METHODS OF DETECTION AND ISOLATION OF TROPHOBLAST CELLS FROM TRANS-CERVICAL SPECIMENS – A HISTORICAL OVERVIEW

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Abstract

Trophoblast cells can be detected and isolated from the cervical epithelial cells obtained via various techniques of trans-cervical samples collection such as a mucus aspiration, endocervical lavage, or standard cervical brushing in the early first trimester, even from the 5 weeks' gestation. Isolated fetal cells can be used in the early prediction of fetal sex, prenatal diagnostics of the most common aneuploidies, and any other genetic abnormalities. Nevertheless, the collection of trophoblastic cells has limited efficacy compared to currently used methods of detection of free fetal DNA in maternal circulation or other protocols of invasive prenatal diagnostics available at later stages of pregnancy. In the past years, trans-cervical cell samples were collected mainly in women before planned pregnancy termination. The early trophoblastic cells isolation from women in ongoing pregnancies opens new perspectives for further studies focused on the elucidation of pathophysiology of numerous pregnancy-related complications.

Running title: Fetal cells detection and isolation from cervical samples

Keywords: cervix, pap smear, prenatal diagnostics, trophoblast

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Introduction

The human placenta begins its development at the very early stages of pregnancy, just after the implantation. The fetomaternal compartment of the placenta has both maternal (decidua basalis) and fetal origin (trophoblastic derivatives). The fetal part of the placenta is composed of trophoblastic and non-trophoblastic cellular populations. We can distinguish several specific trophoblastic lineages such as syncytiotrophoblast, cytotrophoblast, and extravillous cytotrophoblast. Either syncytiotrophoblast or extravillous trophoblast cells play an essential role in early placentation, penetration of placental villi through the maternal decidua basalis, and remodeling of uterine spiral arteries. The disturbances in their physiological activity were linked with the genesis of preeclampsia and placental insufficiency [1,2].

According to current recommendations, invasive prenatal diagnostics – amniocentesis or chorionic villus sampling – should be proposed to every pregnant woman at high risk of fetal aneuploidy (relative risk is estimated using the combination of sonography and maternal blood tests) or other genetic abnormalities. A cervical smear is a commonly approved technique in the early screening of cervical dysplasia and atypia in the pregnant and non-pregnant population. Moreover, trans-cervical samples collected via routine brushing, mucus aspiration, or trans-cervical lavage may be used as a source of fetal trophoblastic cells. Isolated fetal cells open new perspectives in early prenatal diagnostics and can potentially elucidate the pathogenesis of the above-mentioned pregnancy-related pathologies [3,4].

This review investigates and summarizes the history of detection and isolation of trophoblastic cells in the trans-cervical samples. Moreover, we tried to describe the current state of knowledge on trophoblastic cells processing methods and their potential utility in early prenatal diagnostics. Finally, we investigated their contribution to the pathogenesis of pregnancy adverse outcomes.

Material and Methods

We searched the PubMed database for relevant references from the first records until December 2021, using the following terms: “trophoblast cells cervix,” “cervical trophoblast cells,” “trophoblast cells Pap smear,” “trophoblast cells cervical smear”. We also searched the database with all the phrases mentioned above using the term “placental” instead of “trophoblast”. We initially excluded articles not related to the review’s aims analyzing the titles and abstracts. We analyzed full texts of papers included in this review. We selected only the abstracts and articles written in English, which could be a possible study limitation.

Methods of detection and isolation of trophoblast cells from trans-cervical samples and their clinical utility

Early history of trophoblast cells identification in cervical smears

The pathologists published the first reports demonstrating the presence of trophoblastic cells in cervical specimens in the early '50s [5,6]. Initial findings revealed that the chorionic tissue described as trophoblastic debris could be detected in women with complete abortions even several weeks after the expulsion of the embryo and placenta remains. That fact has been elucidated by a theory that the subpopulations of placental cells survive deeply in the uterus wall after the physiological delivery or abortion [5].

It was postulated that the residual cells derived from the chorionic tissue detected in the cervical smears could lead to errors in the cytological diagnosis. Arias-Stella noted that the chorionic cells detected in cervical smears often had gigantic atypical nuclei. This apparent feature may lead to problems with the interpretation of histological images – in sporadic cases, even to carcinoma misdiagnosis [6].

Then, in the paper published in 1961, Naib reported that after analyzing numerous cervical smears collected from young women in Baltimore, pathologists described two types of trophoblastic cells – 1) groups of gigantic multinucleated with abundant cytoplasm, and accidentally discovered, 2) the subtype of “large bizarre cells with scanty cytoplasm” mimicking the undifferentiated cervical tumor cells. This specific type of cells was detected in 32 of 30,677 collected specimens. At first, the detected cells were suspected of having a cancerous origin. At the same time, smears from those specimens generally showed the characteristic features of pregnancy – an abundance of intermediate and navicular cellular populations and non-specific inflammation. In most cases – 23 of 32 – the additional invasive surgical procedures were needed to exclude the malignant nature of the identified cells. Interestingly, in none of the described cases, neoplastic diseases were diagnosed. Finally, those “bizarre” cells were established to have a fetal origin in all cases – patients were either pregnant or experienced early spontaneous abortion [7].

Clinical and experimental trials conducted in the '90s

For a long time, from the '60s, there have been no reports about the identification of trophoblastic cells in trans-cervical smears in the PubMed database. In 1991, Frank et al. presented a case report of a patient with abnormal Pap smear results. Their analysis revealed the presence of atypical cells described

as dysplastic cells in her cervical specimen collected one-year postpartum. Detected cells formed the groups and clusters surrounded by the “amorphous pink matrix”. Interestingly, described cells possessed several characteristic features of trophoblastic cells – amphophilic cytoplasm, hyperchromatic nuclei with variably prominent nucleoli, and increased nuclear/cytoplasmic ratios. The invasive diagnostics procedures – colposcopy, cervical biopsy, and endocervical curettage – did not detect any features of malignancy. Surprisingly, the examination of the endocervical samples identified the presence of residual trophoblastic tissue one-year postpartum. The authors concluded that atypical trophoblastic cells could be easily confused with the dysplastic or malignant tissues [8].

Furthermore, Mulvany et al. described a case of the cervical pregnancy in which the malignant-like chorionic cells were detected in the cervical smear. The smear was originally described as inconclusive, and extended invasive diagnostics were required. In that case, the patient underwent colposcopy and cervical conization. Finally, the histologic examination revealed an ectopic pathological pregnancy's remains in the cervix [9]. Then the presence of elusive dysplastic trophoblastic cells in cervical smears was described multiple times in the medical literature as the Arias-Stella reaction or Arias-Stella phenomenon [10–12]. Both terms derive from the name of Javier Arias-Stella (Peruvian pathologist), who, in 1954, first described this intriguing reaction [6].

The first experimental study focused on the detection of trophoblast-derived cells in the trans-cervical swabs was published in 1992. It paved the way for further trials. Griffith-Jones et al. aimed to detect fetal DNA in the cotton wool swabs collected in the first trimester pregnancies. They recruited 33 patients undergoing legal terminations between 9 and 13 gestational weeks. Before the procedure, they collected the trans-cervical swabs from 26 patients. In seven cases, the specimens were obtained through the trans-cervical cell retrieval (TraCeR) procedure that included flushing the lower uterine cavity with saline. To confirm the fetal origin of the collected cells, they planned to detect the Y-chromosome-specific sequences by the polymerase chain reaction (PCR). The presence of fetal cells in the cervical smears was eventually confirmed. Finally, they accurately predicted the fetal sex in the samples (cotton wool swabs) collected from 25 of 26 women. The immunohistochemical analysis detected syncytial trophoblast fragments in the samples obtained by the TraCeR procedure [13]. They concluded that the purification of isolated tissues with immune-magnetic technology could enable the application of more advanced prenatal diagnostics in the future.

In 1994, the French team reported that they managed to determine the fetal sex using the samples of desquamated trophoblast cells non-invasively collected from the cervix in 8/10 analyzed specimens

using the in situ hybridization and in 6/10 cases by DNA amplification [14]. Then, Adinolfi et al. reported in *The Lancet* that it is possible to detect fetal sex, trisomy 18, and RhD DNA sequences by FISH and PCR in transcervical cell samples retrieved by aspiration of cervical mucus before the procedure of invasive chorionic villous sampling at about 10 weeks' gestation and by endocervical lavage between 6 and 13 weeks' gestation [15,16].

In 1995, Briggs et al. conducted a more extensive study that included the analysis of 150 pregnancies. The cervical material was collected between 7 and 17 gestational weeks before planned terminations. The authors compared the efficacy of two methods (flushing of the uterine pole and direct mucus aspiration by swabs) in the transcervical collection of trophoblastic cells. They found that the flushing was associated with a higher ratio of successful detections of visible syncytial vesicles; however, the differences were insignificant (39% vs. 26%). Both methods, FISH, and PCR, were applied to confirm the fetal origin of detected cells. Interestingly, molecular techniques also detected fetal material in the samples devoid of syncytia [17].

At the same time, Bahado-Singh et al. recruited 20 pregnant patients between 7–10.5 weeks' gestation who were admitted for planned pregnancy termination. To detect the presence of trophoblastic cells, they obtained the samples of endocervical lavage using the plastic catheter, which was placed into the cervical canal near to the internal os under the control of sonography. Then, they flushed the cervical canal with 3 mL of saline and aspirated accumulated fluid. Collected cells were suspended in the fixative solution and immediately assessed using light microscopy. Visible trophoblastic fragments were detected in 10/20 samples. Only in 1 out of 5 cases, collected cells were successfully in vitro cultured for 5–7 days in Alpha MEM media with 20% fetal bovine serum and 1% kanamycin – trophoblastic cells were identified using the specific anti-alpha-hCG staining. The study proved that the procedure of endocervical flushing was safe and feasible in the early pregnancy – none of the patients experienced complications of the applied procedure [18].

Also, in 1995, Bulmer et al. provided the first immunohistochemical characterization of trophoblastic cells retrieved from the cervix. They identified two populations of trophoblastic cells – syncytial fragments and single or grouped extravillous cells. While syncytial fragments presented various surface and cytoplasmic reactivity with low molecular weight cytokeratins (5D3) and surface reactivity with human trophoblast protein (NDOG1), extravillous trophoblast cells were unreactive with NDOG5 and anti-fibronectin (BC1) antibodies. Moreover, they reported that numerous maternal squamous and columnar epithelial cells were detected in each specimen – lavages had higher purity than swabs [19].

In 1996, Miller and Briggs published the results of an extensive analysis conducted on the patients who expected elective pregnancy termination. Their study compared two methods of trophoblast collection (trans-cervical intrauterine flushing and mucus aspiration). Both procedures were performed using the embryo transfer catheters. It was discovered that the trans-cervical lavage (17/45) was more effective in collecting syncytial fragments detected by microscopy compared with traditional mucus collection (39/173). The fetal origin of analyzed cells was confirmed using both sex-determining methods and gender-independent trophoblast-specific immunocytochemistry. The fetal sex determined using the transcervical cells in 84% matched with the results obtained from fetal tissue assessments after termination [20]. Miller et al. published a next study focusing on differences between those two methods of cell collection, even with a larger study population. Finally, they did not find significant differences between lavage and mucus aspiration efficacy [21].

Furthermore, Massari et al. detected fetal DNA in 17 out of 39 samples of transcervical lavage. Isolated cells enabled them to correctly predict fetal sex, detect the aneuploidy (trisomy 21), and assess the risk of monogenic diseases such as spinal muscular atrophy and myotonic dystrophy in collected trophoblast cells [22]. Interestingly, it was noted that the collection rate of trophoblastic cells through trans-cervical irrigations was not affected by the location of the placenta in the uterine cavity, fetal gestational age, and maternal age [23]. In the following years, Adinolfi et al. managed to use the trans-cervical cell samples collected through the transcervical flushing or mucus aspiration to the detection of the most common trisomies, sex chromosome aneuploidies, and prenatal diagnosis of monogenic hemoglobin (Hb) mutations in fetuses of parents known to be carriers of Hb mutations. These procedures were conducted in the first or the early second trimester of pregnancy using FISH or PCR reactions. In their analyses, authors used entire collected trans-cervical samples or assessed only selected clumps of trophoblastic tissue, free of maternal debris, isolated by micromanipulation under an inverted microscope [24–27]. Moreover, they reported that the use of X22 nucleotide might have diagnostic value for the detection of trophoblastic cells derived from female fetuses [28].

In summary, studies conducted in the 90's focused mainly on developing efficient trans-cervical cells collection and detection techniques. Multiple studies proved the fetal origin of cells retrieved from the cervix using FISH and PCR methods. The high contamination of the samples with maternal squamous and columnar epithelial tissue was the main limitation of conducted molecular prenatal diagnostics. It was reported that the trophoblastic cells could be manually separated from the ma-

ternal epithelium by micromanipulation. The first promising outcomes in the trophoblast cells isolation paved the way for further investigations in this area. We need to be conscious that at the same time, multiple studies try to evaluate the utility of fetal erythroblasts isolated from the blood of pregnant women and the application of free extracellular fetal DNA detected in maternal serum and plasma in the early molecular prenatal diagnostics [29,30].

Clinical and experimental trials from the 2000s through to present

In 2001, Schueler et al. published an outstanding paper describing the process of trophoblast cells collection from the trans-cervical lavage and uterine blood specimens. The uterine blood was collected from the veins located near the placental attachment site by a trocar needle inserted into a needle guide using the sonographic control. Surprisingly, in comparison to the results of previous studies, they achieved very poor outcomes – 3/23 (13%) – in the detection of trophoblastic cells in trans-cervical lavage samples. Then, they tried to isolate fetal cells from the uterine blood using two methods: depletion (success in 11/18 cases) and positive immunomagnetic cell separation (success in 3/45 cases). Trophoblast cells were identified using the colocalizing with antibodies specific for epithelial cells (cytokeratin) and those characteristic for trophoblastic lines (plasminogen activator inhibitor-1 (PAI-1), human placental lactogen (hPL), and insulin-like growth factor II (IGF-II)), or by in-situ hybridization with several specific oligonucleotides that detected the cytoplasmic mRNA sequences [31]. In general, the study results were disappointing, deprecating the role of trans-cervical cell collection in prenatal diagnostics.

Cioni and Bussani et al. reported that the intra-uterine lavage is more effective in collecting fetal trophoblastic cells (about 80-95% correct fetal sex prediction) than the method of trans-cervical mucus aspiration by the cytobrush (only about 65% efficacy). Interestingly, both methods were significantly less efficient in male sex prediction. For instance, the mucus aspiration method has only about 30% efficacy in male sex prediction and about 90% in female sex prediction, which could be elucidated by the fact that the collected trans-cervical samples lack an appropriate number of fetal cells for molecular analyses. The authors reported that the maternal contaminant in micromanipulated samples is very rare (only 1-2%) [32–35].

Bulmer et al. described a new method of trophoblast cells isolation at early stages of pregnancy from endocervical lavage samples. Using the specific expression of human leukocyte antigen G (HLA-G) expression in cytotrophoblastic cells, they immunolabelled the isolated specimens with HLA-G specific antibodies and then transferred them onto slides.

HLA-G positive cells were detected in about 50% of the slides. In four cases, stained cells were isolated from the maternal epithelium using the laser microdissection and shown to have a fetal origin using the quantitative fluorescent-PCR (QF-PCR) method [36].

In the following years, several other studies tried to isolate fetal cells from the cervical canal in early pregnancies, using the swab, mucus aspiration, and lavage methods in women before an elective pregnancy termination. In general, the trophoblastic cells collection rate was still unsatisfactory. The isolation of fetal cells performed by a combination of immunostaining (HLA-G, cytokeratin-7 (KRT7)) and micromanipulation methods had been reported to have the highest potential feasibility in trophoblastic cells recovery and for future prenatal diagnostics. Furthermore, in about 2005, the FISH method was almost entirely replaced by the QF-PCR in the molecular prediction of the most common aneuploidies and fetal sex-determining techniques [37–43].

Imudia et al., in their pilot study, published in 2009, retrieved the trans-cervical samples containing trophoblastic tissue using the routine cervical canal brushing. After the collection, specimens were placed in the fixative transporting solution used in the liquid-based cytology. Collected cells were labeled with the HLA-G antibody – extravillous trophoblast marker antibody – and transferred onto laboratory slides. Extravillous trophoblast cells were detected in 35/37 specimens from a normal pregnancy. Approximately 1 in 2000 cells was positively labeled with marker antibody. Moreover, they found significantly more trophoblastic cells in samples collected from normal pregnancies than the specimens collected in patients with ectopic pregnancy or blighted ovum [44]. Grabar confirmed previous observations that the quantity of isolated trophoblastic cells could be used in the differential diagnosis of normal pregnancy and missed abortion or tubal pregnancy [45]. Interestingly, extravillous trophoblast cells obtained by cytobrush showed the tendency to endoreduplication [46].

In 2014, Bolnick et al. shared the results of their groundbreaking study in which they reported isolating about 1000 trophoblast cells from each recruited patient. They revealed that such a number of trophoblastic cells is suitable for a wide range of molecular analyses. They collected the trans-cervical mucus samples using the standard endocervical canal brushing technique. Collected cells were labeled with specific HLA-G antibodies and secondary immunomagnetic particles, enabling them to process cell sorting using the magnetic-activated cell sorting (MACS) method. They named their method of trophoblastic cells isolation as a trophoblast retrieval and isolation from the cervix (TRIC). Using immunocytochemistry, they characterized the immunophenotype of isolated cells. Isolated cells were positive for three markers characteristic for

all trophoblast lineages (human chorionic gonadotropin beta subunit (β -hCG), hPL, KRT7), and several specific protein markers of extravillous trophoblast subpopulation (HLA-G, VE-cadherin (CDH5), platelet endothelial cell adhesion molecule precursor (PECAM1), integrin- α 1 (ITGA1), and matrix metalloproteinase 9 (MMP9)). Isolated cells were successfully applied in the DNA integrity and fetal sex analyses [47].

On the other hand, Mantzaris et al. publish less optimistic data on the trophoblast cells isolation using the NDOG1 antibody and following laser capture microdissection. They identified syncytial fragments in 78% of the obtained samples [48]. Furthermore, Pfeifer et al. reported that using the single-cell laser-assisted microdissection isolation method, they isolated trophoblastic cells in each sample obtained by cytobrush. However, the exact cell yield (about 2–12 cells per 2ml of fixative solution) was unsatisfactory [48].

In the following years, the “TRIC” research team published multiple papers which improved our understanding of the biology of trophoblastic cells isolated from the cervix. For instance, they conducted a first case-control study that analyzed pregnancy outcomes in patients after collecting trans-cervical specimens. They compared the trophoblastic expression of several biomarkers – endoglin (ENG), FMS-like tyrosine kinase-1 (FLT-1), α -fetoprotein (AFP), pregnancy-associated plasma protein-A (PAPP-A), galectin-13 (LGALS13), galectin-14 (LGALS14), and placental growth factor (PGF) – associated with adverse pregnancy outcomes in patients with early pregnancy loss and those with uncomplicated term delivery. Their results indicated the increased AFP, ENG, and FLT-1 and reduction in PAPP-A, LGALS14, and PGF expression in patients with adverse pregnancy outcomes [49]. Moreover, they found significant alterations in the PAPP-A, FLT-1, ENG, AFP, PGF, and LGALS14 expression in extravillous trophoblast cells obtained between gestational weeks 6 and 20 from women who, in later pregnancy, developed preeclampsia or fetal growth restriction compared with normal pregnancies [50]. Analyzing samples obtained from 224 women, they concluded that the trophoblastic cell yield is unaffected by the maternal age, body mass index, parity, and gestational age between 5 and 20 weeks of pregnancy. Interestingly, trophoblast yield was significantly decreased in patients with early pregnancy loss [51]. They also informed that the TRIC method could be used for early fetal sex prediction in congenital adrenal hyperplasia (CAH) carriers. They proposed that early male sex prediction (prior to week 7) can avert the necessity of dexamethasone treatment in patients at risk of CAH [52]. Finally, in 2019, they provided the results of transcriptomics analysis of the cells isolated by TRIC, which demonstrated that the transcriptomic profile of extravillous trophoblast cells

is distinct from that of maternal cervical cells [53]. Further analyses of transcriptomic profiles in patients with uncomplicated pregnancies and those who experienced pregnancy-related pathologies such as preeclampsia or fetal growth restriction can elucidate the mechanisms of those conditions.

Interestingly, Bailey-Hytholt et al. developed a new technique of enrichment of trophoblastic cells isolation from cervical samples using the differential settling of the cells in polystyrene wells. They reported that their method could provide 700% enrichment in trophoblast cells and remove 91 ± 3% of the cervical cell population [54]. Lee et al. proposed optimizing the TRIC isolation protocol by testing another fixation method. They found that the post-fixation in formalin could be more effective in trophoblast cells isolation than the pre-fixation method in alcohol solution [55].

Conclusions and future perspectives

Years of ongoing trials proved that the trophoblast cells could be detected and isolated from the squamous and glandular cervical epithelial cells obtained via various techniques of trans-cervical samples collection in the early first trimester, even from the 5 weeks' gestation. Nonetheless, the collection of trophoblastic cells has limited efficacy compared to currently used methods of detection of free fetal DNA circulating in maternal blood or other protocols of invasive prenatal diagnostics. In the past years, trans-cervical cell samples were collected mainly in women before planned pregnancy termination [3,4,56,57]. The early trophoblastic cells isolation from women in ongoing pregnancies and correlation of laboratory results with pregnancy outcomes opens new perspectives for further studies focused on the elucidation of pathophysiology of pregnancy-related complications.

Ethical approval

The conducted research is not related to either human or animal use.

Acknowledgements

Not applicable.

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Conflict of interest statement

The authors declare they have no conflict of interest.

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