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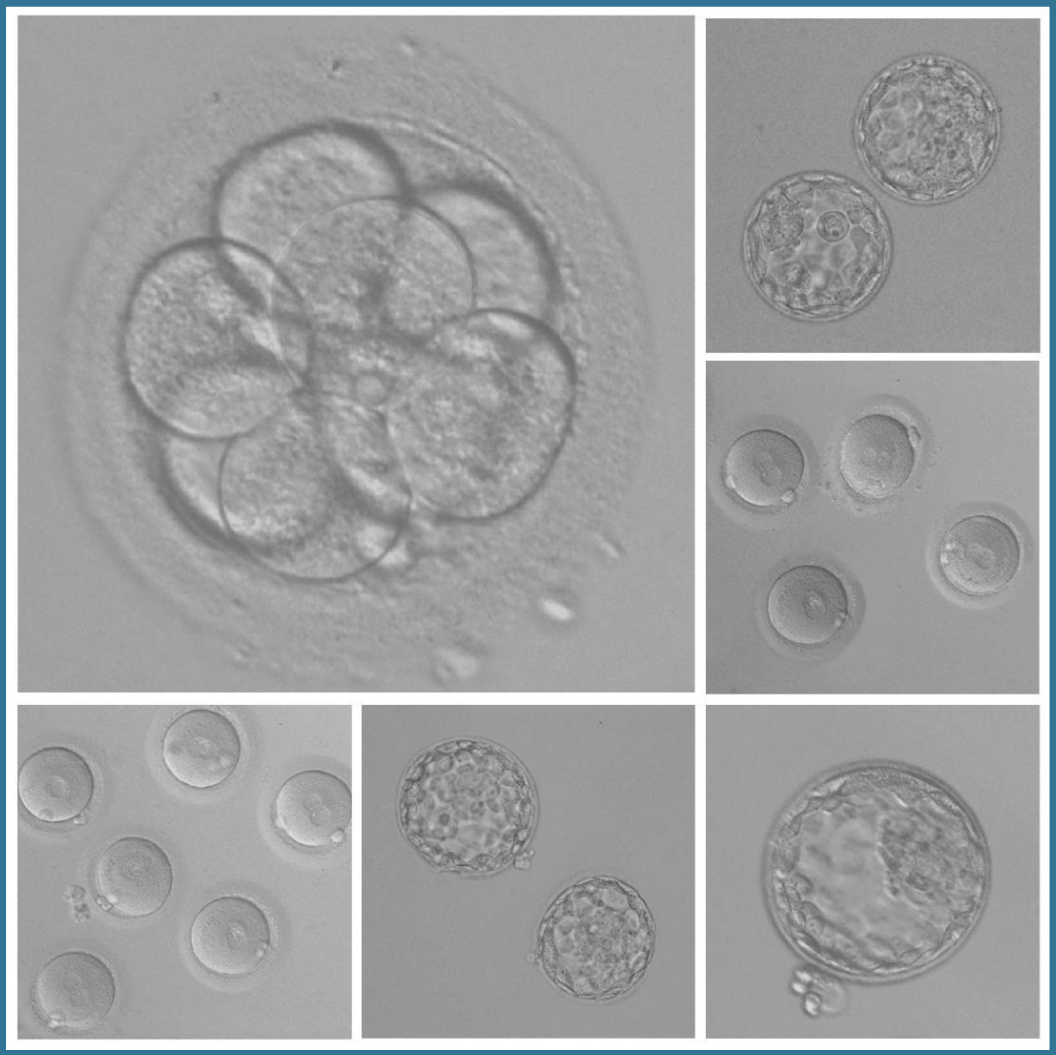

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The Journal of Reproduction

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Editorial

Asch-Schuff Ricardo Héctor[1], 0000-0001-5743-7121.

Assisted Reproductive Medicine (ART), more than any other specialty in medicine, has evolved in the last three decades into a multidisciplinary subject, which not only initiates with the beginning of life but also has recently equaled and perhaps surpassed, the possibility of producing a normal, healthy baby compared to natural fertility.

At the onset of the history of this specialty, very few Centers existed around the world and were placed in very affluent locations that were able to make large investments in the opening of specialty clinics. Nowadays, this specialty has also evolved to be more universal and almost all countries have several clinics of excellence that offer their people treatments in a non-discriminatory fashion.

At the same time that society at large has become much more open and respectful toward minority groups, Assisted Reproductive Medicine has become more inclusive to all groups of society, including people of the LGTBQ+ community that desire to have their own progeny.

ART nowadays is formed by multiple associations with different fields of science such as molecular genetics, chemistry, engineering and physics (such as in Artificial Intelligence), philosophy including bioethics, and psychology, among many others. ART is today a combination of Academics studying their corner of laboratory work, private enterprises involved in the creation of new tools and machinery, and in many countries, governmental intervention to facilitate treatments for the more undeserved economical population.

The Journal of Reproduction is a new Project that aims to offer its readers the most advanced topics of clinical science, research, and new areas of investigation in the field of Assisted Reproduction.

The Journal of Reproduction will be an online production, appearing quarterly, and, will be free of charge to all potential writers interested.

I want to personally thank all the members of the Editorial Board, a Dream Team of experts from all over the world, who have accepted to join in this new adventure without hesitation.

Also, to our Editorial Committee that will be working hard reviewing the initial incoming manuscripts, I extend my sincere appreciation.

We hope that this enterprise will be a successful one to gain a place in the literature of this fascinating specialty of medicine.

Welcome to all.

Asch-Schuff Ricardo Héctor

Editor in Chief

[1] Instituto Mexicano de Alta Tecnología Reproductiva, México.

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CONTACT:
Asch-Schuff Ricardo Héctor.
drrasch@thejournalofreproduction.com
Instituto Mexicano de Alta Tecnología Reproductiva, México. Sierra Mojada No. 340 Col. Lomas de Chapultepec 1era sección, CDMX. C.P. 11000, MEXICO.
Phone: +52 55 5540 2218.

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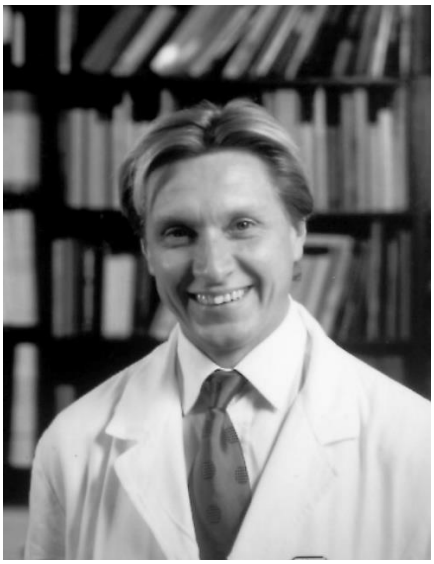
In Memoriam



It is never pleasant to write an obituary, especially about someone that one esteems and admires very much. This is the case with Professor Wilfried Feichtinger.

At the onset of The Journal of Reproduction, I wanted to honor one of the most significant pioneers in the area of world reproduction, my colleague and dear friend for over 30 years, Wilfried Feichtinger.

Wilfried passed away on June 3, 2021, but his legacy will remain forever among us.

His incursion into different areas of Assisted Reproduction and Human Biology has provided us with methods and techniques that we use daily such as ultrasound-guided transvaginal oocyte retrieval, and laboratory techniques such as vitrification and embryo culture.

I could go on and on mentioning his scientific and clinical contributions to the field of Reproduction, but the literature speaks for itself with his numerous manuscripts, organization of International Congresses, and books on our specialty.

I would like to make a note about Wilfried the person. He was the most charismatic colleague, an avid lover of life in its best sense. I believe we were together at least in fifteen different countries for professional conferences. Everything became fun and enthusiastic when Wilfried was around. His elegance as a person was only paralleled by his excellent qualities as an opera singer, and his love for nature.

When we were around him, not only did we learn so much from him but it was always a joyous occasion.

We will all miss this great professional from our field, a great friend and human being.

It is a great pleasure that his son Michael, who follows Wilfried's steps in research and clinical work in Reproduction, is the principal author of the main article of this initial issue of The Journal of Reproduction.

You would be so proud Wilfried!

Asch-Schuff Ricardo Héctor

Editor in Chief

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Non-Invasive Preimplantation Genetic Testing: a Laboratory Review

Vaccari Enrico, Ph.D.

Vaccari Enrico, Ph.D.[1]; Martins Marion, M.Sc.[2]; Feichtinger Michael, M.D., Ph.D., EFRM[1]
,0000-0001-6453-9281.

ABSTRACT

Since the presence of cell free DNA (cfDNA) was reported in blastocoel fluid and spent culture medium the possibility to introduce a non-invasive preimplantation genetic testing (niPGT) as a new tool in assisted reproductive technology (ART) has been investigated by different groups.

A variety of different approaches have been used until now to collect, extract, amplify and analyse embryonic DNA from BF, SCM and eventually both together. However, success rates in term of amplification, ploidy concordance, specificity and sensitivity vary widely among different papers.

This review aims at summarizing those different methodologies.

niPTG offers many advantages compared to the traditional biopsy method especially regarding safety of embryos and future children, nevertheless despite the growing amount of data, optimized and high reproducibility protocols are as for now, missing and clinical replacement is still not possible, and it should still be only classified as a screening method for optimizing noninvasive embryo prioritization.

KEYWORDS: non-invasive preimplantation genetic testing, cell-free DNA, spent culture medium, blastocoel fluid.

ABBREVIATIONS

cfDNA – cell free DNA.
BF – blastocoel fluid.
SCM – spent culture medium.
niPGT – non-invasive preimplantation genetic test.

MANUSCRIPT

Introduction

Since the first papers reporting the presence of cfDNA (cell-free DNA) in BF (blastocoel fluid) and SCM (spent culture medium) were published in 2013 [1], [2] an increasing interest has been set into the possibility to establish solid tools that allow the genetic screening of embryos without the necessity of biopsy.

As aneuploidy in human embryos is common and can result in implantation failure or miscarriage, preimplantation genetic testing (PGT) is used worldwide with the aim of improving pregnancy rates in

[1] Wunschbaby Institut Feichtinger, Lainzerstrasse 6, 1130 Vienna, Austria.
[2] Kinderwunsch im Zentrum, Brüdergasse 3, 3430 Tulln an der Donau, Austria.

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CONTACT:
Feichtinger Michael, M.D., Ph.D., EFRM.
michael.feichtinger@wunschbaby.at
Wunschbaby Institut Feichtinger
Lainzerstrasse 6, 1130 Vienna, Austria.
Phone: +43 1 8777 775.

poor IVF-prognosis patients, for example patients of advanced maternal age and recurrent pregnancy loss.

PGT nowadays is a standard procedure and is commonly performed through biopsy of trophectoderm cells of the expanded blastocyst from day 5 to day 7 of culture, or the biopsy of blastomere on the third day of embryo development. Additionally, polar body biopsy on day 1 after fertilization can reveal meiotic maternal aneuploidies. All those procedures are highly invasive, may compromise the embryonic development and consequently the embryo implantation potential and raise questions about long-term effects on the offspring´s health. From a more practical point of view, all the biopsy procedures require highly trained personal, special instrumentation, they are time consuming, they add risks into the lab workflow and significantly increase the treatment costs for patients.

The origin of cfDNA and the biological mechanisms responsible in both BF and SCM are still controversial. Nevertheless multiple studies in the last years have shown that cfDNA present in the BF and in the SCM is informative, and even if the clinical replacement of classic PGTA is not yet possible, it could provide a safer, easier and more economic source of genetic material for analysis in the future.

Cell Free DNA in Blastocoel Fluid

The first work introducing a minimally invasive preimplantation genetic testing (miPGT) in which BF was aspirated from blastocyst (blastocentesis) was published in 2013 followed from great interest of the potential of the new technique [1]. Blastocentesis is considered to be not detrimental for the embryo and used by many laboratories prior to blastocyst vitrification [3]. It is a well-established method that nevertheless still requires highly skilled personal and is both time and cost consuming. The sampling procedure has been described in detail: it consists of an ICSI pipette that enters the blastocoel cavity through the TE on the opposite side of the inner cell mass (ICM), ideally in the point of contact between two TE cells to minimize the amount of crossed cytoplasm and the possibility of cell damage. Through a slightly negative pressure, the fluid is collected until the embryo is fully collapsed, paying great attention to avoid the aspiration of any cellular material present inside the blastocoel. After the collection, the ICSI pipette is gently retracted and the specimens can be directly transferred to a PCR tube or released in a small drop of medium and then collected into a PCR tube before cryopreservation ([1], [4]–[9]). The reported BF´s aspired volume vary among different works between 0.005 µl and 1µl. Blastocentesis has been applied in different studies during years for both PGT-A and PGT-M with different degree of amplification success (35% to 88%) among different studies[1], [4]–[8], [10], [11] and independently from the genetic approach used, probably due to the restricted amount of DNA present in BF. The low amplification rate obtained and the resulting difficulty to reproduce data, with the consequent risk to have undiagnosed embryos strongly suggest the necessity of additional studies on the potential of BF in clinical use in order to optimize and standardize protocols for sampling, amplification and analysis of BF-cfDNA. Furthermore, blastocentesis needle aspiration protocols do not reduce cost and do not simplify the laboratory workflow compared to the traditional TE biopsy.

Cell Free DNA in Spent culture medium

The analysis of cfDNA in SCM is a potential alternative for the non-invasive preimplantation genetic testing of embryos in IVF and several publications in the last years explored this possibility. cfDNA has been reported to be detectable already on SCM of embryo on day 2 -3 of development [2], [10] and it has been shown that the amount of cfDNA increases during embryo culture, however not all cfDNA has embryonic origin [13]. In particular contamination with maternal DNA seems to be the main source of non-embryonic DNA in SCM. Xu and colleague published in 2016 [14] a comparison between 42 SCM of donated blastocyst and their subsequent whole embryo analysis. Embryos generated by ICSI and vitrified on day 3 were thawed and placed in 25µl single drop culture until day 5. On that day, after 2 days of culture, 5 to 20µl of SCM have been collected. A modified multiple annealing and looping based amplification cycles (MALBAC) followed by NGS was used to perform genome analysis. The corresponding D5 whole embryos were used as gold standard to evaluate the chromosome screening results from the culture media. They were able to amplify DNA in 100% of cases obtaining a ploidy concordance of 86%. The paper published by Shamonki and colleague in 2016 [15] first tried to assess whether PGT is possible by testing for cfDNA in SCM comparing the results with trophectoderm biopsy performed on the same embryos. Embryos were cultured in single 15µl media droplets from d3 until the biopsy (d5 or d6), assisted laser hatching was performed to facilitate the extrusion of TE and to allow the expulsion of cfDNA. Using the SCM in their proof-of-concept paper they were able to detect cfDNA in 55 out of 57 cases. Our group in 2017 ([16]) published a paper in which we demonstrated the presence of cfDNA in SCM after uninterrupted embryo culture after polar body biopsy from d1 to day d5/6. In this case 18 out of 22 samples could be amplified from 5µl of SCM and resulted in a 72% ploidy concordance rate. Ho and colleagues in 2018 ([17]) showed that cfDNA is detectable in SCM from thawed 2PNs stage obtained by ICSI. The zygotes have been cultured in a continuous and uninterrupted way until d3 or d5 of

embryo development. Both informativity and ploidy concordance were higher (39% vs 80.4%) and more accurate (56.3% vs 65%) in d5 samples. Vera-Rodriguez and colleagues ([13]) report a high percentage of maternal DNA in SCM, collected from embryos cultured after AH in single 25µl droplets until day 5, which can be the reason of low ploidy concordance between TE and SCM DNA analysis (33%). Conversely Huang and colleagues ([18]) reported extremely promising results in a cohort of 52 donated blastocysts previously biopsied and vitrified, then thawed and kept in culture for 24 hours. 10µl of SCM was collected from each sample. The results of the WGA (MALBAC) analysed through NGS have been compared with TE biopsy results and with the sequencing result of the corresponding whole embryo. The concordance rates for both embryo ploidy and chromosome copy numbers were higher between whole embryo and niPGT-A than the classical TE biopsy, suggesting that niPGT-A could be less prone to errors associated with mosaicism and that it could be more reliable than PGT-A in discovering aneuploidies. In 2019 Yeung published ([19]) one of the largest data sets on the subject: the authors did not modify any of their laboratory routine, performing assisted hatching and medium change on d3 of development and collecting SCM samples (3µl out of 30 µl) on the day of TE biopsy resulting in comparable results between niPGT-A and TE biopsy.

During the years different studies have been published: despite a big variety of embryo origin (ICSI/IVF), culture condition (single/double step, media type, drop size, volume of SCM collected) and embryo manipulations (assisted hatching, vitrification/thawing, zona pellucida removing) no significant differences have been found in term of amplification and concordance; conversely increasing the contact time between embryo and culture medium through the culture extension until d6 and d7 seems to improve both informativity and concordance of SCM with the TE biopsy. Rubio and colleagues ([20]) reported a significant increase of concordance rates between ploidy and sex from 78.7% to 84%, and an increase of sensitivity and specificity from 94.5% to 95.2% and from 71.7% to 82.1% respectively in embryos maintained in culture until d6/7 compared to d5.

Of paramount importance in niPGT is to avoid sample contamination with maternal and external DNA, and it is one of the big challenges of the technique. To minimize contamination several different strategies have been suggested: careful denudation before ICSI or after IVF, serial washes on the time of media changes with new capillaries for each embryo. In 2020 the largest study to date assessing ploidy concordance per embryo assessing traditional PGT and niPGT was published by Rubio and colleagues ([21]). In this prospective, observational, multicentre study TE and SCM from 1301 day 6-7 blastocyst were collected and analysed in 8 different centres. Data resulted in an overall ploidy concordance of 78.2% between cfDNA and TE analysis; when stratified by culture media used or incubators model concordance rates did not show significant differences. The authors point out the importance of the laboratory routine in obtaining good amplification rates and ploidy concordance. Especially they focused their attention in timing of SCM collection indicating in day 6 of development the ideal time for DNA sampling independently of embryo developmental stage on day 5 allowing the embryo to stay in contact with the medium for at least 40 hours. niPTG-A in this paper was applied to all d6 embryos that reach the blastocyst stage prolonging of 1 day the permanence in culture of d5 expanded blastocyst that normally would have been cryopreserved. The embryos were cultivated in small (10µl) medium droplets and the medium was changed on the 4th day of development. Special attention have been raised on careful denudation of oocytes and extensive washing in three sequential 20µl drops of fresh medium before media change on day 4 to minimise the possibility of samples contamination. Lledo and co-workers noticed in their study ([22]) that the results of niPGT-A are independent from the technique used for chromosomal analysis. They amplified 92 SCM samples with two different WGA protocols (MALBAC and Sureplex) obtaining genetic information in 92.4% of cases regardless of the method used; in 95.2% of the cases they reported consistency in the diagnosis. Furthermore they were able to investigate the causes of the discrepancy between niPGT-A and classic PGT-A using the TE of aneuploid donated embryos. In 22,2% of the cases the differences were due to embryo mosaicism and in 55,6% of cases due to DNA contamination (maternal origin) raising the attention on the protocols used to obtain contamination-free SCM. Hanson and colleague ([23]) collected 166 SCM samples from 30µl drops where embryos were cultivated, after AH, from day3 and day4 until blastocyst biopsy (day 5 to day 7). They compared the data obtained with NGS platform reporting 37.3% of amplification failure on niPGT-A samples and 40.4% of ploidy discordance with TE results. Due to the experimental design, they had in the cohort of SCM samples that were in contact for different durations with the embryos from 1 (dish change on day 4 and biopsy on day 5) to 4 days (dish change on day 3 and biopsy on day 7). They noticed statistically significant higher rates of DNA amplification with the increase of the time in culture of the embryo: after 1 day of exposure they amplified only 1 sample out of 26. After 2 days 36/63 samples where amplified, after 3 days 56/66 and finally all the samples collected after 4 days were amplified (11/11). The discrepancy in the whole chromosome aneuploidies between niPGT-A and PGTA (40.4%) anyhow didn´t show any statistically

relevant correlation with the day of contact. Using 75 previously vitrified donated blastocyst Yin and colleagues ([24]) were able to compare the analysis of SCM with TE and the whole embryo. After warming, blastocysts were placed in single 25µl drops and the SCM was collected after 24 hours of culture. The blastocyst were then biopsied and TE and the remaining embryos separately collected for the analysis. In a first phase of protocol optimization the thawed blastocysts were kept in contact 8 hours with the medium before the collection of SCM: the NGS profiles obtained after WGA were noisy providing the evidence of poor DNA samples and suggesting the necessity to increase to 24 hours the culture time. After this adjustment the group reported a good informative amplification rate (78.7%) nevertheless a significant lower percentage than their corresponding TE and WE groups. No statistically significant different ploidy concordance rate between WE and SCM (89,8%) and between WE and TE (94.8%) were reported, nevertheless they reported remarkable differences in full concordance rate (including mosaicism and segmental aneuploidies) between the two groups: WE to SCM 32.2% and WE to TE 69.3%. In order to assess the potential of zona pellucida (ZP) and its associates transzonal projection as source of cfDNA contamination, 6 ZP were placed in media drop and kept in culture for 24 hours. The media samples analyse showed amplification failure eliminating the possibility of ZP and associated projection as source of DNA in SCM. Interesting findings have been shown by Shitara and colleague ([25]) that compared niPGT-A results with classical PGT-A using outgrowth embryos as golden standard. 20 donated blastocysts have been thawed and placed in culture for 1 day (day 5 blastocyst) or 3 hours (day 6 blastocyst) before zona pellucida removing (embryo exposed to acidic Tyrode, Kitazato) and TE biopsy. After biopsy embryos were kept in long-term culture up to day 10 when the centre of the embryo was biopsied and sampled. Even though the study included only a small sample size the authors report a better autosomal chromosomal concordance between niPGT-A to outgrowth (56.3%) than between PGT-A to outgrowth (43.8%). They report as well for niPGT-A group a better sensitivity (100%), specificity (87,5%), positive predictive value (88,9%) and negative predictive value (100%) compared with the PGT-A group (sensitivity 87,5%, specificity 77.8%, PPV 87.5%, NPV 75%) stating that cfDNA in SCM reflects the chromosomal status of both ICM and TE, whereas the TE biopsy only reflects the chromosomal status of TE, thus the SCM could reflect the ploidy of the blastocyst better than TE biopsy samples. An further group in 2021 (Chen et al. 2021 [26]) published a study where whole embryo was used as a reference to calculate the ploidy concordance of SCM and TE. 265 embryos derived from ICSI and cultured from day 3 to blastocyst stage were donated and used for subsequent analysis. From 20 to 25µl of SCM were amplified with a MALBAC WGA strategy and then subjected to NGS. Using the NGS result from WE as the gold standard to evaluate PGT and niPGT performances they showed no differences in sensitivity (TE 89.6% vs SCM 86.5%), specificity (80.0% vs 73.1%), negative predictive value (92.8% vs 90.0%) and positive predictive value (72.9 % vs 65.9%).

The authors identified three main reason for low proportion of maternal contamination and higher concordance rates obtained in their study compared to Vera-Rodriguez [13]: (i) thorough precaution in removing as many as possible cumulus-corona radiata cells and extensive embryo rinsing on the time of medium change; (ii) MALBAC WGA technique; (iii) the utilization of whole embryos as gold standard for comparison, considering the fact that TE cells karyotype could be not fully representative of the WE. Like Rubio and colleague Chen group concluded that niPGT-A is a good rule-in assay and might be used for prioritizing embryos for transfer.

Collection of cfDNA from Spent culture medium and Blastocoel Fluid

The reported PCR amplification rates achieved from SCM are inferior to those using cellular biopsy and the potential presence of genetic contamination is a significant concern. In 2018 Kuznyetsov and colleague ([27]) published a study where they suggest a new approach to obtain sufficient embryonic DNA for aneuploidy screening with a non-invasive methodology. They combine the collection of SCM with BF. Expanded blastocyst were collapsed by single laser shot at the junction of TE cells allowing the content of blastocoel to flow into the culture medium. 100% of their samples, obtained after thawing previously cryopreserved embryos and maintained in culture for 24hours (28 blastocyst), were informative and the concordance with TE and WE was 87,5% and 96,4% respectively. Also the samples obtained from freshly cultured embryos from d4 to d5/6 were 100% informative and the ploidy concordance between niPGT and TE was 100%. In order to minimize the risk of contamination in fresh culture samples they modified the culture protocol introducing a more careful denudation and a medium drop change on day 4 of culture after extensive embryo wash. In 2020 the same group published a paper ([28]) where they report factors affecting accuracy of minimal invasive preimplantation genetic testing for aneuploidy (mi-PGT-a): according with their results blastocyst morphology has no effect neither on cfDNA quantity found in SCM + BF or in WGA-DNA fragment. According with this finding blastocyst morphology has no effect on informativity and concordance of mi-PGT-A compared with classical TE biopsy and analysis: on a total of 102 samples 88.2% where informative and the

overall ploidy concordance rate was 97,8%. In the same study they propose a new protocol for the whole genome amplification (WGA): in order to reduce the risk of maternal contamination by residual cumulus/corona cells, it avoids the cell lysis step and in this study on 43 blastocysts it showed no difference in terms of informative amplification and ploidy concordance analysis in case of WGA with or without lysis and compared with TE biopsy.

In combining BF and SCM, Li and colleague showed that DNA concentration in SCM together with BF were sufficient to obtain DNA amplification in 97,5% of cases with a ploidy concordance with TE and WE of 76,3% and 78.9% respectively. Several other studies describing collection of cfDNA from SCM together with BF have been published until now ([29]–[33]): anyway, a low number of samples were included in those papers and embryos were subjected to heterogeneous manipulation (vitrification, thawing, TE before or after cfDNA sampling) in addition to artificial collapsing. The genetic analysis results were compared with TE and WE and the results were heterogeneous in terms of informativity and ploidy concordance, nevertheless they never drop under 87,5% and 70%. Jiao and colleague collected SCM and BF from donated and thawed embryos kept in culture for 15 hours. They obtain 100% of amplification rate, 90.5% clinical concordance for aneuploidies and 100% clinical concordance for chromosomal structural rearrangements with embryos NGS analysis. Notably they developed a modified MALBAC approach to reduce the time of WGA and library preparation to 2.5 hours, introducing the possibility to undergo a fresh blastocyst transfer after PGT ([29]). In 2022 Tsai and colleague published a study where cfDNA where collected from SCM and BF using a routine IVF laboratory workflow. They used both IVF (64%) and ICSI (36%) as fertilization methods, the embryos were cultivated in a time-laps system in sequential media (change on d3). They reported an amplification rates consistent with those reported in previous studies but with a drop in the overall ploidy concordance to 67.7% ([34]). The ploidy concordance was higher in ICSI samples compared to IVF but the difference was not statistically significant and like shown by Rubio ([21]) sensitivity and specificity are not affected by the fertilisation methods used.

Discussion

Recently, after pioneering studies reported the presence of cfDNA in both BF and SCM, niPGT methods have been suggested and shown promising potentiality as an alternative to classical PGT. ([1], [2], [4], [15]). Although the biological mechanisms responsible for the presence of embryonic DNA in SCM and BF are elusive, a growing body of literature has demonstrated the possibility to collect, detect and amplify cfDNA and to use it for clinical application. Some authors suggested that embryonic cfDNA comes both from ICM and TE as results of apoptosis mechanisms happening during pre-implantation development, making it superior to traditional invasive PGT, since TE might be non-representative of the whole embryo due to mosaicism ([17], [25], [27]). Different studies report that the amount of cfDNA available in SCM is directly related with the time of culture of embryos in culture drops, obtaining better amplification rates and concordance after cfDNA sampling on day 6/7 of culture ([17], [18], [20]). Anyway the ideal time for sample collection has not been established yet and there are concerns about the risk connected with the prolongation of culture time regarding the potential negative effects that it could have on embryos viability and developmental potential ([23]). In the last years an interesting approach has been used from different groups to increase the amount of cfDNA present in SCM: a single laser pulse between two TE cells generated an artificial blastocyst shrinking, considered not armful for the embryo, that resulted in the flow of BF into the culture drop. Encouraging results have been achieved in terms of amplification rate in different studies, nevertheless most of them used prior vitrified and then donated embryos as samples. It is possible that the use of vitrified-thawed embryos introduced a bias, due to the possible damage of the freezing-thawing method, resulting in increased levels of DNA in the medium ([27]–[31]). Small media (10-15µl) droplets have been used in different works in order to obtain higher cfDNA concentration and improved DNA amplification rates but the necessity of this protocol modification is still debate [18], [20], [21], [29], [33]. Maternal DNA contamination is one of the main issues that must be solved to avoid misdiagnosis using niPGT. Gentle and careful denudation is of paramount importance to avoid contamination with cumulus and granulosa cells, together with serial extensive washing steps of embryos before transferring them to the culture drops and on the day of culture media change. Each embryo must be manipulated singularly using new capillaries to avoid cross-contamination between samples. Particular attention during all laboratory procedure and sterile condition must be used to prevent contamination from external DNA sources ([18], [21], [27]). From a laboratory point of view, it has been reported that concordance rates between PGT and niPGT is not influenced neither by the culture media used nor by incubator brand, potentially allowing the introduction of this technology in any IVF laboratory without the need to have any new instrumentations [21].Blastocysts laser collapse and assisted hatching have been used in order to increase the amount of cfDNA and its release into the culture drop. The results obtained look promising, and some of the highest niPGT amplification rates have been reported, indicating that sampling

SCM and BF together might be a valid approach to the DNA collection, nevertheless results must be evaluated and confirmed by further studies ([27]–[29], [35]).

CONCLUSIONS

cfDNA of embryonic origin is present in SCM and in BF and can be used to obtain genetic information. However, at present the application of niPGT in routine clinical setting has to be viewed critically due to the lack of clinical data. Further studies using larger sample sizes and laboratories condition that reflect the clinical practice should be encouraged in order to define new standardized laboratory protocols widely accepted. Since the biological mechanisms involved are at the moment not clear, more studies that try to solve the mystery of the origin of cfDNA are fundamental and necessary to improve genetic methodologies used for amplification and analysis of cfDNA and result interpretation. A lot of work needs to be done before niPGT can be used in clinical programs as a diagnostic tool. Nevertheless it is a very promising approach, which can be already considered a good tool in clinical practice to prioritise the order of transfer and possibly reduce the time to conceive for couples undergoing assisted medical reproduction treatments, without imposing biopsy related risks for the embryos and the deriving new-borns related with classical PGT approach.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

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Metabolic syndrome with involvement of the male reproductive system

Saldívar Cerón Héctor Iván

Saldívar Cerón Héctor Iván[1,3], 0000 0002 9125 9100; Castañeda Ramírez Ari Evelyn[1], 0000 0002 1465 8255; Quiñones Lara Efrén[1]; 0000 0001 5577 0908; Vargas Camacho Jorge Arturo[1], 0000 0002 7727 1576; López Desidero Nely Gisela[1,2], 0000 0002 5107 6158.

ABSTRACT

Metabolic syndrome is an endocrine disorder that involves systemic alterations of intermediate metabolism, cardiovascular, hormonal, and reproductive systems. It is characterized by central obesity, elevated blood pressure, insulin resistance, and dyslipidemia, which lead to the development of type 2 diabetes mellitus, cardiovascular and renal diseases. At the clinical level, the central axis of patients with metabolic syndrome focuses on the prevention of cardiorenometabolic comorbidities, however, it's not common that the physician addresses the issue of male reproduction and its implications. Currently there is increasing evidence that patients with metabolic syndrome have low levels of testosterone, GnRH, FSH and LH and high levels of estrogen which impacts sperm quality, fertility, and sexual health, which can undoubtedly be reversible upon remission of the metabolic syndrome. This review addresses clinically how metabolic syndrome impacts the male reproductive system, addressing male fertility, testicular endocrine function, and sexual health.

KEYWORDS: Metabolic syndrome, fertility, testosterone, sperm quality, sexual health.

MANUSCRIPT

Introduction

Metabolic syndrome (MetS) is a group of endocrine disorders caused by excess fatty tissue characterized by visceral obesity, insulin resistance, hyperglycemia, hypertriglyceridemia, atherogenic dyslipidemia (increased LDL/VLDL cholesterol and decreased HDL cholesterol), high blood pressure, microalbuminuria, prothrombotic and proinflammatory state etc. Chronically, metabolic syndrome promotes the development of type 2 diabetes mellitus, cardiovascular complications, chronic kidney damage, cancer, and infertility [1]

It is estimated that the prevalence of MetS in the world ranges between 20-25% in the adult population. However, in Mexico the prevalence of MetS almost doubles the global results (25% vs 41%) [2,3].

Although the diagnostic criteria overlook the reproductive function as a parameter of the MetS, there is increasing evidence that recognizes this as a disruptor of male fertility, in part due to alterations in the hypothalamic-pituitary-testicular axis presenting low levels of GnRH, FSH, LH and testosterone and high levels of estrogens which negatively impact spermatogenesis resulting in a decrease in seminal volume, sperm concentration, DNA fragmentation and finally alterations in fertility [4].

[1] Carrera de Médico Cirujano, Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México, Tlalnepantla 54090, México.
[2] Red de Medicina para la Educación y Desarrollo y la Investigación Científica de Iztacala (Red MEDICI), Carrera Médico Cirujano, Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México, Tlalnepantla 54090, México.
[3] Laboratorio 14, Unidad de Biomedicina, Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México, Tlalnepantla 54090, México.

NOTE: The numbers following the affiliation markers are the author's ORCID iD.

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CONTACT:
Saldívar Cerón Héctor Iván.
ivansaldi@iztacala.unam.mx.
Avenida de los Barrios 112, 54090
Tlalnepantla de Baz, México.
Phone: +52 55 7980 1550.

In this review we focus on the effects of MetS on the male reproductive system, including endocrinological disorders, male fertility, and sexual health.

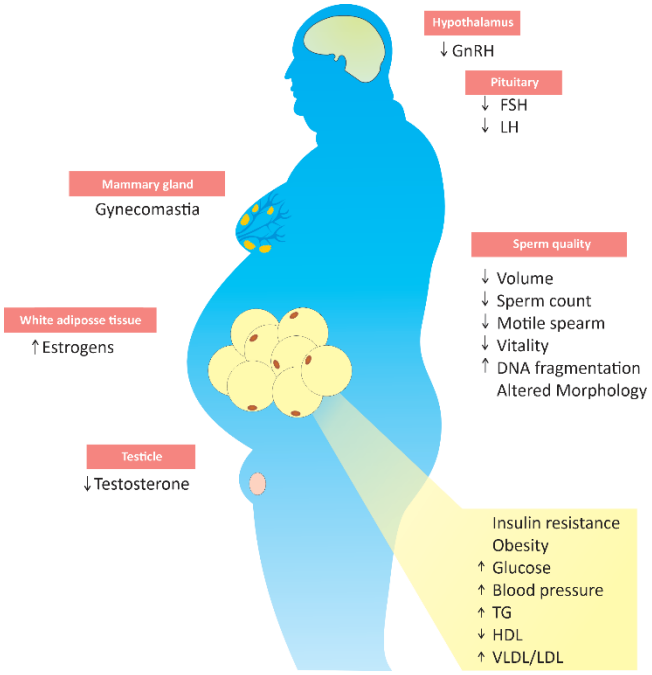

Figure 1. The impact of the metabolic syndrome on male fertility.

Metabolic syndrome and Sperm Quality

The causal relationship between MetS and male fertility is not new. The molecular mechanisms involved in male infertility caused by MetS have been known for decades. However, in the MetS primary care consultation there is a lack of interest in knowing the Male fertility status compared to the approach given to female fertility, this is partly since male fertility continues be a taboo subject [5].

Evidence shows that patients with MetS have negative effects on sperm quality, showing lower seminal volume, decreased total sperm count, decreased motility and vitality, and high levels of sperm DNA fragmentation. It is imperative to take a complete medical and sexual history as well as perform a thorough physical examination in addition to the semen analysis to provide a complete diagnostic assessment. Early diagnosis of MetS may provide an advantage of comprehensive management to promote short-term remission. Reduction of body weight and alleviation of MetS improves the parameters of sperm quality, showing a significant increase of semen volume, sperm motility and motile sperm count. On the other hand, it has also been observed that treatment with aGLP-1 enhances weight loss and can be used for preserving sperm quality in the long term, contrary to what was thought that liraglutide therapy was detrimental to sperm concentration and motility. Finally, there is still discrepancy about the relationship between bariatric surgery and sperm quality, since some studies report a null or worse influence, while others report an improvement after bariatric surgery, so further long-term studies are required period to elucidate the impact of bariatric surgery on sperm quality [6-10].

Metabolic syndrome and its alteration in the hypothalamus pituitary testis axis

The are many endocrinology alterations that can cause male infertility since it has been observed that patients with MetS present hypogonadism because of alterations in the hypothalamus-pituitary-testicle axis (HPG axis). Adipokines have been shown to be functionally involved in the HPG axis. Plasma levels of some adipokines are associated with specific metabolic states. In men with MetS, aromatase enzyme activity is markedly increased, causing excessive conversion of androgens to estrogens. Therefore, gonadotropin secretion by the adenohypophysis is decreased through negative feedback loop inhibition of the HPG axis, having a direct impact on Leydig cells and Sertoli cells, leading to a significant decrease in testosterone production and spermatogenesis, which then impacts further through drops in gonadotropin-releasing hormone (GnRH) pulses [11-13].

Weight loss and gain have been associated with reciprocal changes in luteinizing hormone (LH) and testosterone concentrations, suggesting the reversibility of secondary hypogonadism. Thus, weight loss due to lifestyle changes is associated with a significant increase in LH and testosterone levels [14].

It has been seen that physical exercise (PhyEx) has an impact on the HPG axis, normalizing the infiltration of macrophages related to a high-fat diet and increasing the expression of steroidogenic enzymes, which results in increased levels of LH and the production of testosterone [15].

Regarding drug therapy, previous studies have shown that exenatide plus dapagliflozin combination therapy increased SC lactate production, which has been reported to improve testosterone levels and spermatogenesis [16].

Metabolic syndrome and aromatase activity

The excess of visceral adipose tissue generates a decrease in the serum concentration of sex hormone binding globulin (SHBG), inhibin B (INHB) and levels of free and total concentration of testosterone, however it increases the conversion of testosterone to 17B-estradiol (E2) through aromatase, which is increased. It has been found that there is a relationship between

the increase in aromatase activity and the increase in adipose tissue, which generates a greater conversion of testosterone to E2, resulting in hyperestrogenism. [17-19].

Being a proinflammatory state, obesity generates insulin resistance, so the body tries to maintain homeostasis by producing hyperinsulinemia; which in this case causes a reduction in the hepatic synthesis of SHBG, a glycoprotein which decreases the activity of sex hormones; therefore, when SHBG is decreased, there is a greater activity of E2. INHB is a growth factor that acts by inhibiting FSH, thus stimulating the secretion of testosterone. Leptin resistance decreases kisspeptin expression in the rostral periventricular region of the third ventricle as well as in the arcuate nucleus, thus decreasing GnRH release, and thus FSH, LH, and T secretion [20-22].

Hyperestrogenism includes clinical signs of gynecomastia, sexual dysfunction, loss of body hair, low libido, low sperm quality, fatigue, weight gain and loss of muscle mass, hypogonadism and atrophy of testicles, osteoporosis, hot flashes, decline in cognition and memory loss [23-26].

Although testosterone replacement therapy alleviates the effects of metabolic syndrome by improving insulin and leptin sensitivity, reducing adiposity, and increasing muscle mass, treatment with testosterone is not recommended because it causes hypothalamic axis blockade. -hypo-testis showing defects in spermatogenesis. However, the use of aromatase inhibitors (Letrozole and anastrozole), selective estrogen receptor modulators (Tamoxifen and clomiphene), and LH analogs (Human Chorionic Gonatrophin) have been reported to suppress the conversion of testosterone to estrogens, compete by the estrogen receptor or simulate the effect of LH, respectively, positively impact spermatogenesis, also increase testosterone levels and decrease estrogen levels, relieving the effects of metabolic syndrome on male reproduction, however, the impact of these long-term treatment on fertility and male hormonal regulation needs to be studied [27-30].

Sexuality in metabolic syndrome

It is important to highlight that there are not only physiological aspects that condition sexual deterioration; obesity in these patients prevails a high rate of dissatisfaction with their body image that is altered increasing the frequency of psychiatric conditions such as depression, mood disorders and the use of antidepressants; impairing sexual function. Other added factors that influence the negative results of sexual function and self-esteem are advanced age, lack of marriage, depressive symptoms, fatigue, low energy level, lack of interest, difficulty getting aroused, reaching orgasm and other functional difficulty. Use of antidepressants (except bupropion, trazodone, nefazodone, and mirtazapine) was also found to be associated with less frequent sexual desire. Questionnaires have been used in patients with obesity to collect data focused on symptoms of depression and their consequences, such as the Beck Depression Inventory version 1 and the IWQOL-Lite; where the participants supported the need to reduce their food intake to lose weight, due to associated mood disorders, because that leads to dysfunctional physical activity and sexual limitation. Carrying out physical activity and a hypocaloric diet together with adequate treatment decreases the severity of erectile dysfunction and improves self-esteem, interpersonal relationships, and quality of sexual life. Previous studies have associated depression with candidates for patients seeking bariatric surgery. Finally, it is an analysis that shows deterioration of different predisposing psychosocial factors that continues in the search for future research [30-35].

CONCLUSIONS

Metabolic syndrome is an endocrine disorder that involves systemic alterations of intermediate metabolism, cardiovascular, hormonal, and reproductive systems. There is sufficient evidence showing that men with MetS present infertility and endocrinological disorders of sexual hormones, the main factor is an excess of adipose tissue that secretes adipokines (increase in leptin and decrease in inhibitor B) that deregulate the hypothalamus-pituitary-testis axis (decrease in GnRH, FSH, LH and testosterone and increase in circulating estrogens). It is common to observe low sperm quality, showing lower seminal volume, decreased total sperm count, decreased motility and vitality, and high levels of sperm DNA fragmentation, gynecomastia, sexual dysfunction, loss of body hair, low libido, low sperm quality, fatigue, weight gain and muscle loss, hypogonadism and testicular atrophy, osteoporosis, hot flashes, decreased cognition and memory loss as well as changes in sexual health presenting a high rate of dissatisfaction with their body image reflected in depression, mood disorders and the use of antidepressants. Despite everything, these conditions are reversible, since there are more and more clinical trials showing that weight loss and remission of MetS, whether through diet, exercise or pharmacological and/or surgical treatment, improves sperm quality, increased testosterone levels and decreased estrogen (restoration of the hypothalamus-pituitary-testis axis) and improvement in sexual function. However, in clinical practice it is rare to observe an evaluation of the male reproductive system in patients who are MetS both physically and psychologically, which is why we strongly recommend that physicians take a complete

medical and sexual history of all patients who have been diagnosed with MetS.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

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Concentration of maternal biochemical markers: Complications during pregnancy and their effect on the detection of trisomy 21 in the first trimester, by combined test (study carried out at a private clinic in CDMX)

Luján-Irastorza Jesús Estuardo

Luján-Irastorza Jesús Estuardo[1], 0000-0002-4986-7698; Durand-Montaño Carlos[1]; Hernández-Ramos Roberto[1]; Ávila-Pérez Felipe de Jesús[1]; Ávila-Rebollar Daniela[1]; Loof-Esquivel Mónica Stéphanie[1]; Villa-Jiménez Catalina[3]; Angulo-Rujano Francis Erika[4]; Valdez-Chávez Teresita de Jesús[1]; Arcos-Hernández Héctor[4]; García-Cruz Valeria[1]; Vargas-Hernández Víctor Manuel[2].

ABSTRACT

Aneuploidies are alterations that affect the number of chromosomes. Historically, a combination of markers has been used that includes: maternal age, concentrations of biochemical markers and ultrasonographic markers during pregnancy, as well as screening in the first trimester in order to detect Trisomy 21 (T21).

Objective:

To identify if biochemical markers such as Pregnancy Associated Placental Protein A (PAPP-A) and Beta human Chorionic Gonadotropin (β-hCG) are altered by complications during pregnancy and if this leads to an increased risk of developing T21 in pregnant women with healthy babies (combined test).

Material and method:

Retrospective, observational and cross-sectional study, included 73 pregnant women. Anthropometric data from the mother and the newborn were collected. A combined test was performed in the 1st trimester and biochemical markers were recorded in maternal serum (β-hCG AND PAPP-A) and ultrasonographic markers [Nuchal Translucency (NT)]. In addition, the evaluation of Cell-Free Fetal DNA (cffDNA) in peripheral blood was performed to identify aneuploidies and a pathological study of the placenta.

Results:

In the group of patients with intermediate risk for T21, there was a higher prevalence of complications during pregnancy [fetal growth restriction (4.5%), preterm labor (4.5%), miscarriage (9.09%) and death (9.09%). In the same way, patients with intermediate risk presented a higher prevalence of placental alterations such as calcifications, atrophy, congested dilated vessels, hemorrhage, hematomas, Chorioangioma, extravasation of erythrocytes and villus infarcts.

[1] Clínica de PRONATAL (Hospital Bité Médica). Prolongación Paseo de la Reforma 19, Santa Fe, Paseo de las Lomas, Cuajimalpa de Morelos, 01330 Ciudad de México, CDMX.
[2] Clínica de Salud Femenina. Insurgentes Sur 03810 Ciudad de México, México.
[3] Horizontes clínica de fertilidad. Calle Josefa Ortiz de Domínguez 538, Coaxustenco, 52140 Metepec, Méx., Metepec.
[4] Clínica FertiFetal, Salud femenina/Reproductiva/Prenatal. Mayorazgo #130, alcaldía Benito Juárez, Hospital San Angel Inn Universidad, consultorio 744, 03339, Ciudad de México, CDMX.

NOTE: The numbers following the affiliation markers are the author's ORCID iD.

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CONTACT:
Luján-Irastorza Jesús Estuardo.
jlujan05@hotmail.com
Prolongación Paseo de la Reforma 19,
Santa Fe, Paseo de las Lomas, Cuajimalpa
de Morelos, 01330 Ciudad de México,
CDMX.
Phone: +52 55 2129 2609

Conclusions:

The development of placental alterations and the presence of complications in pregnancy could modify the peripheral blood concentrations of β-hCG and PAPP-A, and alter the results of the combined test for T21.

KEYWORDS: Pregnancy, trisomy 21, biochemical markers, combined test, Cell-Free Fetal DNA.

MANUSCRIPT

Introduction

Aneuploidies are alterations in the number of chromosomes that can occur in 0.5% of live newborns, in the form of monosomies or trisomies. The best known monosomy is Turner Syndrome [monosomy 23 (M23), only affects women]. The most studied trisomies are: Patau syndrome [trisomy 13 (T13)], Edwards syndrome [trisomy 18 (T18)] and Down syndrome [trisomy 21 (T21)]. The prevalence of the latter varies according to the literature consulted. For T13 the prevalence is between 1/12,000 to 1/29,000, for T18 from 1/6,000 to 1/8,000; and for T21 in 1 in 1,000 live births [1, 2, 3, 4]. A risk factor that increases the prevalence of aneuploidy is maternal age, with a prevalence of 1 in 1,000, 1 in 2,500, and 1 in 8,000, in women aged 20 years at T21, T18, and T13 (week 12 of gestation) respectively. The risk factor increases in women over 35 years of age, with a prevalence of 1 in 250, 1 in 600 and 1 in 1800, respectively, reporting a prevalence of trisomy 21 up to 30% in women older than 35 years [5].

Historically, for the detection of T13, T18 and T21, a combination of markers has been used that take into account maternal age, concentrations of feto-placental biochemical markers in maternal serum, including Alpha-fetoprotein (AFP), Beta Human Chorionic Gonadotropin (β-hCG), Inhibin A, unconjugated estriol (E3), Pregnancy-Associated Plasma Protein A (PAPP-A), ultrasonographic markers such as Nuchal Translucency (NT), absence or hypoplasia of the nasal bone, reverse wave of the ductus venosus, and tricuspid regurgitation, among other "soft markers" that allow reporting rates of up to 96% with a false positive rate of 2.5% for T21 [4, 5, 6, 7, 8, 9, 10]. Table 1 shows the different screens focused on T21 detection, which use different combinations of the aforementioned markers and their detection rate.

EVALUATION METHOD	Detection rate (%)	False Positive Rate (%)
ME	30	5
First trimester		
MA, TN	75 - 80	5
MA, β-hCG and PAPP-A	60 - 70	5
MA, TN, β-hCG AND PAPP-A (combined test)	85 - 95	5
Combined test, nasal bone or tricuspid flow or venous duct	93 - 96	2.5

Table 1. Different methods for detection of trisomy 21.
MA: Maternal Age, NT: Nuchal Translucency, β-hCG: β-Human Chorionic Gonadotropin and PAPP-A: Plasma Protein A associated with pregnancy. Table modified from Kypros, 2011.

Nowadays, cffDNA is added to screening tools (it is a screen or predictor of T13, T18 and T21) in the first trimester with a high detection rate. Being a Non-Invasive Prenatal Test (NIPT), it has further reduced the use of invasive methods such as: chorionic villus biopsy or amniocentesis. At the same time, the associated risk of fetal loss (0.6 to 2%) with these tests is decreased. It has been observed that cffDNA increases sensitivity for T13, T18 and T21 of 93.8, 97.4 and 99.3%, with specificity for T13, T18 and T21 of 99.98, 99.98 and 99.96% respectively, and in all cases a false positive rate of <1% [11, 12, 13, 14, 15, 16, 17, 18, 19]. Finally, chorionic villus biopsy or amniocentesis used to confirm the results of these two non-invasive tests (combined test and cffDNA) continues in indicated cases.

On the other hand, the concentrations of maternal biomarkers can be altered by different degrees of placental dysfunction that result in perinatal complications in patients without chromosomal abnormalities or with neural tube defects [20]. This is because maternal biochemical markers may reflect fetal placental function, as well as endocrine, immunologic, and endothelial dysfunction. In this sense, altered levels of β-hCG and PAPP-A in

peripheral blood can be associated with maternal vascular malperfusion, Fetal Growth Restriction (FGR), Placental Abruption (PA), Premature Detachment of Membranes (PDM), preeclampsia, hypertension, Preterm Birth (PB) and Pregnancy Loss (PL) [21, 22, 23].

Therefore, the objective of this work is to analyze whether the calculated risk, in the first trimester for T21 by the Combined Test, is influenced by the presence of placental alterations that modify the concentrations of maternal biomarkers and their association with adverse perinatal outcomes such as FGR, PA, PDM, preeclampsia, hypertension, PB and PL.

Material and Method

Retrospective, observational and cross-sectional study, which included 73 pregnant women who underwent prenatal care at the PRONATAL Clinic (Mexico City), between 2016-2021. The included patients were scheduled between weeks 11 and 13+6 of gestation to undergo first-trimester screening (Combined Test), which allowed them to classify and identify their risk of having a child with T21. Maternal characteristics, clinical history, obstetric history, biochemical markers in maternal serum (β-hCG AND PAPP-A) and ultrasonographic markers (NT) were recorded by the specialist in maternal-fetal medicine from the Fetal Medicine Bité Clinic (Hospital Bité Médica, Mexico City). The data obtained were added to The Fetal Medicine Foundation software, for the calculation of risks in the first trimester. With the above, 3 groups were formed: 1) LR-T21: Low risk of having a child with T21, 2) IR-T21 Intermediate risk of having a child with T21 and 3) HR-T21: High risk of having a child with T21.

Regarding cffDNA to detect aneuploidies, it was indicated when the results obtained in the combined test resulted in intermediate or high risk for T21, or when patients with low risk for T21 requested it. cffDNA was evaluated in peripheral blood by a private laboratory [Target DNA-based technology: (DANSRTM, FORTE), DANSRTM analysis fragments from the specific chromosomes and SNP analysis distinguishes maternal from fetal DNA and quantifies the fetal DNA].

Baseline anthropometric data of the mother were collected at each consultation by the nursing team, taking into account data such as: age, weight, height and Body Mass Index (BMI), as well as vital signs (blood pressure in each arm, heart rate, etc.). The same as for newborns in the Labor, Delivery and Recovery (LDR) unit (Hospital Bité Médica, Mexico City), taking into account weeks of gestation (WoG), weight, height, APGAR score 1m and APGAR score 5m.

The histopathological study of each placenta was carried out in the Bité Médica Department of Pathology using the formalin fixation technique, paraffin process and hematoxylin/eosin staining.

Informed consent was recorded for each test performed on each patient and personalized genetic counseling was offered.

Inclusion criteria:

- Pregnant between 11 and 13+6 weeks of gestation.
- Had complications during pregnancy (FGR, DPP, PDM, preeclampsia, hypertension, PL and GL)
- Patients with:
    - Complete files.
    - Combined test.
    - cffDNA study.
    - Placental pathology at birth.

Exclusion criteria:

- *Patients who did not accept their inclusion in the study.*
- *cffDNA study with "no detected" result.*

Statistical analysis: Maternal data: age, weight, height and body mass index (BMI) and newborn (weeks of the pregnant, weight, height, APGAR m1 and APGAR m5), are reported with mean ± standard deviation (SD) and were evaluated using a Student's T. For its part, the prevalence of: 1) T21 by Combined Test (CT) (low, intermediate and high risk), 2) T21 by cffDNA and 3) placental pathology, is reported in Percentage and number of total individuals [% (n/N)], evaluated using a Chi2, being statistically significant in all cases a difference ≤0.05 for "p". The statistical package SPSS version 25 was used.

Results

This work includes 73 patients aged 36.3 ± 3.8 years, who were divided according to the result of the CT in the first trimester of pregnancy, which includes Low Risk (LR-T21), Intermediate (IR-T21) and High for T21 (HR-T21).

This classification was defined in part by first-trimester ultrasonographic and biochemical markers included for the combined test, reporting an increase in nuchal translucency thickness and peripheral blood concentrations of β-hCG. In addition, a decrease in PAPP-A concentrations in peripheral blood, placing 6.4% (12/73) of pregnancies in HR-T21 and 30.1% (22/73) in IR-T21, surpassed by 53.4% (39 /73) that

resulted with LR-T21 (Table 2). Only 25% (3/12) of HR-T21 (combined test) were positive for T21 in the cffDNA study [100% (3/3)], confirmed by performing karyotype in these 3 cases [100% (3/3)] (Table 2).

After classification, no statistically significant difference was observed between groups made up of the following parameters evaluated, but a numerical difference was observed, showing that HR-T21 were younger compared to IR-T21 and LR-T21 (34.9±3.9 vs 36.7±3.4 and 37±4.3); IR-T21 presented lower weight compared to HR-T21 and LR-T21 (57.6±7.6 vs 61.2±11.6 and 61.5±7.8) and LR-T21 presented greater height (1.64±0.06 vs 1.61±0.06 and 1.61±0.07) and BMI (23.6±3.1 vs 22.03±0.8 and 22.6±3.7) compared to IR-T21 and HR-T21 (Table 2). For its part, the highest prevalence of nulliparity was presented by LR-T21, followed by IR-T21 and ending with HR-T21 (35.8, 22.7 and 8.3%, respectively) (Table 2).

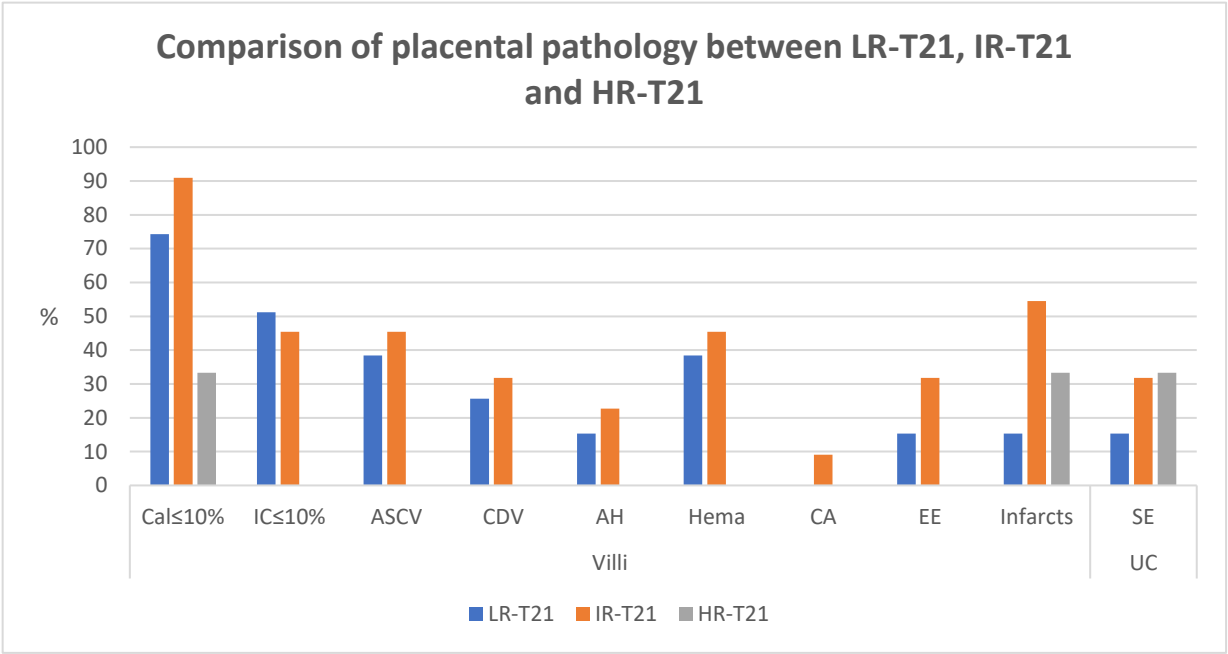
Patient and gestational characteristics in the entire cohort and low, intermediate and high-risk groups					
N		73			
Age (years, Mean ± SD)		36.3 ± 3.8			
		Low	**Intermediate**	**High**	**p**
Trisomy 21 (Risk in combinated test)		53.4% (39/73)	30.1% (22/73)	16.4% (12/73) *	≤0.05
Trisomy 21 (Positive in cffDNA)		0% (0/39)	0% (0/22)	25% (3/12)	NA
Mean cffDNA fetal fraction (Mean±SD)		11.3 ± 3.3	10.8 ± 4.4	8.3 ± 2.5	>0.05
Confirmation by karyotype		-	-	100% (3/3)	NA
Maternal and gestational characteristics					
Age (years, Mean±SD)		36.7 ± 3.4	37 ± 4.3	34.9 ± 3.9	>0.05
Weight (kg, Mean±SD)		61.2 ± 11.6	57.6 ± 7.6	61.5 ± 7.8	>0.05
Height (m, Mean±SD)		1.64 ± 0.06	1.61 ± 0.06	1.61 ± 0.07	>0.05
Median BMI (kg/m^2)		23.6 ± 3.1	22.03 ± 0.8	22.6 ± 3.7	>0.05
Nulliparity		35.8% (14/39)	22.7% (5/22)	8.3% (1/12)	>0.05
Combined test (Gestation Week, Mean±SD)		12.8 ± 2.4	13.2 ± 1.9	13.4 ± 1.3	>0.05
Prenatal screening and testing	**NT (mm)**	1.7 ± 0.5	1.6 ± 0.52	2.2 ± 1.4**	≤0.05
	β-hCG (MoM)	1.7 ± 1.5	1.8 ± 1.1	2.1 ± 1.3	>0.05
	PAPP-A (MoM)	0.8 ± 0.6	0.7 ± 0.5	0.6 ± 0.4	>0.05
Pregnancy and delivery outcome					
Preeclampsia		-	-	8.3% (1/12)	NA
FGR		5.1% (2/39)	4.5% (1/22)	-	>0.05
Gestational age		38.5 ± 1.5	38.5 ± 0.8	37.2 ± 2.1***	≤0.05
Live birth		100% (39/39)	81.8% (18/22)	75% (9/12)	>0.05
PB<34 wk		-	4.5% (1/22)	0	NA
Induced abortion		-	-	25% (3/12)	NA
PL		-	9.09% (2/22)	-	NA
Obito		-	9.09% (2/22)	-	NA
Birth weight		3010.1 ± 358.3	2972.8 ± 318.3	2853 ± 749.3****	≤0.05
Birth size		48.8 ± 1.9	48.8 ± 2.1	47.3 ± 2.2	>0.05
APGAR 1m		8.8 ± 0.4	9	8.6 ± 0.5	>0.05
APGAR 5m		9.5 ± 0.5	9.5 ± 0.5	9.6 ± 0.5	>0.05

Table 2. Patient and gestational characteristics in the entire cohort and low, intermediate and high-risk groups

* Significant difference when comparing HR-T21 vs LR-T21 and IR-T21 (p≤0.05, Student's T), **Significant difference when comparing HR-T21 vs LR-T21 and IR-T21 (p≤0.05, T of Student), *** Significant difference when comparing HR-T21 vs LR-T21 and IR-T21 (p≤0.05, Student's T), **** Significant difference when comparing HR-T21 vs LR-T21 and IR-T21 (p≤0.05, Student's T).

Among the alterations that occurred during pregnancy, only one patient in HR-T21 developed preeclampsia (8.3%), one with IR-T21 presented PB (4.5%), two patients with LR-T21 (5.1%) and one patient with IR-T21 (4.5%) presented FGR, two patients with IR-T21 had PL (9.09%) and two with IR-T21 ended in death (9.09%) (Table 2).

In contrast, the prevalence of live births was higher in patients with LR-T21 [100% (39/39)], followed by patients with IR-T21 [81.8% (18/22)] and finally, by patients with HR-T21 [75% (9/12)], remembering that the abortions in this group were induced (Table 2). From the above, the newborns of patients with LR-T21 showed a weight of 3010.1±358.3, which was statistically higher than that presented by patients with intermediate risk (2972.8±318.3) and high for T21 (2853±749.3). Regarding height and APGAR (1m and 5m), there were no significant differences between the patients of the different risks for T21 (Table 2).



Graphic 1. Cal≤10%, Calcifications ≤10%, IC≤10: Ischemic Changes ≤10%, ASCV: Atrophic Small Chorionic Villus, CDV: Congested Dilated Vessels, AH: Acute Hemorrhage, Hema: Hematoma, CA: Chorioangioma, EE: Extravasation of Erythrocytes, SE: Stromal Edema and CU: Umbilical Cord. p≤0.05, Chi^2.

Finally, the histopathological findings of the placentas showed that IR-T21 presented a numerical increase in the prevalence of Cal≤10% [90.9% (20/22) vs 74.3% (29/39)], ASCV [45.4% (10 /22) vs 38.4% (15/39)], CDV [31.8% (7/22) vs 25.6% (10/39)], AH [22.7% (5/22) vs 15.3% (6/39)] , Hema [45.4% (10/22) vs 38.4% (15/39)], CA [9.09% (2/22) vs 0% (0/39)], EE [31.8% (7/22) vs 15.3 % (6/39)], infarcts [54.5% (12/22) vs 15.3% (6/39) and SE [33.3% (7/22) vs 15.3% (6/39)], compared to LR-T21. In parallel, IR-T21 had a higher prevalence of Cal≤10% [90.9% (20/22) vs 33.3% (4/12), IC≤10% [45.4% (10/22) vs 0% (0/ 12)], ASCV [45.4% (10/22) vs 0% (0/12)], CDV [31.8% (7/22) vs 0% (0/12)], AH [22.7% (2/22)) vs 0% (0/12)], Hema [45.4% (10/22) vs 0% (0/12)], CA [9.09% (2/22) vs 0% (0/12)], EE [31.8% (7/22) vs 0% (0/12)] and Infarcts [54.5% (12/22) vs 33.3% (4/12)], compared to HR-T21. On the contrary, LR-T21 presented a higher IC≤10% [51.2% (20/39) vs 45.4% (10/22)], compared to IR-T21 (Graphic 1).

Discussion

One of the main objectives of the CT is to identify the risk of having a child with Down Syndrome (T21), a result that can be complemented with the performance of the cffDNA, giving the doctor the opportunity to propose the performance of an amniocentesis, either of amniotic or chorionic villus fluid. In the particular case of this study, only 25% (3/12) of HR-T21 (CT) were found to be positive for T21 in the cffDNA and karyotype tests of embryonic remains (Table 2). In addition to this, 100% of RB-T21 and RI-T21 (CT) were negative at T21 by cffDNA analysis, which ruled out the performance of invasive tests (Table 3). For its part, the prevalence of women with IR-T21 and HR-T21 may be due to the fact that 67.7% of the population is ≥35 years old, maternal age associated in the literature with an increased risk of chromosomal abnormalities in offspring [24].

As already described in the introduction to this research, the increase in peripheral blood

concentrations of β-hCG and the decrease in PAPP-A can place pregnant women at greater risk of giving birth to a child with T21 by combined test [20], as can be seen in Table 2 of this work. However, we observed that 100% (22/22) of the patients with IR-T21 and 66.6% of the patients with HR-T21 by CT, in the cffDNA test, were negative for T21. Situations in which the concentrations of β-hCG and PAPP-A could not reflect the risk for T21, but that the fetoplacental function is related to alterations such as FGR, PA, PDM, preeclampsia, hypertension, PB and PL [20].

In our case, higher concentrations of β-hCG and lower concentrations of PAPP-A in RI-T21, may be associated in 23.18% with complications that occurred during pregnancy [FGR (4.5%), PP (4.5%), PG (9.09%) and Death (9.09%)]. Coinciding with Antsasklis P. et al., 2019 [25], who in their review found that PAPP-A concentrations ≤0.2 (MoM) in the first trimester of pregnancy increase the risk of developing FGR, preeclampsia and death, López A. et al., 2016 [26], a retrospective study that included 285 patients found that PAPP-A concentrations (5th percentile) ≤ 0.4 MoM in the first trimester increased the risk of PL, FGR, hypertensive diseases, hypertension and gestational diabetes. In the case of β-hCG, Park H. et al., 2014 [27], observed significantly higher levels of β-hCG (1.66 vs 1 MoM) in patients who developed preeclampsia, compared to control, Mikat B. et al., 2012 [28], find, in a study conducted on 155 pregnant women, that first trimester concentrations of β-hCG were higher in patients who developed preeclampsia later in pregnancy and Rivas M. et al., 2014 [29], in a study that brought together patients with preeclampsia, FGR, PB and PL, to form a group with maternal perinatal complications (CPM, n=10), observed an increase in β-hCG (0.76 vs 0.60 MoM) in the first trimester, in comparison to control. In contrast, Karahasanovic A. et al., 2014 [30], detected a significant reduction in β-hCG levels (0.75 vs 0.97 MoM) in 161 women with preeclampsia, Pornwattanakrilert W. et al., 2020 [31], in work that included 500 women with PL, observed a decrease in β-hCG concentrations, compared to the control group (1.12 vs 1.23 MoM) and Litwińska E. et al., 2017 [23], in a study that included 94 pregnant women with early preeclampsia (n=22), late preeclampsia (n=29) and FGR (n=43), found no significant difference in β-hCG concentrations (1.08, 1.25 and 1.12 vs 1.14 MoM) when compared to control.

For its part, in the literature the development of alterations during pregnancy such as FGR, preeclampsia, death, hypertension and gestational diabetes are associated with macroscopic and microscopic placental changes [32, 33]. Findings in studies such as the one carried out by Vedmedovska N. et al., 2011 [34], in patients with FGR, a higher incidence of alterations in villi, such as thickening of the trophoblastic basement membrane, infarcts, thrombi and hematomas, compared to the control group. Voicu N. et al., 2020 [33], in a study carried out on 30 placentas of women with FGR, found a higher prevalence of macroscopic changes (fibrin deposits, calcifications and infarcts) and microscopic changes (massive infarcts caused by vascular ischemia, fibrin deposits intra and extravenous, calcifications and vascular thrombosis), when compared with a group of patients who did not develop FGR and who presented thrombophilias, Ogge G. et al., 2011 [35], in a retrospective case-control study that included 8307 women who gave birth after 20 weeks of gestation found that women who developed preeclampsia had a higher incidence of alterations in placental villi (infarcts, fibrin, distal hypoplasia, agglutination and syncytial knots), Devisme L. et al. 2013 [36], in a retrospective study of control cases that included 173 women who developed preeclampsia observed a greater presence of syncytial knots, infarcts, basal decidual vasculopathy, hi villous permaturation and placental erythroblastosis, compared to control.

Regarding Sarafzadeh A. et al., 2018 [37], in a study that included 118 patients who had PB, they found that only 10% presented syncytial knots, chorioangiosis and microcalcifications, Azizi M. et al., 2014 [38], in a case-control study of 100 placentas obtained from patients who had PL reported a greater presence of calcifications, inflammatory lesions and thrombi, compared to the control group. Odendal H., 2021 [39], in review, refer to the pathology of 13 placentas of late abortions (19-25 SDG), in which a prevalence of PA of 46% and acute chorioamnionitis of 30% could be observed. In turn, Lema G. et al., 2020 [40], in a case-control study, which included 96 patients with gestational loss at week 33.8±3.2, showed a higher incidence of vascular alterations in the uterus and placenta (73 vs. 3.4%). In addition, acute chorioamnionitis (8 vs 4%), when compared to the control group. For this reason, it could be thought that the higher prevalence of Cal≤10% [90.9% (20/22) vs 74.3% (29/39)], ASCV [45.4% (10/22) vs 38.4% (15/39)], CDV [31.8% (7/22) vs 25.6% (10/39)], AH [22.7% (5/22) vs 15.3% (6/39)], Hema [45.4% (10/22) vs 38.4% (15/39)], CA [9.09% (2/22) vs 0% (0/39)], EE [31.8% (7/22) vs 15.3% (6/39)], Infarcts [54.5 % (12/22) vs 15.3% (6/39) and SE [33.3% (7/22) vs 15.3% (6/39)], in HR-T21 when compared with LR-T21, could be the cause by which, the patients were classified in IR-T21, despite not developing complications during pregnancy (FGR, PA, PDM, preeclampsia, hypertension, PB and PL).

CONCLUSIONS

The concentrations of biochemical markers such as β-hCG and PAPP-A, as part of the first trimester

combined test, are a good tool that can allow for the identification of T21.

The development of placental alterations such as small villi, atrophic chorionic villi, with calcifications, congested dilated vessels, acute hemorrhage, hematomas, chorioangioma, extravasation of erythrocytes, infarcts and stromal edema, in addition to the presence of alterations or complications, such as fetal growth restriction, preterm delivery, spontaneous abortion and death, could alter the peripheral blood concentrations of β-hCG and PAPP-A, which, when used to calculate the risk in the Combined Test, would place groups of patients in IH-T21 and HR -T21, really reflecting some alterations or complications during pregnancy, but not the presence of a fetus with T21.

Finally, it is recommended to perform cffDNA to reinforce the result obtained in the Combined Test that will allow the doctor to make a decision.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

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Follicular flushing increases the number of oocytes retrieved in women with diminished ovarian reserve and serum progesterone levels of ≤ 5.0 ng/ml post surge

Alkon-Meadows Tamar

Alkon-Meadows Tamar, MD, MSc [1], 0000 0001 8866 8379; Hernández-Nieto Carlos, MD[1]; Sandler Benjamin MD[1]; Cassis-Bendeck Deborah[1]; Luna-Rojas Martha, MD[1].

ABSTRACT

Objective

To evaluate the utility of follicular flushing increase the number of oocytes retrieved in women with diminished ovarian reserve (DOR) and serum progesterone of ≤ 5.0 ng/ml the day after final oocyte maturation induction.

Design

Retrospective study.

Subjects

Women <4O years, with DOR, underwent controlled ovarian stimulation for an invitro fertilization cycle (IVF) were separated into two groups: Group 1: follicular flushing (n=35) Group 2: direct aspiration (n=33).

Main outcomes measured

Our primary outcome was the number of retrieved oocytes. Secondary outcome measures included the total procedure time, mean oocyte/follicle retrieval rate, mean number of metaphase II (MII) oocytes, MII rate, mean number of fertilized oocytes, and blastulation rate.

Results

A total of 288 oocytes were retrieved: 59.0% from the flushing group and 41.0% from the direct aspiration group. The mean number of oocytes retrieved by aspiration and subsequent flushes was significantly higher than the number retrieved from direct aspiration (4.4 ± 0.96 versus 2.64 ± 1.00, P = 0.003). The oocyte/follicle rate was also significantly higher in women in Group 1 vs Group 2 (68.3% vs 48.7%, p=0.001). No significant differences were observed in the total number of MII, oocyte maturation rate, fertilization rate nor blastulation rate. The retrieval procedure time was higher among those who underwent follicular flushing, with an estimated increase of 6.5 minutes (14.0 ± 2.5 versus 7.5 ±1.7, P = 0.003).

[1] Reproductive Medicine Associates on New York-international, Mexico, CDMX.

NOTE: The numbers following the affiliation markers are the author's ORCID iD.

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CONTACT:
Alkon-Meadows Tamar, MD, MSc.
talkon@rmany.com, tamaralkon@gmail.com
Paseo de la Reforma 2693, Lomas de Bezares, 11910, Ciudad de México, CDMX.
Phone: +52 55 2167 2515.

Conclusions

The results of this analysis in the poor responders found that women with DOR and post surge P4 levels of ≤ 5.0 ng/ml may benefit from follicular flushing on the number of oocytes retrieved.

KEYWORDS: Follicular flushing**,** Oocyte yield**,** DOR.

MANUSCRIPT

Introduction

As the number of oocytes available for in vitro fertilization (IVF) is a determinant of the cumulative chance of a patient to achieve pregnancy[1], it is important to retrieve the maximum number of oocytes from a given number of follicles that have developed in response to medication. Accordingly, in patients with poor ovarian response, the use of follicular flushing and double lumen needles has become popular, the purpose of follicular flushing is to increase the oocyte yield, possibly by improving the detachment of the cumulus–oocyte complex (COC) from the follicular wall and decreasing the risk of oocyte retention within the follicle[2,3].

Oocyte maturation and embryo development are controlled by intra-ovarian factors such as steroid hormones. Progesterone (P4) exists in the follicular fluid, and it is known to mediate luteinizing hormone (LH)- initiated periovulatory events through autocrine/paracrine mechanisms that help mediate granulasa cell luteinization and oocyte maturation[4]. More importantly, a rise in P4 levels is associated with an adequate follicular rupture[4]. We hypothesize that lower P4 levels (≤ 5.0 ng/ml) after oocyte maturation induction is a reflection of impaired physiological periovulatory mechanisms required for the oocyte release from the follicular wall, and, by flushing the follicle it may facilitate oocyte detachment from the COC. The aim of this study is to determine the utility of using P4 levels the day after surge as a cutoff point to establish if woman with diminished ovarian reserve (DOR) undergoing an oocyte retrieval could benefit from this practice.

Materials and Methods

Study design and patient population

This retrospective, single center study included women <4O years, with DOR diagnosis according to ESHRE Bologna criteria described poor ovarian response as the presence of two out of three of the following criteria: advanced maternal age, previous poor ovarian response (three oocytes or fewer after conventional stimulation), an abnormal ovarian reserve test (AFC 5–7, AMH <0.5-1.1 ng/mL)[5] who underwent IVF from October 2019 through January 2022 and had serum P4 levels of ≤ 5.0 ng/ml the day after trigger. Cohorts were separated into two groups: Group 1: follicular flushing; Group 2: direct aspiration (control).

Cases of patients harboring chromosomal rearrangements, undergoing preimplantation genetic testing for monogenic defects (PGT-M) and/or using donor gametes were excluded from the analysis.

Stimulation protocol

Patients underwent controlled ovarian hyperstimulation (COH) for IVF as previously described[6]. Briefly, the COH protocol was selected at the discretion of the reproductive endocrinologist and involved the administration of follicle-stimulating hormone (FSH) and human menopausal gonadotropin (hMG) with a gonadotropin-releasing hormone (GnRH) agonist downregulation protocol with leuprolide, a GnRH antagonist protocol, or a microflare protocol with leuprolide acetate. These protocols have been described previously[7]. Follicular development was monitored using transvaginal ultrasonography. When at least two follicles reached 18 mm in diameter, final oocyte maturation was induced with either hCG (5000–10,000 IU, Choragon, Ferring Pharmaceuticals, Mexico, CDMX), recombinant human chorionic gonadotropin (250–500 µg, Ovidrel, EMD Serono, Mexico, CDMX) or a dual trigger with 2 mg of leuprolide acetate (Lucrin, AbbVie Laboratories, Mexico, CDMX) and 1000-5000 IU of hCG. For all cases, the level of serum progesterone the day after trigger was measured. All P4 serum levels were measured with electro-chemi-luminescence analysis utilizing an in-site 'Cobas e-601'VR (Roche Diagnostics, Mexico) (measuring range =0.03–60 ng/ml, Intra-assay variation = 1.1% and Inter-assay variation = 0.99). Thereafter, patients underwent vaginal oocyte retrieval under transvaginal ultrasound guidance 36 h after oocyte maturation was triggered.

Oocyte retrieval

The follicular flushing group puncture was performed with aspiration and follicular flushing using a 35 cm double-lumen 17-gauge needle (Cook1 EchoTip1 Double Lumen Aspiration Needle K-OPSD-1735-B-L). The flushing system inclusive of the needle was pre-filled with pre-warmed and equilibrated flushing medium before each procedure. The initial aspirated follicular fluid was collected in a tube, if no oocyte was identified, follicular flushing was repeated until an oocyte was retrieved or up to a maximum of three times.

The direct aspiration group puncture was performed following the department's standard protocol, with a 35 cm single-lumen 17-gauge needle (Cook1 EchoTip1 Single Lumen Aspiration Needle 1735). The follicular fluid was collected in tubes without differentiating between the follicles. For both groups aspiration of the follicular fluid was performed using a vacuum pump set at -130 mmHg). All retrievals were performed by one physician to minimize surgical variability. The physician was experienced in both flushing and direct aspiration techniques.

Laboratory procedures

The oocytes and embryos were cultured in the same type of media as that used for follicular flushing. All metaphase II (MII) oocytes underwent either intracytoplasmic sperm injection (ICSI) or conventional insemination (CI). Embryos were cultured to the blastocyst stage as previously described[6].

Outcome measures

The primary outcome was the total number of retrieved oocytes. Secondary outcome measures included the total procedure time (time of transvaginal insertion of the retrieval needle into the first ovary to the removal of the needle from the second ovary), mean oocyte/follicle retrieval rate (number of COC seen by ultrasound divided by the number of aspirated follicles), mean number of MII oocytes, MII rate, mean number of fertilized oocytes, and blastulation rate.

Statistical analysis

Groups were compared using Student's T, Mann–Whitney U and Chi-squared when appropriate. Results were expressed as percentages, means and SDs. Adjusted odds ratios (aORs) with 95% CI's were calculated using a multivariate logistic regression analysis to adjust for confounding variables. Statistical analyses were performed using SAS version 9.4 (SAS institute Inc., Cary, NC, USA). All p-values were two-sided and were considered significant if less than < 0.05.

Regulatory approval

This retrospective study was approved by the local institution (RMA) Institutional Review Board, Inc.

Results

In total 68 women were included in the cohort (group 1, n= 35 women; group 2, n= 33). Patient demographic characteristics and COH parameters are described in Table 1. No significant differences were found in mean patient's age, BMI, baseline FSH, AMH, baseline antral follicle count dose of gonadotropins used, serum progesterone and estradiol levels the day of ovulation trigger, day of ovulation trigger, serum progesterone and estradiol levels post trigger, and COC among cohorts.

	Group 1 (n=35)	Group 2 (n=33)	P value
Age at retrieval (years)	38.6 ± 3.5	38.4 ± 3.1	0.66
BMI (Kg/m2)	24.1 ± 3.5	23.3 ± 3.9	0.52
AMH	0.5 ± 0.2	0.6 ± 0.3	0.11
Baseline FSH (IU/mL)	10 ± 4.0	9.5 ± 3.5	0.44
Baseline antral follicle count	12.4 ± 6.8	11.9 ± 6.0	0.39
Male age	40.6 ± 2.5	41.6 ± 2.5	0.68
Cycle characteristics			
Cumulative GND dose (Units)	3690 ± 1301	3166 ± 1339	0.35
Day of ovulation trigger	13.0 ± 1.6	12.6 ± 2.4	0.22
Surge E2 (pg/mL)	1295.1 ± 1036.8	1214.3 ± 1148.7	0.09
Surge P4 (ng/mL)	0.9 ± 0.5	0.9 ± 0.4	0.08
Post surge E2 (pg/mL)	1174.1 ± 1236.3	1017.5 ± 1154.3	0.07
Post surge P4 (ng/mL)	3.6 ± 1.2	3.8 ± 1.1	0.65

Table 1. Demographic and cycle characteristics of patients.

Note: Data presented as percentages, mean and ± standard deviations, unless stated otherwise. BMI= body mass index; AMH = anti Mullerian hormone; FSH = Follicle stimulating hormone; GND = gonadotropins; E2 = estradiol; P4= progesterone; COC=cumulus–oocyte complex.

A total of 288 oocytes were retrieved: 170 (59.0%) from the flushing group and 118 (41.0%) from the direct aspiration group. All patients who underwent retrieval had oocytes retrieved (Table 2). The mean number of oocytes retrieved by aspiration and subsequent flushes was significantly higher than the number retrieved from direct aspiration (4.4 ± 0.96 versus 2.64 ± 1.00, P = 0.003). In the flushing group, 21 oocytes (12.3%) were retrieved at the initial aspiration, 115 (67.6%) in the first flush, 27 (15.9%) in the second, and 7 (4.2%) in the third. The oocyte/follicle rate was also significantly higher in women in Group 1 vs Group 2 (68.3% vs 48.7%, p=0.001). No significant differences were observed in the total number of MII, oocyte maturation rate, fertilization rate nor blastulation rate (Table 2). The retrieval procedure time was higher among those who underwent follicular flushing, with an estimated increase of 6.5 minutes (14.0 ± 2.5 versus 7.5 ±1.7, P = 0.003).

	Group 1	Group 2	P value
Avradge ocytes retrieved	4.4 ± 0.9	2.64 ± 1.0	0.003 *
Oocyte/follicle retrieval rate %	68.3	48.7	0.001 *
Number of MII	3.3± 1.3	1.9±2.3	0.18
Oocyte maturity rate %	77.1 (132/170)	70.3 (83/118)	0.46
Fertilization rate %	78.0 (103/132)	77.1 (64/83)	0.08
Blastulation rate%	67.9 (70/103)	65.6 (42 /64)	0.66

Table 2. Cycle outcomes among poor responders who received direct aspiration versus follicular flushing at oocyte retrieval.

Note: Data presented as percentages, mean and ± standard deviations, unless stated otherwise. MII= Metaphase II oocytes.

*= Statistical significance is defined as p < .05.

Discussion

This is the first study to establish post surge P4 levels as a cutoff point to determine whether patients with DOR could benefit from follicular flushing. Our findings suggest that patients with P4 levels of ≤ 5.0 ng/ml who underwent follicular flushing had higher oocyte yield compared with direct aspiration. It is also the first to suggest that lower P4 levels (≤ 5.0 ng/ml) after final oocyte maturation induction may be a result of compromised mechanisms associated to the release of the oocyte from the follicular wall as demonstrated by lower oocyte recovery rate.

The use of follicular flushing remains controversial. Prior trials and meta-analyses found no benefit of flushing on the number of oocytes retrieved.[8-10] However, these studies are limited by not having a control group, each follicle was punctured a single time and aspiration occurred followed by flushing. Oocytes retrieved early were attributed to direct aspiration and oocytes retrieved later were attributed to flushing. Some of these trials attempted to address this issue by counting oocytes retrieved in the first flush as resulting from aspiration alone. Randomizing patients to flushing versus non-flushing is the best way to compare these approaches, and to address the potential for inaccurate attribution of oocytes retrieved to aspiration or flushing. Our study found that women with DOR and lower P4 levels (≤ 5.0 ng/ml) after final oocyte maturation induction benefit from follicular flushing, possibly by detaching the oocyte from the COC.

Several studies have reported higher oocyte yield following follicular flushing in poor responder patients managed with semi natural cycle IVF[14]. Likewise, Van Wolff et al. reported two-fold higher oocyte yield and transferrable embryos with follicular flushing[15]. The same group conducted a well-designed adequately powered RCT directly comparing aspiration and follicular flushing in their mono-follicular IVF patients after gonadotropin free ovarian stimulation. Higher oocyte yield, mature oocytes and fertilization rates were noted with follicular flushing[16]. Similar to our findings, we demonstrated higher number of oocytes retrieved, however, no differences were noted in the MII nor blastulation rates.

For the quantitative outcome procedure duration, our findings show a significantly prolonged procedure time for follicular aspiration when using flushing. This finding comes as no surprise and confirms previous meta-analyses for normal response IVF patients[11-13]. The mean increase is only 6.5 minutes, which is likely of no clinical relevance for the patient in anesthesia time. Follicular flushing, however, increases the effort for the team (preparation and equilibration of the flushing medium), as well as the financial burden, as double lumen needles and flushing media are costly.

Our study distinguishes itself as it was performed at a single, high-volume academic center with a team of embryologists all uniformly trained, thereby reducing the inherent variability that may arise from multicenter studies. Oocytes retrievals were carried out by one physician, reducing the inherent intra-operator variability that may arise from distinct surgical techniques.

Notwithstanding our best efforts to avoid biases, some shortcomings and limitations exist in the analysis. The most notable limitation is its retrospective design, which increases the chance of selection bias, the small sample size, selected progesterone cutoff value and progesterone assay techniques compared to other ART centers may limit the external validity of our findings. A prospective randomized controlled trial is urged in order to analyze the real benefit of this novel intervention in patients with DOR. As for now, this study serves as a proof of concept and description of this technique for follicular flushing in women with serum P4 levels of ≤ 5.0 ng/ml the day after trigger aiming to improve ART outcomes in this group of patients with adverse prognosis during their family-building journey.

CONCLUSION

The results of this analysis in the poor responders found that women with DOR and post surge P4 levels of ≤ 5.0 ng/ml may benefit from follicular flushing on the number of oocytes retrieved.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

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Effects of autologous Platelet-Rich Plasma treatment on thin endometrium in patients undergoing frozen-thawed embryo transfer cycles

Oliveros Ceballos Adán

Oliveros Ceballos Adán[1], 0000 0003 0825 7831; Mejía Jiménez Emanuel[2]; Solano Arellano Eduardo[3]; Aguilar Segovia Luz Elena[4].

ABSTRACT

Objective

The objective of this study is to determine whether the treatment of a thin endometrium with autologous platelet-rich plasma (PRP) prior to an embryo transfer increases positive reproductive outcomes in cycles where implantation failure is attributable to a thin endometrium, during the endometrial preparation cycles.

Material and Methods

This is a cohort, prospective and interventional study, carried out in a private facility. Women between 32 and 45 years old were included, with a history of two or more failed IVF/ICSI (In vitro fertilization/Intracytoplasmic sperm injection) cycles and a thin endometrium (<7 mm), the cycles were with their own ovules or from donors not older than 28 years, with or without alterations in the seminal samples of their partners.

Twenty-five women were enrolled in this study, the patients were treated with an intrauterine infusion of autologous PRP 2 or 3 times during the menstrual cycle on days 8, 10 and 12 of endometrial preparation for their frozen-thawed embryo transfer (FET) cycle, and Embryo Transfer (ET) was performed 3 to 5 days after the final autologous PRP infusion. A total of 25 patients underwent FET.

Results

Of 25 patients included in this study, the mean endometrial thickness after PRP treatment was 8.6 mm. The average increase in endometrial thickness was 1.76 mm this difference was statistically significant when compared with the previous cycles for each patient, respectively. The results of the cycles with PRP infusion treatment were compared with the previous results obtained in the same group of patients. The implantation, clinical pregnancy, and live birth rates (LBR) in the PRP treatment cycle were 24.59, 56, and 48%, respectively. Implantation, clinical pregnancy, and LBR in the control cycle were 0%. Implantation, clinical pregnancy, and LBR were significantly higher in the PRP treatment cycle than in the control cycle. Age, BMI, number of embryos transferred, and number of good quality embryos transferred were not significantly different.

[1] Scientific director of the Institute of Reproduction and Gynecology of Acapulco (IREGA), Acapulco de Juárez, Guerrero, México.
[2] Laboratory embryologist at Institute of Reproduction and Gynecology of Acapulco (IREGA), Acapulco de Juárez, Guerrero, México.
[3] Laboratory Andrologist at the Institute of Reproduction and Gynecology of Acapulco (IREGA), Acapulco de Juárez, Guerrero, México.
[4] Responsible of the Reproduction Techniques Program at the Institute of Reproduction and Gynecology of Acapulco (IREGA), Acapulco de Juárez, Guerrero, México.

NOTE: The numbers following the affiliation markers are the author's ORCID iD.

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CONTACT:
Oliveros Ceballos Adán.
director@irega.com.mx
Cerrada de Cumbres 104, Fraccionamiento Farallón CP.39690, Acapulco, Guerrero, México.
+52 74 4449 2772.

Conclusions

The present study revealed a noticeable improvement in endometrial thickness after the PRP treatment considering the history of the patients, however, more studies are needed to elucidate the molecular basis of the PRP action mechanism on the endometrium and to support the results obtained and generate more solid evidence on the beneficial effect of autologous PRP treatment on the thin endometrium.

KEYWORDS: Thin endometrium, platelet-rich plasma (PRP), recurrent implantation failure, frozen-thawed embryo transfer, endometrial receptivity, endometrial thickness.

MANUSCRIPT

Background

Since the first introduction of IVF (in vitro fertilization) and ET (embryo transfer), technology has evolved rapidly and the pregnancy rate with FET (frozen-thawed embryo transfer) has increased significantly. However, thin, or damaged endometrium remains an unresolved problem in the treatment of infertility patients. Several treatments have been tried to restore endometrial receptivity, including the administration of exogenous estrogens, vitamin E, vaginal sildenafil citrate, and pentoxifylline (1). Patients with a thin endometrium who do not respond to the abovementioned treatment do not foresee many options and it is known that an endometrium with a thickness of less than 7 mm is not optimal for an embryo implantation and it is associated with a low pregnancy rate (2). Recently, progress has been made in the treatment of damaged or thin endometrium with the use of therapies that promote cell proliferation, including stem cell therapy (3). However, there are still unresolved issues related to the safety of using bone marrow-derived stem cells (4).

Autologous PRP is an alternative known for its safety. Analogous platelet products have been used since the 1970s and have become more popular since the 1990s (5). Platelets are known as the blood component that plays a crucial role in hemostasis. During the healing process, α-granules within platelets secrete growth factors, cytokines, and chemokines. These various secreted proteins have paracrine effects on myocytes (6), tendon cells (7), mesenchymal stem cells of different origins (8), chondrocytes, osteoblasts (9), fibroblasts and endothelial cells, stimulating migration, cell proliferation, angiogenesis and, consequently inducing tissue regeneration (10).

The first study on PRP for the treatment of thin human endometrium in vivo was published in 2015 (11). Four studies followed up and concluded that PRP is an influential treatment for patients with thin endometrium (12, 13, 14,15). The studies stated that autologous PRP promotes endometrial growth and improves pregnancy outcomes. However, the number of patients was small, and they did not provide enough information on the type or concentration of PRP they used. It is recognized that the effectiveness of PRP treatments can vary depending on the concentration of platelets (16). In the present study, we assessed the effect of a platelet-rich infusion treatment on the thin endometrium of patients with a history of failed IVF/ICSI cycles with respect to pregnancy rates.

Materials and Methods

We conducted a prospective and interventional cohort study. The patients were recruited from November 2017 to October 2021 at the Acapulco Institute of Reproduction and Gynecology (IREGA). Women with a history of two or more failed IVF cycles and thin endometrium in previous cycles were included in this study. The inclusion criteria were as follows: (a) age 32 to 45 years at the time of the procedure, (b) endometrial thickness <7 mm at the end of estrogen administration in FET, (c) two or more failed IVF/ICSI cycles, (d) more than two cycles of prior therapy to increase endometrial thickness, such as hysteroscopic adhesiolysis with subsequent hormone replacement therapy (HRT), the use of high-dose estradiol valerate, transvaginal administration of sildenafil , or combination of pentoxifylline with vitamin E, (f) at least one good quality frozen embryo available for transfer, and (g) signed informed consent form. The exclusion criteria were patients with an endometrial thickness greater than 7 mm at the end of estrogen administration in FET cycles, adenomyosis, endometrial uterine pathology such as polyps and submucosal fibroids, and patients with thrombophilia.

Preparation of Autologous PRP

On each day of PRP treatment administration, with the patients fasting, 18 mL of venous blood was extracted using 30 mL syringes coated with 1.25 mL of citrate-dextrose anticoagulant solution A (TERUMOBCT, USA). Blood samples were centrifuged at 1120 G for 3 min in a KITLAB Ck-12 centrifuge (DESEGO, Mexico). The buffy coat and plasma just above the buffy coat were collected, and 0.7-1.0 mL of PRP was produced and infused into the uterine cavity.

Autologous administration of PRP and ET

Intrauterine autologous PRP administration was performed in the FET cycle parallel to estrogen administration. The patients began taking a daily dose of 4 to 8 mg of estradiol valerate (Primogyn, Bayer,

Germany) from day 2 of the menstrual cycle to prepare the endometrium. The first autologous PRP infusion was performed on cycle day 10 and repeated at 3-day intervals until endometrial thickness reached 7 mm. PRP was administered into the uterine cavity using an embryo transfer catheter within 1 hour from the completion of the PRP centrifuge preparation. The syringe containing the PRP was connected to the embryo transfer catheter and the PRP was infused, this procedure was subsequently confirmed on ultrasound.

Ultrasound was used to measure endometrial thickness on day 2 of the menstrual cycle and each day of autologous PRP administration until the ET. The ET was performed 3 days after the final autologous PRP administration. When the endometrial thickness reached 7 mm or more, 50 mg of strong yellow body (HORMONA, Mexico) as progesterone were administered intramuscularly (IM), plus 20 mg piroxicam (Senosiain, Mexico) every 12 hours, until the day of the ET. The serum level of β-hCG was measured from peripheral blood 2 weeks after the ET. A follow up ultrasound was conducted to those with positive β-hCG results another 2 weeks later to confirm clinical pregnancy. Clinical pregnancy was defined as the presence of an intrauterine gestational sac. All patients received luteal support with Utrogestan, a 200mg micronized progesterone (Besins Manufacturing, Belgium) after the ET. If the transvaginal ultrasound showed a gestational sac and embryonic heart beats 4-6 weeks after the ET, luteal support was continued until week 10 of gestation. Obstetric follow up was given to clinical pregnancies up until the outcome of at least one alive newborn.

Comparison of results between treatment and previous cycles

In the same patient group variables of the previous cycles were compared with those of the PRP treatment cycle. Clinical outcomes were also compared between cycles, cycles with endometrial thickness >7mm without the need for treatment against cycles with PRP treatment. Primary results were ongoing pregnancy rate and LBR. Secondary results were implantation rate, clinical pregnancy rate, and the increase of endometrial thickness compared to the previous cycle.

Statistical analysis

The descriptive analysis was performed by the mean and standard deviation for quantitative variables and frequency, proportion was used for categorical variables. Statistical analysis of the outcome measures and associated clinical variables was performed with XLSTAT version 2020, with Student's t-test and $\chi 2$. A value of $p < 0.05$ was considered statistically significant.

Results

A total of 25 women were recruited, and all of them underwent ET. No patients were lost to follow up and all data on the 25 women were collected. The average age of the patients was 38.6 years at the time of treatment. The mean duration of infertility was 3.45 years. The mean number of failed IVF cycles was 1.83. The mean endometrial thickness on the final day of estrogen administration of the previous cycles was 6.84 mm (Table 1).

PARAMETER	PREVIOUS CYCLE (n=25)	PRP TREATMENT CYCLE (n=25)	P VALUE
Age (years)	37.11 ± 4.24	38.60 ± 4.31	0.867
BMI (Kg/m2)	26.03 ± 3.31	26.09 ± 3.21	0.974
Duration of infertility (years)	3.45 ± 3.95		-
Number of previous failed cycles	1.83±0.55		-
AMH (ng / ml)	2.69 ± 1.88	2.20 ± 1.22	0.342
FSH (mUI / ml)	6.57 ± 1.63	6.56 ± 1.70	0.769
LH (mUI / ml)	4.94 ± 1.92	5.03 ± 1.96	0.859
Infertility cause			
Female factor (n, %)	8 (32)	7 (28)	0.762
Masculine factor (n, %)	8 (32)	9 (36)	0.803
Number of oocytes retrieved.	12.31 ± 6.6	12.56 ± 6.3	0.709
Fertilization method			
IVF (n, %)	17 (68)	16 (64)	0.748
ICSI (n, %)	8 (32)	9 (36)	0.819

Table 1. Baseline characteristics of patients.

The average number of transferred embryos in each patient was 2.44 (2 or 3) in the PRP treatment group. Grading of embryos at the cleavage stage was performed using the parameters established at the Istanbul Consensus Workshop on Embryo Evaluation (17). Blastocysts were classified using the Gardner classification system.

A good grade embryo was defined as a grade I or II cleavage stage embryo with six or more cells and a blastocyst score of 3BB or higher. The ET in the morula stage was not recorded. The gestational sac was confirmed in 56% (n = 14) of the patients. One patient had a miscarriage at 9 weeks of gestational age. Another patient had a heterotopic pregnancy, with a spontaneous abortion of the intrauterine fetus at 7 weeks of gestation. The LBR was 48% (n = 12). All ongoing pregnancies resulted in live births without obstetric complications. The mean endometrial thickness after PRP treatment was 8.6 mm. The average increase in endometrial thickness was 1.76 mm, this difference was statistically significant. The results of the PRP treatment cycle were compared with the previous results obtained in the same group of patients. The implantation, clinical pregnancy, and LBR in the treatment cycle were 24.59, 56, and 48%, respectively. Implantation, clinical pregnancy, and LBR in the control cycle were 0%. Implantation, clinical pregnancy, and LBR were significantly higher in the treatment cycle than in the control cycle. Age, BMI, number of embryos transferred, and number of good quality embryos transferred were not significantly different. The PRP treatment outcomes are summarized in Table 2.

PARAMETERS	PREVIOUS CYCLE WITHOUT PRP TREATMENT (n=25)	PRP TREATMENT CYCLE (n=25)	P VALUE
Endometrial thickness on the last day of endometrial priming (mm)	6.84 ± 0.87	8.60 ± 1.12	<0.0001
Fresh/frozen cycle	4/21	0/25	0.114
Autologous/heterologous cycle	11/14	13/12	0.852
Number of transferred embryos	2.29 ± 0.53	2.44 ± 0.49	0.869
Number of good quality embryos transferred	1.8 ± 0.43	1.7 ± 0.47	0.786
Implantation rate (n, %)	0/58(0)	15/61(24.59)	<0.0001
Clinical pregnancy rate (n, %)	0/25(0)	14(56)	<0.0001
Ongoing pregnancy rate (n, %)	0/25(0)	12(48)	<0.0001
Live born rate (n, %)	0/25(0)	12(48)	<0.0001

Table 2. Comparison of results between PRP treatment and previous cycles.

Correspondingly, the results of the PRP treatment cycle were compared with the standard results of a patient group without the need for PRP treatment. Implantation, clinical pregnancy, and LBR were not significantly higher in the PRP treatment cycle compared to the traditional cycles. Age, BMI, endometrial thickness, number of embryos transferred, and number of good quality embryos transferred were not significantly different, however, there was a statistically significant difference in the proportion of autologous and heterologous compared cycles. The treatment results are summarized in Table 3.

PARAMETERS	STANDARD CYCLE WITHOUT PRP TREATMENT (n=47)	PRP TREATMENT CYCLE (n=25)	P VALUE
Age (years)	36.28 ± 4.59	38.60 ± 4.31	0.567
BMI (Kg/m^2)	26.14 ± 3.49	26.09 ± 3.21	0.969
Endometrial thickness on the last day of endometrial priming (mm)	9.05 ± 1.12	8.60 ± 1.12	0.762
Fresh/frozen cycle	7/40	0/25	<0.0001
Autologous/heterologous cycle	35/12	13/12	0.157
Number of transferred embryos	2.30 ± 0.64	2.44 ± 0.49	0.520
Number of good quality embryos transferred	1.6 ± 0.6	1.7 ± 0.47	0.701
Implantation rate (%)	37/159(23.27)	15/61(24.59)	0.237
Clinical pregnancy rate (%)	24/47(51.06)	14/25(56)	0.982
Ongoing pregnancy rate (%)	22/47(46.80)	12/25(48)	0.913
Live born rate (%)	22/47(46.80)	12/25(48)	0.913

Table 3. Comparison of results between the group of patients with PRP treatment and standard cycles with patients with a normal endometrium.

Discussion

The optimal endometrium thickness is one of the critical factors for the successful implantation of the embryo. Accordingly, the priming of the endometrium has been considered a critical step for ET.

In this study, there were no differences in other clinical characteristics, such as age, infertility duration, number of failed IVF/ICSI cycles, and embryo grading, and number of embryos transferred according to pregnancy outcomes. For this reason, there are no prognostic factors that expect successful results with PRP treatment. However, this result could be due to a small number of patients and further studies with a larger number of subjects are needed to confirm this finding.

The efficacy and safety of autologous PRP have been reported in many fields of medicine, but few clinical trials exist to determine the role of PRP (18). The molecular mechanisms of PRP therapy in endometrial proliferation are currently not well understood. We hypothesize that intrauterine infusion of PRP helps expand the thin endometrium and improve the pregnancy rates. PRP is a relatively new treatment applied to improve endometrial thickness in women with a thin endometrium. It appears that PRP is safe due to the autologous nature derived from the patient's own blood.

Chang et al. reported the efficacy of intrauterine PRP infusion for endometrial growth in women with thin endometrium for the first time. Five patients with a history of thin endometrium (on the day of hCG administration) were recruited into the study. PRP was infused into the uterine cavity on the tenth day of the endometrial preparation cycle. If the endometrial thickness did not increase during the next 72 hours, the PRP infusion was infused 1 to 2 times in each cycle. When the thickness of the endometrium reached >7 mm, embryos were transferred. Successful endometrial expansion and pregnancy were observed in all patients after PRP infusion (12).

The LBR was noted in two previous studies (14,15). The first study reported 26.3% live births after PRP treatment, and the second study's live birth rate was 38.2%. The variation in LBR between the previous and current studies may be due to the difference in patient characteristics.

In this study the results of the PRP treatment cycle were compared with the reproductive outcomes of a patient group without the need for PRP treatment. Age, BMI, endometrial thickness, number of embryos transferred, number of good quality embryos transferred, implantation rate, clinical pregnancy rate and live births were not significantly higher in the treatment cycle with PRP compared to cycles without treatment, however, if there was a statistically significant difference in the proportion of the autologous and heterologous cycles when comparing both groups. A higher response was noted on the number of cycles with oocyte donation in the treatment group, which could represent alone a positive effect on our results not only due to in endometrial thickness increase, but

also to a hypothetical grow in the proportion of euploid embryos transferred, considering that a greater proportion of these are derived from younger donor ovules. A shortcoming of this study was the small group of patients, and insufficient information on the platelet concentration in each infused plasma preparation, however it is known that autologous blood plasma that has been enriched for platelets to approximately 4- 5 times more than circulating blood is able to upregulate Leukemia inhibitory factor (LIF) expression in endometrial stromal cells (19), and upregulating LIF expression could improve endometrial receptivity. It is also suggested that PRP may exert some impact in enhancing trophoblast placentation. PRP can stimulate proliferation and regeneration with many growth factors and cytokines, including Platelet derived growth factor (PDGF), Transforming growth factor (TGF), vascular endothelial growth factor (VEGF), Epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin-like growth factor I, II (IGF I, IGF II), interleukin 8 (IL8) and connective tissue growth factor (CTGF).

Endometrial receptivity is controlled by dynamic and precise molecular and cellular cytokine events, transcription factors, and genes. Currently, PRP infusion is progressively used in various fields of medicine such as nerve injury, osteoarthritis, chronic tendinitis, bone repair and regeneration, heart muscle, alopecia, plastic surgery, and oral surgery, but there is limited experience in obstetrics and gynecology. For the first time, Chang reported the efficacy of intrauterine PRP infusion for endometrial growth in women with thin endometrium. In that trial, PRP was infused in 5 women with inadequate endometrium who had poor response to conventional therapy during FET cycle. Adequate endometrial thickness is a major factor for implantation and pregnancy, so women with persistent thin endometrium often do not undergo ET. Several methods have been described for the preparation of the endometrium, but there is still no gold standard. A number of investigators reported that granulocyte colony-stimulating factor (G-CSF) promotes endometrial growth, because this cytokine stimulates the differentiation and proliferation of granulocyte neutrophils and can induce endometrial proliferation and growth, and, therefore, improve pregnancy outcomes (21, 22). In agreement to this hypothesis, a local infusion of PRP containing various growth factors and cytokines can improve endometrial growth and receptivity.

CONCLUSIONS

The present study was conducted to determine the effects of autologous PRP treatment on the thin endometrium of women in preparation por ET, and the results obtained represent a noteworthy improvement considering the history of the patients. Even though more studies on the molecular basis of this PRP treatment are needed to reveal the exact mechanism of action and obtain more solid evidence on its beneficial effect in patients with thin endometrium. For this reason, we suggest additional clinical trials should be conducted in this context. Autologous PRP treatment for endometrial priming is a safe procedure, with minimal risks of infectious disease or immunological reactions since it is made from autologous blood samples.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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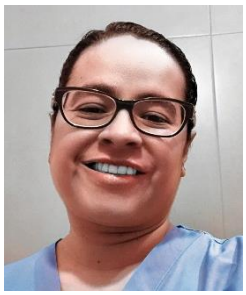
Discordant non-invasive prenatal test, train of thought when having positive ultrasound markers



Fragoso-Benítez Marcela

Fragoso-Benítez Marcela[1], 0000 0002 4218 9951; Noriega Jiménez Miguel Ángel[2], 0000 0002 5746 0150; Ballescá Estrada Adriana[3]; 0000 0003 0365 335X; Espinosa Arce Andrea Fernanda[4], 0000 0002 2584 8900.

ABSTRACT

Here we present a clinical scenario and the approach taken by the laboratory and medical staff in a private Reproductive Genetics laboratory in Mexico City.

We received a maternal blood sample for non-invasive prenatal testing that resulted in high risk for trisomy 18. Confirmatory amniocentesis was performed and amniotic fluid qfPCR showed an euploid fetus. In this case report we discuss our approach to provide accurate genetic testing for the couple.

KEYWORDS: NIPT, Trisomy 18, first trimester.

MANUSCRIPT

A sample from a 10.5 gestational age pregnancy with ultrasound positive markers was sent to our laboratory for non-invasive prenatal testing (NIPT); the sample was processed as usual obtaining a high risk of trisomy 18 in a female fetus. Notification for the requirement of a confirmatory qfPCR testing was done and amniocentesis was accepted by the mother. This confirmatory testing was processed as usual and resulted in 46,XX, confirming the female fetus but discordant for aneuploidies. Because of contradictory results a case study was initiated and an interview with the parents was scheduled.

Parents were a healthy young couple, ages 31-33, first grade cousins. Ultrasound was reported at week 10 with small nasal bone and heart murmur, those were the indications for the NIPT. Genetic counselling was focused on different possibilities:

- First, the possibility of maternal contamination in the amniocentesis sample. We recommended to perform Short Tandem Repeat testing (STR) comparing both samples, fetus versus mother.

- Then, the possibility of a false positive non-invasive prenatal testing but with an alternative diagnosis occurring on the fetus hence the alterations observed in the ultrasound, and considering monogenic diagnosis that may be or not related to the

[1] Medical Director, GD Technologies.
[2] Medical Geneticist, GD Technologies.
[3] Former Laboratory Director, GD Technologies.
[4] Former Laboratory Supervisor, GD Technologies.

NOTE: The numbers following the affiliation markers are the author's ORCID iD.

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CONTACT:
Fragoso Benítez Marcela.
marcela.fragoso@grupo-bio.com.
GD Technologies, San Luis Tlatilco 5,
Fraccionamiento Industrial. Naucalpan, Edo.
Méx. CP53370, México.
Phone: +52 55 1328 1328.

consanguinity. For this, we recommended follow up and structural second trimester ultrasound, at first.

• And of course, the possibility of placental mosaicism or maternal mosaicism.

Parents decided to follow the pregnancy, regardless the risks addressed during the genetic consultation. STR testing discarded maternal contamination of the amniocentesis sample, therefore the other possibilities increased their chance. Different testing was proposed to the parents, but because of the costs, and because for monogenic recessive disorders where no potential risks were identified during the genetic consultation, the recommendation was following up, second trimester ultrasound and genetic consultation of the new born. A third party, recommended the couple repeating the NIPT which was discouraged out by us, but at the end, the couple decided to follow the third-party recommendations and we lose contact with them.

Prenatal diagnosis is an informative tool to provide patients enough information for their reproductive choices (1), however since in Mexico not all gynaecologists, obstetricians or reproduction health workers have an adequate training in genetic counselling, some patients may be disinformed about the optimal studies, in this case the patients were offered a second non-invasive prenatal testing, which of course, would not be able to address different information.

Current guidelines of most colleges of Genetics and Obstetrics recommend prenatal screening to all women regardless their age (1), however, the selection of the type of screening may represent a great difference in costs, which in low income countries such as Mexico could compromise the reproductive autonomy of the couple. Some studies, for example Gekas et.al and Ohno et.al., (1, 2) address that when NIPT requires confirmatory testing would represent cost effective as compared when use as a diagnostic tool, and is as well cost-effective in high-risk patients due to maternal age 35 or greater (3). In fact, some studies in general population have shown economic justification (4). In low income countries such as ours, these topics should be start to be discussed, and all genetic testing should be accompanied by genetic counselling to determine the optimal follow up. In our laboratory, considering the cost of invasive procedures, the confirmatory testing is offered for free.

Discordant NIPTs is strictly referred to as those being different from a fetal karyotype. False positive cases are more frequent than false negative in a ratio of 27:1 (5). False positive cases main reasons include maternal copy number variation, confined placental mosaicism, positive vanished twin, maternal mosaicism, and maternal malignancy. Whereas false negative requires further investigation, most cases reported are due to complex chromosomal formulas: true fetal mosaicism, discordant abnormal formulas between NIPT and fetus and/or placenta, placental complex mosaicism, etc. (6). However, due to the emotional impact of a positive result, false positives should be addressed differently, especially when ultrasound markers or abnormality are present.

Cases of termination of pregnancy after a positive NIPT result but without a confirmation test have been reported up to 6.2-19.6% of cases (6), raising up the need of having a genetic counselling before the, as sensitivity rates are known to be 99% with false positive below 1%, but positive predictive value is limited to 40-90% (6), and the knowledge of the mechanisms involved in discordant NIPTs should be brought to the table on priority, to address the need of further testing and/or follow up. Pre and post-test genetic counselling should be mandatory, to all women choosing NIPT.

Unfortunately, further studies of placenta, maternal chromosomal abnormalities, and/or fetal mosaicism in false positive cases are limited, not only because these tests are often invasive, but because the normal outcome of the baby diminishes the need of answers and the need to pay for further testing; this type of practices have also hind to establish biostatistical values for NIPTs in aneuploidies not concerning 13, 18, 21, X and Y chromosomes, as well for CNVs.

Beulen et al. (7) have questioned the clinical utility of non-invasive prenatal testing in ultrasound anomalies, they reported normal NIPT results in 89.2% of performed tests with ultrasound abnormalities, however their population included cases in which whole single aneuploidies may not be the main diagnostic consideration or where NIPT would indeed not be recommended by a geneticist, for example, ultrasound with multiple important abnormalities such as holoprosencephaly, multiple pregnancies, etc. It is of enormous importance that although NIPT has demonstrated to be a good test for single whole chromosomal aneuploidies, NGS is not intended to be a substitute neither for karyotype, especially in the case of rearrangements with minimal gain/loss nor NIPT is at this point available to discard other genetic abnormalities that may be implicated in an abnormal ultrasound. Guidelines upon how to test pregnancies with ultrasound abnormalities should be agreed by colleges but should not discard NIPT.

It is very important to remember that teratogenic and monogenic disorders are responsible of most part of the congenital diseases/complexes and/or syndromes and there is no yet a protocol or tests to address them all (5); indeed, chromosomal

abnormalities are age-related in a population that has an increasing age of maternity and therefore, NIPT as well as other screening tests are available for this population, there is still no test 100% reliable.

CONCLUSIONS

Though NIPT is a reliable test for prenatal diagnoses, certain cases require not only consolidation of guidelines but also specialized counselling with a geneticist. Cases in which NIPT has a discordant result but in the presence of ultrasound abnormalities should be of special interest of looking for optimal testing, follow up with the consideration of cost-effectiveness and securement of reproductive autonomy.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

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