

BOVINE EXTRACELLULAR VESICLES CHANGE
THE DYNAMICS OF HUMAN DECIDUAL
STROMAL CELLS DECIDUALIZATION

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Abstract

Decidualization of the endometrium is essential to healthy pregnancy. It is governed by a network of signals, including signalling via extracellular vesicles (EVs). Decidualization is often studied in cultured decidual stromal cells (DSC). However, a standard component of the culture medium in this model is bovine serum, which is abundant in EVs that can hypothetically interfere with the decidualization process. In this study we investigated the effect of depleting the medium of exogenous EVs.

Human DSC were differentiated with progesterone, β -estradiol and cAMP in a regular and bovine EVs-removed medium for 10 days. Secreted prolactin, a decidualization marker, was assayed by Alinity method and *prl* transcription was analyzed via real-time qPCR.

A disparity of DSC decidualization efficacy with and without bovine EVs under the same deciduogenic stimuli was revealed. A sustainable production of PRL (range: 96 ± 16 mIU/L to 140 ± 35 mIU/L) without bovine EVs contrasted to initial 2-fold higher PRL release and significant loss in the decidualization by day 10 with EVs. *Prl* gene expression remained the same in both conditions showing posttranslational regulation. Bovine serum EVs and factors dysregulate the quality of the long-term DSC response causing a collateral bias in the model of decidualization.

Key words: decidual stromal cells, in vitro decidualization, prolactin, progesterone, cAMP, extracellular vesicles, in vitro model

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Introduction. Human endometrial stromal cells possess the inherent ability to undergo a process of differentiation called decidualization, during which they transform into decidual stromal cells (DSC) – a distinct cell type capable of sustaining a microenvironment suitable for embryo implantation. This process begins in the mid-secretory phase of the menstrual cycle, under the effects of progesterone and estrogen, in the area around the spiral arteries [1].

Progesterone and estrogen bind to progesterone receptors (PRs) and estrogen receptors (ERs), which act as ligand-associated nuclear transcription factors and lead to a plethora of cellular changes to both morphology and metabolism in the DSC. The cells enlarge and round up, and a number of genes become differentially regulated, including those supporting angiogenesis, immune cell recruitment and activity, and tissue remodelling, thus forming the decidual niche. The importance of PRs and ERs for the decidualization of the endometrium, as well as maintaining pregnancy, has been shown in knock-out mouse models and human cell and tissue studies alike [2]. During decidual transformation of endometrial stromal cells, the transcription complex of progesterone/PR and several decidualization-related factors (FOXO1, C/EBP β , etc.) bind directly to the promotor regions of many target genes, including the decidual prolactin (PRL), whose secreted product proportionally reflects the state of decidualization. The increase of decidual PRL in the developing maternal part of the placenta leads to its progressive accumulation in the amniotic fluid by week 22 of gestation, which subsequently declines [3,4]. Physiologically linked to pregnancy, the decidual PRL specifically accounts for processes in the feto-maternal interface. Many cell types, such as amniotic epithelium, trophoblasts, and immune cells express receptors for PRL, which implicates PRL in immune modulation, trophoblast invasion, and angiogenesis, and also in osmoregulation by controlling electrolytes' exchange through fetal membranes [3]. Sustaining PRL secretion by DSC is crucial for the success of pregnancy, as has been shown in female PRL or PRL-receptor knock-out mice [5,6]. In humans, dysregulated decidual maturation manifests in diminished production of PRL and leads to the occurrence of infertility, pregnancy loss or high maternal and fetal morbidity conditions such as preeclampsia. It is suspected that aberrant decidualization in early gestation lies at the core of impaired trophoblast invasion and failure to widen spiral arteries, which results in reduced placental perfusion and diminished supply of oxygen and nutrients to the fetus [7,8]. Accumulating evidence shows that the decidual transformation, including progressive angiogenesis and epithelial and stromal maturation, in many instances is the primary coordinator of embryo implantation and pregnancy outcome. The process involves multicellular interactions of different types of cells leading to dynamic tissue remodelling [9]. The molecular aspect of this process is the paracrine signalling via soluble factors in lipid enclosed membrane compartments (30–1000 nm in diameter) called extracellular vesicles (EVs). EVs are secreted by all cell types and are essential signals for cell communications. Their cargo contains proteins,

RNAs, DNAs and lipids, which are delivered to recipient cells by receptor binding, membrane fusion, and downstream signalling [10].

Laboratory experimentation with human primary cells is a fundamental technology applied in studies of biomedical sciences. Cell maintenance in culture requires from 2 to 20% supplementation of bovine serum to the growth media, which presents an indispensable source of vitamins and growth factors. The widely used in vitro model for DSC decidualization uses 2% bovine serum in the culture medium. However, given the involvement of EV signalling in decidualization, we questioned whether the presence of bovine EVs, which transport bioactive molecules, could influence the phenotype and functionality of the cultured cells and even the experimental readout – as has been found in other cases [11,12]. In this study, therefore, we aimed to elucidate the efficiency of DSC response in the conditions with depleted levels of exogenous bovine EVs during decidualization. We chose to use PRL secretion by DSC as our main marker. We suggest a modification in the standard culture protocol to avoid possible cross-species interference, which has so far been scarcely investigated.

Material and methods. Collection of human decidual tissue. Decidual tissue was obtained from patients who underwent elective termination of pregnancy (week 6–11 of gestation) after approval by the institutional review board of Vita Hospital, Sofia, Bulgaria (1-23-NI/01.04.2023), and signed consent from participating patients. Decidual stromal cells were isolated as previously described [13], and the cell purity of cultures was validated at above 96% by flow cytometry using markers for mesenchymal stem cells classification [14].

DSC maintenance and induction of decidualization in culture. DSC were maintained in RPMI 1640 (PAN-Biotech GmbH, Germany) without phenol red, supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, USA) and 100 U/ml Penicillin, 100 µg/ml Streptomycin, 0.25 µg/ml Amphotericin B (PAN-Biotech) at 37 °C and 5% CO₂. DSC (~ 95% confluency) were decidualized with 1 µmol/L medroxyprogesterone acetate (MPA), 10 nmol/L β-estradiol (E) and 0.5 mmol/L 8-bromo-adenosine-3',5'-cyclic monophosphate (cAMP) (Sigma-Aldrich, USA) in RPMI 1640 with charcoal-stripped 2% FBS and 1% Penicillin/Streptomycin/Amphotericin B containing medium, which was changed every 48 h for 10 days. A control was performed with equimolar ethanol, the solvent of the hormones. DSC were differentiated in regular decidualization medium (R-DM) with bovine EVs and modified decidualization medium (M-DM) with depleted bovine EVs to uncover the effect of exogenous EVs. DSC were collected on day 10 of culture and viable cells were counted using 0.2% Trypan blue (Gibco, USA), which is excluded by live cells.

Depletion of bovine EVs from M-DM. The medium was centrifuged at 2000 × *g* for 30 min at RT to precipitate the large serum proteins. It was then placed in 100 kDa cut-off filter Amicon Ultra-15 (Merck, Germany) and centrifuged at 3680 × *g* for 20 min with a flying rotor. The bovine EVs and proteins

with molecular weight > 100 kDa were discarded with the cut-off fraction. The obtained flowthrough was used as M-DM. Deciduogenic factors (MPA, E and cAMP) were added to the medium prior to culture.

Prolactin measurement in culture supernatant. Forty-eight-hour supernatant from DSC cultures was collected on days 4, 6, 8, and 10 and secreted prolactin (PRL, ng/ml) was evaluated using Alinity i Prolactin Reagent Kit 07P66 (Abbott, USA). Data was converted to mIU/L per 1×10^4 cells.

Prolactin gene expression by mRNA extraction and qPCR. Total RNA was isolated by TriReagent (Sigma, USA) according to the manufacturer's recommendations and quantified by NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). Copy DNA was synthesized from RNA (1 μ g/sample) using RevertAid First Strand cDNA Synthesis Kit and random hexamers (Thermo Fisher Scientific). Real-time qPCR was performed using GoTaq[®] qPCR Master Mix (Promega Corporation, USA) in 10 μ L reactions on a Stratagene Mx3005P machine (Agilent Technologies, USA) at the conditions: 95 °C for 5 min, followed by 30–35 cycles of denaturation at 95 °C for 30 s, annealing at 62 °C for 30 s and elongation at 72 °C for 40 s [13]. The relative expression of *prl* gene was calculated by the standard curve method and normalized to β -*actin* house-keeping gene.

Statistics. Statistical significance of differences between mean values was assessed using Student's paired *t*-test or one-way repeated-measures ANOVA, followed by Tukey's post hoc test. Statistical analyses were performed using Graph-Pad Prism 8 (version 8.4.2.), and a value of $p \leq 0.05$ was considered statistically significant. Results are reported as mean $\pm$ standard error of the mean (SEM).

Results. Decidual transformation of endometrial stromal cells was driven in culture by R-DM containing MPA, E and cAMP [13]. During the course of treatment, DSC enlarged and acquired round morphology, which are signs of the decidual reaction (Fig. 1A control and Fig. 1B decidualized DSC at day 10). To investigate the decidualization capacity of DSC in the absence of EVs and large proteins (> 100 kDa) from bovine serum, M-DM was used. The differentiation response of DSC in paired R-DM and M-DM cultures was assessed by decidual PRL secretion (mIU/L per 10^4 cells) from day 4 to day 10. DSC mounted a distinct decidual response, which ranged between 17 mIU/L and 429 mIU/L of PRL (Fig. 1C, $n = 8$), and baseline levels (< 0.1 mIU/L, data not shown) in controls of both conditions. DSC decidualization without bovine EVs showed steady and sustainable PRL release (mean $\pm$ SEM: 140 ± 35 mIU/L at day 4 and 96 ± 16 mIU/L at day 10) throughout the timepoints. However, in the presence of bovine EVs, DSC demonstrated a spiking release of PRL at day 4 (227 ± 153 mIU/L, $\#p = 0.0291$), which was followed by a significant 3- to 4-fold decline at day 8 (77 ± 19 mIU/L, $**p = 0.0016$) and day 10 (52 ± 34 mIU/L, $***p = 0.0002$). Comparison of decidual response at day 10 with and without bovine EVs (96 ± 44 mIU/L, $\#\#p = 0.0098$) revealed a significantly higher PRL level in the latter. Furthermore, we verified whether the reduced PRL levels in R-

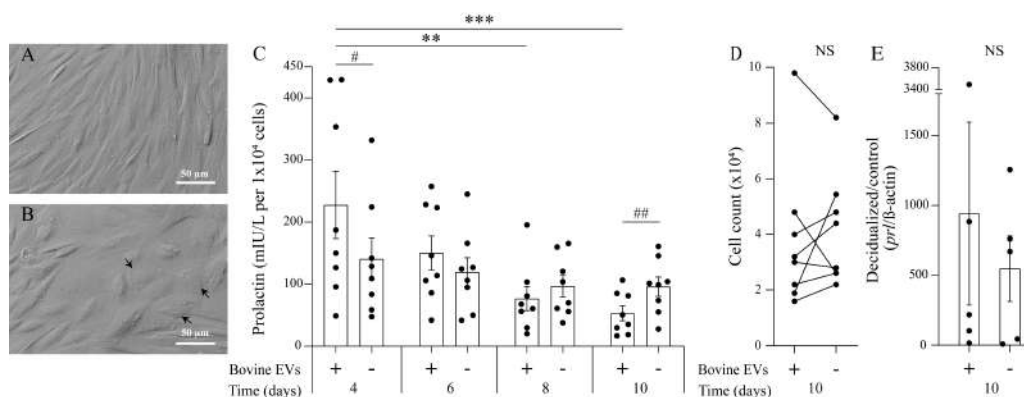


Fig. 1. Comparison of differentiation capacity of DSC induced in R-DM versus bovine EVs-depleted M-DM conditions. Bright field images of: A) control culture of DSC; B) decidual response in DSC (arrows) under decidualogenic condition in R-DM at day 10. Scale bar, 50 μ m. C) A timepoint quantification (at day 4, 6, 8, and 10) of PRL secretion by DSC in R-DM and M-DM. PRL levels are measured by Alinity method in ng/ml, converted in mIU/L and standardized per 10⁴ cells. The error bars represent mean $\pm$ SEM, * p < 0.05, ** p < 0.01 and *** p < 0.001 (One-way Anova test followed by Tukey's multiple comparison); # p < 0.05, ## p < 0.01 (paired t -test). D) Absolute counts of viable DSC cultured with and without bovine EVs are defined at day 10 using 0.2% Trypan blue staining. E) *prl* gene expression in decidualized DSC in the presence and absence of bovine EVs is normalized to the control cells at day 10; NS – not significant (paired t -test)

DM at day 10 were caused by a different survival rate of DSC in the cultures. Absolute count of vital DSC was determined by Trypan blue exclusion staining. The result showed unaffected cell viability in the presence of bovine EVs (Fig. 1D), which suggests a specific inhibition of differentiation capacity of DSC. To address whether the regulation of PRL is influenced at transcription level, *prl* gene activity was investigated at day 10 (Fig. 1E). The quantity of *prl* transcripts was similar irrespective of the presence of bovine EVs, suggesting that the regulation of PRL takes place at translational but not at transcriptional level. Overall, the modified model for DSC decidualization demonstrates a beneficial effect of the stripping of bovine EVs, which leads to pronounced stability of DSC state at least up to ten days compared to the regular protocol for DSC differentiation.

Discussion. DSC are a specific stem cell type in the endometrium directly subordinated to steroid sex hormones [15]. They prevail and progressively differentiate when pregnancy ensues. Although accessible and widely applied, in vitro decidualization is a challenge due to the transiency of the state [16]. Decidualization of DSC is exclusively dependent on the presence of MPA and cAMP factors, with the latter strongly amplifying their biochemical response [1]. Standard decidualization of DSC is performed in medium with 2% bovine serum. By default, bovine serum is rich in EVs and proteins, which may encode disparate signals modifying cell functions [11]. Therefore, the putative interference of bioactive

EVs from bovine serum in the decidualization response of human DSC in vitro was prevented by stripping EVs from M-DM medium using PRL as an efficiency indicator for DSC differentiation [8]. DSC reveal a disparity of decidualization efficacy between R-DM and M-DM, even when subjected to the same deciduogenic stimuli of MPA, estradiol and cAMP. Our results show a sustained production of PRL up to 10 days in the absence of bovine EVs, which is not the case when DSC decidualized in R-DM. A substantial fluctuation in the time and magnitude of PRL secretion is registered, with the highest PRL release at day 4 and significant loss in the decidualization state by day 10 in the presence of bovine EVs. This suggests an additive contribution of unknown serum factors in R-DM, which cause fast induction of decidual response in DSC. Endometrial stromal cells in decidual state up-regulate the expression of PRL receptor throughout the early pregnancy [17]. It can be speculated that in decidua PRL receptor expression makes DSC susceptible to their own PRL and by this mechanism a synchronization of decidual integrity is achieved. Concomitantly, PRL is shown to exert an autoinhibition of its own production when it is in high concentrations during decidualization [18]. Therefore, we can hypothesize that the initial fast elevation of PRL from DSC sends negative feedback for global DSC dedifferentiation in R-DM cultures. Our result indicates that in the first four days of deciduogenic treatment bovine serum factors excessively dysregulate PRL secretion, and thus influence the quality of the long-term DSC decidual response. However, no difference in *prl* gene activity in DSC between R-DM and M-DM conditions is established, indicating intrinsic regulation of secreted PRL at post-translational level.

Conclusions. Secretory decidual PRL encodes the quality of endometrial decidualization. It is one of the paracrine arms for maternal-fetal cell communications, and therefore fundamental for placentation [3, 4]. Due to a lack of suitable animal models that can legitimately represent DSC differentiation in humans, the endometrial/decidual stromal cell culture models remain the only experimental option for obtaining insights into the decidual process. In this respect, it remains essential to remove highly variable xenogeneic serum that can reprogram cell fate [12] and skew biomedical modelling. Three main advantages can be identified after purification of culture medium from bovine EVs in the M-DM model:

- 1) conserved viability of DSC in stimulating conditions,
- 2) intercellular communications only with human EVs from DSC carrying native signals,
- 3) achieving a sustained state of decidualization in the course of time.

These findings validate the efficiency of this decidualization model for wider use in the medical sciences.

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