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Growth factors, gene activation and cell recruitment: From intraovarian condensed platelet cytokines to de novo oocyte development

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Growth factors, gene activation, and cell recruitment:Exploring connections between intraovarian insertion of autologous condensed platelet cytokines and de novo oocyte development
Journal of Clinical and Translational Research

Dear Dr Sills,

Reviewers have now commented on your paper. You will see that they are advising that you revise your manuscript. If you are prepared to undertake the work required, I would be pleased to reconsider my decision.

For your guidance, reviewers' comments are appended below and attached to this email.
The editorial board requests that you pay particular attention to the comments from reviewer #3. This expert in the field raised significant concerns and recommended a reject verdict. The editorial board would like to extend the opportunity to the authors to rebut and revise, also in light of the minor revision and accept recommendation by the other reviewers.

If you decide to revise the work, please submit a list of changes or a rebuttal against each point which is being raised when you submit the revised manuscript. Also, please ensure that the track changes function is switched on when implementing the revisions. This enables the reviewers to rapidly verify all changes made.

Your revision is due by Feb 06, 2022.

To submit a revision, go to https://www.editorialmanager.com/jctres/ and log in as an Author. You will see a menu item call Submission Needing Revision. You will find your submission

record there.

Yours sincerely

Michal Heger
Editor-in-Chief
Journal of Clinical and Translational Research

Reviewers' comments:

Reviewer #2: The authors summarized the clinical responses to specific cytokine-dependent gene activation pathways likely needed to induce oocyte differentiation. Platelet-rich plasma and/or condensed plasma cytokines of platelet origin, which are injected into older ovarian tissue, is a promising new application for ovarian rejuvenation.

Separation of key factors from platelets will be better used for ovarian rejuvenation.

Reviewer #3:
The manuscript is a medical hypothesis on the platelet rich plasma on the ovarian follicle growth.

The nomenclature and terms used in this manuscript were confused, such as "ovarian rejuvenation' and oocyte recruitment"; recruitment and differentiation of oocytes vs reprogramming; undifferentiated oocyte stem precursors; primordial germ cells vs precursor cells; etc..

The figure legend was not clear and could not be correlated to the text.

There are also few major limitations in this manuscript:
1. The authors did not clearly indicate their hypothesis on which stage of follicle? There are four stages of follicular growth since the resting primordial follicles to the preovulatory follicles: initiation; early follicle growth; selection of one follicle from a pool of selectable (≥2 mm) follicles; and maturation of the preovulatory follicle. (A. Gougeon Human ovarian follicular development: From activation of resting follicles to preovulatory maturation Annales d'Endocrinologie 71 (2010) 132-143)
2. The authors did not clearly indicate their hypothesis on which layer of ovarian tissue? Almost all publications regarding the effectiveness of PRP on ovarian follicle growth was based on the injection of PRP into ovarian medulla stroma. However, most of immature and growing ovarian follicles located at the cortical area of ovary. Recent work has indicated the whole scale subcortical administration of PRP on the purpose to restore the follicular growth in early menopausal women (Hsu, Menopause 2021; 28, No. 6, pp. 660-666).
3. They did not cite proper and enough references. The impact of various cytokines, growth factors (derived from platelets) on the folliculogenesis has been hypothesized in several publication in recent one decade. Most of the cytokines, growth factors mentioned in this manuscript have been indicated in previous publications (Annales d'Endocrinologie 71 (2010) 132-143; Endocr. Rev. 2015, 36, 1-24.; E. Scott Sills and Samuel H. Wood Bioscience Reports (2019) 39 BSR20190805; Endocrines 2021, 2, 15-27.).
4. The hypothesis in this manuscript was based on previous works of the authors including BMP-11. However, the data obtained in the mouse and nonhuman animals must be considered

with caution before extrapolation to humans since they may be irrelevant because of strong differences existing between human and mouse folliculogenesis.
5. The effect of oocyte itself. As the oocytes secret several GF, such as GDF 9; BMP which might modulate the growth of surrounding granulose cells and another oocytes.
6. What's the effect of various cytokines, growth factors (derived from platelets) on the gonadotropic hormones FSH and LH? Recent microarray results and the Ovarian Kaleido¬scope Database reveal that FSH regulates the expression of ~500 target genes in granulosa cells that support follicle maturation.
7. What's the effect of various cytokines, growth factors (derived from platelets) on the rate-limiting enzyme in estrogen biosynthesis, P-450 aromatase (aromatase); increased progesterone production, P-450 cholesterol side chain cleavage (SCC); expression of 3β-hydroxysteroid dehydrogenase (3β-HSD)?
8. Did those factors the authors mentioned related to the follicular depletion or atresia or apoptosis of follciles? This is important as estimated that more than 90% of the ovarian reserve escapes by apoptosis mainly during infancy and early adulthood.
9. The achievement of follicle maturation includes a sophisticated program of regulatory mediators of both somatic and germ cell origin, primordial germ cells (from yolk sac) and certain immune cells (from dorsal aorta) and some somatic cell types. Somatic cells originating from the primitive gonad surround the oogonia, forming rudimentary ovarian follicles. What's the effect of various cytokines, growth factors (derived from platelets) on this issue?
10. Upstream contributions by ovarian stem cells (OSC) may be possible under conditions enabled by growth factors released by platelet-rich plasma (PRP-GFs). It was not clear whether the major issue or target of the PRP explained in this manuscript was ovarian stem cells or primordial germ cells. From most of recent clinical data, the restoration of ovarian follicular growth and ovulation could be reached in 3-6 months and even shorter period. It would need more time for ovarian stem cells to the stage of preovulatory follicles and ovulation.

Reviewer #4: The manuscript is well written and reviews the myriad growth factors and cytokines that may play a role when PRP is injected in the ovaries. It is a welcome review that adds to the increasing literature on this potentially important add-on for women with poor ovarian reserve.

There is additional documentation related to this decision letter. To access the file(s), please click the link below. You may also login to the system and click the 'View Attachments' link in the Action column.

Authors' response

J Clin Translation Res D-21-00191R *cover letter*

Dear Prof. Heger, 10th January 2022

Thank you for this critique of our recent submission. The manuscript has now been revised, updated, resubmitted, and specifically marked by line# in response to reviewers' comments, as noted below:

Reviewer #2: The authors summarized the clinical responses to specific cytokine-dependent gene activation pathways likely needed to induce oocyte differentiation. Platelet-rich plasma and/or condensed plasma cytokines of platelet origin, which are injected into older ovarian tissue, is a promising new application for ovarian rejuvenation.
Separation of key factors from platelets will be better used for ovarian rejuvenation.

Yes, the concept of differential effect from specific PLT cytokines makes sense. Our paper focuses on the difficulty of disentangling these multiple signals (growth factors), and then attributing cell- or tissue-specific responses for each. Within reproductive biology, this work may claim priority to frame this challenge especially regarding which PLT products are most relevant and how they might work together to reverse menopause—at least temporarily.

Reviewer #3:
The manuscript is a medical hypothesis on the platelet rich plasma on the ovarian follicle growth.

The nomenclature and terms used in this manuscript were confused, such as "ovarian rejuvenation' and oocyte recruitment"; recruitment and differentiation of oocytes vs reprogramming; undifferentiated oocyte stem precursors; primordial germ cells vs precursor cells; etc..

We regret any misunderstanding. For now, there are still open questions which our work brings into sharper focus: It expands on the theory that autologous PLT cytokines, when used for intraovarian insertion, may explain effects observed clinically by moving OSCs into a differentiation pathway to *de novo* oocytes. We agree that uncertainty on 'ovarian rejuvenation' definition exists, but the nomenclature fits with our previous work (see Ref# 8 and 39).

The figure legend was not clear and could not be correlated to the text.

Without specific examples as guidance, unfortunately satisfactory reshaping may be difficult. However, we take this critique seriously and to remove doubt, sub-cortical placement is now stated in the legend. It shows not just PLT processing options, but also where the sample ideally should be inserted.

1. The authors did not clearly indicate their hypothesis on which stage of follicle? There are four stages of follicular growth since the resting primordial follicles to the preovulatory follicles: initiation; early follicle growth; selection of one follicle from a pool of selectable (≥2 mm) follicles; and maturation of the preovulatory follicle. (A. Gougeon Human ovarian follicular development: From activation of resting follicles to preovulatory maturation Annales d'Endocrinologie 71 (2010) 132-143)

This assessment is valid, but the proposed sequence above operates ONLY with adequate ovarian reserve. Our paper addresses a different problem, where older/menopausal ovaries cannot enable anything described above (see p.4, lines 10-11). We emphasize upstream factors, where ovarian induction via PRP could set the stage for follicular maturation. 'Ovarian

rejuvenation' thus aspires *a priori* to awaken ovarian target, otherwise inactive and nonresponsive, to begin follicular growth & selection. This is clarified at p.10 lines 1-4. We also added Gougeon (2010) to our bibliography as Ref# 3 and appreciate the suggestion.

2. The authors did not clearly indicate their hypothesis on which layer of ovarian tissue? Almost all publications regarding the effectiveness of PRP on ovarian follicle growth was based on the injection of PRP into ovarian medulla stroma. However, most of immature and growing ovarian follicles located at the cortical area of ovary. Recent work has indicated the whole scale subcortical administration of PRP on the purpose to restore the follicular growth in early menopausal women (Hsu, Menopause 2021; 28, No. 6, pp. 660-666).

This reflects back to the 'graphical abstract', mentioned earlier. There the OSC pool is shown sub-cortically (in red) and also noted in the legend. That localization is discussed at p. 6, lines 8-9 with extensive coverage in Ref# 8. Surgical technique and equipment fall outside the scope of this work, as we published on this previously (see Refs# 39 & 46). Of note, the Hsu paper (2021) describes *laparoscopy* for ovarian PRP. Depth of ovarian tissue injection is difficult/impossible to judge by laparoscopy vs. TV-USG guided insertion, the latter affording interior structural information and thus is far more anatomically precise.

3. They did not cite proper and enough references. The impact of various cytokines, growth factors (derived from platelets) on the folliculogenesis has been hypothesized in several publication in recent one decade. Most of the cytokines, growth factors mentioned in this manuscript have been indicated in previous publications (Annales d'Endocrinologie 71 (2010) 132-143; Endocr. Rev. 2015, 36, 1-24.; E. Scott Sills and Samuel H. Wood Bioscience Reports (2019) 39 BSR20190805; Endocrines 2021, 2, 15-27.).

No action seems indicated on this. With >45 references, our work embraces recent data from animal investigations and human clinical IVF experience. In contrast, the 2021 *Endocrines* paper (above) is a review, presenting no original data. The 2019 *Biosci Rep* publication is ours, cited as Ref# 8.

4. The hypothesis in this manuscript was based on previous works of the authors including BMP-11. However, the data obtained in the mouse and nonhuman animals must be considered with caution before extrapolation to humans since they may be irrelevant because of strong differences existing between human and mouse folliculogenesis.

We should acknowledge the BMP-11 data were from Zhou *et al* (see ref#18), not our own group. The referee is correct to call out 'strong differences' across some mammals. Our paper is careful to discriminate animal vs human research, to avoid this confusion. Unfortunately, where PLT growth factor research is concerned, sometimes the only data available come from animal experimentation - although we hope to help address this soon.

5. The effect of oocyte itself. As the oocytes secret several GF, such as GDF 9; BMP which might modulate the growth of surrounding granulose cells and another oocytes.

But all hinges on any competent oocyte to manifest such secretion—hence the putative role of PLT-derived cytokines to begin the process. Any autocrine effects would of course be stalled at nil without the egg itself. Since our manuscript centers on signaling from autologous PLTs, secondary (local) contributions made by other sources (*i.e.*, oocyte) were considered

extraneous. However, we do concur that development would eventually permit production of estradiol, as stated at p.6, line 16. Note this theme is also portrayed in the 'graphical abstract'.

6. What's the effect of various cytokines, growth factors (derived from platelets) on the gonadotropic hormones FSH and LH? Recent microarray results and the Ovarian Kaleido¬scope Database reveal that FSH regulates the expression of ~500 target genes in granulosa cells that support follicle maturation.

This is a powerful question perhaps best suited for neuroendocrine colleagues. Our paper is about intraovarian PRP/platelet cytokine therapy, and how specific GFs may upregulate genetic controls within dormant ovarian tissue. A causal link may indeed exist between reanimated granulosa cells post-PRP injection, and FSH levels, but this fell outside the scope of our non-pituitary paper.

7. What's the effect of various cytokines, growth factors (derived from platelets) on the rate-limiting enzyme in estrogen biosynthesis, P-450 aromatase (aromatase); increased progesterone production, P-450 cholesterol side chain cleavage (SCC); expression of 3β-hydroxysteroid dehydrogenase (3β-HSD)?

Most of these remain poorly characterized, inviting more study. We do discuss E_2 (see p.6, line 16) but the reviewer brings attractive targets for future work. By example, a link may exist between genes for RUNX2 and aromatase, as found in mouse [see Jeong *et al*. The gene for aromatase, a rate-limiting enzyme for local estrogen biosynthesis, is a downstream target gene of RUNX2 in skeletal tissues. *Mol Cell Biol* 2010;30:2365].
We recently found (Dec 2021) a previously unknown RUNX2 mutation, unrelated to ovarian PRP [Sills & Wood. Phenotype from SAMD9 mutation at 7p21.1 appears attenuated by novel compound heterozygous variants at RUNX2 and SALL1. *Global Med Genetics* 2022: *in press*], so additional characterization of this mutation is underway.

8. Did those factors the authors mentioned related to the follicular depletion or atresia or apoptosis of follciles? This is important as estimated that more than 90% of the ovarian reserve escapes by apoptosis mainly during infancy and early adulthood.

No longitudinal data exist on how follicular depletion/atresia might progress after ovarian PRP. We did address this knowledge gap in the 2019 *Neuroendocrinol Lett* publication (see Ref # 43).

9. The achievement of follicle maturation includes a sophisticated program of regulatory mediators of both somatic and germ cell origin, primordial germ cells (from yolk sac) and certain immune cells (from dorsal aorta) and some somatic cell types. Somatic cells originating from the primitive gonad surround the oogonia, forming rudimentary ovarian follicles. What's the effect of various cytokines, growth factors (derived from platelets) on this issue?

Yes, as the referee correctly notes in query #4 (above) conclusions based on non-human experimental cell models might not generalize to clinical practice. Much developmental anatomy teaching is supported by animal experiments. The sequence for ovary, follicle, to oocyte was given in our earlier work (see Ref# 8).

10. Upstream contributions by ovarian stem cells (OSC) may be possible under conditions enabled by growth factors released by platelet-rich plasma (PRP-GFs). It was not clear whether the major issue or target of the PRP explained in this manuscript was ovarian stem cells or primordial germ cells. From most of recent clinical data, the restoration of ovarian follicular growth and ovulation could be reached in 3-6 months and even shorter period. It would need more time for ovarian stem cells to the stage of preovulatory follicles and ovulation.

Extent of these 'precursors' is hotly debated, and underscores the reason for our manuscript. It acknowledges a need to delineate the action pathway/s facilitating *de novo* oocyte development, beginning with action on sub-cortical OSCs. Here we propose a linkage with PLT-associated cytokines, as inducers for specified genes committing those cells to become functional follicles, and later, oocytes. How long does this require? Again, the referee is probably right on this – 3mo may be too brief for this to generate anything clinically useful, if animal gamete data may be cautiously taken as a benchmark. This was explored in our dataset on patient subjective response after ovarian PRP (see Ref# 43).

We hope you agree that the work now is scientifically stronger as the above points are addressed. After reflecting on the comments, the wording of the title has also been cut down, edited to be more concise. Please let me know if any additional input is needed, as this will receive priority attention here to move forward quickly.

Respectfully,

ES SILLS

2nd Editorial decision
09-January-2022

Ref.: Ms. No. JCTRes-D-21-00191R1
Growth factors, gene activation and cell recruitment: From intraovarian condensed platelet cytokines to de novo oocyte development
Journal of Clinical and Translational Research

Dear authors,

I am pleased to inform you that your manuscript has been accepted for publication in the Journal of Clinical and Translational Research.

On a personal note: very solid rebuttal Scott. Much appreciated.

You will receive the proofs of your article shortly, which we kindly ask you to thoroughly review for any errors.

Thank you for submitting your work to JCTR.

Kindest regards,

Michal Heger

Editor-in-Chief
Journal of Clinical and Translational Research

Comments from the editors and reviewers: