RESEARCH LETTER

Characterization of microRNA exosome content from endometrioma wall in vitro culture

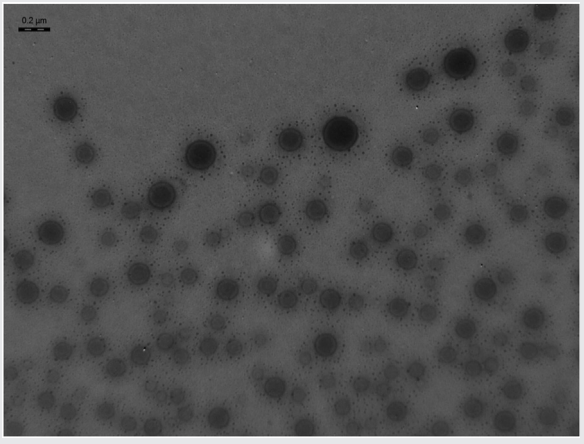
OBJECTIVE

Endometriosis is a chronic inflammatory gynecological disorder characterized by endometrium-like tissue outside the uterine cavity, including the ovaries (1), causing severe chronic pelvic pain and infertility. Endometriomas are filled with dark brown endometrial fluid, produced by ectopic endometrial glands over time. This elicits a chronic inflammatory reaction from surrounding ovarian tissue, leading to formation of a fibrotic pseudocapsule (2). This pathological process is detrimental to the ovarian follicle pool, which undergoes massive atresia in response to different stimuli, such as free iron and reactive oxygen species (3). The mechanisms of damage to the ovarian reserve, which also generates chronic pain and a toxic pelvic environment conducive to multifactorial infertility, are not yet fully understood.

Exosomes, a subpopulation of extracellular vesicles, are attracting increased attention as key players in paracrine signaling and damage mediation. They are nano-sized endogenous cell-derived carriers charged with transporting and delivering specific cargo like RNA, proteins, and lipids (4). Ongoing research into exosomes highlights their potential for revolutionizing diagnostics and investigations into cell damage mechanisms across various disorders, including endometriosis (5). However, there are no published data on exosome isolation from the endometrioma wall.

Our objective was to demonstrate the feasibility of endometrioma wall in vitro culture to mimic the native environment of ovarian endometriosis. We retrieved, isolated, and characterized exosomes and their microRNA (miRNA) cargo

FIGURE 1



Morphological appearance of exosomes isolated by ultracentrifugation. Particles showed a round-oval shape, in line with the literature, often with a thin and highly electron-dense membrane. Transmission electron microscopy image. Original magnification, ×20,000. Bar = 0.2 µm.

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TABLE 1

The 50 most widely expressed microRNAs sorted by count per million in exosomes secreted by ovarian endometriomas.

Name	CPM
hsa-let-7b-5p	386084.1368
hsa-let-7a-5p	275589.895
hsa-miR-143-3p	164862.3702
hsa-miR-320a-3p	85387.71619
hsa-let-7f-5p	83422.85767
hsa-let-7c-5p	80205.3138
hsa-miR-335-5p	74053.68683
hsa-let-7i-5p	71954.14042
hsa-let-7g-5p	67597.47158
hsa-miR-29a-3p	62114.00215
hsa-miR-199a-3p	55442.11023
hsa-miR-26a-5p	51533.52069
hsa-miR-424-5p	35878.035
hsa-miR-130a-3p	32430.72945
hsa-miR-125b-5p	31858.52603
hsa-miR-16-5p	31354.98701
hsa-miR-10b-5p	29307.37907
hsa-miR-423-5p	23446.25538
hsa-miR-26b-5p	22646.0509
hsa-miR-151a-5p	21854.64955
hsa-miR-30e-5p	21310.61614
hsa-miR-21-5p	17434.59818
hsa-miR-193a-5p	16578.05367
hsa-miR-30d-5p	15141.38291
hsa-miR-34a-5p	13984.65168
hsa-miR-29c-3p	11831.40618
hsa-miR-24-3p	11385.96783
hsa-miR-497-5p	10988.06637
hsa-miR-574-5p	10679.95683
hsa-miR-191-5p	10275.89318
hsa-miR-100-5p	10267.09005
hsa-let-7d-5p	8724.781748
hsa-miR-126-3p	8564.564789
hsa-miR-125a-5p	8524.950706
hsa-let-7e-5p	8458.927234
hsa-miR-30a-5p	8259.096192
hsa-miR-660-5p	7771.402812
hsa-miR-532-5p	7131.41529
hsa-miR-28-3p	5344.379981
hsa-miR-99a-5p	4389.240419
hsa-miR-320c	4133.949661
hsa-miR-376a-3p	3165.605405
hsa-miR-151a-3p	2971.936553
hsa-miR-320d	2765.943321
hsa-miR-126-5p	2463.995975
hsa-miR-93-5p	2383.007183
hsa-miR-99b-5p	2376.844992
hsa-miR-195-5p	2350.435604
hsa-miR-98-5p	2247.438987
hsa-miR-103a-3p	2198.141461

CPM = count per million; miR = microRNA.

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generated in vitro in an attempt to gain new insights into diagnosis, prognosis, and treatment monitoring.

EXPERIMENTAL DESIGN

Human endometrioma wall biopsy specimens were collected in accordance with the Declaration of Helsinki guidelines and approved by the ethics committee of Cliniques Universitaires Saint-Luc and the Université Catholique de Louvain (ref: 2020/14AOU/410). Biopsy specimens from eight reproductive-age women (age range, 24–47 years) undergoing laparoscopic cystectomy were cut into small fragments (3 mm × 3 mm) and seeded with 20 μL Matrigel inside 48-well plates in multiplicate (5 pieces per drop). They were then cultured with Dulbecco's Modified Eagle and Ham's F-12 nutrient mixture medium (1:1) (Thermo Fisher Scientific, Waltham, MA) enriched with 5% extracellular vesicle-depleted fetal bovine serum and 1% penicillin-streptomycin (Thermo Fisher Scientific). Viability was assessed using calcein or ethidium homodimer-1 assays (Invitrogen, Waltham, MA) on cultured tissue after 4 days. The supernatant collected at the end of the culture underwent centrifugation (Sigma 2-16PK, Merck, Rahway, NJ) at low speed (2,000 rpm) to remove all cell debris, and then differential ultracentrifugation (LE-80K, Beckman Coulter, Brea, CA) for 20 minutes at 17,300 rpm twice and 90 minutes at 40,000 rpm twice. The goal was to eliminate apoptotic bodies (500–5,000 nm) and microvesicles (200–1,000 nm size range) and obtain a pellet enriched in exosomes (50–200 nm) (5). These purified particles were then investigated by nanoparticle tracking analysis for exosome size and concentration using ZetaView (Particle-Metrix, Dusseldorf, Germany), transmission electron microscopy on a 200-mesh Formvar or carbon copper grids (Merck) for morphology, and flow cytometry to detect exosome biomarkers. To explore their content, total RNA was subjected to Qiagen miRNA next-generation sequencing using NextSeq (Illumina Inc., San Diego, CA).

RESULTS

Viability tests after culture found fewer than 10% dead cells in all samples, validating the feasibility of the in vitro culture protocol (data not shown). Exosomes could be purified from endometrioma wall culture, showing a 50–200 nm size range (179.5 ± 72) and an 8–26 x 10^{10} particles/mL concentration range (17.8 x 10^{10} ± 12.4 x 10^{10}), according to nanoparticle tracking analysis. They appeared as cup-shaped structures, often with a thin and even more electron-dense membrane on transmission electron microscopy (Fig. 1). Flow cytometry confirmed that 80% of particles per sample were positive for exosome transmembrane markers (phycoerythrin anti-human CD9 antibody and/or allophycocyanin anti-human CD81 TAPA-1, BioLegend, San Diego, CA). Sequencing results revealed the distribution of small RNAs in endometriomas. miRNAs, mapped using the miRBase database, represented 58% of total small RNAs. Among the most widely expressed miRNAs (Table 1), 11 were already known to play a role in follicle development and oocyte maturation (Table 2). Six of them belong to the let-7 family, an established target of the antimüllerian hormone that affects oocyte quality, recruitment, and growth of follicles by restricting growth factors and the effect of gonadotropins. Of all predicted target genes among selected miRNAs and their significant pathways acquired from the miRPathDB (https://mpd.bioinf.uni-sb.de/about.html) and miRDB miRNA target prediction databases (https://mirdb.org/mirdb/index.html), those already known to impact the ovarian reserve are shown in Table 3.

CONCLUSION

Having proved the feasibility of in vitro culture of the endometrioma wall, we were able to isolate and characterize the generated exosomes. miRNAs carried by exosomes from patients with endometriomas may be involved in mechanisms

TABLE 2

MiRNAs previously identified as playing a role in follicle development.

miRNAs	References	Function
Let-7 family (let-7a, b, c, d, e, f)	Qin et al. (6), 2019 Caponnetto et al. (7), 2021	• Targeting antimüllerian hormone • Association with oocyte quality • Involvement in biological processes related to follicle development and oocyte maturation
miRNA-21	Fu et al. (8), 2017	Regulation of GC apoptosis and follicle development
miRNA-320	Yin et al. (9), 2014	Overexpression of miRNA-320 inhibits E_2 synthesis and proliferation of GCs by targeting E2F1
miRNA-335	Yao et al. (10), 2018	• Abundance of miRNA-335-5p linked to endocrine abnormality and increased number of growing follicles • Suppression of GC proliferation
miRNA-423	Caponnetto et al. (7), 2021	• Involvement in biological processes related to follicle development and oocyte maturation • Role in regulation of the ovarian response
miRNA-574	Caponnetto et al. (7), 2021	Involvement in biological processes associated with follicle development and oocyte maturation

E_2 = estradiol; GC = granulosa cell; miRNA = microRNA.

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of damage to the ovarian reserve. Thorough analysis of miRNA exosome content and predicted target genes may be a promising starting point for a better understanding of endometrioma-mediated follicle loss.

CRediT Authorship Contribution Statement

Margherita Zipponi: Writing – original draft, Methodology, Formal analysis, Data curation. **Dong-Yun Lee:** Conceptualization. **Christina Anna Stratopoulou:** Methodology. **Luciana Cacciottola:** Conceptualization, Writing – review & editing. **Marie-Madeleine Dolmans:** Writing – review & editing, Supervision.

Declaration of Interests

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TABLE 3

Predicted target genes and significant pathways known to impact the ovarian reserve.

Target genes	Gene description	References	Function
STARD13	StAR-related lipid transfer domain containing 13	Sun et al. (11), 2015	Role in yolk and follicle development
IGF2BP1	Insulin-like growth factor 2 mRNA binding protein 1	Mu et al. (12), 2022	Regulation of GC growth and follicle development
PGRMC1	Progesterone receptor membrane component 1	Peluso (13), 2013 Peluso et al. (14), 2018 Peluso (15), 2022 Will et al. (16), 2017	• Involvement in the premature demise of the adult population of primordial follicles and the ability of progesterone to regulate ovarian follicle growth • Mediation of both antiapoptotic and antimitotic effects in GCs
ADAMTS	ADAM metallopeptidase with thrombospondin type 1 motif	Brown et al. (17), 2006 Brown et al. (18), 2010 Yung et al. (19), 2010	• Physiological role in folliculogenesis • Essential to normal ovulatory tissue morphogenesis • Remodeling of follicle structure in the ovulating follicle by mediating degradation of matrix barriers to the infiltrating vasculature
CDK	Cyclin-dependent kinase	Ataei-Nazati et al. (20), 2022	Role in the primordial-to-primary transition crucial to ovarian follicle development
E2F	E2F transcription factor	Yao et al. (10), 2018	Regulation of GC proliferation and steroid production after binding to miRNA-320 in follicle development
TET	TET methylcytosine dioxygenase	Sagvekar et al. (21), 2022	Potential alterations to transcriptional regulation of TETs, resulting in DNA methylation changes in GCs

GC = granulosa cell; miRNA = microRNA; mRNA =messenger RNA; TET = Ten-eleven translocation.
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