyzed the gene expression levels in males as compared to females, used as control. The study was performed on umbilical cords of 6 males and 6 females all characterized by spontaneous vaginal delivery in healthy women aged 25 to 35 years (all at the first delivery). Since the epigenetic related gene, DNMT1 was evaluated, we defined specific features of the samples which are important in order to ensure repeatable and more easily interpretable results, and to eliminate possible confusing factors related to lifestyle.

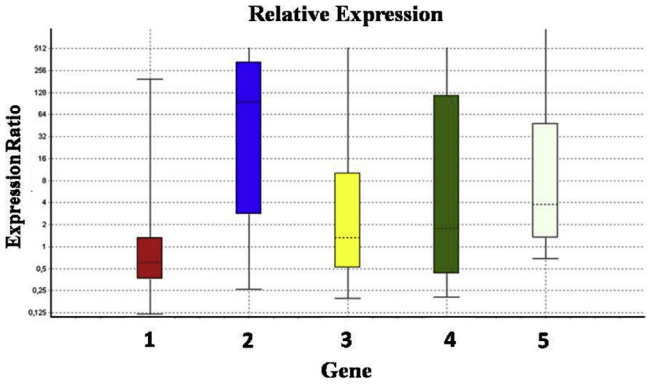

Fig. 1. Boxplots representing the gene expression analysis of the target genes evaluated in WJ-MSCs of males compared to WJ-MSCs of females. The graph shows the expression of the males genes compared to the females, used as control. 1 = SOX2; 2 = OCT4; 3 = NANOG; 4 = C-MYC; 5 = DNMT1.

Table 1
All primers used (Invitrogen)

Primer name	Forward	Reverse
OCT4	GAGGAGTCCCAGGACATCAA	CATCGGCCTGTGTATATCCC
SOX2	CCGTTCATGTAGGTCTGCGAGCTG	CAACGGCAGCTACAGCATGATGC
NANOG	CATGAGTGTGGATCCAGCT	CCTGAATAAGCAGATCCAT
C-MYC	GGACGACGAGACCTTCATCAA	GCACCGAGTCGTAGTCGAG
DNMT1	GACGTCCGAGCGTCACACA	CTCCTTGGGCCGCGCATCAT

Table 2
Five genes analyzed (SOX2, OCT4, NANOG, C-MYC, DNMT1) were identified with a number from 1 to 5, using HPRT1 (number 6) as a normalizer.

Parameter Value							
Iterations 200							
Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
1	TRG	1.0	1.225	0,284. 19,592	0,138. 190,687	0.963	
2	TRG	1.0	66.649	1,642. 668,425	0,355. 45.374,38	0.014	UP
3	TRG	1.0	3.242	0,458. 40,712	0,226. 1.119,751	0.303	
4	TRG	1.0	9.470	0,359. 189,998	0,237. 54.119,218	0.255	
5	TRG	1.0	12.168	1,037. 213,446	0,758. 7.459,442	0.028	UP
6	REF	1.0	1.000				

Interpretation
[1]sample group is not different to control group P(H1) 0,963.
[2]is up- regulated in sample group (in comparison to control group) by a mean factor of 66,649 (S.E. range is 1,642. 668,425). Sample group is different to control group P(H1) 0,014.
[3]sample group is not different to control group P(H1) 0,303.
[4]sample group is not different to control group P(H1) 0,255.
[5]is up- regulated in sample group (in comparison to control group) by a mean factor of 12,168 (S.E. range is 1,037. 213,446). Sample group is different to control group P(H1) 0,028.

In particular the donors were all Caucasians, with normal body mass index values; the criteria of recruitment were spontaneous birth, donors free from drugs, smoking and diseases. Body weight gain during pregnancy was within 10 kg (22 lb) and all of the donors had a full-term pregnancy. Their geographical origin was also assessed: all pregnant came from the northern part of Sardinia island (Italy).

Figs. 1–3 show the relative expression of genes analyzed in WJ-MSC isolated from the umbilical cord of 6 males and 6 females, with males compared to females, used as a control. The mean newborn weight was 3.446 gr (7,716 lb) ± SD 0.417 for males, and 3.442 gr (7,495 lb) ± SD 0.357 for females. The results show that there are no significant differences in the expression levels of SOX2, NANOG, C-MYC between males and females (Fig. 1). Fig. 2 and 3 describe the gene expression analysis of the pluripotency related gene OCT4 and of the epigenetic related gene DNMT1 respectively. The expression levels of OCT4 and DNMT1 were significantly increased in males compared to females (Figs. 1–3). The results show that epigenetic controlling factors, responsible for the maintaining of the chromatin methylation state, are involved in gender gene expression differences observed in our samples.

Discussion

In the present study we evaluated whether WJ-MSCs could exhibit gender differences, affecting stemness related gene expression. Here, we showed that OCT4 gene expression was significantly higher in cells isolated from males, as compared to stem cells from females Wharton jelly (Figs. 1,3). OCT4 translation

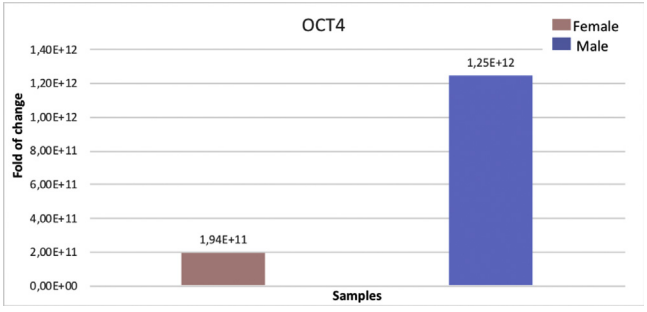

Fig. 2. Gene expression analysis of OCT4 in WJ-MSC harvested from males compared to WJ-MSC from females. The graph shows the expression of the males genes compared to the females, used as control.

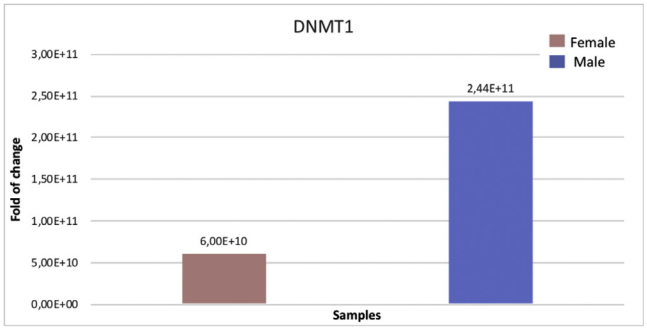

Fig. 3. Gene expression analysis of DNMT1 in WJ-MSC from males compared to WJ-MSC from females. The graph shows the expression of the males genes compared to the females, used as control.

is suppressed post-transcriptionally as germ cells differentiate toward the male lineage and enter mitotic arrest: the repression of the core machinery regulating pluripotency is a robust and early event involved in the differentiation of the male germ cell lineage [31]. It is hypothesized that active repression of pluripotency is required for fetal male germ cell differentiation and that failure of this mechanism could make germ cells susceptible to tumor formation [31]. Expression of OCT4, NANOG, and SOX2 varies within stem cells according to the original source [32–34]. Furthermore, cytosinephosphate-guanines (CpGs) within the promoters of some OCT4/SOX2 target genes, including NANOG, are methylated in *in vitro* cultured spermatogonial stem cells, while the promoters of OCT4 and SOX2 are unmethylated and partially methylated, respectively [33]. Here, we demonstrated that the upregulation of OCT4 gene expression is consistent with the upregulation of DNMT1, an epigenetic gene crucial for maintaining methylation status during DNA replication [35], unravelling a sex-dependent epigenetic modulation of this stemness regulating gene. Methylation of mammalian genomic DNA at 5 loci in cytosine residues via DNA methyltransferases (DNMTs) is an important epigenetic modification, which plays central regulatory roles in the control of cell physiology, including embryonic development, cell reprogramming, spermatogenesis [36]. DNMT1 functions as a major maintenance methyltransferase in vivo, it prefers hemimethylated CpGs, being critical for the maintenance of methylation patterns during DNA replication [36]. OCT4 was previously found to be a transcription factor controlling DNMT1 [36]. Our results seemed to indicate that OCT4 and NANOG directly bind to the promoter of DNMT1 and enhance its expression, a regulatory mechanism previously described by other

Authors [37]. Methylation represents a unique epigenetic program that complements other regulatory mechanisms to ensure the expression of appropriate genes in ESCs [37]. OCT4 induces DNMT1 expression and maintains DNA methylation after each round of cell cycle, thereby decreasing the expression of genes associated with senescence and developmental regulators and keeping cells in proliferative and undifferentiated states [38–39]. An interesting difference between meiosis in the two sexes is that in males, the two rounds of meiotic division of spermatogenetic cells are continuous, while during oogenesis oocytes are blocked in the diplotene/dictyate stage of the prophase of the first meiotic division until puberty, when cell pools are cyclically recruited to grow [40]. Then, they become oocytes to acquire the specific competence to resume meiosis and progress to the metaphase of the second meiotic division [40]. It is likely that OCT4 could fulfill primary function during the first steps of germline differentiation [41]. Its expression in undifferentiated spermatogonia in the testis could prevent the differentiation of these cells. OCT4 upregulates DNMT1 through direct binding to its promoter, and inhibit cell differentiation [28]. Numerous genes that play roles in spermatogenesis have been identified [26,29]. Genes related to DNA methylation have been highlighted as crucial genes responsible for spermatogenesis impairment since DNA methylation is a key event for normal spermatogenesis. In summary, we described for the first time an interesting network connecting stem cell pluripotency, through OCT4, epigenetics and gender differences. Our results showed greater expression in males than in females of the OCT4 and DNMT1 genes (Fig. 4). These results disclosed novel landscape in the field of regenerative medicine and stem cell capability to acquire a specific phenotype in relation to the donor. Other authors previously described a less capability of males MSCs in restoring tissue damages [6]. Here we hypothesized that these differences may be caused by the higher expression of OCT-4 pluripotency gene and DNMT1, found by us in males-WJ-MSCs, leading to a higher and quicker differentiation process, thus depleting stem cells sources for future regeneration. Nevertheless, our findings added new insights on the molecular signature of MSCs from Wharton jelly deriving from males, which could be used as a future diagnostic tool to define features connected to spermatogenesis impairment.

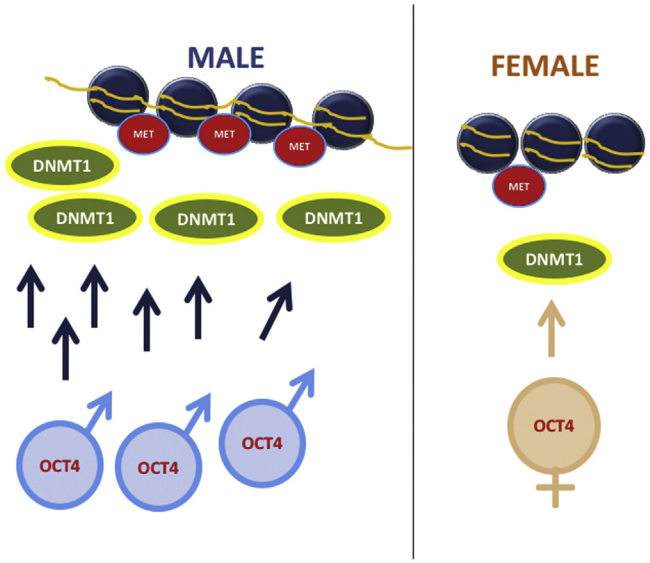

Fig. 4. Gene expression networking of OCT4 and DNMT1 in WJ-MSCs from males compared to WJ-MSCs from females.

Conflicts of Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ejogrb.2018.12.028.

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