REVIEW ARTICLE

Ovarian Rejuvenation

Suada Tinjić[1], Džihan Abazović[2], Dušica Ljubić[3], Svetlana Vujović[4], Danilo Vojvodić[5], Tatjana Božanović[6], Aleksandar Ljubić[7]

Abstract

Bckground: Premature ovarian insufficiency (POI) occurs in 1% of cases in women aged 35–40 years and a large number of women of this age are faced with the problem of reduced reproductive ability or losing reproductive ability and a number of other symptoms affecting overall health and quality of life. It has been proven that many genetic and external factors lead to the Hippo signal pathway disruption and follicle growth disruption, resulting in amenorrhea and menopause.

Methods: Our study provides concise summary of published data about experimental evidences for the restoration of reproductive ovarian function in women with compromised reproductive health. The database used was Pubmed where full text articles and English-written reviews published between 2004 and 2018 were preferred. The MeSH (Medical Subject Headings) terms used were 'ovarien rejuvenation'," ovarien follicle activation', 'female germline stem cells', 'stem cell therapy' and 'ovarien insufficiency', either alone or in combination. The references of the articles were also considered when searching for the most relevant articles.

Results: Exposure of ovarian tissues to autologous concentrated growth factors and autologous stem cells results in the Hippo signal path disruption and stimulation of revived follicle growth and improvement of ovarian reproductive function. *In vitro* ovarian activation represents autologous genetic treatment of the gonadal tissue to restore reproductive and endocrine ovarian function. Among the sleepy follicles, ~ 0.1% of the follicles were selected for activation. Since patients with POIs (primary ovarian insufficiencies) have <1,000 residual follicles, growth factors and maternal cells can activate sleepy follicles. SEGOVA acts on the intracellular signaling system. The use of the SEGOVA method (ovarian *in vitro* activation by autologous growth factors and autologous stem cells) leads to regeneration and improvement of the reproductive function of the ovaries.

Conclusion: *In vitro* ovarian activation represents autologous genetic treatment of gonadal tissue to restore reproductive and endocrine ovarian function. Oogenesis depends on the proper genetic control. Ovarian function is achieved by the formation of ovarian cells, which is associated with a specific hormone activity. The primitive status of primordial follicles is characterized by communication with the surrounding granulocyte cells and numerous mechanical and chemical factors that control the progression of their cell cycle. Autologous growth factors and autologous stem cell therapy activate genetic pathways, initiate, and promote the development and differentiation of ovarian cells, resulting in improved endocrine status and ovarian function.

Keywords: *In vitro* activation, Ovarian rejuvenation, PRP, Segova, Stem cells, Tissue engineering.

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Introduction

Ovaries are specific organs, with very important reproductive and endocrine roles. Premature aging of the ovary may develop as a result of various hereditary, immunological, infectious, endocrine, and iatrogenic causes, which leads to the disease—premature ovarian failure (POF).

Natural cessation of the ovarian function occurs on an average at about 50 years of age, at which time, postmenopause occurs. Menopause occurs when so few viable follicles remain that the ovaries are no longer able to keep maturing and releasing eggs in response even to very high levels of luteinizing hormone and follicle stimulating hormone (LH and FSH). The monthly cycle comes to an irregular halt, and the ovaries' production of estrogen plummets, leading to the classic menopausal symptoms: hot flashes, insomnia, mood changes, and loss of vaginal lubrication, tissue elasticity and pH, as well as to the loss of bone mass and quality that culminates in osteoporosis.

Menopause shares much in common with major age-related health problems, inasmuch as they all result from the accumulation of cellular and molecular damage in our tissues over time. As it is well known, damage of certain organs or tissues causes the diseases. The ovary ages almost 50% faster than other tissues and organs of the body. Most likely, the nature linked reproductive and endocrine role in this manner, to prevent reproduction in an advanced age, with a greater risk for the mother and fetus.

[1]Department of Gynaecology Polyclinic, Korak do Života, Tuzla, Bosnia and Herzegovina

[2]Renova Hospital, Serbia

[3]MediGroup, Serbia

[4,6]Medical School, University of Belgrade, Serbia

[5]Military Medical School, Serbia

[7]MediGroup, Serbia; Dubrovnik International University, Croatia

Corresponding Author: Suada Tinjić, Department of Gynaecology Polyclinic, Korak do Života, Tuzla, Bosnia and Herzegovina, Phone: +387 62 424 837, e-mail: drtinjic@ginekologija.ba

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Oogenesis

For decades, it has been believed that the woman's reproductive and endocrinologic potential was entirely dependent on the size of the stock (pool) of primordial follicles in the ovary.[1] The paradigm that has prevailed in the scientific world about the existence of a consistent number of primordial follicles, established during embryonic and fetal periods, was in many ways changed by works done by Tilly's group, demonstrating the existence of germline or oogonial stem cells.[2]

Physiology—Activation, Differentiation, Maturation

Human ovaries contain follicles as basic functional units. The total number of follicles is determined early in life, and follicle depletion leads to reproductive senescence. Human follicles begin the development during the fourth month of fetal life, and each human ovary contains ≈400,000 follicles at birth. Unknown intraovarian mechanisms activate a small number of dormant primordial follicles at a rate of ≈ 1,000 per month to initiate growth, and follicle depletion occurs at menopause when <1,000 follicles remain.[3] For follicles not activated, the default pathway is to remain dormant for years or decades. Once activated, primordial follicles develop through the primary and secondary stages before acquiring an antral cavity.[4] After gonadotropin stimulation, a few antral follicles reach the preovulatory stage and respond to the preovulatory gonadotropin surge to release mature eggs for fertilization.

The Hippo-YAP pathway mediates the control of cell proliferation by contact inhibition as well as other attributes of the physical state of cells in tissues. Several mechanisms sense the spatial and physical organization of cells, and function through distinct upstream modules to stimulate Hippo-YAP signaling: adherens junction or cadherin–catenin complexes, epithelial polarity and tight junction complexes, the FAT-Dachsous morphogen pathway, as well as cell shape, actomyosin, or mechanotransduction. Soluble extracellular factors also regulate Hippo pathway signaling, often inhibiting its activity. Thus, the Hippo-YAP pathway senses and responds to the physical organization of cells in tissues and coordinates these physical cues with classic growth-factor-mediated signaling pathways.

Reduction of the number of primordial follicles leads to failure of ovarian function and, ultimately, to menopause. Reduction of the number of primordial follicles is associated with cellular and molecular damage of the ovarian tissue, which gradually reduces the possibility of tissue to perform its functions. Removal or correction of these defects by biotechnological regenerative processes may lead to recovery of ovarian function.

Therapy

As already mentioned, it is considered that menopause has a lot in common with the main health problems related to aging, which is the result of the accumulation of the cellular and molecular damage within tissues over time. Damage caused by aging gradually degrades the capacity of each tissue to perform its normal function with time. Contemporary attempts to maintain female fertility and delay or eliminate symptoms of the menopause use biotechnological methods to repair or replace damaged cells (especially egg-cells) and tissues (follicles), whose degradation, associated with aging, leads to menopause.

Growth Factors—Autologus Platelet-rich Plasma (PRP) Therapy

PRP is a serum which includes platelets (and lymphocytes), coagulation factors, and proteins. The platelets do not have nuclei, but they contain nearly a thousand signaling proteins, small granules (α, δ, and λ). In 50 up to 80 α granules contained in one platelet, there are almost 30 types of growth factors (GF) related to hemostasis. These growth factors are also produced in other tissues, such as adipose tissue or liver, and they have a very short half-life.

Platelet levels in PRP are 5–8 times higher than the physiological level. During the wound healing, platelets' response is important for the initiation of the process. In addition to the procoagulant effect, they are an important source of growth factors, such as platelet-derived growth factor (PDGF), transforming growth factor-b (TGF-b) 1 and 2, and vascular endothelial growth factor (VEGF). Adding calcium chloride and thrombin to PRP automatically activates the alpha granules. Their activation causes the release of the aforementioned biological growth factors, PDGF, TGF-B, VEGF, insulin-like growth factor I, epidermal growth factor (EGF), and epithelial cell growth factor. The most active of these factors is PDGF, which stimulates chemotaxis, mitogenesis, and the replication of stem cells. In addition, the PDGF stimulation of VEGF enhances angiogenesis, by stimulating fibronectin, it increases cell proliferation and modeling. The activity of other cytokines TGF-b1 and -b2 is associated with an increase in the activity of mesenchymal stem cells (MSCs).[5] A certain number of studies have pointed out the importance of leukocytes in PRP. On the one hand, their combat against infection is important, and, on the other hand, immunoregulation. Leukocytes produce large amounts of VEGF. Considering the abundance of platelet stimuli, VEGF, fibroblast growth factors, and inhibitors endostatin and thrombospondin-1, additional VEGF from leukocyte origin may be of the crucial importance for angiogenesis.[6] At the beginning of this decade, Spanish authors were attempting to improve the chances of reproductive success of autotransplanted ovarian tissue by adding PRP to substrate. At that time, the announcement about birth of the first child from pregnancy resulted from the preparation of the mesosalpinx tissue with PRP for acceptance of its own unfrozen ovarian tissue (previously removed during repeated surgery for spread endometriosis) was made.[7] In an elegant study, Iranian scientists have demonstrated the effect of PRP on oogenesis. This study proved positive effects of PRP on growth and survival of isolated early human follicles in a 3D culture system. PRP promotes the development of isolated human primordial and primary follicles to the preantral stage.[8] This year, the group from the USA and the UK has published a hormonal improvement, and oocytes and embryos obtaining in four patients after intraovarian PRP applications, and one pregnancy. Evidence of improved ovarian function was noted in all who received intraovarian PRP, possibly as early as 2 months after treatment.[9] This group also conducts a PRP-based autologous treatment used in combination with a stimulated IVF sequence and preimplantation genetic screening to treat infertility in women experiencing menopause, perimenopause, and POF. Spanish authors have made an announcement of four pregnancies achieved using this rejuvenation method.[10]

Stem Cell Therapy

Stem cell therapy is a direct way to combat losses of viable oocytes. The theory of "No new eggs" was first published in 1951 by the influential anatomist Solly Zuckerman, at the University of Birmingham, UK. He claimed that women cease to produce oocytes after birth. The loss of cells over a certain limit, due to the regular activation of a certain number of them during each period, leads to a loss of mutual communication among the oocytes, follicles, and regulatory centers in the brain. It is followed by atrophy of the follicles and the cessation of the sex hormones production, which leads to menopause.

Awakenng "Oogonial Stem Cells"

No new eggs' theories have been strongly challenged in the last decade, mostly by studies of Jonathan Tilly. He began to question

the conventional wisdom on this subject when he conducted studies in mice at different ages, and found that the math on the follicle counts did not add up: when he compared the rate at which follicles were degenerating with age with the actual number of remaining follicles at a given age, the aging mice had more remaining follicles than could be accounted for by simply taking the number of follicles present early in life and subtracting the number that his studies showed were being lost every year to degenerative aging processes. Moreover, cases were being reported of women who had regained their fertility after having had it destroyed by cancer therapy: months or years after being rendered infertile and suffering with menopausal symptoms, women who had received bone marrow transplants would spontaneously begin cycling again, with several cases of spontaneous pregnancies reported. Then in 2006, Tilly reported the results of a surprising study that explained these phenomena. An earlier study by Tilly had suggested that stem cells in the bone marrow transplants were homing to the ovaries and somehow developing into new, donor-derived follicles. But when other investigators repeated his study using more precise ways of tracking those cells, they found that while the transplanted cells were indeed reaching the ovaries, they were behaving more or less like blood cells, with no evidence of the donated cells developing into follicle or egg cells. In his 2006 study, Tilly found a possible way to resolve that contradiction. He simulated chemotherapy in mice, making them infertile, and then gave them bone marrow transplants, using bone marrow from mice whose cells had been modified to produce a fluorescent protein that allows scientists to track them and their genetic progeny. Then he housed these treated mice with males and let them breed. But consistent with his critics' findings, the newborn mice did not bear the telltale fluorescent protein in their cells, indicating that the pups could not have been generated from egg cells that were derived from the bone marrow transplant cells.

In 2012, Tilly performed painstaking studies of adult mouse and human ovarian tissue, and reported the presence of a rare population of cells in the ovaries that expressed genes in a pattern similar to the primitive egg cell precursors that are found in embryos, and that had the ability to replicate. And after isolating and culturing these cells, Tilly was able to get them to develop into cells that bore multiple hallmarks of being actual egg cells. To test the human cells' ability to actually develop into egg cells, Tilly tweaked them to express a fluorescence tracking protein, and nested them in with ovarian tissue left over from women of childbearing age who had recently undergone sex-reassignment operations. Then, he took the cocultured mystery cell/ovarian tissue cultures and injected them together into mice. Remarkably, the two tissue types together developed into follicles containing egg cells that expressed the fluorescent-labeling protein, consistent with the idea that these "oogonial stem cells" (OSCs) really do have the potential to develop into egg cells under the right conditions.[11–13] Using the induced pluripotent stem cells (iPS) technology, in 2012, Japanese scientists were able to differentiate the deep skin layers cells to the embryonic stem-like cells in the mice.[14] These researchers have taken advantage of genetic stimulation to form iPS in the early embryonic cells, which later became oocyte precursor—primordial germ cell-like cells (PGCLCs). These cells are brought into contact with the ovarian tissue embryonic cells without germ cells, thus creating a laboratory reconstructed ovary. After transplantation of reconstituted ovarian tissue to mouse, the production of early-stage egg cells commenced in it. Using stimulation and IVF, functional oocytes and embryos were obtained, and with the use of surrogate mothers, they produced viable offspring. Currently, technologies for the corrections of developmental clock of differentiated cells are developed, providing them with the possibility of dedifferentiation with developmental potential of embryonic stem cells. Further differentiation into specific cells is induced by chemicals, followed by exposure to a structured environment to direct maturation in a certain direction. For now, the research with this approach has progressed the most, in the area of diabetes type I and Parkinson's disease treatments.

Tissue Engineering

This approach focuses on the replacement or modification of an organ or tissue. A group of scientists from the Stanford University School of Medicine and St. Marianna University School of Medicine in Kawasaki, Japan, have reported the ability to generate new follicles from ovarian tissue from women with primary ovarian insufficiency. After preliminary studies in mice, the researchers surgically took ovarian tissue from 27 such women, and divided the tissue into smaller units. The separation of the tissue reduced the expression of a regulatory gene called Hippo, whose normal function is to prevent excessive growth of tissues. With Hippo signaling turned down, a cascade of growth factors in the ovarian tissue was unleashed, and follicles began to grow. They then bathed the tissue with a drug that activates AKT, a signaling molecule that promotes survival and inhibits defensive "cellular suicide" (apoptosis). Consistent with what they had seen in mouse studies, this treatment causes dormant "proto-follicles" in the tissue to become active, further promoting follicle growth. The researchers then transplanted this reactivated tissue with its newly generated follicles underneath the thin outer membrane of the fallopian tubes. Over the course of the next 6 months—and, in several cases, within weeks—eight of the women began experiencing the growth of new follicles from the reimplanted tissue. Patients were treated women with FSH and human chorionic gonadotropin (HCG), and, after IVF procedure, delivery of the healthy baby was reported.[15] The Japanese group showed that the fragmentation of mouse ovaries leads to actin polymerization and interruption of Hippo signal. Disruption of this signaling pathway leads to the increased expression of downstream growth factors, promotion of follicle growth, and maturation of oocytes. After laparoscopic ovariectomy, parts of the ovarian cortex were vitrified. After thawing, in the framework of preparation for autografting into mesosalpinx, the fragmentation and exposure of AKT by simulating substances (Stanford based) was done during 2 days. Laparoscopic autotransplantation of the activated tissue in mesosalpinx was done in this study. After the transfer, three pregnancies occurred: one of which ended in miscarriage, while the two children born with normal outcome, until the year 2015.[16]

The Segova approach, published by our group, consists of PRP growth factor ovarian therapy, and stem cell ovarian therapy and *in vitro* ovarian activation.

Segova PRP Therapy

In the Segova PRP process, special systems and machines for the separation of specific cell lines are used, which allows for the increase of the concentration of the desired cells (and, therefore, of the growth factors gained from them) up to 18 times the initial concentration. This approach differs from the majority of other ovarian PRP therapies. The majority of systems for the separation of growth factor-rich cells are based on specially prepared vials containing blood samples. The blood samples are then put in a centrifuge at a specific rate of rotation for a given time. This usually

increases the concentration of cells up to 2 or 3 times. Segova PRP process allows us to choose the appropriate concentration for each individual patient, which is usually about 7 or 8 times more than the initial concentration. The effect of the growth factor depends on the concentration of it in the active substance introduced back into the patient. The use of sophisticated technology, similar to that used for the separation of stem cells, allows for a significantly higher concentration and effect of PRP as part of the Segova program.

Segova Stem Cell Therapy

Major difficulty with stem cell therapy is to maintain cell viability, properties, and function of stem cell before and after implantation *in vivo*. Once stem cells are isolated from the native tissue environment, they quickly lose the niche and function they had when growing in culture dishes. In addition, they shorten cell lifespan because of over-expansion *in vitro*. Furthermore, cellular DNA tends to be unstable during long-term culture. Such cells implanted in the host lead low rates of cell survival, and poor outcomes in-growth, homing, differentiation, and paracrine effects. Segova program overcomes these problems by performing autologous stem cell therapy without the incubation.

In Vitro Activation

Segova *in vitro* activation uses cortical ovarian biopsy to obtain tissue. Conservative laparoscopic surgery with partial decortication instead of ovariectomy is performed, allowing orthotopic instead of heterotopic approach during re-transplantation. Second, instead of chemical (Stanford based) stimulation of AKT pathway, autologous PLRP growth factors are used. Third, bone marrow mesenchimal stem cells are obtained at the same time. The second laparoscopic operation is avoided, using needle injection under color Doppler ultrasonic guidance, for ovarian autologous orthotopic transplantation. During re-transplantation procedure, bone marrow concentrate, along with mesenchimal stem cells, is injected into the ovary. We have obtained four healthy newborns (out of 36 postmenopausal patients), using a combination of PRP, MSCs , and autologous *in vitro* activation. Our team has made the announcement about obtaining the first embryo using the genetic tissue engineering, with the use of autologous growth factors for the activation of the AKT genetic pathway.[17]

Cell Encapsulation

The rejuvenation biotechnologies we have explored so far involve replacing egg cells, or whole follicles, or even whole ovaries with new tissue, which would restore both fertility and normal, youthful hormone production. However, the two cell populations involved in the production and release of release sex hormones under the orchestration of FSH and LH are part of the follicle itself, and their release is not directly tied to ovulation. If these cells could be replaced and maintained in the ovaries, they could potentially carry on producing sex hormones and maintain the normal system of feedback between the ovaries, those hormones, and the regulatory centers in the brain, even with no egg cell replacement. Scientists in the USA have started the development of implants of healthy follicular cells into small encapsulated devices. These devices are separating the passage of hormones through the membrane transplanted cells from the recipient's own cells, but also allow. A system like this would allow the implanted cells to respond to LH and FSH by producing sex hormones in the normal, regulated way, and for those sex hormones to be released in the blood, while keeping the transplanted cells isolated from the body's immune system. Engineered multilayer ovarian tissue secretes sex steroids and peptide hormones in response to gonadotropins. The advantage of such a system is that the implanted cells could be taken from any donor, without the worry of immunological rejection, since the patient's immune system would never "see" the donated cells. Chinese scientists have published their results with the ovarian cell microcapsule system. Most notably, there are now several such systems being tested in human clinical trials as a way to transplant fresh insulin-producing β-cells into patients with diabetes, replacing cells destroyed by autoimmune disorders or by decades of overtaxing their capacity. Although it is still early days, this research could well lead to day when women can have their physiologically regulated, youthful hormone levels and balance restored and maintained throughout their lives.[18,19]

Future

Accumulating evidence now suggests that a novel cell-free therapy, MSCs—secreted exosomes, might constitute a compelling alternative because of their advantages over the corresponding MSCs. There is also the phenomenon of mitochondrial transfer from MSCs to neighboring stressed cells, which thereby aids in cellular repair and regeneration of different organs.[20,21]

Conclusion

Using *in vitro* ovarian activation with autologous growth factors and autologous cell stem cells in patients with POF, their own ovarian cells were successfully obtained. *In vitro* activation has yielded successful results in patients with infertility, after *in vitro* ovarian activation, mature oocytes have been created and after *in vitro* fertilization and embryo transfer (IVF and ET), ending the pregnancy with healthy children. The method can also be applied to patients with POF who do not want to achieve reproductive function but to restore the function of hormones and diminishing the quality of life and general health. *In vitro* ovarian activation has a future in the treatment of POF, and less invasive procedures should be developed.

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