Communication

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Entropy and mechanistic concepts after intraovarian platelet-rich plasma: Experimental considerations for local tissue responses mediated by NF-κB and TNF-α

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Abstract: The negative slope of human ovulatory fidelity begins with a high follicular fund balance with gradual withdrawals over time. This depletion of reserve is a sentinel event foreshadowing severe reproductive loss and, eventually, systemic aging. Conversely, the youthful ovarian phenotype requires coordination among endothelial, granulosa, immune, perivascular, stromal, and perhaps germline stem cells. This diverse tissue matrix theoretically can be modified by platelet (PLT)-derived moieties, but this awaits experimental confirmation in adult ovaries. Indeed, while the cellular entropy states of menopause and low reserve could follow a final common pathway, cell kinetics are not always irreversible within this set. We propose an experimental design to track nuclear factor κB (NF-κB) oscillations, tumor necrosis factor-alpha (TNF-α), selected gene expression, apoptosis, and key immune/inflammatory actions as entropy drivers antagonizing reproductive fitness. Since NF-κB and TNF-α are discharged in activated PLT releasate (or react to its cargo proteins), our investigation audits response markers pre- vs post-injection of processed platelet-rich plasma (PRP), connecting discrete signals to transcriptional output, cell function, and ovarian cytoarchitecture. This may reveal intraovarian PRP operating as a local entropy rectifier, with organ field function shifting to support oocyte competence incidentally, where "menopause reversal" is merely a beneficial secondary effect.

Keywords: reproductive biology, ovarian function, PRP, extracellular matrix, entropy

Introduction

Initially developed by Clausius and refined later by Boltzmann [1], the concept of entropy expresses system order as measured by uncertainty or unpredictability. Young cells in "healthy order" are expected to display low entropy, while tissue aging reflects deterioration of physical structures such as misfolded/deformed RNA, DNA, or proteins, with harmful downstream effects in tissues and organs [2]. Entropy can be reversed during early development, growth, and under metabolic conditions where energy is expended to attenuate disorder [3–5]. Common to other mammalian organ systems [6], the adult human ovary demonstrates nonlinear dynamics characterized by multi-stability, hysteresis, and transitions across different metabolic states (i.e., ovulatory vs non-ovulatory functions). The fertility vs infertility divide may thus be classified within the ovarian field as a stochastic process where reproductive potential is optimized at an entropy minimum, while subfertility expands with a declining capacity to maintain orderliness over time. Manipulating this biostat would be tantamount to resetting the female biological clock.

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Current models of cellular aging portray DNA damage accreting with lost regenerative capacity, more genomic infidelity, and eventually, cell loss without replacement (death). For the ovary, early diminishments of reserve inescapably presage a final functional collapse at menopause. Of note, this process is only partially heritable (<50%) and underscores the importance of non-genetic factors in ovarian aging [7]. For example, twins sharing the same household may acquire dissimilar age-related epigenetic changes, revealing an energy system possibly subject to modification [8,9]. If this paradigm is correct, it invites speculation on possible external inputs like intraovarian platelet-rich plasma (PRP) able to alter the functional dynamic and influence clinical outcomes (i.e., recover reserve by reducing entropy).

Experimental program

Following IRB approval, written informed consent would be obtained from patients (age, 20–55) already scheduled for standard GYN laparoscopy for benign indications [10]. This study is powered by <1 g of tissue obtained from each ovary by standard punch biopsy (Figure 1). No hormone use within 6 months would be confirmed before enrollment [11]. Deidentified ovarian samples are maintained fresh as the experimental substrate and cultured at 37°C/5% CO_2, based on a previously published complex tissue protocol [12]. While a signal multiplier array incorporated a protein–protein interaction model for platelet-derived bFGF and VEGF [13], it failed to include guidance on methods or study design to report on cellular response. Here, the reagent used as the external stimulus is matched-autologous PRP processed with calcium gluconate activation [14].

We propose information on ovarian nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) dynamics to be supplied from DNA binding at κB enhancer motif sequences found in NF-κB target genes, as described by others [15]. DNA-protein binding may be measured either by the electromobility shift assay technique or by quantitative ELISA [16,17]. Alternatively, a fluidic chip system could be used whereby DNA, histone/protamine, and transcription factor NF-κB are observed to record how PRP cytokine inputs alter DNA-protein configurations [18]. Transcriptional activation data also can be quantified using an NF-κB consensus promoter sequence linked to a "reporter gene" and luciferase assay [19,20]. Differences in TNF-α levels pre- vs post-PRP exposure would be marked by quantitative immunoassay (Promega; Madison, WI). Recording changes in TNF-α will improve knowledge of how this pleiotropic cytokine may impact differentiation, proliferation, and survival

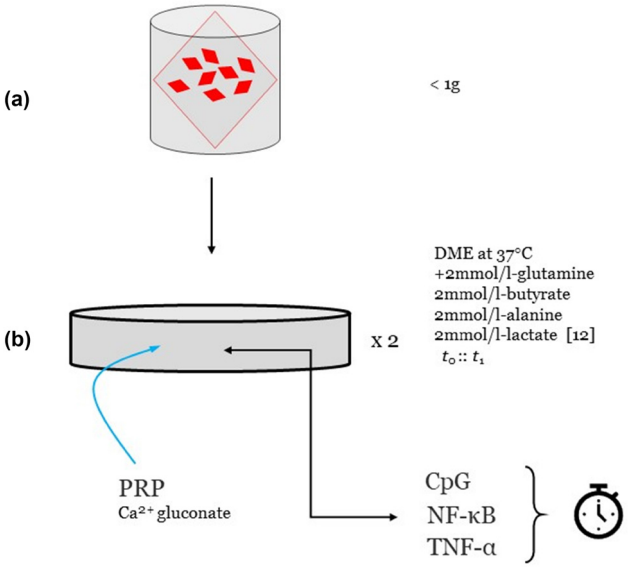

Figure 1: Laboratory schematic for planned study of 5′ – C – phosphate – G – 3′ (CpG) islands, nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), and tumor necrosis factor-α (TNF-α). Fresh ovarian tissue (a) would be obtained via laparoscopic biopsy (bilateral) from qualified volunteers (age, 20–55) and incubated [12]. Baseline assessments of study parameters at t_0 are followed by dosing with matched, autologous PRP (b) after Ca^{+2} gluconate activation [14]. As PRP incubations run as duplicates, concentrations would be reduced by ½ in each corresponding arm.

after PRP. Although unlikely, short-term PRP effects on 5′ – C – phosphate – G – 3′ (CpG) islands may quantify expression noise attenuation post-stimulus [21]. Since the duration of any PRP impact on NF-κB, TNF-α, or CpG islands is unknown, the endpoint for experimental culture termination is recursive and must be delimited by data not currently available.

In addition to functional immunohistochemical findings, ovarian cells can be imaged by stereological analysis [22] to note potential differences in cytoarchitectural elements by the Delesse principle [23]. As each patient would contribute two biopsies, the plan is to run these in duplicate to validate observed findings (Figure 2).

The older ovary: Special features

Unlike most other adult endocrine organs, the human ovary begins to show functional decline relatively early (approx. 30–35 years). It is generally agreed that this negative slope describes a follicular pool which recedes soon after menarche [24,25]. Losses in ovarian reserve can bring adversity to those wishing to conceive as well as for patients disinterested in fertility (i.e., symptomatic menopause) [26].

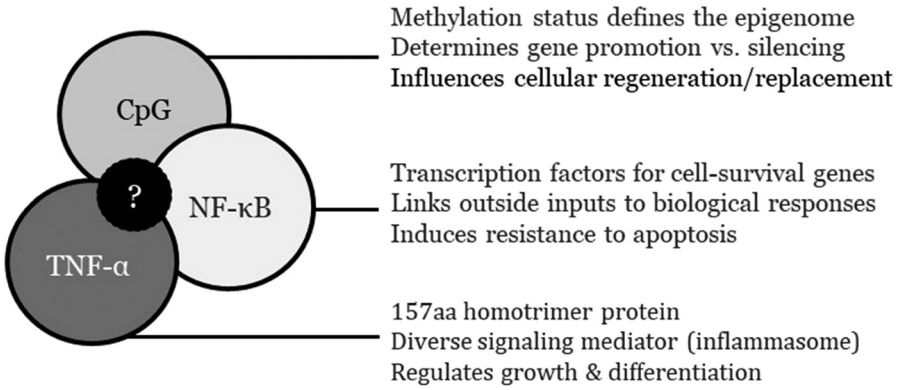

Figure 2: Truncated descriptions of CpG, NF-κB, and TNF-α as rationales for experimental measurements in adult human ovarian tissue cultured with matched, autologous activated PRP. Shared interactions among sampled parameters (center) require clarification in an ovarian context, which may be provided during the proposed study.

Against this background, if epigenetic changes act as a perimenopause trigger [27], one theory supports the concept of ovarian compromise as among the first detectable signs of aging [28]. As such, the adult human ovary would be merely the most fragile unit evincing senescence traits, manifesting more broadly later [29].

Fundamentally this is a degenerative and entropic sequence typified by poor tissue homeostasis [30]. Primordial ovarian follicles emerge during the fetal period and their programmed decline with age – imperceptible in the beginning – has been well documented [31]. Indeed, early animal experiments with transplanted young ovaries into aged recipients fixed the central role of competent ovarian tissue [26] with good order and minimal entropy. Follicular density loss is perhaps the most conspicuous structural feature of the aging ovary, as collagen gradually displaces hormonally active elements with fibrillin-1 and EMILIN-1 vanishing over time. Such changes offer a substructural insight into tissue disorder, as the ovary becomes less pliable and more fibrotic with approaching menopause. Unsurprisingly, significantly more elastin is observed in menopausal ovary biopsies where HRT is used vs no HRT [32]. Rigidity of the granulosa compartment thus resists follicle growth commensurate with progressive entropy, becoming more inclined to quiescence [32].

The variety of platelet cargo proteins [33] meeting ovarian entropy opens the possibility to impact several loci, where PRP might manipulate imbricated signal networks driving perfusion, HOX regulation, *N*-glycan post-translational modification, adjustment of voltage-gated ion channels, telomere stabilization, optimization of SIRT3, or ribosome and mitochondria recovery [34]. Another example is transcription factor FIGLA, which directs expression of Gdf9, Lhx8, Nobox, Sohlh1&2, and Taf4b as controllers of oocyte growth and differentiation [35]. In a murine model, FIGLA knockdown severely squelches meiosis to cause oocyte apoptosis. Non-operation of any regulatory member yields follicles overtaken by fibrosis, with downregulation of genes preferentially expressed in oocytes [36].

Since TNF-α is known to upregulate NF-κB [37] and the NF-κB signaling system coordinates Gdf9 actions [38], this draws notice to cytokines of PLT source, either mimicking FIGLA or boosting its function. For the follicular unit and its local support matrix, where remodeling, regeneration, and/or proliferation effects are presumably induced by PRP [39,40], clarification of CpG, TNF-α, and NF-κB roles can provide important information for reproductive biologists. Most cell processes are not indifferent to such interstitial effects: Membrane tension and cytoskeletal focal adhesions [41] set the stage for ovarian function, although these have yet to be evaluated in human ovarian tissue post-PRP dosing.

In this regard, computer-assisted 2D fluorescence imaging (Analytical Technologies; Singapore) of the ovarian cytoskeleton and juxta-follicular components can assist in documenting and classifying changes associated with local PRP injection, similar to techniques described recently [41]. Others have detailed PRP-integrated alginate gelatin composites where PLT cytokines "seed" cell behavior, form vascular endothelial cells, and order macrophage polarization in a paracrine manner [42]. While activated PRP may impact structures extraneous to the granulosa compartment, more research is needed to build on recently reported responses [34,41]. If parallel connective tissue effects are verified after ovarian PRP use as outlined here, this would help explain the restorative results observed in its early clinical use [14,33,43,44].

Ovarian signaling response after PRP

With attention to ovarian cellular entropy, experimental evidence exists [45] that NF-κB oscillations are involved in governing immune responses, cellular growth, development, and apoptosis. These NF-κB actions are driven by inhibitory proteins κB and IκB, where an inverse relation has been reported between pulse frequency and quantity of IκB [45,46]. NF-κB actually embraces an entire transcription factor family, responsible for vital immune and inflammatory actions [47]. This includes NF-κB1, NF-κB2, RelA, RelB, and c-Rel, as transcription mediators of key target genes [48]. In general, NF-κB proteins are sequestered in the cytoplasm by inhibitory proteins typified by ankyrin repeats [49,50]. NF-κB enhances expression of pro-inflammatory genes, including those for cytokines and chemokines, and participates in inflammasome regulation. NF-κB also orchestrates survival, activation, and differentiation of inflammatory T cells [49]. In eukaryotes, the IκB-NF-κB module ideally operates like a signal transduction unit, where inputs are external stimuli conducted by membrane receptors, and its outputs are signals channeled to the nucleus to regulate gene expression [46]. NF-κB may convey specificity of contextual information via quantitative features of its signaling dynamics [51], where greater metabolic noise correlates with unwanted entropy as seen with aging and disease. It may be possible to establish that intraovarian PRP elicits a local squelching of such signals using this research design.

The use of fluorescent-tagged proteins would enable tracking of dynamic NF-κB traffic, where its nuclear localization has been confirmed along with an oscillation period near 90min–an observation in reasonably close alignment with results predicted by mathematical models of NF-κB signaling [52,53]. Subsequently, NF-κB has become perhaps the best-known exemplar of pulsing or oscillating genetic circuits mapped by active imaging [54–56]. Pulsed TNF-α stimulation also affects gene expression in a target-specific way, providing another connection for signal dynamics and target gene expression [57]. As expected, these ordered oscillatory patterns are largely absent in high entropy states, although platelet cytokines can promote nuclear translocation of NF-κB and upregulate mRNA expression of NF-κB-dependent mediators outside the ovary [58]. Direct measurement of similar induction in human ovarian tissue awaits confirmation, and the present approach would help meet this need.

Several Kyoto Encyclopedia of Gene and Genome processes are known to be preferentially boosted in young mammalian follicles compared to aged ovaries, representing attractive areas open to therapeutic enhancement. For example, leucocyte-rich platelet-rich fibrin mediates NF-κB signaling, toll-like receptor signaling, and MAPK signaling via T-cell receptor signaling pathway, and other platelet derivatives are closely involved in the JAK-STAT signaling network to upregulate STAT1 [59]. How platelet cargo proteins interact with follicular surface markers after PRP dosing intersects with entropy studies, here, as membrane order and structure directly impact homeostasis via ion transport and governance of signaling pathways [34,60].

Cytokine effects on cellular entropy

The biochemical work of the adult human ovary exhibits transitional fluctuations where a closed compartment should uniformly reach a stable state. This is not a violation of the 2nd Law of Thermodynamics because dampened oscillations by intermediate species can form *en route* to equilibrium, even as overall Gibbs free energy (G^0) decreases [61,62]. Here, G^0 unifies enthalpy and entropy with change in free energy, ΔG, being the combination of enthalpy with the product of temperature and entropy:

$$\Delta G^0 = \Delta H^0 - T\Delta S^0,$$

where H is the enthalpy, T is the temperature, and S is the entropy; heat is not involved/added in life models, and thus $\Delta H^0 = 0$. Protein synthesis lowers cellular entropy by amino acid polymerization (e.g., cell growth also reduces entropy), making G^0 for protein synthesis net positive [63]. Although perhaps not applicable to every ovarian process, hydrophobic effects also contribute to lowered entropy of aqueous solutions [64]. As our hypothesis for intraovarian PRP posits that activated PLT-derived signals operate as entropy modulators, any growth stimulated by this treatment would involve protein synthesis with augmented amino acid synthesis, where required components are actively assembled from the dispersed field (cytosol). Reestablishing ordered signaling, if confirmed here, would agree with earlier work connecting entropy ("serial irregularity" by mathematical network analysis) and reproductive fitness [65].

Cell division in eukaryotes is also orchestrated in part by M-phase Promoting Factor (MPF), a heterodimeric protein kinase with Cdk1 (kinase subunit) and cyclin B (regulatory-targeting subunit) constituents [66]. Interestingly, MPF activity was first discovered in a reproductive context where (amphibian) oocytes and embryos were studied [62,67,68]. While MPF and NF-κB share a common signaling

coordinator in "wee1" [69], understanding the interplay among these mediators could be improved using the proposed study design.

There are innumerable nodes in regulatory networks where outside signaling could be relevant, especially NF-κB, which normally regulates biological processes as a function of dynamic oscillation [46,70]. If re-ordering of NF-κB (i.e., nuclear sequestration) were documented in our experimental program after activated PRP, then feedback on regulatory genes and NF-κB activation may be contingent on nuclear localization as facilitated by PLT cytokines. These signals, acting either individually or in concert, would tend to realign the prevailing energetics of protein synthesis soon after interfacing with ovarian tissue.

Intraovarian PRP: Epigenetic impacts

Molecular processes essential to normal ovarian function are indices of epigenomic competency as communicated via stochastic feedback signals [71]. As menopause nears, noise and entropy gradually overtake these well-ordered oscillations. Low ovarian reserve is a clinical problem where no uniform initiating factor is likely causative, but oligoovulation and non-responsiveness to gonadotropins comprise a familiar presentation. How (or if) cytokines of PLT origin may shift methylation status by CpG audits represents another technique to gauge intraovarian PRP actions.

DNA methylation plays a major role in gene expression, and how this changes with aging and disease is becoming better characterized. Most age-related DNA methylation drift is attributed to adult stem cell replication, yet there is controversy with respect to methylation changes being strictly from proliferation errors or due to other factors relevant to non-proliferating cells [9,72]. Any net gain/loss here ("methylation drift") results in genomic instability. For example, CpG sites can experience methylation loss over time to activate retrotransposons. Conversely, hypermethylation with age can occur within or near unmethylated CpG islands [9]. Age-related DNA methylation drift is highly conserved and inversely proportional to lifespan [73]. Age-related DNA methylation entropy as measured by Jensen-Shannon distribution [74] affects up to 25% of detectable CpG sites. This has been checked as a function of age in blood, heart, kidney, liver, lung, muscle (skeletal), and spleen [9], and our experimental program seeks to expand this to include the ovary post-PRP dosing. Epigenetic clocks are often based on just one sample site, so accuracy is limited to the source on which it was trained [29]. Epigenetic clocks developed from machine learning tools have reported informative CpGs by regressing a transformed version of chronological age on a set of CpGs [75,76]. While this number may be large to provide high accuracy, even a limited number of CpGs can offer adequate robustness to depict methylome properties [29]. DNA methylation drift and increased entropy with age are caused by – and are markers for – stem cell replication in adult tissues [9]. The experimental program described here should generate new data, at least indirectly, on how PRP may influence markers of ovarian cellular entropy.

PLT factors as activators & transport modulators

For the adult human ovary, its component cells appear to function before a backdrop of phenoptosis – eventual cell death pre-programmed by the genome. Normal ovarian metabolism generates many toxic by-products and errors, to amplify this, causing cellular damage as seen in other tissue systems. While damage accumulation is a spontaneous entropy-driven process, the kinetics are not necessarily irreversible and are subject to genetic and environmental modification [77]. In a murine model, an epigenome/metabolome/epigenome paradigm has proven useful to explain the functional crosstalk needed for cellular differentiation [49]. Being the first term in the set, epigenome status is rightly cast as a key player in cell-fate decisions for both embryonic and adult tissues [31,78]. While adjusting the epigenome via extracellular inputs is possible, the extent to which different stimuli can push this is unresolved. NF-κB is activated by various stimuli and can reprogram the epigenome by promoting latent enhancers, but this depends on whether NF-κB is oscillatory or not. Tonic (non-oscillatory) NF-κB signaling opens chromatin [79] by sustained disruption of histone-DNA interactions, triggering latent enhancers to express immune response genes. Previously unknown temporal aspects may fix a transcription factor's capacity and range for epigenetic reprogramming [80]. Environmental cues influencing the epigenome include nutritional inputs, as a high-fat/high-carbohydrate diet was recently found to prompt nuclear translocation of NF-κB p65 factor transcription in surface epithelium cells of rabbit ovary, an intervention with harmful ovarian reserve results [81].

PRP is a known suppressor of inflammatory NF-κB, where reduced doxorubicin-induced phosphorylation of

IκB and NF-κB has been described [42]. Models correlating nuclear NF-κB with mRNA expression give predictions of high accuracy at single-cell resolution [82]. Specific to its intraovarian use, autologous PRP does appear to foster improved tissue response, although further clarification is required to define local signal modulation effects.

Discussion

Stimuli overlapping with PLT cytokines have been reported to enable recovery of ovarian tissue post-injury [83]. For example, when paeoniflorin (a bioactive glucoside) was used after ischemia-reperfusion injury, protective ovary tissue effects were accompanied by increased levels of TNF-α, IL-1-β, IL-6, and NF-κB p65 [83]. A brief TNF flash would be akin to burst exposure after intraovarian PRP, which has been shown to "jump start" expression of NF-κB target pro-survival genes in other contexts [84]. As a controversial intervention, intraovarian PRP has proven difficult to manage in multicenter clinical trials given nonuniform patient screening, sample preparation, and injection technique. As activated PRP puts a small cytokine bolus into an inactive or senescent ovarian tissue field where metabolic or endocrine responses await characterization, the proposed study aims to address these deficiencies (Figure 2).

If a PRP contribution to reduced ovarian entropy were to be convincingly proven using this experimental design, it would join existing feedback and oscillatory research where similar conclusions were advanced. For example, bolus estradiol (E_2) increases growth hormone (GH) release substantially, while an abrupt GH spike stimulates fast internalization of its receptor to evoke second-messenger nuclear signaling [85]. So, both magnitude and pattern are central to physiologic signaling as the nonresponsive (older) ovary is characterized not by highly-ordered, information-rich pulsation, but rather by high entropy, non-cyclic, disordered monotone field. Just as supplementary E_2 can improve receptiveness, at least temporarily, to signaling elsewhere [86], a yet-to-be-defined PRP component may likewise restore ovarian reproductive capacity by effects within its local regulatory milieu after injection.

There are potential limitations with our design which warrant comment. First, while the collapse of ovarian function with advanced maternal age may reflect slow replacement of normal follicular processes with (inactive) fibrosis, the problem of why this happens remains unsolved. If it occurs due to subtle dampening of molecular signaling, then the two markers proposed here (NF-κB and TNF-α) may be inferior to different, unchosen mediators. We do not have a full picture of which feedback circuits fail first in diminished ovarian reserve. The notion that ovarian decline is a critical, irreversible indicator serving to flag an entropy threshold may also be incomplete. Indeed, this protocol is shared before launch to gain from early input where others may use or improve the design. From this, perhaps better clinical delivery approaches will lead to enhanced efficiency and reduced costs to patients. As currently configured, intraovarian PRP is still impractical and too expensive for mainstream use compared to much lower cost conventional therapies [87]. As data become available from our centers and elsewhere [88], further investigation will better define how to optimize intraovarian PRP (e.g., methodology comparisons or dose-finding studies).

In the meantime, as an adjunct to *in vitro* ovarian cell imaging, fully utilized mathematical models should start to settle the problem of how activated PRP affects cellular entropy or local microarchitecture. The method can estimate concentrations/copy numbers of NF-κB to define how it binds to inhibitors, as well as clarify its effects on target gene mRNA levels [55] secondary to PLT releasate. Because the NF-κB regulatory circuit is complex [71], providing data on the local ovarian NF-κB system holds great investigational and clinical promise. Given the clinical responses reported from independent, multicenter experience with intraovarian PRP [33,43,44], the proposed study design can be a first step to elucidate a relevant mechanism.

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