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SHORT COMMUNICATION

Extracellular vesicles in human follicular fluid do not promote coagulation

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Abstract Body fluids contain extracellular vesicles expressing tissue factor on their surface and serve as an additional trigger for coagulation. During the menstrual cycle ovarian tissue restoration is mandatory and it is unknown whether follicular fluid might provide procoagulant substances. Within an observational study, follicular fluid from women undergoing IVF/intracytoplasmic sperm injection (ICSI) was analysed by fluorescence-activated cell sorting (FACS), electron microscopy, resistive pulse sensing (RPS), nanoparticle-tracking analysis (NTA) and fibrin generation tests (FGT). The presence of extracellular vesicles, especially CD9-positive extracellular vesicles in follicular fluid, was proven. However, clotting tests revealed no procoagulant properties of the detected extracellular vesicles.

KEYWORDS: extracellular vesicles, exosomes, follicular fluid, microparticles, ovary

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Introduction

In various body fluids, extracellular vesicles express tissue factor on their surface, thus serving as an additional trigger for coagulation that is thought to play a role for minimisation of blood loss (Berckmans et al., 2011). Most recently, our group was able to detect extracellular vesicles in human ejaculate by fluorescence-activated cell sorting (FACS) and transmission electron microscopy (TEM) which were highly procoagulant and strongly triggered the extrinsic pathway of coagulation (Franz et al., 2013). During ovulation and rupture of the mature follicle the ovarian tissue faces injuries which have to be restored every month. Therefore, this study questioned whether extracellular vesicles in follicular fluid might trigger coagulation in order to minimize ovarian tissue damage, as these extracellular vesicles are released at exactly the time and place of the injury caused by the follicle rupture.

Methods

Study population

The Human Investigation Review Board of the Ruprecht-Karls-University, Heidelberg approved this study on 21 June 2010 (reference number: S-075/2010). Samples were collected from nine patients undergoing ovarian stimulation with gonadotrophin-releasing hormone agonist. On average follicular fluid from eight mature follicles was pooled per patient.

Sample collection and preparation

Follicular fluid was obtained from all patients at the day of oocyte retrieval and follicular fluid from multiple follicles was pooled. According to International Society for Extracellular Vesicles (ISEV) guidelines, samples were centrifuged twice at 1550 *g* for 20 min at 20°C to remove residual tissue. After each centrifugation the supernatant was collected and aliquots of 300 µl were snap-frozen in liquid nitrogen and stored at −80°C until use.

FACS

FACS was performed on samples from nine patients. Fluorescein isothiocyanate (FITC)-labelled annexin V as well as phycoerythrin (PE)-labelled immunoglobulin G_1 (IgG_1) and IgG_{2b} were obtained from Immuno Quality Products (Groningen, The Netherlands), anti-CD9-, -CD14- and -epidermal growth factor (EGF)-receptor-PE from BD Biosciences (Heidelberg, Germany), anti-CD24-FITC from AbD Serotec (Kidlington, Great Britain), anti-CD45-PE from Sanquin (Amsterdam, The Netherlands), anti-heat shock protein (HSP)70-FITC from MBL International (Woburn, MA, USA) and anti-L1CAM-Alexa647 from DKFZ, Heidelberg, Germany. Diluted antibodies were centrifuged at 18,890 *g* for 5 min at 20°C before being used to remove antibody aggregates. Isolation of extracellular vesicles and FACS (FACSCalibur, Beckton Dickinson, Heidelberg, Germany) were performed as previously described (Franz et al., 2013). FACSCalibur by light scatter detects single vesicles of approximately 500 nm and larger. In detail, samples were thawed, 250 µl were centrifuged at 18,890 *g* for 30 min at 20°C (Mikro 22R, Hettich Zentrifugen, Tuttlingen, Deutschland) and supernatant (225 µl) was removed. The pellet was resolved in 225 µl PBS-citrate (Citrate 0.32% w/v), centrifuged again under the same conditions and supernatant (225 µl) was removed. The remaining extracellular vesicle suspension (25 µl) was adjusted with PBS-citrate to a volume of 100 µl. Then, 5 µl was added to PBS-$CaCl_2$ (35 µl) and incubated with annexin V (pre-diluted, 5 µl) and a specific antibody (pre-diluted, 5 µl). After 15 min, antibody-binding-reaction was stopped by the addition of 900 µl PBS-$CaCl_2$.

Fibrin generation test (FGT)

FGT was performed on samples from four patients. As a source of clotting factors, endogenous vesicle-depleted pool plasma from healthy volunteers ($n = 400$) was used. Antibodies against factor VII(a) and factor XI(a) were obtained from Sanquin (Amsterdam, The Netherlands). FGT was applied as previously described (Franz et al., 2013). Optical density at λ = 405 nm was measured in a Spectramax Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) at 37°C for one hour (Berckmans et al., 2011).

TEM

TEM was performed on samples from two patients. Isolation and processing of extracellular vesicles including negative staining were performed according to the literature (Berckmans et al., 2011). The electron microscope operated at 80 kV (Philips CM10, Eindhoven, The Netherlands), using a Veleda side-mounted CCD-camera and imaging solutions software (Olympus SIS, Münster, Germany).

Size distribution techniques

Nanoparticle tracking analysis (NTA) and resistive pulse sensing (RPS)

The concentration and size distribution of particles in each sample was measured with NTA (NS500; Nanosight, Amesbury, UK), equipped with an EMCCD camera and a 405 nm diode laser and with RPS (qNano; Izon Science Ltd, Christchurch, New Zealand) using an NP200A nanopore as described previously (Franz et al., 2013).

Statistical analysis

Statistical analysis was performed with SPSS for windows, version 21.0 (IBM, Armonk, NY, USA). Correlations were calculated according to Pearson, non-parametric data was tested using the Mann-Whitney *U*-test. Results are given as mean ± standard deviation (minimum–maximum) ($P < 0.05$ significant).

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