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Multivitamin Supplementation and Fertility Outcome: A Retrospective Single-Center Cohort Study and the Clinical and Medicolegal Value of Nutritional Counseling

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Abstract: Resveratrol can beneficially affect growth and follicle development and lead to improved sperm function parameters in pre-clinical studies, while information from clinical studies is still inconclusive. This study aims to evaluate the biological and clinical impact of a resveratrol-based multivitamin supplement on level II assisted reproduction cycles (IVF and intracytoplasmic sperm injection [ICSI]). A retrospective, case-control study, involving 70 infertile couples undergoing IVF/ICSI cycles, was conducted at the Assisted Reproductive Center, Obstetrics and Gynecology Unit-Villa Sofia-Cervello Hospital in Palermo. The study group underwent pre-treatment with a daily nutraceutical based on resveratrol, whereas the control group received 400 mcg/day of folic acid. Primary endpoints to be evaluated were the number of mature follicles developed (>16 mm), total oocytes and Metaphase II (MII) oocytes retrieved, fertilization rate, number of embryos/blastocysts obtained, and semen quality. Secondary objectives in our evaluation were the duration and dosage of gonadotropins, the starting dose, the number of blastocysts to be transferred and frozen, implantation rate, and, ultimately, biochemical and clinical pregnancy rates. In the study group, a significantly higher number of mature follicles, oocytes, and MII oocytes were collected compared to the control group. In the study group, a higher fertilization rate as well as higher numbers of cleavage embryos per patient, blastocysts per patient, and frozen blastocysts were obtained. In the study group, a shorter administration time and lower dosages of gonadotropins required to reach follicle maturity were also observed compared to controls, with fewer dose adjustments during stimulation compared to the starting dose. No significant differences were found in biochemical or clinical pregnancy rates. A 12-month period of dietary supplementation with a resveratrol-based multivitamin nutraceutical leads to better biological effects on ICSI cycles.

Keywords: multivitamin supplementation; resveratrol; in vitro fertilization (IVF); intracytoplasmic sperm injection (ICSI); nutritional counseling

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1. Introduction

Nutrition has a huge impact on physical and mental health. It is through diet that humans can provide the body with the energy and nutrients needed to perform all its functions. It should therefore not be surprising that food can have a huge influence on reproductive capacity. The link between nutrition and fertility has been widely studied in the scientific field, and there is a lot of evidence in its favor. Healthy eating habits and a lifestyle can greatly enhance one's ability to conceive. Nonetheless, many people often believe they are following a healthy diet, but most of the time their diet is not suitable for improving fertility. A diet aimed at enhancing procreative capabilities, as well as the physical and mental well-being of patients seeking motherhood, should include foods rich in specific nutrients needed for reproduction and hormonal balance, fetal development, and the health of eggs and sperm [1–3]. Despite growing evidence as to the impact of eating habits on human fertility, few studies have examined the public health implications of such an association.

A thorough analysis as to the current scientific evidence on the associations between dietary intake and fertility points to major challenges in the realm of public health. We therefore have set out to review the literature on the relationship between diet and fertility, supported by a retrospective single center cohort study based on our personal experience (IVF Center Villa Sofia Cervello Hospital, Palermo, Italy). This study aligns with currently available research findings and ultimately outlines a set of evidence-based recommendations to address these issues.

A diet relying mostly on unsaturated fats, whole grains, vegetables, and fish has been associated with improved fertility in both women and men. While current evidence on the role of dairy products, alcohol, and caffeine is still inconclusive, saturated fat and sugar have been associated with poorer fertility outcomes in women and men. In addition, obese women and men, i.e., with a body mass index (BMI) $\geq$ 30 kg/m^2 [4], have a higher risk of infertility, as do underweight women (BMI < 20 kg/m^2) [5]. Diet and BMI influence outcomes of clinical infertility treatment. Women who belong to underrepresented minority groups, with lower income and lower education levels overall, have significantly higher rates of infertility than non-Hispanic white women with higher incomes and higher education levels. In light of this, it is worth remarking on the importance of relying on sound nutritional counseling into both clinical guidelines for infertility and national dietary guidelines for people of reproductive age. Further studies on diet, reproductive health, and dietary supplementation can improve our ability to optimize existing fertility programs by providing personalized care to women and men in at-risk groups. Healthy eating habits can greatly contribute to physical and psychological well-being overall, and even increase fertility, in both males and females. Incidentally, infertility can have adverse psychological repercussions as well [6]. A direct consequence is that through diet we can take in the right nutrients needed to support hormonal balance and the health of oocytes and sperm.

2. Materials and Methods

The study centered around 70 patients who were enrolled in the pre-treatment period. Patients took two tablets/day for a period of 9 months in the pre-treatment phase. The study is centered around the collection and recording of a series of information and clinical data according to the times and methods described. Ultimately, the duration of supplementation for the couples that could finally be included in the study, in order to standardize the sample without interruption (and therefore drop-out), was 9 months.

The study lasted for a 12-month period and was subdivided as follows:

Pre-phase of monitoring and enrollment of subjects. In this phase, treatment with a multivitamin compound GENANTE, (S&R Farmaceutici S.p.A., Bastia Umbra, Perugia,

Italy) had begun. Such a compound is a formulation based on trans-resveratrol supported by a magnesium hydroxide matrix, designed to effectively address the solubility problem of resveratrol, thus substantially improving its dissolution rate, solubility, and, ultimately, bioavailability.

Observed duration period was 270 days during which patients took two tablets each day. Table 1 lays out patient characteristics for each group.

Table 1. Patient characteristics between treatment and control groups.

Patient Characteristics	Treatment Group	Control Group	*p*-Value
Age (mean)	31.5	30.5	n.s.
BMI (mean)	24.6	24.9	n.s.
AMH (mean)	1.4	1.4	n.s.

2.1. Inclusion Criteria

The study only included infertile women aged 18 to 42 years, with a BMI between 18 and 30 kg/m^2, with fewer than 3 previous oocyte retrievals, basal FSH < 10 IU/L, number of antral follicles (ranging 2–10 mm in size) > 10, patients diagnosed with "poor ovarian response" (less than 3 oocytes retrieved in previous attempts or who had an estradiol peak at the end of stimulation < 500 pg/mL), and a regular uterine cavity for the expected reproductive function. In addition, patients who underwent hysterosalpingography, hysterography, or hysteroscopy performed no earlier than 12 months after the procedure; had hematological and biochemical parameters within normal limits; and with euthyroidism (with or without treatment) were included in the study. No restrictions regarding the indication of infertility were implemented.

2.2. Exclusion Criteria

The following exclusion criteria were applied: primary or secondary ovarian failure, previous episodes of ovarian hyperstimulation syndrome, presence of ovaries inaccessible to oocyte retrieval, persistent ovarian cyst > 20 mm, presence of sactosalpinx, heterologous fertilization, contraindication to pregnancy, atypical genital discharge of unspecified cause, uncontrolled thyroidism, presence of neoplasms, severe alteration of liver or kidney function, or intake of drugs that may interfere with the study.

2.3. MAP Intervention Protocol

Patients underwent ovarian stimulation treatment with gonadotropins (recombinant FSH, urinary FSH, FSH + LH) starting from the 2nd–3rd day of the menstrual cycle. The initial dose may vary between 150 and 300 IU, in relation to the ovarian reserve and BMI, as previously assessed. On the 5th day of stimulation, follicular ultrasound monitoring began evaluating the ovarian response. Dosages were adjusted in relation to the outcome of the ultrasound results. In the presence of at least 2 follicles with a diameter of 18 mm, human chorionic gonadotropin was administered for the final induction of ovulation. After 34–36 h, vaginally performed oocyte collection was performed under ultrasound control and local vaginal anesthesia.

Collection of Biological Material: The oocytes were deprived of the cumulus oophorus and subsequently classified by maturity, in accordance with the ESHRE 2012 guidelines [7]. A high-quality oocyte, in metaphase II (MII), was defined as a round oocyte, of normal size, with a regular polar body in the perivitelline space, a homogeneous ooplasm without irregularities, and an adequate thickness of the zona pellucida [7]. These were subsequently fertilized using the IVF/ICSI procedure. Then, 18 h after insemination, fertilization was verified with the formation of pronuclei. Then, the egg was left in culture to verify regular

cell replication. All embryos obtained were re-evaluated on the 2nd day, regardless of the day chosen for transfer. The reassessment consists of the consideration of the number of developed blastomeres, the percentage of fragmentation (Grade A: ≤10%; Grade B: >10% to ≤25%; Grade C: >25% to ≤50%; Grade D: >50%), and the appearance of cell division (typical or non-typical), in accordance with consolidated guidelines [8]. The standard ICSI procedure was performed using sperm from the male partner. Embryo transfer was performed between days 3 and 5 after collection of the oocytes, taking into account the characteristics of the patient and the embryo.

Only embryo transfers from fresh cycles were considered. Beta hCG levels were measured to diagnose pregnancy. Clinical pregnancy was established by the presence of a gestational sac, an embryonic pole, and cardiac activity on transvaginal ultrasound, after dosing of beta hCG performed 14 days after the transfer. Finally, luteal phase support was achieved by administering 50 mg/day of intramuscular natural progesterone (ProntogestV R, IBSA, Lugano, Switzerland) or 800 mg/day of micronized progesterone (ProgeffikV R, Effik Italia, Milan, Italy) and maintained until the day of hCG measurement.

3. Results

3.1. Primary and Secondary Outcomes

Primary outcomes

- Effects, intended as biological differences, of GENANTE® treatment on biological parameters deriving from the study of oocyte quantity and quality.

Secondary outcomes

- Differences between the two groups in terms of gonadotropin dosage, in the timing of administration/dose adjustments.
- Improvement of the associated reproductive process; i.e., biochemical and/or clinical pregnancy.

3.2. Statistical Analysis—Primary Endpoint Analysis

The analysis conducted on the number of mature follicles (>16 mm) highlighted a mean value of 9.4 in the treated group and 5.8 among the controls. The difference of 3.6 (95% CI: from 2.6 to 4.5) was statistically significant ($p < 0.0001$).

Total oocyte count showed a mean value of 9.0 in the treated group and 5.3 in the control group. The difference of 3.7 (95% CI: 2.8 to 4.7) was statistically significant ($p < 0.0001$). The number of M2 oocytes showed a mean value of 7.3 in the treated group and 3.9 in the control group. The difference of 3.4 (95% CI: 2.6 to 4.2) was statistically significant ($p < 0.0001$).

The fertilization rate was 5.7 in the treated group and 2.8 in the control group. The difference of 2.9 (95% CI: 2.3 to 3.6) was statistically significant ($p < 0.0001$). Table 2 outlines a comparison analysis of primary endpoints between treatment and control groups.

Table 2. Comparison analysis of primary endpoints between treatment and control groups.

				95% CI		*p*-Value
Primary Endpoints	**Treatment Group (Mean Value)**	**Control Group (Mean Value)**	**Difference in Mean Values**	**Min**	**Max**	
Number of mature follicles	9.4	5.8	3.6	2.6	4.5	<0.0001
Total oocyte count	9.0	5.3	3.7	2.8	4.7	<0.0001
Number of M2 oocytes	7.3	3.9	3.4	2.6	4.2	<0.0001
Fertilization rate	5.7	2.8	2.9	2.3	3.6	<0.0001

3.3. Secondary Endpoint Analysis

The analysis of the duration of gonadotropins showed a mean value of 9.3 in the treated group and 9.5 in the control group. The difference of −0.2 (95% CI: −0.36 to −0.04) was statistically significant at the 5% level ($p < 0.05$). The analysis of gonadotropin dosage showed a mean value of 191.8 in the treated group and 234.6 among the controls. The difference of −42.9 (95% CI: from −65.4 to −20.4) was statistically significant ($p < 0.001$).

The number of frozen blastocysts showed a mean value of 3.4 in the treated group and 1.2 in the control group. The difference of 2.2 (95% CI: 1.8 to 2.7) was statistically significant ($p < 0.0001$). Table 3 lays out a comparative analysis of secondary endpoints between treatment and control groups.

Table 3. Comparative analysis of secondary endpoints between treatment and control groups.

				95% CI		*p*-Value
Secondary Endpoints	**Treatment Group (Mean Value)**	**Control Group (Mean Value)**	**Difference in Mean Values**	**Min**	**Max**	
Duration of gonadotropins	9.3	9.5	−0.20	−0.36	−0.04	<0.05
Gonadotropin dosage	191.8	234.6	−42.9	−65.4	−20.4	<0.001
Starting dose	174.6	198.8	−24.1	−49.4	1.1	n.s.
Transferred blastocysts	1.4	1.2	0.17	−0.001	0.34	0.05
Frozen blastocysts	3.4	1.2	2.2	1.8	2.7	<0.0001
Beta hCG	0.34	0.31	0.03	−0.13	0.19	n.s.

Primary endpoints: The study group showed higher numbers of mature follicles, total oocytes retrieved, and M2 oocytes retrieved compared to controls. The study group also showed a higher fertilization rate as well as higher numbers of cleavage embryos per patient, blastocysts per patient, and frozen blastocysts.

Secondary endpoints: The study group was administered a lower total amount of gonadotropins during stimulation compared to the control group.

The time of administration of gonadotropins, necessary to reach follicle maturity, was shorter in patients belonging to the study group compared to controls; it can also be observed that patients in the study group underwent fewer adjustments to the initial dosage of gonadotropins (less frequent adjustments of the "starting dose"), compared to controls. No significant differences in biochemical or clinical pregnancy rates were found between the two groups.

4. Discussion

The World Health Organization (WHO) considers the absence of conception after 12/24 months of regular unprotected sexual intercourse as a pathological condition [9]. In such cases, infertility is diagnosed, which can be male, female, or couple-related infertility. In Italy, the infertility rate is roughly 15%, compared to a world average that ranges from 10 to 12%. Although infertility is often associated with women, male physiological factors reportedly account for ~25% of cases, which highlights the need to focus on both partners [10,11]. Thus, it is now quite common to use the phrase "couple infertility". Infertility rates have remained high despite the increase in the use of assisted reproductive technologies (ART) in recent years [12]. For this reason, research has long sought to identify modifiable risk factors for infertility, and nutritional factors have long been investigated. Evidence points out that diet and nutritional supplementation with nutraceuticals may play an important role in modifying fertility-related outcomes in both men and women. The

link between diet and fertility has been extensively researched over the years. A research study published in 2007 [13] has had a strong impact on subsequent examinations. In this study, 17,544 women with no history of infertility were followed for 8 years while they were trying to achieve pregnancy.

The study found that a tailored "fertility diet" model was associated with a lower risk of infertility due to an ovulatory disorder. The proposed diet included a higher consumption of monounsaturated fats rather than trans fats; plant-based rather than animal-based protein sources; low-glycemic carbohydrates; high-fat dairy products; and multivitamins and iron from plants and supplements.

A combination of five or more low-risk lifestyle factors, including diet, weight control, and physical activity, was found to be associated with a 69% lower risk, although the impact of rigorous dieting and exercising is still being investigated in terms of its potential benefits and contraindications as well [14].

The result of the study therefore demonstrated that a "fertility diet" model can favorably influence fertility in healthy women, as well as prevent most cases of infertility due to ovulation disorders. Particularly significant in that regard are the studies conducted by Erasmus MC-University Medical Center Rotterdam [15] and the one published by the *Journal of Nutritional & Environmental Medicine* [16], both of which found eating habits and healthy nutrition to be crucial factors in both female and male fertility. The access to in vitro fertilization (IVF) techniques for women at an increasingly advanced age is due to the tendency in our society to try to have children at an increasingly advanced age, when fertility is reduced. In fact, the reproductive capacity of the couple declines with age. Aging reportedly has a role in mitochondrial dysfunction [17]. Energy production through the mitochondrial respiratory chain is necessary in various phases of female gametogenesis [18].

During oocyte maturation, the number and functionality of mitochondria rapidly increase to prepare for the energy expenditure associated with early embryonic development [19]. Many fertility-related parameters are connected with mitochondrial metabolic status [20]. Some natural substances can effectively modulate mitochondrial activity. For instance, resveratrol has been reported to modify mitochondrial activity at various levels: transcriptional, through the expression of the SIRT family (sirtuin NAD-dependent deacetylase); functional, through the modulation of the respiratory chain; and epigenetic [20]. All such mechanisms positively affect resistance to apoptosis and result in greater energy availability [19].

Trans-resveratrol is poorly bioavailable due to reduced absorption mainly related to its low solubility and rapid metabolism characterized by its conversion to glucuronidated and sulfated compounds, which are generally inactive and with high renal clearance [21]. According to the Biopharmaceutical Classification System (BCS) [22], resveratrol belongs to class 2. It is part of those molecules characterized by low water solubility (about 30 mg/L), but with high membrane permeability (logP~3.1) [23]. In humans, following oral administration, resveratrol is detected in plasma after about half an hour, indicating that its absorption begins already at gastric level and reaches a peak plasma concentration sub-micromolar. Recently, a formulation named REVIFAST (Vemax Pharma, Belgrade, Serbia) has been developed based on the interaction of trans-resveratrol with magnesium hydroxide which, by modifying the gastric dissociation profile, increases its bioavailability. REVIFAST is able to solubilize faster and in larger quantities than trans-resveratrol alone, with significant advantages in biopharmaceutical terms. From a physical point of view, REVIFAST is a solid dispersion, i.e., a new formulation in which the active ingredient relies on support by high-safety inorganic material (magnesium hydroxide), which improves its

performance without any chemical alteration in the structure of the natural product, thus making it possible to fully harness the pharmacological properties of this molecule.

Vitamin B12 (cyanocobalamin) is necessary for the metabolism of odd-chain fatty acids and therefore necessary for energy metabolism and cell proliferation. Supplementation with folic acid and vitamin B-12 has been associated with positive reproductive outcomes in both natural pregnancies and those following treatment with assisted reproductive technology (ART) [24], and preliminary data show benefits even in terms of follicular output rate (FORT) in patients who took multinutrient supplementation (omega-3, coenzyme Q10, folic acid, selenium, vitamin E, and catechins) [25]. Particularly, vitamin B6 (pyridoxine) affects various biochemical reactions involving both the metabolism of amino acids and that of various other compounds. Higher levels of vitamin B6 have been found in fertile women compared to infertile women [26]. Vitamin D3 is well known for its role in maintaining calcium homeostasis and promoting bone mineralization. Growing evidence points to a correlation between vitamin D deficiency and decreased female fertility [27]. A 2024 study [28] shows how children of women with low levels of vitamin D during pregnancy were characterized by increased activity of the reproductive axis and faster postnatal growth of the genital organs. Minipuberty is a term that refers to the temporary and sex-specific activation of the hypothalamic–pituitary–gonadal axis, which is involved in the development of male and female genital organs. The study, the first of its kind, has taken into account minipuberty in the offspring of vitamin D-deficient women. Three matched groups of girls were included in the study population: those born to women with vitamin D deficiency (25OHD concentration less than 50 nmol/L), daughters of women with vitamin D insufficiency (25OHD concentration between 50 and 75 nmol/L), and daughters of healthy females (25OHD concentration between 75 and 150 nmol/L). Salivary concentrations of estradiol, progesterone, 17-hydroxyprogesterone, and androgens, as well as urinary concentrations of FSH and LH, were analyzed during the first 18 months of life (once a month in the first 6 months, bimonthly between months 6 and 12, and every three months thereafter). The size of the reproductive organs, ovaries, uterus, and breasts was assessed at every subsequent examination. In daughters of mothers with vitamin D deficiency, FSH, LH, and estradiol concentrations were higher and detectable for a longer period of time, while ovarian volume, uterine length, and breast diameter were found to be larger than in the other groups. Within the same detection period, females born to women with vitamin D deficiency had higher FSH levels than the daughters of healthy women, and such groups also showed different breast diameter. Such findings point to a low vitamin D status during gestation as a factor determining more pronounced and long-lasting activation of the reproductive axis and are also associated with an increase in the size of sexual organs, the extent of which is linked to vitamin D deficiency levels. The role of vitamin D deficiency in metabolic disorders and infertility in women with polycystic ovary syndrome (PCOS) has been supported by recent research data [29,30]. Both insulin resistance (IR) and endometrial receptivity seem to be favorably affected by vitamin D supplementation. On the other hand, excessively high levels of vitamin D seem to have a detrimental role on oocyte development and embryo quality. Therefore, we encourage low-dose supplementation (400–800 IU/day) especially in women with vitamin D deficiency who have metabolic disorders such as PCOS. Regarding reproductive health, we recommend vitamin D supplementation in selected populations, only during specific times of the ovarian cycle, to support the luteal phase. However, currently available clinical studies published so far are still somewhat inconclusive as to supplementation dosage and timing and further studies are needed to corroborate such correlations [31].

In women with a history of endometriosis, vitamin C and vitamin E supplementation has been linked to improved fertility rates. A 2021 study [32] enrolled 60 women

of reproductive age (15–45 years) with pelvic pain in this triple-blind clinical trial. They had stages 1–3 of laparoscopically confirmed endometriosis. Participants were randomized to either group A (n = 30), which was administered the combination of vitamin C (1000 mg/day, 2 tablets of 500 mg each) and vitamin E (800 IU/day, 2 tablets of 400 IU each), or group B (n = 30), which was administered placebo pills daily for 8 weeks. After treatment with vitamin C and vitamin E, a significant reduction in MDA and ROS was found compared to the placebo group. No significant decline was found in total antioxidant capacity after treatment. However, the severity of pelvic pain (p-value < 0.001), dysmenorrhea (p-value < 0.001), and dyspareunia (p-value < 0.001) significantly decreased in the treatment group after 8 weeks of supplementation. By virtue of their antioxidant capacity, vitamin C and E can "turn off" endometriosis, thus improving fertility prognosis in women undergoing treatment. Another recent study funded by the Division of Reproductive Endocrinology and Infertility at the University of North Carolina at Chapel Hill, evaluated the usefulness of omega-3 administration in women for the purpose of preserving fertility [33]. The study, which was not a randomized controlled trial, pointed to women who used omega-3 supplements as a more health-conscious population. We attempted to address this issue by adjusting our model for several factors. Additionally, the omega-3 fatty acid supplements used by TTC participants included several types and brands with varying dosages of omega-3 fatty acids. Women reported the type of supplement they were taking, but not the concentration of omega-3 in that supplement. Therefore, no comparison can be drawn between the dosage or a dose-response relationship in this study. However, omega-3 supplementation may represent a feasible and inexpensive modifiable factor for improving fertility. Randomized controlled trials are needed to further investigate the benefits of omega-3 supplementation for women seeking natural conception.

Folate plays an essential role in regulating amino acids and nucleic acids at the metabolic level and in one-carbon metabolism. Folate must be obtained from the diet, and supplementation is strongly recommended in populations at risk of deficiency due to of specific conditions. Folic acid is the synthetic form of the vitamin, usually incorporated into foods and supplements. In the body, it must be reduced to the bioactive folate derivative (6S)5-MTHF by cellular metabolism. Folate deficiency is linked to many health problems, such as neurological disorders, and may increase the risk of cardiovascular disease [34]. Patients of childbearing age and pregnant women, in addition to women with MTHFR polymorphism, exhibit the highest rates of folate deficiency. Folate supplementation is substantially helpful for the inhibition of neural tube defect (NTD) in pregnancy and for lowering homocysteine levels. Moreover, (6S)5-MTHF supplementation in pregnancy is advisable instead of folic acid, thanks to its ability to bypass the polymorphic enzyme blockade of folic acid metabolism. The use of (6S)5-MTHF can also mitigate the risk of deleterious effects of unmetabolized folic acid (UMFA) associated with the use of a supraphysiological dose of folic acid [34].

Methylation regulates numerous biochemical/physiological stages, and the importance of folates and one-carbon cycles for proper methylation has frequently been overlooked. Adding FA through nutrition has proved useful to lower NTD rates during pregnancy. However, the generation of circulating UMFA is now reason for concern, since it can compete heavily with the natural "active" folate metabolite, 5-MTHF, particularly in neonates. When assessing physiological effects by measuring serum folate values, fluorescence-based assays gauge UMFA along with other folate molecules, which can lead to results and misleading conclusions. Most IVF (in vitro fertilization) units recommend the intake of FA supplements before ART cycles, even in in high doses (5 to 15 mg) at times. It is therefore essential to take into account the possible impact of UMFA on germ cells in the long run. UMFA-induced sperm alterations in animal sperm have also been observed, and

such alterations appear likely to occur in humans as well. Differential sex-related abnormalities are clearly linked to varying degrees of gradual resetting of epigenetic marks between males and females [35–39]. It is worth focusing on how germ cells may be impacted, since the consequences/effects may not be easily perceived [40–43]. The effects on gametogenesis will only become apparent in the next generation, after reproduction of the offspring, about 25 years later. Furthermore, depending on the sex, epigenetic abnormalities can skip a generation and then resurface.

Synthetic FA can be an issue for MTHFR SNP carriers (especially for newborns) from a biochemical and physiological standpoint, since the initial phases of FA metabolism are already compromised. 5-MTHF can get over the initial strict metabolic phase and does not result in the accumulation of unmetabolized compounds; still, it is advisable to stich to a physiological dose of approximately 500 μg/day, which may be raised during the first trimester of pregnancy. In couples presenting " idiopathic infertility", the paternal effect should not be overlooked [44,45]. Routine testing of both partners for MTHFR SNPs is indicated, as treatment with 5-MTHFR may be substantially useful.

A 2022 randomized, double-blind, controlled study [46] has corroborated the potential value of myo-inositol and melatonin supplementation in PCOS patients undergoing IVF cycles. Polycystic ovary syndrome (PCOS) can bring about anovulation in women of reproductive age and is one of the pathological factors involved in IVF failure. In fact, patients with PCOS generally have poor quality oocytes, hence the need to rely on treatment aimed at improving the oocyte quality for such patients. Myo-inositol [47] and melatonin have been shown to be effective predictors of positive IVF outcomes, correlating with high oocyte quality. A 2022 review article [48] also points out that dietary supplementation of melatonin and vitamin D, by reducing oxidative stress and turning off chronic inflammation, could have a favorable effect on fertility, ensuring good reproductive function. Melatonin is an interesting compound, as is vitamin D, which has a pleiotropic activity and responds to light–dark cycles. From a scientific perspective, melatonin has an antioxidant value action capable of crossing the blood–brain barrier, tackling inflammation and interacting with the intestinal microbiome. Thus, a melatonin imbalance can signal a "dark deficiency", just as vitamin D levels can indicate whether or not a person has a "light deficiency". Doctors should therefore provide both nutritional and lifestyle recommendations to optimize melatonin intake for their patients.

A recent 2024 study [49] has pointed to a lower expression of melatonin receptors, in addition to reduced serotonin synthesis, as significant factors in "ovarian aging". The chief melatonin receptors that were accounted for are the MT1 and MT2 types. The study centered around the expression of genes and synthesis of MT1 and MT receptors, in addition to serotonin synthesis in the ovaries during ontogenesis. Histological materials from the ovaries of newborns; women of young and advanced reproductive age; and premenopausal, menopausal, and postmenopausal patients were analyzed. RT-PCR and immunohistochemistry were used to analyze MTI and MT receptors and serotonin expression and synthesis. Both serotonin synthesis, and MTI and MT2 receptors in the ovaries, have been found to significantly decrease during ontogenesis, with the sharpest decrease being reported in samples obtained from one-year-old infants, pubescent girls, and menopausal women. Moreover, a considerable 2.3- to 7.6-fold dip in the expression of the MTNRIA and MTNR1B genes in the ovaries has been reported in one-year-old infants, adolescents, and middle-aged women. Such data are highly valuable in order to gain a deeper understanding of the fundamental mechanisms through which aging affects the female reproductive system, and to further our search for molecules that predict aging, thus preserving fertility.

The value of nutrition plans as major factors for infertility patients has been supported by clinical guidelines, hence their relevance is clinical as well as medicolegal [50,51]. As a matter of fact, any therapeutic pathway aimed at restoring fertility or implementing a MAP plan enabling patients to achieve parenthood must be deeply rooted in evidence-based guidelines, best practices, and recommendations in order to be sound from a medicolegal standpoint [52]. Nutritional counseling is therefore of utmost importance in order to maximize the possibilities of successfully treating infertility. As recent findings published in the *Journal of the American Medical Association* show, a lower likelihood of pregnancy loss has been linked to pre-conception healthy dietary patterns before infertility treatment [53]. In light of such highly significant correlation, any failure to provide counseling as to such requirements could contribute to unfavorable outcomes and even result in litigation and negligence-based malpractice, especially ones possibly associated with poor or lacking nutrition [54,55]. From that perspective, dietary/nutritional counseling ought to be prioritized for patients with infertility to the same extent that other forms of reproductive counseling are implemented [56,57], particularly in light of the toll rising obesity rates are taking on fertility [58–60]. Against the backdrop of the post-COVID-19 pandemic era, it is worth mentioning the destructive impact of the pandemic on both fertility and infertility treatments [60–64]. Nutritional counseling should therefore be acknowledged as a key tool in making up for the losses brought about by the pandemic [65,66], and any such intervention needs to be thoroughly documented and provable, should litigation arise from adverse outcomes. It is in fact worth bearing in mind that particularly under civil statutes, the onus is placed on the doctors and facilities to prove compliance with evidence-based guidelines and recommendations [54,67,68]. Nutritional counseling is in fact a process with unique complexities [69], hence it needs to rely on highly trained professionals with a solid grounding in medical/clinical as well as psychological and behavioral sciences (e.g., evaluating and adapting to each patient's motivation and awareness/knowledge levels, seeking involvement from patients and maintaining such an involvement, and varying between counseling formats to best suit the profile of all involved) for each patient's needs to be met as effectively and beneficially as possible [69–71]. It is therefore worth encouraging the framing of more comprehensive, evidence-based global and regional weight control guidelines, in addition to including nutritional counselling indicators and criteria as an integral part of quality-of-care frameworks and blueprints both at the antenatal care and postnatal care levels.

Study Strengths and Weaknesses

The chief strength of this study is its focus on supplementation with a nutraceutical that has contributed to increasing reproductive outcomes, without significant side effects. This aspect is still somewhat under-researched and takes on even greater relevance in light of lower and lower birth rates, in Western countries especially. The main limitation has to do with data selection bias, which can influence the representativeness of the data with confounding and causality factors. In such respects, retrospective studies are usually particularly vulnerable because of the difficulties in controlling all the variables. This is in contrast to prospective studies, where a more rigorous analysis of the variables themselves can be framed and better control of the confounding effects can be obtained. In retrospective studies, such a range of control is limited. The outcomes can therefore be measured and assessed in a non-uniform way, which may result in substantial differences in the conclusions.

5. Conclusions

Pre-treatment adherence to a fertility-friendly diet has been linked to better chances of achieving a pregnancy, and even live birth, in patients undergoing ART. A 9-month period of dietary supplementation with a resveratrol-based multivitamin nutraceutical leads to better biological effects on ICSI cycles. In light of the worrisome declining fertility rates in Western countries (which are in large part influenced by socioeconomic factors beyond the scope of this manuscript), it is essential to maximize the opportunity to achieve pregnancy and parenthood for those who want to start a family. From that perspective, nutritional counseling is to be deemed an extremely valuable component of a multidisciplinary effort, aimed at providing strictly tailored personalized strategies to overcome infertility whenever possible. Such an undertaking needs to be deeply rooted in evidence-based guidelines and validated recommendations, so as to ensure the best therapeutic pathway as well as medicolegal viability of any such intervention.

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Review

Resveratrol and Female Fertility: A Systematic Review

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Abstract: Resveratrol is a natural polyphenolic compound that may have multiple influences on human health, including antiaging, anti-inflammatory, anti-neoplastic, antioxidant, insulin-sensitizing, cardioprotective and vasodilating activities. Growing evidence also suggests a potential positive effect of resveratrol on female fertility. The aim of the present study was to collate and appraise the scientific literature on the relationship between resveratrol and female fertility. We systematically searched Medline, PubMed, Web of Science and Embase from the databases' inception (1951, 1951, 1947 and 1900, respectively) until 9th May 2024. All in vivo or in vitro retrospective or prospective studies reporting the effects of resveratrol interventions on women's fertility were included. We ultimately incorporated twenty-four studies into a systematic review with a narrative summary of the results; of those studies, nine were performed on women seeking natural or assisted fertility, and fifteen were in vitro studies performed on human cells and tissues in different stages of the reproductive cascade. The current literature, though limited, suggests that resveratrol may play a role in female infertility. Specifically, it may significantly and positively impact reproductive outcomes, owing to its potential therapeutic effects improving ovarian function. Further studies are now needed to better understand resveratrol's effects and define the optimal dosage and periods of intake to maximize beneficial effects, as well as to prevent adverse outcomes on implantation, subsequent pregnancy and the fetus.

Keywords: resveratrol; 3,5,4-trihydroxystilbene; female fertility; female infertility

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1. Introduction

Resveratrol (3,5,4-trihydroxystilbene) is a natural polyphenolic compound present in a variety of plants, foods and drinks [1]. In plants, it is produced in response to ultraviolet radiation, injury, fungal or bacterial infection and it is predominantly found in the skin of grapes, blueberries, raspberries, mulberries and peanuts [2]. Resveratrol may have many effects on human health, including antiaging, anti-inflammatory, anti-neoplastic,

antioxidant, insulin-sensitizing, cardioprotective and vasodilating activities [3]. In particular, this antiaging activity is carried out through resveratrol's ability to improve cellular mitochondrial activity and trigger a series of molecular mediators capable of counteracting some of the most important metabolic mechanisms of aging [4–6]. Moreover, resveratrol exhibits antioxidant properties by scavenging free radicals and enhancing the activity of antioxidant enzymes. Additionally, it exerts anti-inflammatory effects through a variety of signaling pathways inhibiting pro-inflammatory cytokines [7]. Growing evidence suggests a potential positive effect of resveratrol on the infertile population, particularly females, by potentially enhancing ovarian function, improving oocyte quality and exerting protective effects against age-related fertility decline and polycystic ovary syndrome (PCOS) [8]. In particular, in vitro, resveratrol inhibits proliferation and androgen production by theca–interstitial cells, exerting a cytostatic but not cytotoxic effect on granulosa cells, while decreasing aromatization and vascular endothelial growth factor expression [8]. Interestingly, in vivo, resveratrol treatment has been found to reduce the size of adipocytes and improve estrus cyclicity in an acyclic rat model of polycystic ovary syndrome (PCOS), also increasing the ovarian follicular reserve and prolonging the ovarian life span [9]. Another study found that after resveratrol treatment, mitochondrial membrane potential and ATP content in oocytes of aging mice was increased, resulting in the restoration of oocyte quality without adverse effects in the animals or their offspring [10]. At low doses, resveratrol activates SIRT1, an NADH-dependent deacetylase, able to program cellular energy metabolism through transcriptional regulation of the pGC-1α gene, a master regulator of mitochondrial metabolic activity, suggesting the ability of resveratrol to optimize such activity [11]. SIRT-1 can activate LKB1 by deacetylation; LKB1 phosphorylates and activates AMP-dependent kinase (AMPK) in a reciprocal positive feedback relationship. Indeed, activated AMPK itself can increase the concentration of NAD+ by promoting further activation of SIRT-1. Both AMPK and SIRT-1 are involved in the activation of PGC-1α, which is responsible for the co-activation of nuclear respiratory factors (NRF-1 and NRF-2), which induce transcription of genes involved in mitochondrial biogenesis and mitochondrial transcription factor A (TFAM). This process is directly involved in mitochondrial DNA replication [11]. Ragonese et al. confirmed in vitro and ex vivo the mitochondrial activity of resveratrol on granulosa cells, showing that activation of SIRT-1 and AMPK by resveratrol promotes increased mitochondrial membrane potential, ATP production and mitogenesis. This suggests the impact of resveratrol as an energy enhancer for granulosa cells, which are essential for oocyte development and maturation [12].

Interestingly, a limited body of literature also suggests a role of resveratrol in enhancing male fertility by improving testicular function [13] and sperm quality [14]. However, there is no consensus on the usage and posology of resveratrol. Moreover, literature on the relationship between resveratrol and female fertility is contrasting. For these reasons, the aim of this systematic review was to explore the impact of resveratrol on female fertility, examining data extracted from both in vivo and in vitro studies performed in females and human cells or tissues.

2. Methods

This systematic review with a narrative summary of the results adhered to the PRISMA [15] and MOOSE [16] statements and followed a structured protocol available under reasonable request from the corresponding author.

2.1. Search Strategy

Two investigators (AB and DP) independently conducted a literature search using the Medline, PubMed, Web of Science and Embase databases from their inception (1951, 1951, 1947 and 1900, respectively) to the 9th of May 2024. The following search strategy was used: "(3,5,4′-Trihydroxystilbene" OR "3,4′,5-Stilbenetriol" OR "trans-Resveratrol-3-O-sulfate" OR "SRT 501" OR "cis-Resveratrol" OR "Resveratrol" OR "trans-Resveratrol" OR "Resveratrol-3-sulfate") AND ("oocytes" OR "oocytes development competency" OR

"fertilization" OR "blastulation" OR "pregnancy" OR "live birth" OR "oocyte quality" OR "embryo" OR "embryo quality" OR "in vitro fertilization" OR "fertilisation in vitro" OR "IVF" OR "ICSI" OR "assisted reproduct*" OR "reproduct* medic*"). The references of the retrieved articles together with the proceedings of relevant conferences were hand-searched to identify other potentially eligible studies for inclusion in the analysis missed by the initial search or any unpublished data. The literature search, assessment of inclusion and exclusion criteria, quality assessment of studies and extraction of data were independently undertaken and verified by two investigators (AB, DP). The results were then compared, and in the case of a discrepancy, a consensus was reached with the involvement of a third senior investigator (LS). There was no language restriction applied.

2.2. Types of Studies; Inclusion and Exclusion Criteria

Studies included in the present review had to meet the following criteria: (1) the study design was retrospective, cross-sectional, prospective, a randomized clinical trial (RCT) or another experimental design; (2) the population included women seeking fertility; (3) resveratrol supplementation was included as the intervention or an exposure group; (4) the study contained a female control group not treated with resveratrol supplementation; (5) the study reported effects of resveratrol on women's fertility.

Studies were excluded if they had a study design different from those stated in the inclusion criteria, focused on males, contained no data on resveratrol supplementation or did not investigate any aspect of fertility.

2.3. Data Extraction and Statistical Analyses

For each eligible study, two independent investigators (AB, DP) extracted the following data: name of the first author and year of publication, article type, study design, sample size, sample characteristics, intervention, outcome measures and findings.

2.4. Outcomes

The primary outcome was the effect of resveratrol supplementation on female fertility. Secondary outcomes included in vitro findings related to the effect of resveratrol on fertility cascade processes.

2.5. Assessment of Study Quality

For human studies two independent authors (DP, AB) assessed study quality using the Newcastle–Ottawa Scale (NOS) [17]. The NOS assigns a maximum of 9 points based on three quality parameters: selection, comparability and outcome. As per the NOS grading in past reviews, we graded studies as having a high (<5 stars), moderate (5–7 stars) or low risk of bias (≥8 stars) [18]. Notably, for in vitro studies, there is no general consensus and standard tool for assessing study quality.

2.6. Assessment of the Certainty of Evidence

To ascertain the certainty of the evidence, the Grading of Recommendations Assessment, Development, and Evaluations (GRADE) framework was used [19].

3. Results

As shown in Figure 1, we initially found 395 possibly eligible articles. After removing 366 papers through title/abstract screening, 29 were retrieved as full text. Of the twenty-nine full-text articles, five studies were excluded—four because they focused on male fertility and one because it assessed resveratrol levels without supplementation—leaving twenty-four studies published between 2010 and 2024 to be included in the systematic review [12,20–42]. Among these, nine were performed on women seeking natural or assisted fertility [20–28], and fifteen were in vitro studies performed on human cells and tissues in different stages of the reproductive cascade [12,29–42]. Most of the studies (n = 10) were conducted in Europe, nine in Asia, three in South America, one in the Middle East and one in Oceania. The nine

in vivo studies included a total of 9563 participants, and descriptions of the characteristics of these studies and their main findings are reported in Table 1. The main results are varied and not always consistent. Resveratrol induced a reduction in the expression of the vascular endothelial growth factor and hypoxia-inducible factor 1 genes in the granulosa cells. The number of mature oocytes, cleavage rate, fertilization rate and fertility rate were not significantly different, but the high-quality oocyte rate and high-quality embryo rate were higher in the resveratrol group [20]. It also induced modification in the miRNome reflecting transcriptomic and proteomic modification in granulosa cells. The number of fertilized good-quality oocytes increased in treated women, and a significant anticorrelation between miR-125 fold change values and biochemical pregnancy was present [21]. Resveratrol treatment was associated with statistically significant increases in the follicle output rate and follicle-to-oocyte index with no difference in the number of oocytes retrieved, biochemical pregnancy, clinical pregnancy or live birth rates [22]. The time needed to control blood pressure in resveratrol-treated women was significantly reduced, while time before a new crisis was extended. The number of treatment doses needed to control blood pressure was lower in treated women. No differences in maternal or neonatal adverse effects were observed between the two groups [23]. Again, resveratrol supplementation was associated with significantly higher numbers of oocytes and MII oocytes retrieved, a higher fertilization rate, more cleavage embryos per patient, more blastocytes per patient and more cryopreserved embryos. No significant differences in biochemical or clinical pregnancy, live birth or miscarriage rates were revealed, but a trend toward a higher live birth rate was revealed in the resveratrol group [24]. Resveratrol treatment promoted remodeling of the scarred uterus, regeneration of the endometrium and muscular cells and vascularization. It also improved the pregnancy rate compared with patients receiving placebo [25]. No difference in systolic or diastolic parameters between treated and control-group obese women were reported. All blood chemistry parameters improved compared to placebo at 30 days and significantly improved at 60 days with respect to placebo. Resveratrol also significantly improved lipid and glucose parameters at 30 to 60 days of treatment [26]. No difference in the treatment of pain in endometriosis was observed [27]. Resveratrol intake was also reported to be strongly associated with a decrease in the clinical pregnancy rate and an increased risk of miscarriage [28].

Table 2 reports the characteristics and the main findings of the in vitro studies.

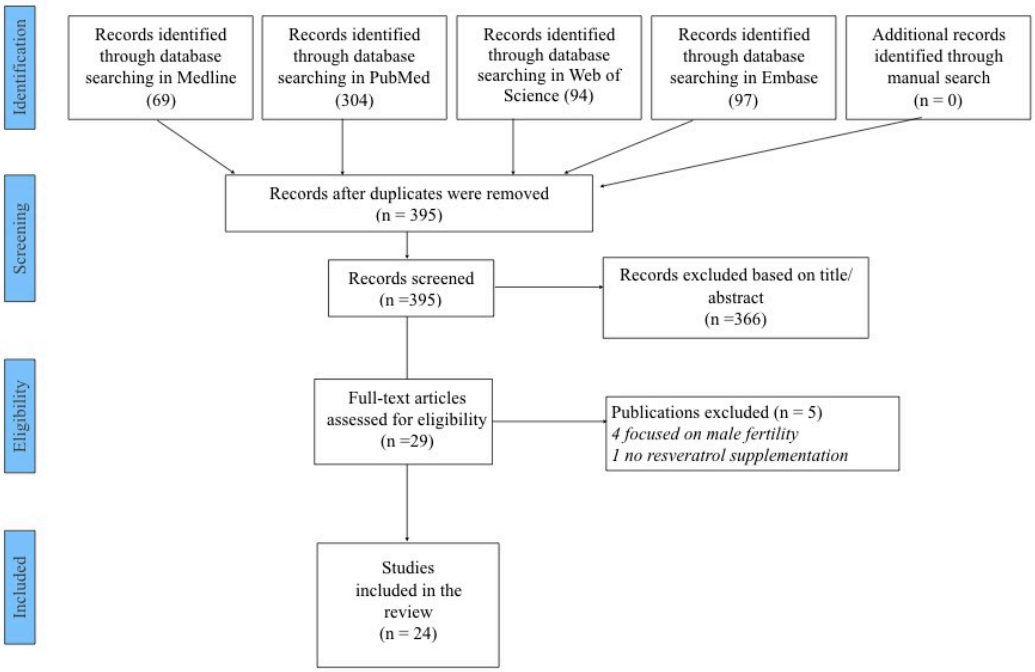

Figure 1. PRISMA flowchart.

Table 1. Main information and findings of included studies on fertility and reproductive outcomes.

Author and Date	Aim	Study Type	Intervention	Outcome Measures	Findings
Ding, 2017 [23]	To evaluate the outcome of treatment combining oral nifedipine and resveratrol against preeclampsia	RCT	400 women with preeclampsia	Time to control blood pressure and time before a new hypertensive crisis, number of doses needed to control blood pressure and maternal and neonatal adverse effects	In the resveratrol group, the number of treatments and time needed to control blood pressure were significantly reduced, while time before a new crisis was extended.
Malvasi, 2017 [26]	To investigate the effect of trans-resveratrol during spontaneous pregnancies in overweight patients	RCT	110 pregnant women aged 25–40 years, gestational age at enrollment between the 24th and 28th weeks and BMI in first trimester between 25 and 30	Blood pressure, total cholesterol, LDL, HDL, triglycerides and blood glucose	All blood chemistry parameters improved compared to placebo at 30 days and significantly at 60 days with respect to placebo. The resveratrol group showed significantly improved lipid and glucose parameters compared to the DC/MI group after 30 to 60 days of treatment.
Mendes da Silva, 2017 [27]	To evaluate resveratrol utilization for reducing endometriosis pain	RCT	44 women (ages 20–50) with laparoscopic diagnosis of endometriosis	Pain assessment using a visual analog scale	Resveratrol is not superior to placebo for treatment of pain in endometriosis.
Ma, 2018 [25]	To investigate the effects of resveratrol in patients with a scarred uterus	Cohort study	78 patients (mean age 30.4) with a scarred uterus, randomly divided into resveratrol treatment (n = 46) and placebo (n = 32) m groups	Uterus scar modeling and fertility	Resveratrol treatment promoted remodeling of the scarred uterus, regeneration of the endometrium and muscular cells and vascularization and improved the pregnancy rate.
Bahramrezaie, 2019 [20]	To assess the effect of resveratrol on the angiogenesis pathway for management of PCOS	RCT	62 ICSI candidates with PCOS	VEGF and HIF1 gene expression, number and quality of mature oocytes and embryos, cleavage rate, fertilization rate and fertility rate	There was a reduction in the expression of the VEGF and HIF1 genes under the effect of resveratrol in the granulosa cells. The high-quality oocyte rate and high-quality embryo rate were higher in the resveratrol group.

Table 1. *Cont.*

Author and Date	Aim	Study Type	Intervention	Outcome Measures	Findings
Ochiai, 2019 [28]	To assess resveratrol's impact on IVF–embryo transfer	Cohort study	8686 embryo transfers	Pregnancy outcomes	Resveratrol intake is strongly associated with a decrease in clinical pregnancy rate and an increased risk of miscarriage.
Gerli, 2021 [24]	To evaluate the impact of resveratrol on ICSI	RCT	101 infertile women undergoing ICSI, aged 18–42, BMI 18–30, normal thyroid function and normal blood parameters, regular uterine cavity	Number of developed follicles, total oocytes, MII oocytes recovered, fertilization rate, number of cleavage embryos/blastocysts, number of embryos for cryopreservation, duration and dosage of gonadotropins, number of embryos per transfer, implantation, pregnancy rates, live birth rate and miscarriage rate	Resveratrol supplementation was associated with significantly higher numbers of oocytes and MII oocytes retrieved, a higher fertilization rate, more cleavage embryos/patient, more blastocytes/patient, more cryopreserved embryos and a higher live birth rate.
Battaglia, 2022 [21]	To evaluate follicular fluid miRNome modification in aged women with a poor ovarian reserve receiving a resveratrol-based supplement for 3 months	Cohort study	12 women 35–42 years old with a poor ovarian reserve (AMH < 1.2 ng/mL, AFC < 5) undergoing IVF treatment	MiRNome modifications and oocytes quality	The number of fertilized good-quality oocytes increased in treated women, and a significant anticorrelation between miR-125 fold change values and biochemical pregnancy was present.
Conforti, 2024 [22]	To evaluate the effect of resveratrol on the outcome of IVF	RCT	70 women >35 years with good ovarian reserve (AMH > 1.2 ng/mL)	Follicle output rate, follicle-to-oocyte index, number of oocytes retrieved, biochemical pregnancy, clinical pregnancy and live birth rates	Resveratrol treatment was associated with a statistically significant increase in the follicle output rate and follicle-to-oocyte index.

AFC = antral follicle count; AMH = anti-Müllerian hormone; DC = D-chiro-inositol; HIF = hypoxia-inducible factor; ICSI = intracytoplasmic sperm injection; IVF = in vitro fertilization; MI = myo-inositol; PCOS = polycystic ovary syndrome; RCT = randomized clinical trial; VEGF = vascular endothelial growth factor.

Table 2. Main information and findings of included studies on in vitro outcomes.

Author and Date	Aim	Sample Size and Characteristics	Intervention	Outcome Measures	Findings
Schube, 2010 [38]	To evaluate the effect of resveratrol against oxLDL-induced damage to granulosa cells	Granulosa cells obtained from patients undergoing in vitro fertilization	Cells were treated with 150 µg/mL oxLDL alone or with 30 µM resveratrol for 36 h	Measurement of oxidative stress markers, cell vitality and activity	Resveratrol protected granulosa cells by reducing cell death; enhancing mitosis; inducing protective autophagy; reducing oxidative stress markers and reducing expression of LOX-1, TLR4, CD36 and heat-shock protein 60. Resveratrol could restore steroid biosynthesis.
Novaković, 2015 [35]	To evaluate the in vitro effect of resveratrol on the oxytocin-induced contractions of term pregnant myometrium and the contribution of different K^+ channels to resveratrol's action	Myometrial samples from 42 nonlaboring women undergoing elective cesarean section in the third trimester of pregnancy, mean age 35.46 years	Resveratrol was dissolved in 70% *v/v* ethanol with further dilution in distilled water before use; working concentrations of ethanol in the bath were <0.01% (*v*/*v*)	Levels of oxytocin-induced contractions of myometrium cells and K^+ channel activity	Resveratrol induced a concentration-dependent relaxation of myometrium contractions. The inhibitory effect of low-concentration resveratrol involves different myometrial K^+ channels. When applied in high concentrations, resveratrol has an additional K^+-channel-independent mechanism(s) of action.
Savchuk, 2016 [37]	To characterize the effects of resveratrol on human fetal adrenal steroidogenesis	Primary cultures of human fetal adrenocortical cells prepared from adrenals of aborted fetuses (GW10–12)	Fetal adrenocortical cells were treated in the presence or absence of ACTH (10 ng/mL) with or without resveratrol (10 µM) for 24 h	Dehydroepiandrosterone, androstenedione and 11-deoxicortisol levels; activity of cytochromes 17αhydroxylase/17,20 lyase and 21-hydroxylase	Resveratrol significantly suppressed synthesis of dehydroepiandrosterone, androstenedione and 11-deoxicortisol by ACTH-activated and unstimulated human fetal adrenocortical cells, which was associated with inhibition of the activity and expression of cytochromes 17αhydroxylase/17,20 lyase and 21-hydroxylase.

Table 2. *Cont.*

Author and Date	Aim	Sample Size and Characteristics	Intervention	Outcome Measures	Findings
Hannan, 2017 [30]	To assess resveratrol's anti-inflammatory and anti-oxidative effects in trophoblast and endothelial cells	NR	Trophoblasts were treated with 0–100 μM resveratrol for 48 h in culture; HUVECs were treated with 0–75 μM resveratrol for 24 h in culture	Measurement of sFlt-1 and sEng or protein expression of peNOS, eNOS or HO-1	Resveratrol reduced sFlt-1, sFlt-1 e15a and soluble endoglin secretion from trophoblasts and HUVECs and reduced mRNA expression of the pro-inflammatory molecules NFκB, IL-6 and IL-1β in trophoblasts. IL-6, IL-1β and TNFα secretion were also significantly reduced. In HUVECs, resveratrol significantly increased mRNA of the antioxidant enzymes HO-1, NQO1, GCLC and TXN but did not significantly alter HO-1 protein expression, while it reduced HO-1 protein in trophoblasts.
Liu, 2018 [31]	To evaluate the effects of resveratrol on oocyte maturation in aged mice and humans	64 women 38–45 years of age undergoing ICSI	3 different concentrations of resveratrol (0.1, 1.0 and 10 mm) or dimethylsulfoxide	Oocyte maturation, fertilization, immunofluorescence intensity of mitochondria and normal morphology	Resveratrol at 1.0 mm significantly increased the first polar body emission rate in oocytes. The immunofluorescence intensity of mitochondria and normal morphology of spindle and chromosome of oocytes undergoing in vitro maturation were notably improved.
Caldeira-Dias, 2019 [29]	To investigate the effect of resveratrol on endothelial cells from women before the development of preeclampsia regarding antioxidant defenses and vasodilator factors	6 samples from women who developed severe preeclampsia and 6 samples from women who had healthy pregnancies	HUVECs were incubated in medium containing 10% (*v*/*v*) plasma from case and control patients and 1 μM trans-resveratrol	Levels of Nrf2, HO-1, GSR, GSH and NO in endothelial cells	Resveratrol prevents alterations in HO-1 and NO markers and improves GSH levels.

Table 2. *Cont.*

Author and Date	Aim	Sample Size and Characteristics	Intervention	Outcome Measures	Findings
Ochiai, 2019 [36]	To assess the effect of resveratrol on HESC decidualization	Endometrial biopsies from patients without overt uterine pathology during the luteal phase	Confluent monolayers were maintained in DMEM/F12 without phenol red containing 2% (*v*/*v*) DCC-FBS and treated with 0.5 mM 8-bromo-cAMP and 1 μM P4 with or without 100 μM resveratrol	Expression levels of prolactin and IGFBP1; cell decidualization	Resveratrol has anti-deciduogenic properties, repressing the induction of the decidual marker genes prolactin and IGFBP1 but also abrogating decidual senescence. Resveratrol blocks differentiation of HESCs into mature and senescent cells by accelerating downregulation of the CRABP2-RAR pathway.
Mestre Citrinovitz, 2020 [33]	To evaluate the effect of resveratrol on decidualization of HESCs	Endometrial biopsies from healthy, regularly cycling women mean age 34.4 years old	At days 3 and 5 of the decidualization, different doses of resveratrol (0 (vehicle treatment), 6.25, 12.5, 25 and 50 μM) were added	Expression levels of prolactin and IGFBP1, cell proliferation and mRNA levels	Resveratrol increased the expression levels of prolactin and IGFBP1, indicating enhanced in vitro decidualization of HESCs. It was accompanied by a decrease in cell proliferation and by changes in the mRNA levels of key cell cycle regulators.
Viana-Mattioli, 2020 [39]	To investigate the effects of trans-resveratrol on oxidative stress and NO production in women with preeclampsia, gestational hypertension and healthy pregnancies	10 blood samples collected for each group from non-smokers women < 34 years	Cells were incubated with 10 μM of trans-resveratrol	ROS production, SIRT1 activity and NO levels	In the gestational hypertension group, resveratrol decreased intracellular ROS and increased their antioxidant capacity, while inhibiting SIRT1 reestablished previous levels. In preeclampsia, inhibition of SIRT1 increased antioxidant activity. Intracellular NO and supernatant nitrite levels were increased by inhibiting SIRT1 in the preeclampsia group.

Table 2. *Cont.*

Author and Date	Aim	Sample Size and Characteristics	Intervention	Outcome Measures	Findings
Wang, 2020 [41]	To investigate the protective effects of resveratrol against oxidative-stress-induced damage in trophoblasts	A human first-trimester extravillous trophoblast cell line purchased from a supplier	Cells were pre-treated with 12.5, 25, 50 and 100 μM resveratrol for 24 h, followed by 200 μM H_2O_2 for 12 h	ROS, MDA and SOD levels; cell viability and apoptosis and SIRT1 levels	Pre-treatment with resveratrol significantly ameliorated H_2O_2-induced cytotoxicity, morphological damage, oxidative stress and apoptosis.
Moreira-Pinto, 2021 [34]	To evaluate the direct effects of resveratrol on granulosa cell viability, steroidogenic function and oxidative stress	34 women undergoing assisted reproductive technology, mean age 34 years	Granulosa cells were treated with resveratrol (0.001–20 μM) for different lengths of time (24–72 h)	ROS/RNS levels and granulosa cell viability and steroidogenic function	Low concentrations of resveratrol suggest a protective role reducing ROS/RNS formation after inducement of stress. High concentrations of resveratrol affect granulosa cells' viability and steroidogenic function.
Ragonese, 2021 [12]	To study the effects of resveratrol on the growth, electrophysiology and mitochondrial function of human granulosa cells	7 infertile women undergoing assisted reproductive techniques	Cells were treated with various concentrations of resveratrol (3, 10 and 20 mM) for up to 72 h	Granulosa cell viability and mitochondrial activity; electrophysiological activity of potassium current and calcium concentration	Resveratrol induced mitochondrial activity with a bell-shaped, dose-dependent relationship. It increased ATP production and cell viability and promoted the induction of cellular differentiation. Resveratrol reduced the functional expression of an ultra-rapidly activated, slowly inactivated, delayed rectifier potassium current that is associated with plasma membrane depolarization and that promotes an increase in intracellular calcium.
Yao, 2021 [42]	To examine resveratrol's effect in rescuing defects caused by zearalenone in HESCs during human decidualization	NR	Unspecified resveratrol treatment	ROS levels and glutathione peroxidase 3 gene expression	Resveratrol restored the impaired decidualization process by induction of the anti-oxidative gene glutathione peroxidase 3.

Table 2. *Cont.*

Author and Date	Aim	Sample Size and Characteristics	Intervention	Outcome Measures	Findings
Wang, 2022 [41]	To investigate resveratrol's neuroprotective effect during development	Human induced pluripotent stem cells purchased from a supplier	Cells were treated with 2, 10 and 50 μM resveratrol	Cell proliferation, apoptosis and differentiation	Resveratrol showed neuroprotective effects by promoting neural cell proliferation, inhibiting apoptosis and accelerating the differentiation of germ layers.
Long, 2023 [32]	To examine resveratrol's effects on defects caused by DEHP during human decidualization	NR	After DEHP treatment, cells were treated with resveratrol (RSV) at a cell density of 70%	Cell proliferation and decidualization and the up/downregulation of molecules associated with decidualization	Resveratrol treatment was associated with an upregulation of decidual molecules, confirmed by RNA-seq transcriptome analysis and a quantitative real-time PCR assay.

ACTH = adrenocorticotropic hormone; CRABP2 = cellular retinoic acid-binding protein 2; DEHP = Di-(2-Ethylhexyl) phthalate; DMEM = Dulbecco's modified Eagle's medium; GSH = glutathione; GSR = glutathione reductase; HESC = human endometrial stromal cell; HO-1 = heme oxygenase-1; HUVEC = human umbilical vein endothelial cell; ICSI = intracytoplasmic sperm injection; IGFBP1 = insulin-like growth factor binding protein 1; MDA = malondialdehyde; NO = nitric oxide; NR = not reported; Nrf2 = nuclear factor-erythroid-derived 2-related factor-2; oxLDL = oxidized low-density lipoprotein; ROS = reactive oxygen species; SOD = superoxide dismutase.

No publication bias test was performed. The median quality of the studies performed on human studies was 5.9 (range: 4–9), indicating an overall satisfactory quality. Because of the high heterogeneity, the certainly of this evidence has been rated as moderate.

4. Discussion

The findings of our systematic review suggest a general positive impact of resveratrol on female reproductive health. Although the wide range of aspects considered in the included studies does not allow robust conclusions, the results do suggest a potential positive effect of resveratrol on multiple domains of the female reproductive system. The number and quality of matured oocytes were investigated by four and two studies, respectively. The number was reported as being increased in two [21,24] and having no significant differences in the others [20,22]. Both studies assessing quality reported an increased quality in women who underwent resveratrol supplementation. A possible explanation is the direct action of resveratrol in reducing oxidative stress, protecting mitochondrial DNA from damage and mutations, while enhancing telomerase activity and reducing cellular aging. Moreover, it activates sirtuin 1 (SIRT1), a key molecule in aging, which is typically reduced in aged oocytes, making them vulnerable to oxidative stress [43]. By compensating for this decreased SIRT1 expression, resveratrol may inhibit ovarian aging and extend ovarian lifespan [8,44]. Additionally, it potentially exerts positive effects on PCOS and obesity-related infertility by inhibiting pathways involved in androgen production and reducing inflammation and oxidative stress [45,46]. Moreover, in a rodent premature ovarian insufficiency model induced by chemotherapy or radiotherapy, resveratrol inhibited oxidative stress and inflammatory events in the ovaries by activating the PI3K/Akt/mTOR and NF-κB signaling pathways [47], improving loss of the oogonial stem cells through antiapoptotic effects [48]. In in vitro fertilization treatments, it was observed that resveratrol enhanced oocyte maturation and embryo development to the blastocyst stage in both animals and humans [31] and protected against postovulatory oocyte aging [49]. Fertilization and fertility rates were reported as being improved by Gerli et al. [24] and as having no difference by Bahramrezaie and colleagues [20]. The latter study also reported no difference in cleavage rate but found an increased embryo quality [20]. The follicle output rate and follicle-to-oocyte index were reported as improved in one study [22], and two studies reported no difference in terms of live birth [22,24]. Importantly, there are contrasting data regarding pregnancy rates and miscarriage indicators. The pregnancy rate was reported by five studies, with two indicating an increase [21,25], two no difference [22,24] and Ochiai et al. reporting a decrease [28]. Ochiai and colleagues also reported an increase in the miscarriage rate [28], while Gerli et al. observed no difference [24]. Moreover, during spontaneous pregnancies in overweight patients, resveratrol was found to significantly improve lipid and glucose parameters [26], and the literature suggests that it may be beneficial for the treatment of preeclampsia in combination with oral nifedipine [23]. This contrasting evidence could be explained by the fact that, since implantation requires an inflammatory response with local secretion of proinflammatory cytokines and prostaglandins from the decidualized endometrium, the anti-inflammatory properties of resveratrol might directly suppress embryo implantation. In turn, this could imply an optimization of the supplementation scheme in terms of period, dosage and duration. Finally, no effect in pain relief in women suffering endometriosis was observed [27], although in vitro studies showed antiapoptotic and antiproliferative effects with a possible role in inhibiting the progression of ectopic endometrium [9]. Thus, resveratrol may have therapeutic benefits for treating infertility associated with endometriosis, even without symptomatic effects. Taken together, all these findings do not allow putative conclusions to be drawn, but they spark optimism for the possible use of resveratrol for women seeking fertility. Many questions still need to be addressed, including the dosage, treatment duration, optimal time window and possible side and teratogenic effects. However, additional encouraging results are coming from in vitro studies that also shed light on possible mechanisms of action. Importantly, in vitro studies included in the present review assessed different aspects of the impact of resveratrol

on human cells, indicating an overall potential beneficial effect of supplementation. Interestingly, its antioxidant activity was reported by multiple studies [29,30,34,39,40]. Granulosa cells' viability was also found to be improved by supplementation [12,34,38], and other observed effects included an anti-inflammatory response [30]; oocyte maturation [31]; myometrium relaxation [35]; reduction of dehydroepiandrosterone, androstenedione and 11-deoxicortisol [37]; and neuroprotection [41]. Contrasting results were reported on decidualization. Three studies showed enhancement of in vitro decidualization [32,33,42], while Ochiai and colleagues reported anti-deciduogenic properties [36]. In this case, different methodological approaches, resveratrol doses and timing of administration may explain the contrasting results, highlighting the need to increase efforts in studying this multi-potential supplement. Despite some contrasting evidence, it seems clear that resveratrol may play an important role in female fertility management. Moreover, a limited body of literature also suggests a role of resveratrol in improving testicular function and sperm quality through enhanced protection from reactive oxygen species (ROS), which negatively impacts sperm quality by damaging mitochondrial membranes, impairing sperm motility and increasing sperm DNA damage [13,14].

The considerations drawn in this systemic review should be interpreted in the light of some limitations including the limited number of in vivo studies; the different parameters taken into account by each study and the lack of robust data on dosage, side effects and teratogenic effects. Moreover, although the quality of the studies was satisfactory overall, due to the high heterogeneity, the certainly of this evidence has been rated as moderate. Finally, the present review was not registered in the PROSPERO database.

Resveratrol is generally considered safe and well tolerated when consumed in moderate amounts through diet and in supplemental doses up to 5 g/day for a month [50,51]; however, the safety of high-dose supplementation, particularly over long periods, remains unclear. Potential side effects include gastrointestinal disturbances and interactions with medications. Therefore, it is crucial to conduct further and larger studies to determine the safe and effective dosage of resveratrol for improving female fertility. Considering its limited absorption after oral administration, particular attention should also be paid to resveratrol's bioavailability and, in particular, to the different resveratrol-based technologies that can ameliorate resveratrol's pharmacokinetic characteristics, exploiting its biopharmaceutical potential [52].

5. Conclusions

In conclusion, although it is not possible to define conclusive indications on resveratrol supplementation, the current evidence suggests that its utilization for women seeking fertility and during pregnancy could significantly and positively impact reproductive outcomes, particularly because of its potential therapeutic effects in improving ovarian function. Further studies are needed to better understand resveratrol's potential in women in order to define the optimal doses and periods of intake to maximize beneficial effects and to prevent adverse effects on implantation, subsequent pregnancy and the fetus.

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RESEARCH

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The impact of resveratrol on the outcome of the in vitro fertilization: an exploratory randomized placebo-controlled trial

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Abstract

Background Resveratrol is a natural polyphenolic compound present in plants and red wine with many potential health benefits. This compound has various anti-inflammatory and anti-tumor properties and can improve cellular mitochondrial activity. This trial was designed to evaluate the effect on the outcome of IVF of Resveratrol supplementation in women > 35 years with good ovarian reserve (AMH > 1.2 ng/ml). Women were randomized to receive or placebo or Resveratrol (150 mg per day) for three months preceding the ovarian stimulation (OS). All patients were stimulated with a starting dose of recombinant FSH ranging between 150 and 300 IU according to age and ovarian reserve. GnRH antagonist flexible protocol was adopted for pituitary suppression. Triggering was performed with urinary hCG (10.000 IU).

Results The study was conducted between January 2019 and December 2022 with aa total of 37 cases and 33 controls were recruited. No statistically significant differences in the number of oocytes retrieved, biochemical pregnancy, clinical pregnancy and live birth rates were observed between women treated with resveratrol and control group. A statistically significant increase in the follicle output rate (FORT) and follicle-to oocyte index (FOI) was observed in women treated with resveratrol-based nutraceutical (0.92 *versus* 0.77 [$p = 0.02$], and 0.77 *versus* 0.64 [$p = 0.006$], respectively).

Conclusions Preliminary results from this study indicate that pre-treatment with resveratrol may improve ovarian sensitivity to exogenous FSH, which in turn may decrease the risk of hypo-response to OS in advanced reproductive age women.

Keywords Ovarian sensitivity, FORT, FOI, FSH, POSEIDON, Hypo-response

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Introduction

Resveratrol is a natural polyphenolic compound present in plants and red wine with numerous health properties. Several in vivo and in vitro studies have demonstrated that this compound has various anti-inflammatory and anti-tumor properties [1, 2]. Moreover, resveratrol can improve cellular mitochondrial activity and trigger a series of molecular mediators capable of counteracting some of the most important metabolic mechanisms underlying aging [3]. Mitochondria are major regulators of multiple vital cellular processes, including apoptosis, calcium homeostasis, and adenosine triphosphate (ATP) generation through the metabolic pathway known as oxidative phosphorylation [4]. A significant reduction in mitochondrial DNA levels has been reported in female oocytes in advanced maternal age hypothesizing a fundamental role in embryonic development and in vitro fertilization (IVF) success [5]. Nonetheless, the effect of resveratrol on the ovarian sensitivity is still unknow. The ovarian sensitivity reflects the ability of the ovaries to response properly to exogenous gonadotropin. Women characterized by a reduced ovarian sensitivity (or "hypo-response") are characterized by reduced the number of eggs retrieved despite the normal ovarian reserve markers. Evidence indicates that hypo-response is typically observed in women with reduced IVF prognosis [6–10]. So far, several ovarian sensitivity indicators have been proposed [11]. The main indicators of ovarian sensitivity are summarized in Table 1.

Considering the relevance of ovarian response and aging on IVF success, the aim of the present exploratory randomized placebo-controlled trial was to evaluate the effect of resveratrol-based supplementation on ovarian responsiveness in advanced reproductive age women with normal ovarian reserve markers candidates to IVF.

Methods

Study design

This randomized, single-blind, controlled, single-center, experimental study enrolled infertile women attending in vitro fertilization (IVF) at the IVF Unit of University Federico II, Naples, Italy, from January 2019 and December 2022. The study was approved by the local Ethics Committee of the University of Naples Federico II on January 2019 (n.328/18).

The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guideline [16]. This study was reported fulfilling POSORT and CONSORT recommendations [17, 18]. The study followed the guidelines on infertility testing that emerged from the Harbin Conference [19]. Patients fulfilling the inclusion and exclusion criteria (Table 2) were invited to participate in the study [20–23]; the study was explained to all participants. Both oral informed consent and written informed consent were obtained by all patients included.

Randomization

We employed a stratified randomization scheme together with permuted block randomization. Randomization is then performed using PASS software with package blockrand for each stratum at a 1:1 allocation ratio and with

Table 1 Main indicators of ovarian sensitivity reported in literature

Ovarian sensitivity indicators	How to calculate
Follicle Output RaTe (FORT) [12, 13]	Ratio between number of pre-ovulatory follicles obtained after COS and the number of antral follicles at the beginning of OS
Follicle-to Oocytes Index (FOI) [11, 14]	Ratio between the number of retrieved oocytes and the number of antral follicles at the beginning of OS
Ovarian Sensitivity Index (OSI) [15]	Ratio between number of oocytes retrieved and total dose of gonadotrophins administered

Table 2 Study protocol selection criteria

INCLUSION CRITERIA	EXCLUSION CRITERIA
Age ≥ 35 years	Presence of ovarian neoformations at the time of OS
AMH > 1.2 ng/ml [20]	Body Mass Index (BMI) < 18 and > 28 kg/m^2
Regular menstrual cycle (every 25–35 days)	Polycystic ovary syndrome [21]
Indication for IVF/ICSI: idiopathic, tubal factor, mild and moderate male factor, maternal age	Endocrinopathies (disorders of the hypothalamic-pituitary axis, dysthyroidism or hyperprolactinaemia)
No previous ovarian surgery	Endometriosis III-IV Stage [22]
	Diabetes type I e II
	Severe male factor [23]

block size determined as 2, 4, 6 and 8. The randomization list was prepared by an independent statistician not participating in the recruitment. Sequentially numbered, opaque sealed envelopes are used for allocation. Envelopes receive numbers in advance and are opened sequentially, only after the participant's name has been written on the appropriate envelope. One member of the research team who labelled the containers was aware of the allocation. Researchers were unaware of treatment allocation until the envelope is opened. Treatment was not masked to care providers and investigators but masked to the participants and the outcome assessor.

Clinical and ultrasonographic evaluation

For each patient we collected: age, BMI Kg/m^2, menstrual cycle characteristics, last menstrual period, basal FSH and LH, ovarian reserve markers (AMH and AFC) and number of years of infertility. The ultrasound examinations were executed using a Voluson E8 device (GE Healthcare, Zipf, Austria) with a transvaginal probe and they were all performed by two sonographers (G.G.I and SP) within the fifth day of OS. AFC was assessed using 2D-ultrasound and 3D-volume to reduce interobserver variability [24]. AFC was assessed considering 2–10 mm antral follicles using trans-vaginal pelvic ultrasound (multi-frequency vaginal probe 5.0–7.5 MHz) according to the most recent guideline [25].

Intervention

Patients of study group received 3 months of treatment, before undergoing OS, with a nutraceutical formulation (2 daily capsules) containing *trans*-resveratrol and a form of resveratrol supported on Magnesium dihydroxide (total amount of resveratrol 150 mg) [26, 27], folic acid (400 mcg), vitamin D (25 mcg), vitamin B12 (2.5 mcg), and vitamin B6 (1.4 mg) (GENANTE®, S&R Farmaceutici, Bastia Umbra PG, Italy), while control group received 3 months of treatment with placebo, containing only excipient (microcrystalline cellulose, vegetable Magnesium, Stearate (E470b), croscaramellose sodium (E468), talc (E553b), Silicon Dioxide (E551), Arabic gum (E414) and no active ingredients. The 150 mg/day dose is not based on dose-finding studies. The dose is dependent on the recommendation of European Food Safety Authority (EFSA), which considers 150 mg daily the maximum safe dose for the human population without restrictions [28]. The pretreatment duration of 3 months before IVF was arbitrary, as no studies were available at the time of the study design and motivated by the duration of the transition from pre-antral to mature follicles taking approximately 80–90 days [29]. After completing the treatment, patients undergo OS, oocyte retrieval and fresh embryo transfer.

Patients of both groups performed OS as follows:

- on day 2–3 of the menstrual cycle recombinant FSH (r-FSH) were administrated based on age and ovarian reserve (from 150 to 300 IU daily);
- all patients were treated with flexible GnRH antagonist protocol at a dose of 0.25 mg/day in case of dominant follicle at the ultrasound (mean diameter > 13 mm or estradiol levels > 300 pg/ml);
- in women with at least two follicles of mean diameter > 17 mm, 10.000 IU of hCG or triptorelin 0.2 mg were administered as ovulation inducer, the latter was preferred in the presence of more than 20 follicles during ovarian stimulation, recovery of more than 24 oocytes, and estradiol levels above 3,500 pg/mL [30];
- oocyte retrieval (PU) was performed by ultrasound-guided transvaginal aspiration 34–36 h after the trigger;
- ultrasound-guided Embryo transfer (ET) was performed within 5 days of oocyte yield;
- the luteal phase, except for cases deemed to be at risk of hyper-response, was supported with 400 mg of micronized vaginal progesterone per day.

Outcomes

The primary endpoint was the clinical pregnancy rate (CPR) per aspirated cycle (a pregnancy diagnosed by ultrasonographic visualization of one or more gestational sacs or definitive clinical signs of pregnancy). Secondary endpoints were major efficacy and efficiency endpoints in IVF: number of oocytes retrieved and number of mature oocytes (metaphase II oocytes), mature oocytes percentage, number of cleavage embryos and blastocysts, number of cryopreserved embryos, blastulation rate (for those patients underwent transfer on day five), defined as the percentage of inseminated oocytes reaching blastocyst stage, ovarian sensitivity indicators (FORT, FOI and OSI), estradiol (E_2) levels at peak, duration of stimulation, total dose of gonadotropins, biochemical pregnancy rate (BPR) per aspirated cycle (a pregnancy diagnosed only by the detection of beta hCG in serum or urine), live birth rate (LBR) per aspirated cycle (delivery after 22 completed weeks of gestational age) [31].

Statistical methods

Reproductive outcomes were compared between the resveratrol treated group (Study Group) and Control group. Continuous variables are expressed in terms of mean ± SD or median and interquartile range for parametric and non-parametric data, respectively. Categorical variables are expressed in terms of frequency and

percentage. The distribution of continuous variables was evaluated with the Shapiro test. The two-sided t-test for independent samples was used to assess inter-group differences concerning parametric data. The two-sided Mann–Whitney U test was used to test inter-group differences for non-parametric data, whereas the Chi-square test was adopted to verify differences in terms of categorical data between groups. Results were analyzed using the statistical package SPSS 22 for Windows (Statistical Package for the Social Sciences, IBM, New York). A p-value < 0.05 was considered statistically significant.

Sample size

Since no data were available in the literature at the time of the enrollment, the present study is to be considered an exploratory randomized trial, considering a total sample size of 100 patients (50 per group).

Interim-analysis

In November 2022, an unplanned interim analysis was conducted on the basis of data from all randomly assigned patients. Results indicated a probability of more than 95% that we would find no significant differences in terms of CPR or LBR if we would include more patients in the trial. After considering all the evidence, the research group decided early closure of the trial.

Results

A total of 73 women underwent randomization. Therefore, 40 patients were assigned to the test group and 33 to the reference group (Fig. 1).

Three patients in the study group conceived naturally over the 3 months of therapy and were excluded from the final analysis. Demographic and infertility history data are summarized in Table 3.

The mean age and the BMI of the women enrolled in the study were comparable in the two groups. The two groups were also comparable regarding ovarian reserve

Table 3 General characteristics of enrolled women

	Study Group $n = 37$	Control Group $n = 33$	p-value
AGE (years)	38.66 ± 2.18	38.04 ± 2.38	0.26
BMI (Kg/m^2)	22.94 ± 2.95	22.86 ± 2.57	0.9
AFC	9.74 ± 3.8	10.3 ± 4.07	0.55
AMH (ng/ml)	1.95 (1.52–3.2)	2.15 (1.4–3.67)	0.91
FSH (IU/ml)	6.7 (6.15–7.55)	7.65 (6.15–9)	0.25
LH (IU/ml)	5.8 (3.87–7.42)	5.3 (4.25–6.25)	0.95
Years of sterility	4 (3–6.5)	3 (2-4)	**0.01**

Parametric continuous data are presented as mean ± standard deviation

Non parametric continuous data are presented as median and interquartile range

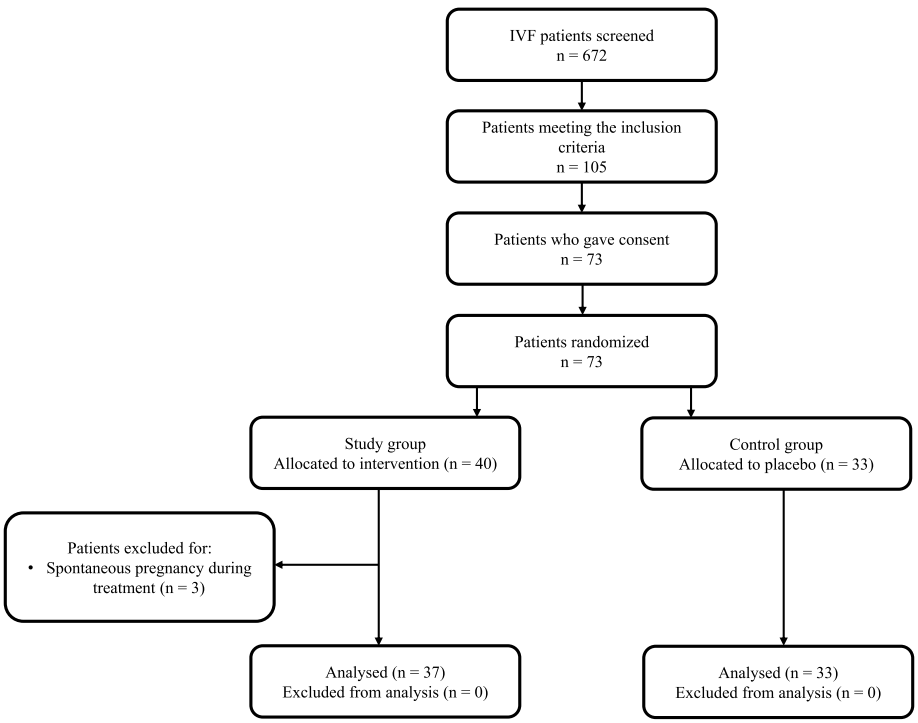

Fig. 1 CONSORT flow diagram

(AMH and AFC), basal FSH and LH. Infertility duration was higher in study group ($p < 0.01$). IVF outcomes are summarized in Table 4. All patients were triggered with 10,000 UI hCG.

The two groups were not significantly different in terms of pregnancy outcomes (BPR, CPR and LBR). The two groups were also similar in terms of mean number of collected oocytes and mature oocytes, percentage of mature oocytes, OSI, total gonadotropin dose and peak estradiol levels. FOI and FORT were significantly higher in the study group than in the control group ($p < 0.02$ and $p < 0.006$, respectively). Furthermore, no significant difference was observed in terms of embryos and blastocyst collected and blastulation rate.

Discussion

In this trial resveratrol pretreatment for 3 months before IVF, while not associated with statistically significant differences in clinical pregnancy rate (primary endpoint), increases ovarian sensitivity to exogenous gonadotrophins in women undergoing OS. Indeed, a significantly increase in FORT and FOI was observed in study versus control group. Consistently an increased, despite not significant, OSI was observed in the study group compared with control group. To the best of our knowledge this is the first time that an effect of ovarian sensitivity is reported after resveratrol pretreatment before OS.

The mechanism by which the resveratrol could increase ovarian sensitivity should be still elucidated, despite several hypothesis could be proposed. The main one might be related to the positive effect exerted by resveratrol on mitochondrial activity. Resveratrol can increase mitochondrial mass in human granulosa cells (GC) through a mechanism involving reduction of voltage-dependent potassium currents, intracellular calcium homeostasis, and regulation of mitomiRNAs [32, 33]. Women with reduced ovarian responsiveness exhibit reduced mitochondrial mass, cholesterol uptake capacity and expression of enzymes involved in steroidogenesis, such as StAR (Steroidogenic Acute Regulatory), 3-beta-hydroxysteroid dehydrogenase (3-beta-HSD) and aromatase, compared with normal responders [34]. Low expression of these enzymes leads to a reduced estrogen and progesterone production even after OS. Since low responsiveness to FSH may correlate with reduced mitochondrial mass, we propose that the enhanced ovarian responsiveness observed in the present study is related to the ability of resveratrol to stimulate mitochondrial biogenesis in GC. Another mechanism of action could be related to the anti-inflammatory and antioxidant properties of resveratrol. Indeed, resveratrol might contrast the negative

Table 4 Ovarian stimulations outcomes, embryos and pregnancies between the two study groups

	Study Group $n = 37$	Control Group $n = 33$	p-value
Total gonadotropin dose (IU)	1973.61 ± 395.14	1907.89 ± 475.08	0.53
E_2 at peak (pg/ml)	1301 (996–2329.5)	1247 (901.5–1836)	0.53
Retrieved oocytes	7.89 ± 3.62	6.4 ± 3.84	0.1
M2 oocytes	6.26 ± 2.28	5.15 ± 2.7	0.1
Mature oocytes	0.89 (0.73–1)	0.88 (0.71–1)	0.76
FORT	0.92 (0.84–1)	0.77 (0.65–0.95)	**0.02**
FOI	0.77 (0.7–0.95)	0.64 (0.49–0.76)	**0.006**
OSI	4.44 (2.34–5.04)	2.86 (1.67–4.48)	0.25
Cleavage embryos/patient	2.16 ± 0.9	1.95 ± 1.14	0.39
Blastocysts/patient	1.47 ± 1.22	1 ± 1.21	0.11
Embryos transferred day 3	14/41 (34%)	15/39 (38.5%)	0.69
Embryos transferred day 5	23/41 (56%)	18/39 (46%)	0.37
Cryopreserved embryos/patient	0.79 ± 0.92	0.65 ± 0.93	0.53
Blastulation rate	0.3 (0.25–0.33)	0.25 (0.17–0.33)	0.45
BPR	9 (24.32%)	10 (30.3%)	0.57
CPR	9 (24.32%)	10 (30.3%)	0.57
LBR	6 (16.22%)	10 (30.3%)	0.16

FORT Follicle output rate, *FOI* Follicle oocytes indexm, *OSI* Ovarian sensitivity index, *BPR* Biochemical pregnancy rate, *CPR* Clinical pregnancy rate, *LBR* Live-birth rate

Parametric continuous data are presented as mean ± standard deviation

Non parametric continuous data are presented as median and interquartile range

Categorical variables are expressed in terms of frequency and percentage

effect exerted by pro-inflammatory environmental factors related to the hypo-response physiopathology [35]. The possibility of improving the sensitivity of the ovary to gonadotropins could be useful in reducing the number of patients with an unpredictable hypo-response to OS, as in the case of POSEIDON groups 1 and 2 patients [12]. POSEIDON's groups 1 and 2 encompass women who had poor (< 4) or suboptimal [4–9] number of oocytes retrieved after a conventional OS despite the presence of an adequate ovarian reserve, defined by an AFC of ≥ 5 and/or an AMH ≥ 1.2 ng/mL. Indeed, retrieval of fewer than 10 oocytes is associated with decreased cumulative live birth rates (CLBR) [36]. Thus, given a patient who fits POSEIDON's groups 1 or 2, the final goal would be to find ways to maximize oocyte yield aiming at obtaining more than 9 oocytes at the end of stimulation [36, 37].

Other studies that have investigated the role of resveratrol in IVF are summarized in Table 5.

A recently published randomized trial [40] reported a statistically significant increase in the number of oocytes retrieved in women pretreated with resveratrol. Despite higher oocytes yield in the study group, our data failed to find statistically significant differences in comparison with the controls. The discrepancy between the two trials could be related to both study populations and sample size. More specifically, our trial investigated the impact of resveratrol in advanced maternal age, which is associated per se with higher risk of suboptimal or poor ovarian response [41]. Indeed, small sample size may explain the reason why, at least in our series, the statistically significant increase in FORT and FOI was not reflected in a significantly higher oocyte yield, however a trend to more retrieved oocytes among the study group ($p = 0,1$) is shown.

In contrast with a retrospective analysis of Ochiai et al. we do not observe a detrimental effect of resveratrol on pregnancy outcome [38]. This could be probably due to the retrospective design, heterogenous population and different IVF protocol adopted in Ochiai study comparing with our trial (mild versus conventional stimulation). Furthermore, the amount of resveratrol prescribed in Ochiai et al. study (200 mg daily) was higher comparing with our trial (150 mg daily). Finally, it should be considered that the age at the oocyte retrieval in Ochiai et al. study was significantly higher in women who were supplemented with resveratrol comparing with control group [38].

The strengths of our study reside in the design, the prospective nature and the selective inclusion criteria involving specifically women with the worst IVF prognosis namely those with advanced reproductive age [42].

The limitation of our study lies in the low sample size. This makes it impossible to speculate on the comparison between the groups in terms of oocytes parameters (es. retrieved and blastocytes) and pregnancy outcomes. Should we have reached a higher enrollment number the positive trend might have been significant in accordance with a previous study Gerli. et al. [41]. Difficulties in achieving the planned sample size were mainly related to the COVID-19 pandemic.

At the time of enrollment, there was no published study on IVF outcomes in patients treated with resveratrol, so a sample size calculation could not be performed. Considering the differences observed in terms of clinical pregnancy rate in our study (24,3% vs 30,3%) a post hoc analysis revealed that we need 865 women per group with an alfa error set at 0.05 and power set 0.80. Nonetheless, the number of women recruited were adequate to detect a difference in terms of FOI and FORT with a power over 90%.

A further concern is that the population selected for the study (women older than 35 years and with good ovarian

Table 5 Published protocols about resveratrol in IVF setting

Authors	Setting	Conclusion
Ochiai et al., 2019 [38]	Cross-sectional retrospective study comparing the outcomes of embryo transfer cycles in women receiving resveratrol supplementation (200 mg/day) continuously with a control group	Resveratrol supplementation during embryo transfer cycles appears to be detrimental for pregnancy outcomes
Bahramrezaie et al., 2019 [39]	Randomized controlled trial comparing the IVF outcomes in PCOS women receiving resveratrol supplementation (800 mg/day) continuously for 40 days before the initiation of ovarian stimulation with a control group	The number of mature oocytes, cleavage rate, fertilization rate, and fertility rate were not significantly different between the two groups, but resveratrol supplementation is associated high-quality oocyte rate and high quality embryo rate
Gerli et al., 2021 [40]	Randomized controlled trial comparing the IVF outcomes in women receiving resveratrol supplementation (300 mg/day) continuously for 3 months before the initiation of ovarian stimulation with a control group	Resveratrol supplementation is associated with significantly higher numbers of oocytes and MII oocytes, higher fertilization rates, and higher numbers of embryos and blastocytes per patient. No significant differences in biochemical or clinical pregnancy, live birth, and miscarriage rates are revealed

Table 6 Unpublished RCT about resveratrol in IVF setting

Identification number	Title
NCT01782911	Effect of Resveratrol on Metabolic Parameters and Oocyte Quality in PCOS Patients Undergoing IVF Treatment
NCT06235294	Effects of Resveratrol Supplementation on Oocyte Quality in Advanced Maternal Aged Women Undergoing in Vitro Fertilization

reserve markers) is the one most likely to benefit from taking the resveratrol-based nutraceutical, and therefore the data should be evaluated in other groups of patients.

The unpublished and ongoing RCT on resveratrol supplementation in IVF available on Clinicaltrials.gov are shown in Table 6.

In conclusion, despite sample size does not allow to address the impact of resveratrol prior OS on clinical pregnancy rate, the preliminary results of this study suggest that such a treatment improves ovarian sensitivity to exogenous FSH at least in women above 35 years of age. Following confirmation of our data, pre-treatment with resveratrol may decrease the risk of unexpected hyporesponse to OS in advanced reproductive age women.

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Authors' contributions
A.C., B.F., and C.A. designed study. S.P., R.D.G., M.Y.R. performed data collection. G.G.I. analysed data. G.G.I. prepared figures and tables. A.C. and C.A. supervised research. A.C., G.G.I., B.F. and C.A. wrote manuscript. All authors reviewed the manuscript.

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Availability of data and materials
Not applicable.

Declarations

Ethics approval and consent to participate
This study was approved by the local Ethics Committee of the University of Naples Federico II on January 2019 (n.328/18).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Publisher's Note

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CASE REPORT
Medicine and Biotechnology

Successful Mestrual Regularity and Spontaneous Pregnancies with a Resveratrol-Based Multivitamin Supplement in Women with Idiopathic Premature Ovarian Insufficiency

Prof. Michele Vignali

Abstract

Premature ovarian insufficiency (POI) is a clinical syndrome defined by loss of ovarian activity before the age of 40 years and is characterized by menstrual disturbance, follicle stimulating hormone (FSH) concentration above 40 IU/l and infertility. In some patients the best option is to conceive spontaneously since many treatment strategies remain unsuccessful or involve eggs donation. In this case report series, we describe the effects of a resveratrol-based multivitamin supplement containing trans-resveratrol, folic acid, vitamin B6, B12 and D, in six women with poor prognosis of pregnancy due to POI and evaluate the achievement of desired conception.

These women, aged less then 40 years, suffered from menstrual irregularities, anovulation and infertility. They all had normal karyotype, and no history of ovarian surgery, radiation exposure or chemotherapy. Blood test showed at least two values of FSH above 40 IU/l.

Four out of six patients with POI conceived after 3-6 months of a resveratrol-based multivitamin supplement, ultimately giving birth to a full-term baby. Regular menstrual cycle was restored in all patients after two to four months the start of treatment. In conclusion the treatment with a resveratrol-based supplement improved menstrual regularity and suggest a useful potential of this supplementation in some cases of POI.

Keywords: infertility, resveratrol, menstrual irregularity, POI

Introduction

Premature Ovarian Insufficiency, also known as primary ovarian insufficiency, is characterized by premature depletion or dysfunction of ovarian follicle (1), with irregular menses (intermittent or unpredictable) before the age of 40. There is no unanimous consensus on what the correct criteria are for identifying POI in adolescent, and delay in diagnosis is common (1). Despite the description of different genetic, immune and iatrogenic factors of POI, the etiology in most cases of this disease are unexplained. POI is characterized by the presence of oligo/amenorrhea in association with menopausal serum level of FSH above 40 IU/l and must be distinguished from natural premature menopause characterized by serum level of FSH very high (between 16 and 134 IU/L), estradiol very low (less than 20 pg/ml), permanent amenorrhea and absence of ovulatory cycles. POI occurs in approximately 1-2% of women aged under 40 years and in 0.1% of women aged under 30 years that present abnormal bleeding pattern (3). Clinical symptoms are similar to premature menopause, including hot flashes, night sweats, vaginal dryness, irritability, difficult on concentration (3). Five to ten percent of women with POI manage to conceive spontaneously while no women affected by premature menopause can conceive naturally.

The diagnosis of POI is psychologically devastating in reproductive-aged women because of deleterious impact on fertility. As long as ovulation is extremely rare and unpredictable in women with POI and none of the ovulation induction regimens have been shown to be effective, treatment strategies are lacking. Successful pregnancy with assisted reproductive technology (ART) rarely occurs in POI patients and the most successful

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method remains eggs donation, with important ethical limitations (3,4).

Recent studies have demonstrated the beneficial effects of resveratrol in humans. Resveratrol has been reported to decrease oxidative stress and attenuate inflammation, and these mechanisms may account for many of its health benefits. Important evidence is also emerging in the field of human reproduction indicating that resveratrol has potential positive effects in older women, PCOS, endometriosis, uterine fibroids and menopause (5). Concerning the oocyte aging, recent research have demonstrated that resveratrol is effective on granulosa cells and impacts positively on the ovarian physiology. Specifically, resveratrol induces granulosa cells proliferation through activation of the PI3K pathway and promotes primordial follicle activation (6). Compared to untreated tissues, a higher proportion of growing follicles in human ovarian tissue culture in the presence of resveratrol has been reported (6,7). However, recently conflicting findings on the actions of resveratrol on decidualization of human endometrial stromal cells (HESCs) have been published. Ochiai et al. demonstrated that resveratrol inhibits decidual transformation of primary cultured HESCs (8) while Mestre Citrinovitz et al. showed that resveratrol enhances decidualization of HESCs in culture (9). Nevertheless, because of the lack of robust data, more studies are required to verify these effects.

Furthermore in premature ovarian failure (POF) animal models, resveratrol effectively improved the ovarian function and the productive capacity of FGSCs via relieving oxidative stress and inflammation and a mechanism involving the hh signaling pathway, suggesting that resveratrol is a potential agent against POF (10).

Case Report Series

In this case report series, we describe the effects of a resveratrol-based multivitamin supplement containing trans-resveratrol (150mg /day in two administrations), folic acid, vitamin B6, B12 and D for at least 60 days, in 6 infertile women, with poor prognosis of pregnancy due to POI, with particular attention to the resumption of regular menstrual flow and/or the achievement of spontaneous conception, during an observation period of 12 months. None of the patients observed could access ART procedures because of an insufficient ovarian reserve whereas every partner has normal seminal test. When POI was diagnosed, all six patients had FSH level above 40 UI/L, irregular menses, normal karyotype, no history of ovarian surgery, radiation exposure or chemotherapy, and a BMI < 30 kg/m2.

Hereafter the description of the individual 6 cases.

Case 1

A 35-year-old female who was diagnosed with POI when she was 34, at the time of the first visit reported vasomotor symptoms, night sweats and being in amenorrhea for six months. The FSH serum level was 39,5UI/L.

After starting resveratrol supplementation, at the second visit two months later, she reported that her period has resumed about six weeks after starting treatment and that vasomotor symptoms and night sweats have disappeared. The FSH serum level in early follicle fase was 13UI/L.. She continued the treatment and returned for a check-up visit after 10 months in which she reported that she was back in amenorrhea. A transvaginal ultrasound was performed and a gestational sac with a viable embryo was recognized. She delivered a healthy full-term baby.

Case 2

A 29-year-old woman presented to our clinic with a 6-month history of oligo-amenorrhea and no vasomotor symptoms or night sweats. Laboratory blood tests performed while the patient was in amenorrhea revealed an elevated serum FSH level (38 IU/l) and AMH 0.01 ng/ml. She was given a diagnosis of POI. Since the patient wished to become pregnant it was suggested to her that egg donation or IVF were her best option to have a child. She chose to attempt conception with her own rather than using donor eggs. She started resveratrol-based multivitamin supplementation and her period was restored after 40 days of treatment. Three months later she conceived a baby naturally, without the need for assisted reproductive technology. This was confirmed by serial serum beta HCG measurements. The pregnancy proceeded uneventfully until the 35th week of gestation when she developed gestational hypertension. The baby was born at 39 weeks and 4 days of gestation, without any serious complications.

Case 3

A 38-years-old patient presented to our clinic reporting that she had been in amenorrhea for 8 months, with vasomotor symptoms and night sweats, and that she wished to become pregnant. She was diagnosed with POI and treated with various hormonal regimens coupled with close ultrasound follicle monitoring. She referred that she had very few ovulatory cycles over the last 2 years. Her serum FSH value at the moment of diagnosis was 55 IU/L. After about 4-months of treatment with resveratrol-based supplement her period resumed. The serum FSH level decreased to 18 IU/L. She continued to have menstrual bleeding every 60 days, but unfortunately, she was unable to get pregnant.

Case 4

A 39-years-old patient presented to our clinic reporting oligo-amenorrhea over the last 2 years and 4 years of infertility. Laboratory blood tests performed at the age of 37, revealed FSH serum level of 45 IU/L and AMH 0.02 ng/ml. The patient reported to have undergone several hormone replacement cycles and ovulation induction cycles and even an unsuccessful trial of intrauterine insemination (IUI) over the last 3 years. She said she was on the waiting list for eggs donation, thus we decided to start a resveratrol-based multivitamin supplementation. Four months after starting the treatment she began to menstruate regularly (every 40days), the serum FSH level decreased to 12,2 IU/L and she was able to conceive after eight

months of treatment with resveratrol-based multivitamin supplementation and delivered a healthy baby after 38 weeks of gestation.

Case 5

A 34-year-old patient came to our observation reporting that she had not menstruated for three months. Her female hormone panel showed serum FSH level of 49IU/mL and AMH of 0,01 ng/ml. Seven-weeks after starting the treatment with resveratrol-based supplement she reported having started menstruating again and a FSH serum level that was decreased to 16 IU/L. She was monitored for one year and her menstrual cycle appeared regularly every 32.4 +4 days, but unfortunately, she was unable to get pregnant.

Case 6

A 30-year-old patient with an 8-month history of amenorrhea and a previous diagnosis of POI at age of 28 presented to our clinic, manifesting her maternity desire. We confirmed diagnosis of POI (FSH value 40,8IU/L) and treated her with a resveratrol-based multivitamin supplement. After about 6 months of treatment her menstrual cycle appeared regularly every 30.4+3.8 days and the FSH value decreased to 11IU/L. This patient underwent to close ultrasound follicle monitoring; spontaneous ovulation occurred and the resulting pregnancy was confirmed by serum HCG and ultrasonography performed 28 days after ovulation. She delivered a healthy baby after 38 weeks of gestation.

Discussion

Several regimens have been employed in the setting of POI to restore menses and to achieve pregnancy however none have been proven to be effective (11,12). Oocyte donation is the most frequent suggested route of treatment in order to get pregnant, nevertheless this practice is not yet available in many countries. There is also no evidence that assisted reproductive technology (ART) without oocyte/embryo donation may improve pregnancy rate of POI patients without any other infertility factors within a spontaneous ovulatory cycle (11,12). Analysis of the six cases presented herein revealed restoration of menstrual cycle flow in all the patients after supplementation with resveratrol for at least 2 months Moreover four out of six patients with a diagnosis of POI successfully conceived spontaneously without the need of assisted reproductive technologies. Only one preclinical study, conducted on animal models, discussed the opportunity of resveratrol supplementation for women with premature ovarian insufficiency (10). The potential pitfall of this present case report is the lack of homogenous classifications of the POI population and the little number of cases. We are aware that case reports cannot decide a therapeutic management but certainly these findings can be useful to pave the way for new treatment hypotheses especially in this selected population (POI) whose treatment is not yet universally defined. Further studies are required to verify these possible beneficial effects of resveratrol observed in this limited case series.

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Table 1. Summary of POI women

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6
Age of POI diagnosis	34	29	38	37	34	28
Age at menarche	12	11	13	13	11	12
Age at initial visit to our clinic	35	29	38	39	34	30
FSH UI/L at diagnosis (medium value)	39,8	38	55	45	49	40,8
FSH UI/L early follicular fase	13	15	18	12,2	16	11
Menses /DAYS (medium value)	55	39	62	43	80	38
Treatments: Resveratrol-based multi supplement	+	+	+	+	+	+
Method for pregnancy	Spontaneous	Spontaneous	-	Spontaneous	-	Spontaneous
Pregnancy outcome	40 wks, female 3900g	39 wks, female 3455g	-	38 wks, female 3100g	-	38 wks, male 3840g

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Article

Resveratrol Treatment Induces Mito-miRNome Modification in Follicular Fluid from Aged Women with a Poor Prognosis for In Vitro Fertilization Cycles

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Abstract: Advanced maternal age impairs reproductive performance, influencing the quantity and the quality of oocytes. Mitochondria dysfunction seems to play a decisive role in conditioning the quality of the female gamete. Different in vitro and in vivo studies, demonstrated the antioxidant and anti-inflammatory activities of Resveratrol and its ability to improve mitochondria function even if the exact mechanism of action has not yet been demonstrated in human oocytes. In this paper, by retrospective analysis, we evaluated follicular fluid (FF) miRNome modification in aged women with a poor ovarian reserve receiving a resveratrol-based supplement the three months before the in vitro Fertilization (IVF) cycle. We found 13 differentially expressed microRNAs (miRNAs) in women treated with resveratrol and specifically miR-125b-5p, miR-132-3p, miR-19a-3p, miR-30a-5p and miR-660-5p, regulating mitochondrial proteins, are able to control metabolism and mitochondrial biogenesis. MiRNA expression differences, observed after resveratrol treatment in FF from women with a poor prognosis for IVF, demonstrated that resveratrol may act on mitomiRNAs to improve follicular microenvironment by transcriptomic and proteomic modifications in granulosa cells.

Keywords: reproductive aging; poor prognosis women; Resveratrol; regulation of gene expression; microRNAs; mitochondria

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1. Introduction

Reproductive aging is a complex biological phenomenon concerning physiological, genetic and molecular changes [1] which begins during the fourth decade of a woman's life, leading to increased infertility and pregnancy risks [2]. Decreasing fertility is primarily due to waves of oocyte atresia whereby growing follicles are continuously induced to undergo cell death with consequent depleting of the non-renewable ovarian reserve established during fetal development [3]. A broad range of progressive changes also occurs in oocytes thus limiting reproductive success. Different alterations in cytoplasmic and nuclear maturation processes have been described and the increase in non-disjunction meiotic errors probably represents one of the most important [4,5]. Mitochondria are the oocyte powerhouse, and through oxidative phosphorylation provide the energy for transcription and translation during oocyte maturation, fertilization, and embryonic development. In

reproductive aging, several mitochondria dysfunctions, such as a decrease in their number, mtDNA damage and membrane potential instability, have been described [6]. Mitochondrial dysfunction, causing ATP deficiency with increased oxidative stress, can contribute to impairments in meiotic spindle assembly, cell cycle regulation, chromosome segregation, embryo development, and implantation [7]. Additional treatments with micronutrients, before IVF cycles, have been demonstrated to protect the follicular microenvironment from oxidative stress, increasing the number of good quality oocytes recovered at the pickup [8]. Thus, finding new strategies aiming at enhancing mitochondrial function to improve oocyte quality and age-related infertility has become the goal of a plethora of studies [6].

Different papers demonstrated that Resveratrol (3,5,4′-trihydroxystilbene), a stilbenic structure polyphenol, that under normal intracellular redox conditions, behaves as a natural antioxidant at low concentrations [9], improves mitochondria function with an induction of genes for oxidative phosphorylation and mitochondrial biogenesis. High-dose resveratrol, instead, may prompt pro-oxidant effects, inducing systemic inhibition of P450 cytochromes and mitochondrial-dependent cell death [9–11].

Moreover, Resveratrol, inducing SIRT1, decreases the Peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1α) acetylation and increases its activity [12,13]. Its antioxidant and anti-inflammatory activities have been shown in different cellular models including human granulosa cells which enhanced metabolic activities, mitochondrial biogenesis and the global electric potential production [14]. In a recent review, the authors discuss the use of resveratrol, and other antioxidant treatments to improve human oocyte and embryo quality, focusing on the mitochondria as their main targets. However, they conclude that the mechanism of action of the treatments has not yet been demonstrated in the human oocyte and highlight the need for further studies in this field [15].

In recent years, different papers have established that polyphenols control the expression of microRNAs (miRNAs) in inflammation, cancer, cell differentiation, and homeostasis. Specifically, it has been demonstrated that in cancer cells, resveratrol treatments decrease the levels of several oncogenic miRNAs while increasing the levels of tumor suppressor miRNAs [16]. In 2017, an article reported changes in miRNA expression linked to the deficiency of Carnitine palmitoyltransferase-2 (CPT2), a mitochondrial enzyme involved in long-chain fatty acid entry into mitochondria for their β-oxidation and energy production. The authors demonstrated that resveratrol treatments can induce changes in miRNA expression linked to CPT2-deficiency [17].

In order to explore the role of resveratrol and its possible action mechanisms in improving female reproductive potential, we analysed miRNA profiles in Follicular Fluid (FF) samples from women of advanced reproductive age and with a poor prognosis for *In Vitro* Fertilization IVF, who have received Resv@MDH based supplementation for three months before undergoing IVF cycles and compared to those of women selected as the control group who had not received any supplementation. Resveratrol is poorly bioavailable because of reduced absorption mainly due to its low solubility and fast metabolism that converts it into glucuronide and sulfates compounds [18]. Solid dispersion of resveratrol supported on Magnesium DiHydroxide (Resv@MDH) has been recently developed to improve solubility and increase the bioavailability of resveratrol [18].

We decided to focus our analysis on FF because it contains different molecules produced by both the somatic and germinal components of the follicle and it has been widely demonstrated that its composition reflects the quality of the female gamete [19]. Moreover, miRNAs are able to influence the complex protein framework with fundamental roles in the numerous pathways working in the ovarian follicle and that are activated during the follicular development, oocyte maturation and acquisition competence [20]; it is also known that miRNA-altered expression can be associated with different reproductive disorders [21–23]. Moreover, a correlation has been reported between changes in miRNA expression and oocyte aging and epigenetics [23]. MiRNome modification in FF from aged women after resveratrol supplementation could demonstrate that the nutraceutical acts on the regulation of molecular pathways related to folliculogenesis and could improve the

reproductive success in women of advanced reproductive age with a poor prognosis for IVF cycles.

2. Materials and Methods

2.1. Patients

Twelve women undergoing IVF treatment were enrolled at the Center of Reproductive Medicine and IVF Unit in Conversano, ASL Bari (Bari, Italy), between July 2019 and December 2019. All patients signed informed consent. The study protocol was approved by the local Ethical Committee (n. 5790). We evaluated the patients who satisfied the following entry criteria: women 35–42 years old with a poor ovarian reserve (AMH < 1.2 ng/mL, AFC < 5, POSEIDON group 4), excluding tumors and/or previous radio-chemotherapy, endometriosis, and severe male factors. Daily nutraceutical supplementation (trade name GENANTE^{TM}) containing Resv@ MDH (total resveratrol 150 mg), folic acid (400 mcg), vitamin D (25 mcg), vitamin B12 (2.5 mcg), and vitamin B6 (1.4 mg) was used. Six women receiving this supplementation in the preceding three months of IVF cycles represent the treatment group and were compared with six women for the same period who did not receive resveratrol supplementation (ctrl, multivitamin supplementation without resveratrol). Ovarian stimulation was performed by administering Human menopausal gonadotropin (Meropur©, Ferring, Milano, Italy) at the starting dose of 300 IU–450 IU per day from the 1st or 2nd day of induced menstruation. According to the ovarian response, serial ultrasound examination and serum routine hormonal measurement (Follicular stimulating hormone, FSH; luteinizing hormone, LH; estradiol, E2, Progesterone, P) every two days, the dose of gonadotropins was adjusted. When the dominant follicle reached 14 mm in diameter, Gonadotropin-Releasing Hormone GnRH antagonist (Orgalutran©, MSD, Merck Sharp & Dohme Corp., Inc., Kenilworth, NJ, USA) was administered. Human chorionic gonadotropin (hCG) 10,000 IU s.c. (Gonasi©, IBSA, Lodi, Italy) was administered when at least two follicles reached a mean diameter of 18 mm. Oocyte retrieval was performed by a transvaginal sonography-guided technique 35–36 h after the triggering of ovulation. Basic and clinical information of all participants are presented in Table 1.

Table 1. Clinical parameters of women enrolled in the study. Values are reported as mean ± standard deviation or percentage (%). *p*-values are based on a two-sample *t*-test. MBI: body mass index; AMH: anti-Müllerian hormone.

Parameters	Study Group	Control Group	*p*-Value
Patients (N°)	6	6	
Age (Years)	38 ± 3.3	39 ± 3	0.29
BMI (kg/m^2)	23.6 ± 3.15	22.16 ± 1.8	0.37
AMH (ng/mL)	0.72 ± 0.32	0.77 ± 0.43	0.31
Antral Follicle Count (N°)	3.83 ± 1	4.16 ± 1	0.29
Gonadotropin dosage (IU)	3425 ± 799	3750 ± 1152	0.28
Stimulation protocol length (days)	10.8 ± 1.2	10.8 ± 1.2	0.5
Follicles (N°)	8.6 ± 6.5	7 ± 4	0.29
MII oocytes (N°)	5.5 ± 3.7	5.8 ± 2.7	0.4
Pregnancy rate (%)	50%	33%	

2.2. Follicular Fluid Sample Collection

FF samples without any flushing were collected during the aspiration of ovarian follicles (Sense™ Single lumen Needle, Vitrolife, Goteborg, Sweden), centrifugated at 2800 rpm for 20 min to remove residual follicular cells and any blood traces. The supernatant, placed into sterile polypropylene tubes, was immediately stored at −20 °C. Only FF samples with no macroscopic evidence of blood were selected.

2.3. RNA Isolation and Precipitation

Total RNA was extracted from 400 µL of follicular fluid samples by using Qiagen miRNeasy Mini Kit (Qiagen, GmbH, Hilden, Germany), according to Qiagen Supplementary Protocol for purification of RNA (including small RNAs) from serum or plasma. The RNA precipitation protocol was performed to increase total RNA yield. Briefly, RNA was first eluted in 200 µL of RNAse-free water and then added to 20 µg of UltraPure Glycogen (ThermoFisher), 0.1 volume of 3 M sodium acetate and 2.5 volumes of ice-cold absolute ethanol and incubated at −80 °C overnight. The day after, RNA was centrifuged and washed three times in ice-cold 75% ethanol and resuspended in 7 µL of RNAse-free water. The spectrophotometer was used to quantify total RNA before and after precipitation.

2.4. MiRNA Expression Profile

The expression profile of 800 miRNAs from follicular fluid samples was analyzed by the NanoString nCounter system assay through the NanoString platform and the nCounter Human v3 miRNA Expression Assay Kits (NanoString Technologies, Seattle, WA, USA), according to the manufacturer's instructions. MiRNA expression profiling was performed on 6 FF samples from women treated with resveratrol and 6 FF samples from women not treated and used as controls. Approximately 100 ng of RNA in a final volume of 3 µL were used. Briefly, samples were processed using the automated nCounter Prep Station; after the hybridization step, they were purified and immobilized on a sample cartridge for quantification and data collection by using the nCounter Digital Analyzer. The nSolver 3.0 software was used for data analysis according to user manual instructions (https://www.nanostring.com/products/analysis-software/nsolver (accessed on 1 January 2022)).

2.5. MiRNA Functional Enrichment Analysis

In order to investigate the potential biological role of DE miRNAs we performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway computational analyses by using Diana-miRPath v3.0 (http://snf-515788.vm.okeanos.grnet.gr/ (accessed on 1 January 2022)) and selected for validated mRNA targets retrieved from Tarbase7.0. The FDR method was applied to select the signaling pathways with a threshold of significance defined by $p \leq 0.05$, a microT threshold of 0.8 and an enrichment analysis method performed by Fisher's Exact Test (Hypergeometric Distribution). The Gene Cards Human Gene Database (https://www.genecards.org/ (accessed on 1 January 2022)) was queried to obtain two different lists of predicted and validated human genes associated with molecular signaling pathways related to the cellular response to oxidative stress and oocyte meiosis. These lists were compared to DE miRNA validated target genes retrieved by miRTarBAse (https://mirtarbase.cuhk.edu.cn/~miRTarBase/miRTarBase_2022/php/index.php (accessed on 1 January 2022)). Only target genes found in both computational analyses- the first performed in the Gene Cards Human Gene Database and the second performed in miRTarbase were selected and used to design the two regulatory networks reporting miRNA-mRNA interactions specific for cellular oxidative stress response and oocyte meiosis that were built by Cytoscape software (version 3.8.2) (https://cytoscape.org/).

2.6. Statistical Analysis

For miRNA profiling analysis, we performed the Volcano Plot and Significance Analysis of Microarrays (SAM) statistical tests by using MeV (Multi experiment Viewer v4.8.1) (http://mev.tm4.org/ (accessed on 1 January 2022)) software. Differentially expressed miRNAs with statistical significance were screened using the threshold log2 fold change ≥ 0.5 (log2FC) and p-values ≤ 0.05 corrected for multiple testing by using the Bonferroni method. SAM statistical test was performed as follows: all statistical tests were computed by applying a two-class unpaired test among log2FC and using a q-value based on 100 permutations; imputation engine: K-nearest neighbors, number of K-nearest neighbors: 10 neighbors. The Benjamini–Hochberg multiple testing correction method for high throughput analyses using a stringent false discovery rate (FDR) limit < 0.05 was applied.

Unpaired *t*-test was applied for DE miRNA expression validation by using GraphPad Prism 6. Statistical significance was assessed by setting the *p*-value cut-off ≤ 0.05. Differences in IVF outcome parameters and Pearson's correlation analyses were computed between the FC values of mitomiRNAs and biochemical pregnancy scores, by GraphPad Prism 6 (https://www.graphpad.com/ (accessed on 1 January 2022)). Statistical significance was established at $p \leq 0.05$. Linear regression analysis was also carried out on GraphPad Prism 6 software only for tight significant correlations.

3. Results

3.1. MiRNA Expression Profiling

A high-throughput miRNA expression analysis of 800 miRNAs was performed in FF samples from women treated with the resveratrol-based nutraceutical supplement and women not treated by using Nanostring technology. The analysis revealed 8 differentially expressed (DE) miRNAs as shown by the volcano plot (Figure 1) and 10 DE miRNAs as revealed by SAM statistical analysis. Only DE miRNAs common to both statistical tests were chosen for further computational analyses: miR-125b-5p, miR-132-3p, miR-19a-3p, miR-30a-5p and miR-660-5p (Table 2 and Figure 2).

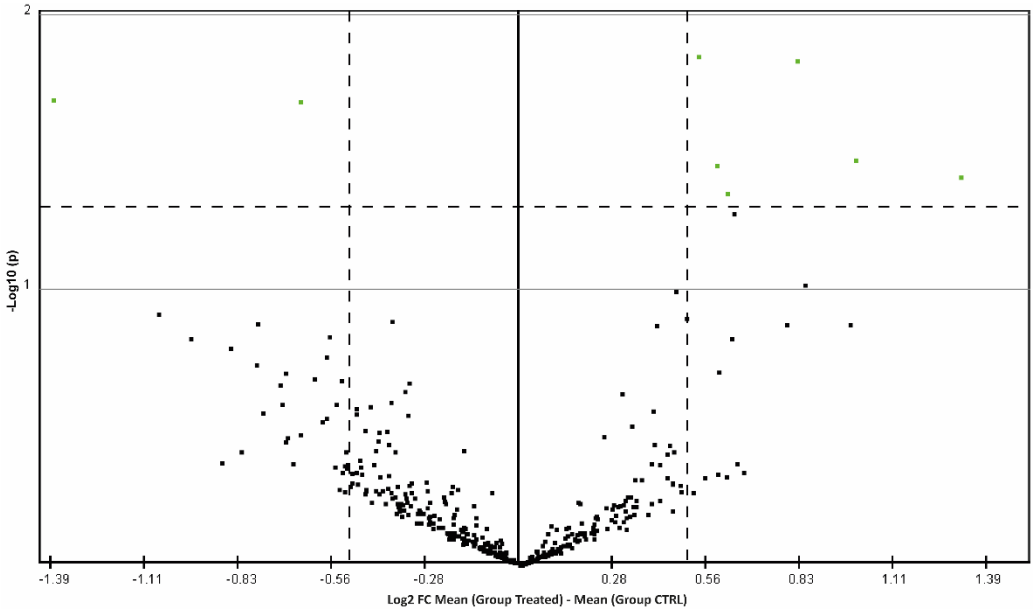

Figure 1. Nanostring miRNA profiling analysis. Volcano plot displaying the differences in fold change (log2FC) of miRNA expression in resveratrol treated FF samples vs. CTRL obtained after data normalization analysis. The *x*-axis indicates differences in log2FC and the *y*-axis indicates the −log10 *p*-value. The horizontal dashed line indicates the threshold for probability of significance ($p = 0.05$) and the vertical dashed lines set the threshold to 0.5 for the difference in FC of miRNA expression. miRNAs whose expression level is at least 0.5-fold different in resveratrol treated FF samples compared to CTRL, with $p < 0.05$ corrected for multiple testing by using the Bonferroni method, are indicated by green dots.

Table 2. List of DE miRNAs in resveratrol treated FF samples vs. CTRL. DE miRNAs were selected according to Volcano Plot and SAM statistical tests. DE miRNAs common to both statistical tests and chosen for further analyses are highlighted in bold. The FC value of each miRNA is reported.

DE miRNAs	Ttest	SAM	Fold Change Treated vs. Ctrl
miR-1180-3p		X	−1.55
miR-125b-5p	**X**	**X**	−1.76
miR-132-3p	**X**	**X**	−2.47
miR-16-5p		X	−1.73
miR-195-5p	X		−1.53
miR-19a-3p	**X**	**X**	−1.5
miR-30a-5p	**X**	**X**	−1.99
miR-30d-5p		X	−1.97
miR-323a-3p	X		1.57
miR-365a-3p + miR-365b-3p		X	−1.79
miR-497-5p		X	−1.41
miR-574-5p	X		2.62
miR-660-5p	**X**	**X**	−1.44

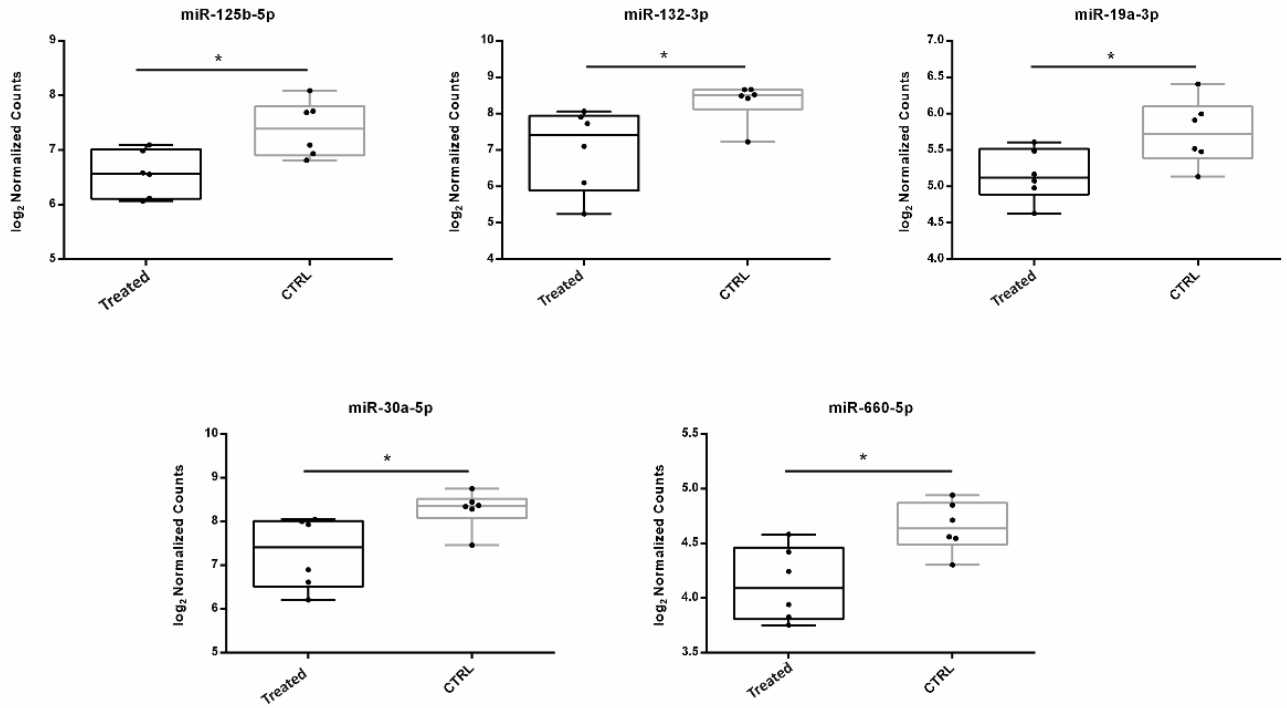

Figure 2. Relative expression of miRNAs in FF samples. DE miRNA relative expression in resveratrol treated FF samples vs. CTRL is shown by box-and-whisker plots. Expression data are represented as log2normalized counts. Significant *p*-values corrected for multiple testing by using the Benja-mini-Hochberg method are indicated by <<*>> (* *p*-value $\leq$ 0.05).

3.2. Functional Enrichment Analysis of DE miRNAs

We investigated DE miRNA functions for molecular signaling pathway enrichment specifically for cellular response to oxidative stress and oocyte meiosis. Functional enrichment analyses showed that the validated target genes of DE miRNAs may regulate several signaling pathways involved in fertility: fatty acid biosynthesis, estrogen signaling pathway, FOXO signaling pathway, p53 signaling pathway, TGF-beta signaling pathway, Hippo signaling pathway and oocyte meiosis (Figure 3). Regulatory network analyses showed that among the DE miRNA target genes, 84 out of 1836 were involved in the

cellular response to oxidative stress (Figure 4), and 37 out of 1836 were involved in oocyte meiosis (Figure 5).

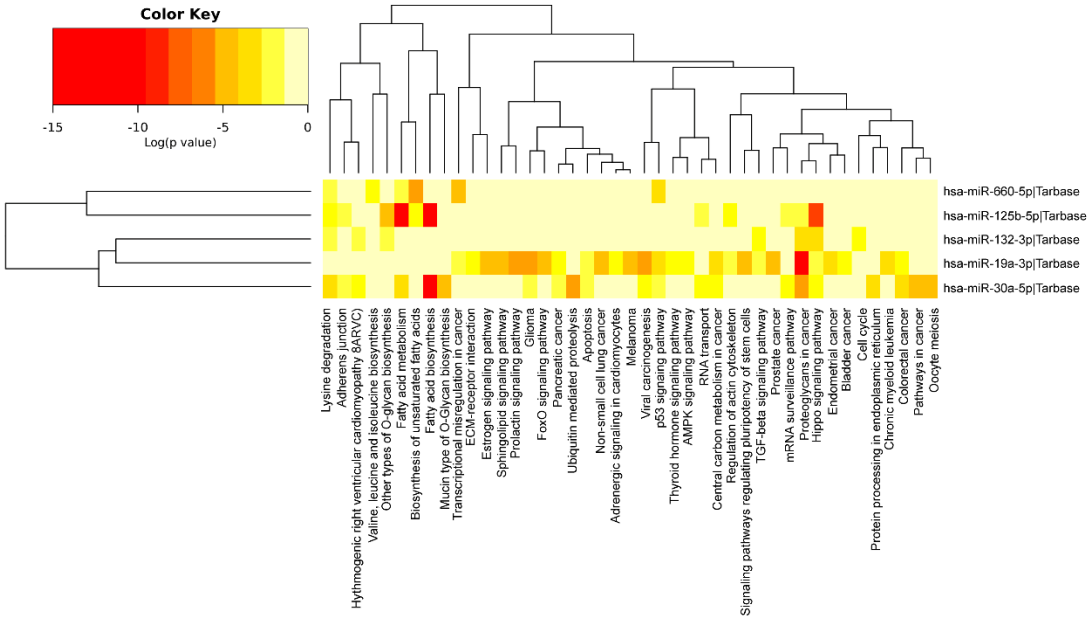

Figure 3. KEGG pathway analysis of DE miRNAs. Functional enrichment analysis of all DE miRNA target genes using KEGG pathway analysis. Log (*p*-value) is indicated by a yellow-red-coloured key.

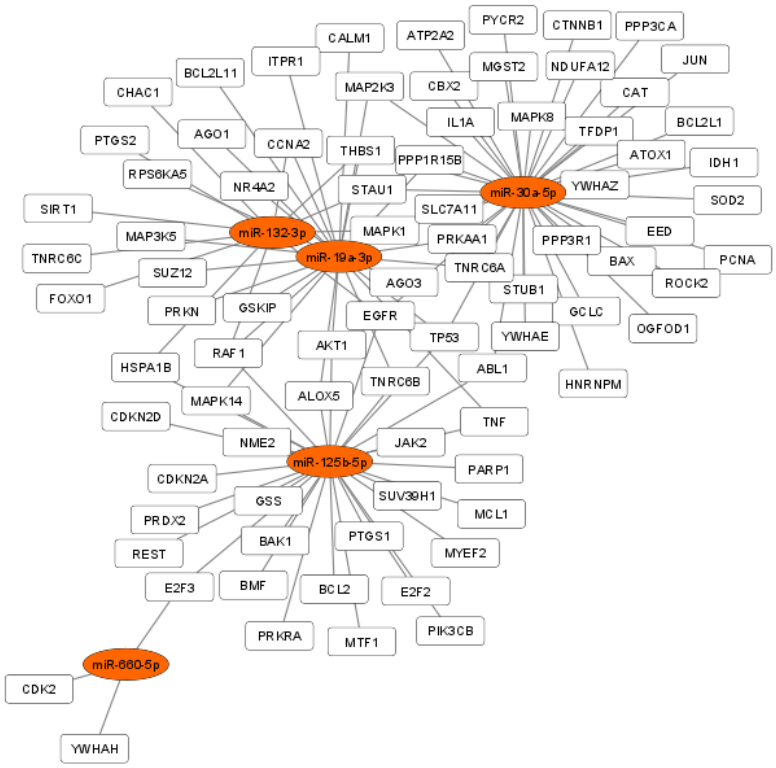

Figure 4. DE miRNA target genes related to the cellular response to oxidative stress. Regulatory network showing the interaction between miR-125b-5p, miR-132-3p, miR-19a-3p, miR-30a-5p and miR-660-5p and their validated mRNA targets. Orange ellipses represent miRNAs and white rectangles represent mRNA target genes involved in the cellular response to oxidative stress.

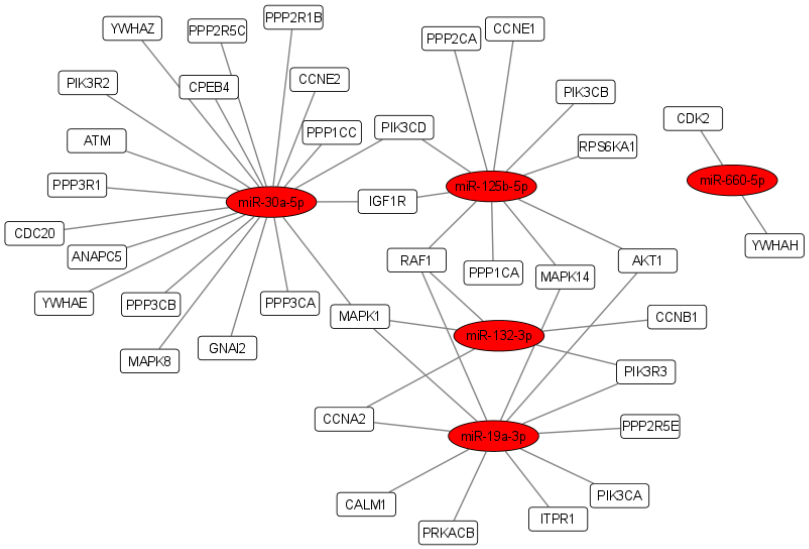

Figure 5. DE miRNA target genes related to oocyte meiosis. Regulatory network showing the interaction between miR-125b-5p, miR-132-3p, miR-19a-3p, miR-30a-5p, miR-660-5p and their validated mRNA targets. Red ellipses represent miRNAs and white rectangles represent mRNA target genes involved in oocyte meiosis.

3.3. Impact of Resveratrol Supplementation on IVF Outcome

The mean number of fertilized good quality oocytes when patients received supplementation was significantly increased from 63% to 80% (Figure 6A). By comparing treated and control patients, we found a significant negative relationship between the biochemical pregnancy scores and miR-125b-5p ($r = -0.91$), a lower negative relationship between the biochemical pregnancy scores and miR-132-3p ($r = -0.43$), whereas there was no correlation between biochemical pregnancy scores and miR-30a-5p ($r = 0.05$) (Figure 6B,C).

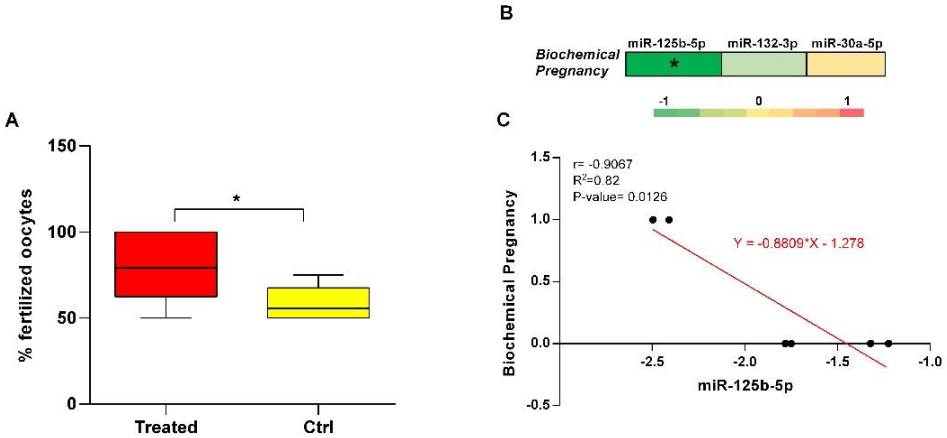

Figure 6. Impact of Resveratrol supplementation on IVF outcome. (**A**) Box and whisker plots showing the number (%) of fertilized good quality oocytes (MII) in treated and control groups. Statistically significant *p*-values ($p \leq 0.05$) are indicated by asterisks. (**B**) Correlation matrix obtained by calculating Pearson correlation coefficients for mitomiR expression (FC) and biochemical pregnancy scores. The correlation values are indicated by a color gradient from green (negative correlation) to red (positive correlation), as shown in the colored bar. Statistically significant *p*-values ($p \leq 0.05$) are indicated by asterisks. (**C**) Scatterplot on miRNAs tightly correlated and showing the best-fit line obtained from linear regression analysis.

4. Discussion

Over the years, a women's ovarian reserve, made up during intrauterine life, is gradually depleted and the quality of oocytes becomes lower for the increase in chromosomal aneuploidies, decreasing mitochondrial quality and impaired balance between oxidative stress and antioxidant defenses [24]. Female reproductive aging reduces fertility and pregnancy outcomes and represents a critical problem in developed countries [25]. Considering that women often have to delay their first pregnancy for professional reasons and Assisted Reproductive Technology (ART) is frequently inefficacious in ovarian ageing, it is becoming increasingly important to design innovative strategies to improve pregnancy success in women over 35 years of age. Resveratrol is a nutraceutical with several therapeutic effects. It has been shown to exert anti-inflammatory and anti-oxidative effects and affect the initiation and progression of many diseases through different mechanisms [26]. Several studies seem to demonstrate that resveratrol improves male and female reproductive function even if the mechanisms of action and the therapeutic effects remain not fully clarified [27]. Recently, in an in vitro model, Ragonese and collaborators demonstrated that resveratrol improved ATP production and cell viability and promoted the induction of cellular differentiation, increasing mitochondrial biogenesis, in granulosa cells [14]. To evaluate the biological effects of the resveratrol nutraceutical in vivo, we investigated miRNA profiles in FF, comparing their expression between aged women treated with resveratrol and untreated controls. We found that the treatment induces important variations of miRNA content in FF and specifically the significant downregulation of miR-19a-3p, miR-30a-5p miR-125b-5p, miR-132-3p and miR-660-5p (Figure 1 and Table 2). It is known that miRNAs can influence mitochondrial activity in different ways. Nuclear miRNAs can regulate mitochondrial proteins encoded by the nuclear genome alternatively, after their translocation inside the mitochondrial matrix, proteins encoded by mitochondrial DNA [28]. MiRNAs could also be transcribed from the mitochondrial genome and after maturation inside the mitochondria, the miRNAs could inhibit mRNA translation in the mitochondrial and cytosolic compartments. There is still little experimental evidence of miRNAs encoded by the mitochondrial genome, instead, to date, about 150 nuclear miRNAs have been detected in mitochondria of different species (MitomiRs). MitomiRs are encoded by the nuclear genome, imported into mitochondria and involved in mitochondrial and nuclear genome regulation. They can regulate metabolism and mitochondrial biogenesis even if, the details of how they are imported into the mitochondria or how they regulate mitochondrial gene expression need to be addressed [29]. Notwithstanding, the role of mitomiRs has been widely demonstrated in the pathogenesis of cardiovascular diseases and neurodegenerative pathologies [29]. Three of the identified downregulated miRNAs, miR-30a-5p miR-125b-5p, miR-132-3p are mitomiRs. MiR-30a-5p can regulate mitochondria fission and apoptosis through TP53 and Dynamin-related protein 1 (DRP1). DRP1, interacting with other proteins, as mitochondrial fission factor (MFF), induces the mitochondrial division and its transcription is promoted by TP53. TP53 is a validated target of miR-30a-5p, accordingly, the downregulation of miR-30a-5p could indirectly upregulate DRP1 enhancing mitochondrion fission [30].

Carnitine-acyl carnitine translocase (CACT) is a critical mitochondrial carrier involved in lipid metabolism. CACT catalyzes both unidirectional transport of carnitine and carnitine/acylcarnitine exchange in the inner mitochondrial membrane, allowing the import of long-chain fatty acids into the mitochondria where they are oxidized by the β-oxidation pathway. Its inactivation, mediated by the upregulation of miR-132, has been demonstrated in obese mice; consequently, the downregulation of miR-132, after resveratrol treatment, could increase fatty acid catabolism which is an important source of energy for the cells [31]. In monocytes, miR-125b-5p reduces mitochondrial respiration through BIK silencing and plays an important role in the repression of Brite adipocyte function by modulating oxygen consumption and mitochondrial gene expression [32].

We propose that miR-30a-5p, miR-132 and miR-125b-5p downregulation, induced by resveratrol treatment in aged women, could improve oocyte quality by enhancing mitochondrial activity and increasing ATP production in granulosa cells. Interestingly, we

found that the number of fertilized good quality oocytes increases in treated women and a significant anticorrelation between miR-125-fold change values and biochemical pregnancy is present (Figure 6). The effect of resveratrol in improving reproductive potential has already been demonstrated both in vitro and in vivo; in fact, it increases mitochondrial activity and biogenesis, in granulosa cell cultures [14] and clinically improves folliculogenesis outcome during IntraCytoplasmic Sperm Injection (ICSI) cycles [33].

Our data suggest that resveratrol treatment induces modification in the granulose cell miRNome suggesting that the different effects of nutriceutical could also depend on the regulation of gene expression mediated by miRNAs. Moreover, different papers reported that resveratrol can modulate miRNA expression in vitro, and among the miRNAs downregulated, miR-125b-5p has been described [34,35]. Of course, miRNAs perform their biological function inside the cells, and their altered expression in biological fluids does not always match their intracellular profile. MiRNA expression levels in biological fluids may be due to both changes in the transcription levels inside the cells producing them and alteration of secretion mechanisms [23,36]. In any case, the expression differences observed in FF after resveratrol treatment surely reflect transcriptomic and proteomic modification in granulose cells.

Functional enrichment analysis on downregulated miRNAs revealed that they may regulate several signaling pathways involved in mitochondria function and female fertility (Figure 2). Fatty acid biosynthesis, estrogen, FOXO, p53, TGF-beta, Hippo signaling pathways and oocyte meiosis represent some of the signaling pathways regulated by the five miRNAs (Figure 3). Interestingly, some literature data confirm these analyses. Zhang et al. investigated steroid hormone concentrations and miR-125b-5p expression in PCOS mouse model. In preantral follicles, inhibition of miR-125b-5p increased the expression of androgen synthesis related-genes and stimulated the secretion of testosterone, while simultaneously downregulating oestrogen synthesis-related genes and decreasing oestradiol release [37]. It is well known that androgens can influence ovarian follicular growth, augment steroidogenesis, promote follicular recruitment and increase the number of primary and preantral follicles [38]. Upregulation of miR-19a-3p has been found associated with an increase in inflammation [39] and its inhibition would seem to mitigate the repression of glycolysis enzymes, glucose uptake and lactate production, and apoptosis induced by an ischemic stroke in neurons [40].

MiR-125b-5p, miR-132-3p, miR-19a-3p, miR-30a-5p and miR-660-5p act synergistically in the networks related to the cellular response to oxidative stress and oocyte maturation and some of their target genes as SIRT1, TNF-a, AKT, have been previously identified as potential effectors of the cellular response to the resveratrol (Figures 3 and 4) [41,42]. SIRT1, acting at different regulation levels, activates the mechanisms of oxidative stress response in human reproductive organs. In the female reproductive system, SIRT1 regulates proliferation and apoptosis in granulosa cells (GCs), and its downregulation is associated with a reduced ovarian reserve [43]. The decrease in its expression causes mitochondrial dysfunction by increasing the Reactive Oxygen Species (ROS), lipid peroxidation and DNA damage in oocytes. In women of advanced reproductive age, SIRT1 by the deacetylation of the FOXO3A transcription factor stimulates the expression of catalase and manganese superoxide dismutase, promoting cell survival under oxidant conditions [44,45]. MiR-132 has SIRT1 as a validated target and its downregulation in granulosa cells could be associated with SIRT1 upregulation, and represent a response, mediated by resveratrol, to oxidative stress.

5. Conclusions

We propose that resveratrol treatment, by inducing modification in the granulose cell miRNome acting specifically on the miRNAs involved in mitochondrial pathways, improves oocyte quality and pregnancy outcome. A better understanding of the roles of nutriceutical on mitochondrial miRNAs will open up the possibility of designing new therapeutic strategies for mitochondrial diseases to improve follicular microenvironment

in women of advanced reproductive age increasing pregnancy success in natural and IVF cycles.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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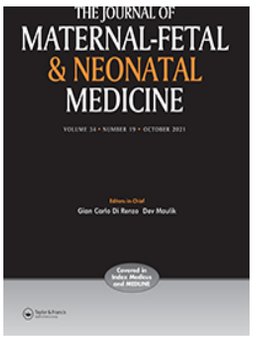

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Biological and clinical effects of a resveratrol-based multivitamin supplement on intracytoplasmic sperm injection cycles: a single-center, randomized controlled trial

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ORIGINAL ARTICLE

Biological and clinical effects of a resveratrol-based multivitamin supplement on intracytoplasmic sperm injection cycles: a single-center, randomized controlled trial

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ABSTRACT

Background: Resveratrol display's positive effects on follicle growth and development in pre-clinical studies while there is scantly information from clinical trials. The aim of this study was to evaluate the biological and clinical impact of a resveratrol-based multivitamin supplement on intracytoplasmatic sperm injection (ICSI) cycles.

Methods: A randomized, single-center controlled trial conducted at the University Center of Assisted Reproductive Technologies involving 101 women infertile women undergoing ICSI cycles was conducted. A pretreatment with a daily resveratrol based nutraceutical was administered to the Study Group; Control Group received folic acid. The primary outcomes were the number of developed mature follicles (>16 mm), total oocytes and MII oocytes recovered, the fertilization rate and the number of cleavage embryos/blastocysts obtained. Secondary endpoints were the duration and dosage of gonadotropins, the number of embryos for transfer, implantation, biochemical, clinical pregnancy rates, live birth and miscarriage rates.

Results: A significantly higher number of oocytes and MII oocytes were retrieved in the Study Group than in Control Group ($p = .03$ and $p = .04$, respectively). A higher fertilization rate ($p = .004$), more cleavage embryos/patient ($p = .01$), blastocytes/patients ($p = .01$) and cryopreserved embryos ($p = .03$) were obtained in the Study Group. No significant differences in biochemical or clinical pregnancy, live birth, and miscarriage rates were revealed, but a trend to a higher live birth rate was revealed in the Study Group.

Conclusions: A 3 months period of dietary supplementation with a resveratrol-based multivitamin nutraceutical leads to better biological effects on ICSI cycles.

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Resveratrol; intracytoplasmic sperm injection; assisted reproductive technologies; embryo; oocyte

Introduction

In recent years, the demand for assisted reproductive technologies (ART) is constantly increasing, with more women encountering challenges with fertility. The reproductive capacity of the couple declines with age [1] and female aging is known to be associated with an impairment of the ART procedure's outcome, with a worsening of number and quality of oocytes, a reduced embryo quality and an increased incidence of miscarriages and embryo aneuploidy [2]. Chromosomal abnormalities depend on spindle instability, telomere shortening, chromosome misalignment, and mitochondrial dysfunction [3,4]. The mechanism of oocyte aneuploidy is not proven, but an energy-dependent mechanism is supposed to play a major role in oocyte development [5]. The production of energy for the metabolic requirements of the oocyte is provided solely by mitochondria and the oocyte has the largest number of mitochondria and mitochondrial DNA (mtDNA) copies of any cell [6]. During its maturation, the number and functionality of mitochondria increases rapidly, to prepare for the energy expenditure associated with the early stages of embryonic development [7]. Aged and unfertilized oocytes are frequently associated with loss of mitochondrial function, mainly due to mtDNA mutations and deletions [8].

The energy metabolism and production of adenosine triphosphate (ATP) occurs through the

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mitochondrial respiratory chain and this is a requirement at various stages of female gametogenesis [9]. ATP production in the follicle is impaired in older women and it has been demonstrated that embryo implantation potential is correlated with the ATP content of the embryo [5]. Granulosa cells (GC) have a critical role in regulating ovarian development and function by cross-talk signaling and crucial energetic support for the oocyte [10].

In a recent research, we showed that resveratrol, a natural polyphenolic compound, detected in a variety of plants, foods, and drinks, such as grapes, nuts, cranberries, and red wine, stimulates mitochondrial number (mitochondrial biogenesis) in GC and increase intracellular ATP levels [11] probably through the expression of the Silent Information Regulator 1 (SIRT1), the mammalian homolog of Sir2 (Silent information regulator 2) in yeast, a NAD-dependent deacetylase sirtuin [12]. All these mechanisms are reflected in better resistance to apoptosis and increased energy availability [13]. Resveratrol also modifies the electrophysiological properties by promoting resting membrane depolarization associated to the ultra-rapidly activating, slowly inactivating potassium current (IKur) currents reduction in h-GCs [11]. A vital interplay between oocytes and their associated GC occurs during follicular growth from the arrested primordial follicle stage to the ovulatory stage [14,15]. It is well established that oocyte development is highly dependent on molecular interactions with somatic cells [16], which provide the oocyte with nutrients that sustain basal metabolic activity and signals that regulate its differentiation [17]. Thus, resveratrol may also improve ovarian functioning and oocyte development *via* energy metabolism crosstalk between GCs and the oocyte, opening the possibility for treatment of infertile women undergoing ART cycles [18,19]. A protection against the reduction of fertility with aging in mice has been already indicated: long-term-oral administration of resveratrol improves healthy follicle number, telomere length, and telomerase activity, as well as oocyte quantity and quality [4,20]. We also reported that a resveratrol-based multivitamin supplement enhances spermatic parameters in men affected by idiopathic infertility [21,22]. Effects of resveratrol on human oocytes maturation, fertilization, and embryo development can be studied only during ART procedures; however, no specific trial has been yet conducted reporting this information. We, therefore, designed a randomized trial, with the aim to evaluate the biological and clinical effects of a resveratrol-based multivitamin supplement in women undergoing intracytoplasmatic sperm injection (ICSI) cycles.

Materials and methods

Study design

This randomized, single-blind, controlled, single-center, experimental study was performed at the Center of Assisted Reproductive Technologies, University of Perugia, Perugia, Italy, from January 2019 to March 2020. The study design was in accordance with the Helsinki Declaration, conforms to the Committee on Publication Ethics (COPE) guidelines. The study protocol was approved by the local Ethical Committee and that of the Aziende Sanitarie della Regione Umbria (n.15188/18/AV) on 20 December 2018 and retrospectively registered in the ClinicalTrials.gov Protocol Registration System (identifier: NCT04386499). The study was conducted according to the CONSORT guidelines [23]. All participating patients signed an approved informed consent.

We enrolled patients diagnosed with infertility with the following inclusion criteria: aged 18–42 years, body mass index (BMI) 18–30 kg/m^2, normal thyroid function and normal blood parameters, regular uterine cavity evaluated by hysterosalpingography or hysteroscopy.

Women were excluded if they had one of the following conditions: couples with severe male factor, women with primary or secondary ovarian failure or who adhered to the Bologna criteria [24], which meant to satisfy at least two of the following features: advanced maternal age ($>$ 40 years), a previous poor ovarian response ($\leq$3 oocytes with a conventional stimulation protocol), an abnormal ovarian reserve test (antral follicle count $<$7 or anti-Müllerian hormone $<$1.1 ng/mL); patients with inaccessible ovaries, ovarian cyst $>$20 mm, sactosalpinx, heterologous fertilization, significant systemic disease or other situations unsuitable for ovarian stimulation.

Randomization

All patients were randomized for no treatment (Control Group) and for treatment with resveratrol (Study Group) with a 1:1 ratio, *via* a sealed envelope with random numbers generated by a computer. The principal investigator generated the random allocation, enrolled patients and assigned patients to intervention. Participants were not blinded to the group assignment. The physicians and embryologists involved in the oocyte retrieval and embryo transfer

were blinded to the group assignments of the participants in the trial.

Intervention

Folic acid 400 μg/day was orally delivered to the Control Group patients while the Study Group received a nutraceutical (2 daily capsules) containing a formulation of sustained resveratrol (Resv@MDH, trademark Revifast® [25,26] and pure resveratrol for a total resveratrol amount of 150 mg), folic acid (400 mcg), vitamin D (25 mcg), vitamin B12 (2.5 mcg), and vitamin B6 (1.4 mg) (GENANTE™, S&R Farmaceutici, Bastia Umbra PG, Italy). Both treatments were started 3 months before the initiation of ovarian stimulation for ICSI procedures and were maintained until the oocyte pick-up.

Ovarian stimulation was carried out using a GnRH antagonist protocol. Gonadotropins, human highly purified FSH (Fostimon®, IBSA, Lugano, Switzerland), human FSH and LH (Meropur®, Ferring Italia, Milano, Italy) from menstrual cycle day 2 or 3, with a daily dose ranging from 150 to 375 UI, were administered. Type and dosage of gonadotropins were decided according to endocrinological patient's features, ovarian reserve, and results of previous attempts. From day 5, determinations of estradiol (E2) and progesterone (P) levels with follicular ultrasound monitoring were conducted to evaluate the ovarian response and follicular development. According to this, gonadotropins dose was daily adjusted.

Oocyte maturation was induced with 10,000 UI of human chorionic gonadotropin (hCG) (Gonasi HP®, IBSA, Lugano, Switzerland) when at least two dominant follicles of 18 mm were found. Patients with more than eight follicles with a diameter $\geq$16 mm were administered a subcutaneous (SC) injection with needle and syringe of 0.2 mg of GnRH agonist (Decapeptyl, Ferring Italy, Milano, Italy) to reduce the risk of ovarian hyperstimulation syndrome (OHSS). In these cases, a segmental approach with a "freeze all" strategy and a delayed transfer was planned. Transvaginal ultrasound-guided follicle aspiration was performed 34–36 h after trigger.

The maturation status of the oocytes was recorded according to ESHRE 2012 guidelines [27]. A high-quality oocyte, metaphase II (MII), was defined as a round, normal-sized oocyte with one regular polar body in the perivitelline space, homogeneous ooplasm without irregularities, and appropriate thickness of the zona pellucida [27]. The standard ICSI procedure was performed using ejaculated sperm from male partner.

All embryos were evaluated starting on day 2 and at the following days according to published criteria [28]: Veeck grades A, B, C, or D. Luteal phase was supported with 50 mg/day of natural, intramuscular progesterone (Prontogest®, IBSA, Lugano, Switzerland) or 800 mg/day micronized progesterone (Progeffik®, Effik Italia, Milano, Italy) and maintained until the day of hCG measurement (14 days after embryo transfer) or the evidence of a clinical pregnancy, in case of positive result. Embryo-transfer was performed between days 3 and 5 following the oocyte pick-up depending on the patient and embryo characteristics. In older women with one or two developing embryos and in women with difficulty to develop a blastocyst demonstrated in a previous attempt, a day 3 transfer was preferred. In all other cases, a day 5 transfer was performed. Only embryo-transfer from fresh cycles were considered. Beta hCG was done for the detection of pregnancy. Clinical pregnancy was established by the presence of a gestational sac, an embryo pole and heart activity by transvaginal ultrasound examination.

Primary and secondary outcomes

Primary outcomes were the number of developed mature follicles (>16 mm), total oocytes, MII oocytes recovered, fertilization rate, number of cleavage embryos/blastocysts obtained, and the number of available embryos for cryopreservation. Secondary outcomes were duration and dosage of gonadotropins, number of embryos per transfer, implantation, biochemical, clinical pregnancy rates, live birth rate and miscarriage rate.

Statistical analysis

A minimum number of subjects calculation (https://clincalc.com/stats/SampleSize.aspx) was performed with a level of 0.05, our sample size yielded sufficient power to detect at least a 20% difference between groups in the primary outcome with over 80% power ($\beta = 0.2$) and an estimated variability of parameter with SD of <30%. Reproductive outcomes were compared between the Resveratrol treated group (Study Group) and Control group with a *post hoc* calculation analysis. To evaluate differences between nonparametric variables which are expressed as frequencies and percentages, we used Chi-square with Yates' correction factor. Reproductive outcomes were compared between the Study group and Control group. An intention-to-treat analysis (ITT) of clinical results on the whole allocated population was performed. The

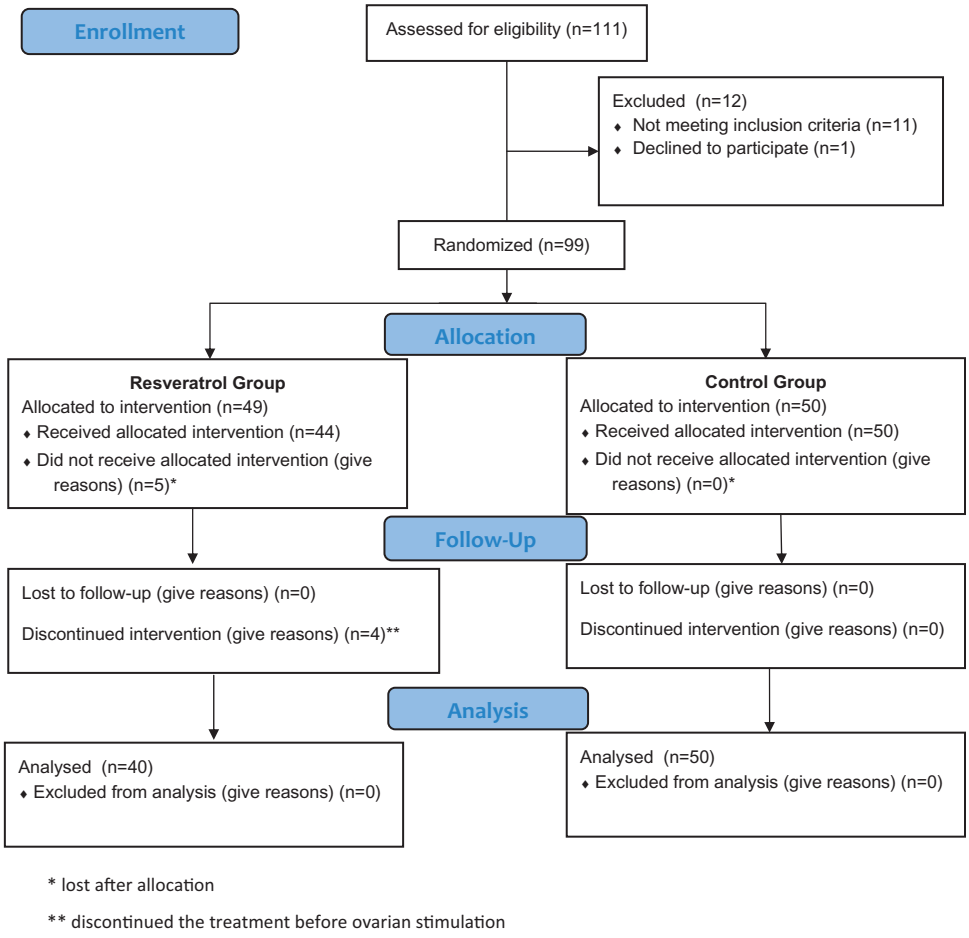

Figure 1. CONSORT flow diagram. Diagram depicts patient enrollment, allocation to different groups (Control and Study Group), follow-up, and analysis.

analyses included also all randomized and exposed women, except for the analyses of fertilization, cleavage embryos, blastocytes, and cryopreserved embryos which are based on patients with at least one oocyte retrieved following ICSI procedure. Analyses were performed using Origin 61version. Statistical significance was defined as a two-sided p value of $<.05$ and are presented as mean ± standard error (SE). Student's t-test was used for normally or near-normally distributed data.

Results

A total of 111 patients were initially considered eligible for the study. Twelve patients were excluded due to the presence of exclusion criteria (severe male factor or patients who adhered to Bologna criteria)and 99 were randomized. Forty-nine patients were allocated in the Study Group, while 50 in the Control Group. In total, five women were lost after allocation and four patients discontinued the pretreatment before starting the ovarian stimulation (all in the Study Group) (Figure 1). No statistical differences were found in the general characteristics of the patients, likewise the two groups were not significantly different even considering the different ovarian stimulation protocol, where preparations with FSH-only or human menopausal gonadotropins were used (Table 1).

Table 2 shows the outcomes of ovarian stimulation for both Groups. A not statistically significant longer stimulation was required for the Study Group ($p = .06$). There were no statistically significant differences in the total gonadotropins dosage used in the ovarian stimulation protocol. At the end of the hormonal treatment a trend to a higher number of leading follicles was shown in the Study Group ($p = .13$), with a significant increase of retrieved oocytes ($p = .03$) and MII oocytes ($p = .04$) in the Study group compared to the control group were found. A higher fertilization rate ($p = .004$), significantly more cleavage embryos, blastocysts ($p = .01$) were obtained and more embryos were cryopreserved ($p = .03$) for patient in the Study Group than in the Control Group. The two groups were similar regarding implantation, biochemical, clinical

Table 1. Patient demographic data, ovarian reserve and stimulation characteristics in the Control and Study groups.

	Control Group (± SE)	Study Group (±SE)	*p*
Patients (*n.*)	50	40	
Age (*n.*)	36.6 ± 0.6	36.1 ± 0.6	.62
BMI (kg/m^2)	22.0 ± 0.6	23.6 ± 0.7	.07
AMH (ng/mL)	2.8 ± 0.3	3.4 ± 0.4	.36
AFC (*n*)	10.8 ± 0.8	11.4 ± 0.8	.24
Basal FSH (mIU/mL)	7.6 ± 0.4	7.9 ± 0.4	.66
Type of gonadotropin			
h-HP-FSH / r-FSH	27 (54%)	23 (57%)	.74
FSH + LH (*n*) (%)	23 (46%)	17 (43%)	

Values are expressed as mean + SE or numbers. BMI: Body Mass Index; AMH: anti-Mullerian hormone; AFC: antral follicle count; FSH: follicle stimulating hormone; h-HP FSH: human highly purified follicle-stimulating hormone; r-FSH: recombinant follicle-stimulating hormone, LH: luteinizing hormone.

Table 2. Gonadotropins, follicles and oocytes in the Control and Study groups.

	Control Group (*n* = 50)	Study Group (*n* = 40)	*p*
Days of stimulation	10.5 ± 0.2	11.2 ± 0.3	.06
Gonadotropins (IU)	2693.4 ± 141.4	2480.6 ± 131.6	.27
Estradiol levels at the day of hCG (pg/mL)	2790.3 ± 567.3	2272.8 ± 378.6	.47
Number of follicles (>16 mm)	9.4 ± 0.6	10.9 ± 0.8	.13
Oocytes retrieved	7.1 ± 0.4	8.7 ± 0.7	.03
Ratio follicles/ oocytes retrieved	0.77 ± 0.02	0.82 ± 0.03	.24
MII oocytes	4.9 ± 0.4	6.4 ± 0.6	.04

Values are expressed as mean ± SE.

pregnancy rate, live birth, and miscarriage rate. A trend to a higher live birth rate was revealed in the Study Group. No adverse secondary effects for the pretreatment protocol or severe OHSS were observed in any of the two groups, but a trend in the reduction of delayed transfer (due to a "freeze all" strategy to prevent OHSS) (2.6% versus 10.6%) was observed in the Study Group (Table 3).

Discussion

This study demonstrated that a pretreatment with a resveratrol based multivitamin dietary supplement is able to provide better biological effects in ICSI procedures. The group of patients who received this supplementation developed a higher number of oocytes, more mature oocytes, with a higher fertilization rate. A higher number of cleavage embryos and blastocysts were also obtained leading to more cryopreserved embryos. Probably due to the small sample size, no significant differences in clinical data (biochemical, clinical pregnancy, live birth, and miscarriage rate) were revealed in the two study groups; however, we could expect a greater cumulative pregnancy rate in the Study Group. Results reported in our study have been achieved with the association of a 3 month pretreatment with resveratrol to the ovarian stimulation protocol. This pretreatment period of 3 months was based on the average duration of oogenesis in which oocytes go through the early stages of maturation in the ovary [29]. These results were similar to those obtained in an *in vitro* maturation (IVM) study, showing that resveratrol, at 1.0 μm in culture medium, significantly enhanced oocyte maturation and fertilization as well as the formation of blastocysts in mice [30]. There are many beneficial effects of resveratrol on humans, including antiaging, antioxidant, anti-inflammatory, insulin-sensitizing, cardioprotective, vasodilating, and anti-neoplastic properties [31]. Important emerging evidence in human reproduction indicate that resveratrol has potential positive effects in older women, PCOS, endometriosis, and uterine fibroids [32], but no reports have been published regarding the effects of resveratrol on oocytes and embryos, with clinical results, during ART procedures.

Only two very recent studies have been published until today evaluating effects of resveratrol on follicles, oocytes and embryos in humans, but they are not comparable with our analysis [19,33]. Ochiai *et al.* evaluated the impact of resveratrol supplementation on pregnancy outcomes in ET cycles, fresh or vitrified-warmed. Data on the effects of resveratrol on ovarian response after stimulation for oocyte retrieval were not collected, as only results regarding the effect on ET have been reported [19]. The outcome showed that resveratrol supplementation was strongly associated with a decrease in clinical pregnancy rate. In that study resveratrol was continued during the luteal phase of the treated cycle and the negative effect was probably due to adverse effects on decidualization of the endometrium, caused by the resveratrol-induced suppression of decidual senescence and deacetylation of significant genes for decidualization [19]. The same group also analyzed the effects of resveratrol in primary culture of human endometrial stromal cells demonstrating its interference with the reprogramming of the retinoic acid signaling pathway and remodeling of the endometrium [34]. It is well known that decidual change in the endometrium may cause implantation failure and recurrent miscarriage [35]. Considering that the half-life of resveratrol is 9–10 h [36], as affirmed by Ochiai et al. [19] it is sufficient to administer resveratrol before and during ovarian stimulation, and to discontinue the intake at the day of oocyte pick up to avoid adverse effects on endometrium. In our trial, resveratrol was given exactly during that period of time and this could explain the different clinical results obtained. However, further clinical studies are required

Table 3. Embryos and pregnancies in the Control and Study groups.

	Control Group ($n = 50$)	Study Group ($n = 40$)	p
Fertilization rate	64.6% (210/325)	75.4% (230/305)	.004
Fertilized oocytes/patient	4.4 + 0.4	5.7 + 0.4	.01
Cleavage rate	94.1% (208/210)	96.5% (222/230)	.08
Cleavage embryos/patient	4.2 ± 0.4	5.7 ± 0.4	.01
Blastocysts/patient	2.3 ± 0.3 (89/38)	3.5 ± 0.3 (104/30)	.01
Transferred embryos/patient	1.8 ± 0.1	1.9 ± 0.1	.3
Embryos transferred day 3	39% (31/79)	27% (20/74)	.11
Embryos transferred day 5	61% (48/79)	73% (54/74)	.11
Cryopreserved embryos/patient	1.1 ± 0.21	1.5 ± 0.3	.03
Implantation rate	25.3% (20/79)	24.3% (18/74)	.96
Biochemical pregnancy rate (BPR)	42.8% (18/42)	43.2% (16/37)	.84
BPR (transfer day 3)	40.0% (6/15)	44.4% (4/9)	.83
BPR (transfer day 5)	44.4% (12/27)	42.9% (12/28)	.91
Clinical pregnancy rate (CPR)	40.5% (17/42)	37.8% (14/37)	.99
Clinical pregnancy rate (CPR)[a]	34.0% (17/50)	28.6% (14/49)	.56
CPR (transfer day 3)	33.3% (5/15)	33.3% (3/9)	.99
CPR (transfer day 5)	44.4% (12/27)	39.3% (11/28)	.70
Live birth rate (LPR)	30.9 % (13/42)	35.1 % (13/37)	.87
Live birth rate (LBR)[a]	26.0 % (13/50)	26.5 % (13/49)	.95
LBR (transfer day 3)	13.3% (2/15)	22.2% (2/9)	.57
LBR (transfer day 5)	40.7% (11/27)	39.3% (11/28)	.91
Delayed transfer rate	10.6% (5/47)	2.6% (1/38)	.31
Delayed transfer rate[a]	10.0% (5/50)	2.0% (1/49)	.1
Miscarriage rate	23.5% (4/17)	21.4% (3/14)	.77
Miscarriage rate[a]	8.0% (4/50)	6.1% (3/49)	.71

Values are expressed as mean ± SE or percentages (numbers in parenthesis).
[a]The statistical analysis was made on the intent-to-treat (ITT) population (Ctrl = 50 and Study Group = 49, for details see Figure 1).

to establish optimal doses and periods of resveratrol intake while preventing adverse effects on implantation, and pregnancy as also suggested in a recent review [37].

The second clinical trial conducted by Bahramrezaie et al. determined the effect of resveratrol on the angiogenesis pathway specifically on the expression of VEGF and hypoxia-inducible factor (HIF) 1 genes in GC of PCOS patients who underwent an ART treatment [33]. According to the results, significant higher rates of high-quality oocytes (81.93 ± 10.81 versus 69.13 ± 18.71; p .002) and high-quality embryos (89.80 ± 11.53 versus 78.83 ± 23.04; p .024) were obtained in the resveratrol group [33].

Several hypotheses coming from basic research could explain the positive effects of resveratrol on oocytes. In regard of the oocyte aging, indeed, recent studies demonstrated that resveratrol effects GCs and, moreover, the ovarian physiology. Specifically, resveratrol induces GCs proliferation through activation of the PI3K pathway and promotes primordial follicle activation [38]. Compared to untreated tissues, a higher proportion of growing follicles has been reported after human ovarian tissue are cultured in the presence of resveratrol [38,39]. The activation of SIRT1 would be a key therapeutic action needed to ameliorate oocyte competence in oxidative stress-mediated ovarian aging [40,41]. Resveratrol is a natural activator of sirtuins able to upregulate the production of SIRT1 in response to oxidative stress [42] and, therefore, its administration may compensate for the physiologic decrease of SIRT1 expression in aged oocytes. *In vitro* studies showed that resveratrol decreases DNA damage, indeed is clearly evident that its addition in culture may have beneficial effects on the ovarian tissue because DNA damage was only 15.2% in those follicles cultured in 2 μM resveratrol versus 55.2% in not enriched culture medium [43]. Regarding animals, in aged mice resveratrol also improved the number of follicles [4]. Resveratrol also effects vascular endothelial growth factor (VEGF), a potent inducer of angiogenesis and vascular permeability that plays a crucial role in ovarian folliculogenesis [44–47].

There are some limitations in our study. First the small sample size of population considered for the analysis. Second, we evaluated only patients who completed the treatment with a fresh transfer. Frozen-thawed ET cycles were not considered and therefore we did not calculate cumulative pregnancy rates per patient. Furthermore, two different protocols of ovarian stimulation were used, with LH or without LH, according to the endocrinological patient's features and to the results of previous attempts, and although a bivariate analysis demonstrated that the two groups were well balanced, this may represent a possible

bias. Regarding gonadotropins, we decided to administer a tailored-dosage to optimize the outcome of the ovarian stimulation: this customized choice may also represent a potential bias of the study.

Conclusions

In conclusion, the treatment with a resveratrol-based multivitamin supplement in infertile patients, before and during ovarian stimulation in ICSI cycles, seems promising to ameliorate the biological patterns related to ovarian aging by enhancing the quality of oocytes and embryos. This study showed no significant differences in the clinical and chemical pregnancy rates by the addition of a resveratrol-base multivitamin supplementation. However, the number of cryopreserved embryos was significantly higher in the Study Group and this may predicted a greater cumulative pregnancy rate from frozen embryos. This is the first randomized, clinical trial and further studies could contribute to reinforce these conclusions.

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Institutional review board statement

The study design was in accordance with the Helsinki Declaration, conforms to the Committee on Publication Ethics [COPE] guidelines. The study was approved by the Ethical Committee of Aziende Sanitarie della Regione Umbria [n.15188/18/AV] on 20 December 2018 and retrospectively registered in the ClinicalTrials.gov Protocol Registration System (identifier: NCT04386499) on 13 May 2020.

Informed consent statement

Informed consent was obtained from all individual participants included in the study.

Disclosure statement

Sandro Gerli, Chiara Della Morte, Margherita Ceccobelli, Monica Mariani, Alessandro Favilli, and Bernard Fioretti declare they have no financial interests. Alessandro Lanti and Rossana G. Iannitti receive a salary from S&R Farmaceutici. Lucio Leonardi is Executive Director of S&R Farmaceutici S.p.A.

Author contributions

Sandro Gerli, Bernard Fioretti, and Rossana G. Iannitti made substantial contributions to the conception or design of the work; Sandro Gerli, Bernard Fioretti, Rossana G. Iannitti, Chiara Della Morte, Margherita Ceccobelli, and Monica Mariani made substantial contributions to the acquisition, analysis, or interpretation of data; Sandro Gerli, Bernard Fioretti, Rossana G. Iannitti, Alessandro Favilli, Lucio Leonardi, and Alessandro Lanti revised it critically for important intellectual content; All authors approved the version to be published

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Data availability statement

All data and materials as well as software application comply with field standards.

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Resveratrol depolarizes the membrane potential in human granulosa cells and promotes mitochondrial biogenesis

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Objective: To study the biological effects of resveratrol on the growth, electrophysiology, and mitochondrial function of human granulosa cells (h-GCs).
Design: Preclinical study.
Setting: Electrophysiology laboratory and in vitro fertilization unit.
Patient(s): This study included h-GCs from seven infertile women undergoing assisted reproductive techniques.
Intervention(s): Human ovarian Granulosa Cell Tumor (GCT) cell line COV434 and h-GCs obtained after oocyte retrieval were cultured in the absence or presence of resveratrol.
Main Outcome Measure(s): Granulosa cells were evaluated for cell viability and mitochondrial activity. Electrophysiological recordings and evaluation of potassium current (IKur) and Ca_{2+} concentration were also performed.
Result(s): Resveratrol induced mitochondrial activity in a bell-shaped, dose-effect-dependent manner. Specifically, resveratrol treatment (3 μM, 48 hours) increased ATP production and cell viability and promoted the induction of cellular differentiation. These biological changes were associated with mitochondrial biogenesis. Electrophysiological recordings showed that resveratrol reduced the functional expression of an ultra rapid activating, slow inactivating, delayed rectifier potassium current (IKur) that is associated with a plasma membrane depolarization and that promotes an increase in intracellular Ca_2^+.
Conclusion(s): The effects of resveratrol on potassium current and mitochondrial biogenesis in h-GCs could explain the beneficial effects of this polyphenol on the physiology of the female reproductive system. These findings suggest there are therapeutic implications of resveratrol in a clinical setting. (Fertil Steril® 2021;115:1063–73. ©2020 by American Society for Reproductive Medicine.)
El resumen está disponible en Español al final del artículo.

Key Words: Resveratrol, mitochondria, granulosa cells, potassium current, membrane potential

The ovarian follicles are the basic units of the mammalian ovary that are implicated in reproductive biology. Each of them contains a single oocyte surrounded by inner layers of granulosa cells (GCs) and outer layers of thecal cells. During folliculogenesis, GCs, which surround the oocyte in primordial follicles, are activated and become proliferating cuboidal cells (1–3). At this stage, GCs proliferate and form multiple layers of somatic cells that enclose the oocyte (cumulus cells) as well as mural GCs that line the follicular wall, resulting in the formation of a secondary follicle. This is followed by the formation of small, fluid-filled cavities within the follicle that coalesce to form the early antral (or tertiary) follicle (4). In the absence of gonadotropin stimulation, these follicles become atretic and disappear from the ovary through an apoptosis process. During

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folliculogenesis, GCs play a critical role in carbohydrate, lipid, and protein metabolisms and provide the appropriate balance of energy required by the oocyte. For example, GCs metabolize glucose into pyruvate, which is then passed to the oocyte for ATP production by oxidative phosphorylation (5). In turn, the oocyte regulates glycolysis in GCs by inducing the expression of glycolytic key genes (6–8). Similarly, within the oocyte-cumulus complex, fatty acid β-oxidation from lipid produces additional ATP for meiotic resumption (9–11) and amino acid turnover (12) to ensure the metabolic needs of the oocyte.

Voltage-gated potassium (Kv) channels are the principal determinants of membrane potential, and their modulation by follicle-stimulating hormone (FSH) and luteinizing hormone (LH) suggests an explanation of the biological effect promoted by these gonadotropins (13–16). Two principal Kv currents have been characterized in mammalian GCs (17): a slowly activating, noninactivating current may be ascribed to KCNQ1 with auxiliary subunit KCNE, defined as a slowly activating, noninactivating current (IKs, 13, 16, 18); and a ultrarapidly activating, slowly inactivating potassium current defined as IKur (16, 18), that represent component overlap carried by homo and hetero-oligomeric Kv1 channel family members (18). Notably, the effect of FSH on GC proliferation was increased by selective blockers of Kv1.3 channel, indicating that the expression of this current can modulate FSH-proliferating action on GCs, without compromising the FSH-enhancing progesterone production (19).

Resveratrol is a natural polyphenol known to have diverse effects depending on physiological, pathological, and pharmacological conditions in various biological systems (20). In rat thecal-interstitial cells, resveratrol showed a potent inhibitory effect on proliferation (21). In ovine ovarian GC tissue cultures, 2 μM of resveratrol induced GC proliferation through activation of the PI3K pathway and promoted primordial follicle activation (22). Resveratrol also affects Vascular Endothelial Growth Factor (VEGF), a potent inducer of angiogenesis and vascular permeability, which play also a crucial role in ovarian folliculogenesis (23). Another important action of resveratrol on GCs is the reduction of oxidative stress and the inhibition of apoptosis, both of which are dependent on the activation of the PI3K/Akt/mTOR signaling pathway, as demonstrated in the rat model of premature ovarian insufficiency (24). Recently, in the human GC (h-GC) immortalized line COV434, resveratrol has been shown to have an antiapoptotic role in a SIRT1-dependent manner, possibly by regulating the ERK1/2 pathway (25). The experimental cell model COV434 has been previously characterized and linked to the preovulatory differentiation status based on the lack of expression of the LH receptor and its ability to respond to FSH stimulation with estrogen (but not progesterone) production (26). Interestingly, treatment with resveratrol of GCs collected from aged cows also increased mitochondrial synthesis, ATP production, and autophagy, leading to an overall improvement in the mitochondrial function and a better development of oocyte growth in vitro (27). It has also been suggested that resveratrol improves mitochondrial quantity by activating the SIRT1/PGC-1α pathway in the human ovarian granulosa-like tumor cell line KGN in hypoxic conditions (28). Given the critical role of energetic metabolism and potassium channels in GCs physiology, and the beneficial effects of resveratrol in folliculogenesis, we studied the effects of this molecule to clarify the underlying mechanism involved.

MATERIALS AND METHODS

Cell Culture

Human ovarian granulosa line (COV434) from a solid primary tumor was obtained from the European Collection of Authenticated Cell Cultures (29) cultured at 37°C with 5% of CO_2 in Dulbecco's modified Eagle medium (DMEM; Euroclone) containing 10% fetal bovine serum (Euroclone), 200 mM glutamine, and 100 U/mL penicillin/streptomycin (Life Technologies). Cells were cultured in 75 cm^2 cell culture flasks (Falcon, Corning) seeded at density of 1.5×10^4 cell/cm^2. For experiments and to maintain cell density, cells were seeded on 35 mm petri dishes or 96-well plates (Falcon, Corning) at a density of 15×10^4 or 5×10^3 cells/well, respectively. After adherence (24 hours after plating), cells were treated with various concentrations of resveratrol (3, 10, 20 μM) for up to 72 hours.

Isolation and Culture of Primary H-GCs

Follicular h-GCs were obtained after oocyte retrieval from seven patients undergoing in vitro fertilization (IVF) during assisted reproductive techniques at the Centre of Assisted Reproductive Technologies, S. Maria della Misericordia Hospital, University of Perugia, Perugia, Italy. All patients participating in this study underwent controlled ovarian stimulation according to a gonadotropin-releasing hormone antagonist or long gonadotropin-releasing hormone agonist protocol. The follicular fluid aspirated from each patient was pooled in conical bottomed 15 mL polypropylene centrifuge tubes and centrifuged at $800 \times g$ for 10 minutes. The supernatant was discarded, and a small amount of saline was added to resuspend the cells. The mixture was transferred to an equal volume of 50% Ficol-Paque plus GE Healthcare (Sigma-Aldrich) followed by centrifugation at approximately $500 \times g$ for 20 minutes. After centrifugation, the GCs were carefully aspirated from the mid-layer using a pipette, washed with saline, and centrifuged for 5 minutes at $800 \times g$; the supernatant was discarded. The collected h-GCs were cultured in DMEM containing 10% fetal bovine serum and 100 U/mL penicillin/streptomycin (30).

Cell Counts. Cell numbers were determined by used the Trypan blue staining method (31). The experimental data was interpreted by Hill equation (equation 1):

$$I_{(|RESV|)} = \frac{A1}{\left[1 + \frac{IC_{50}}{(\mathrm{RESV})}\right]^{h}} + C$$

where $I_{([Resv])}$ represents the inhibition fraction, A_1 and IC_{50} represent the maximum inhibitory and half-inhibitory effects respectively whereas h and C represent the Hill's coefficients and the no inhibitory fraction respectively.

ATP Test. Evaluation of ATP was carried out after 48 hours of treatment with resveratrol. Cell lysates were obtained by applying boiled distilled water to cell cultures to free intracellular ATP. The amount of ATP was determined using the ATP Determination Kit-

Molecular Probes (Invitrogen) following the manufacturer's instructions and measured with a VICTOR 3 luminometer (Perkin-Elmer). Results were expressed as a percentage of variation with respect to control (untreated) cells.

Real-Time Polymerase Chain Reaction (RT-PCR). RNA from COV434 cells was extracted using the Trizol (Invitrogen) method according to the manufacturer's instructions. For gene expression, RNA was reverse-transcribed into first-strand cDNA using the Kit RT2 First Strand and analyzed with the Human Female Infertility RT^2 Profiler PCR Array (Qiagen) using RT2 SYBR Green ROX FAST Mastermix as a reagent (Qiagen) and Real-Time RotorGene 100 (Qiagen) following the manufacturer's instructions. To normalize mRNA levels, the B2M gene was assessed at the same time. The results were expressed as fold change comparing untreated with resveratrol-treated cells. For mitochondrial DNA (mDNA) quantification, the DNA was extracted using RT2 SYBR Green ROX FAST Mastermix (Qiagen) and Real-Time RotorGene 100 (Qiagen). The mDNA level was normalized with HPRT1 gene. The primer used for mDNA was GAGCGATGGTGAGAGCTAAGGT (forward) and CCCTAAAACCCGCCACATCT (reverse); and for HPRT1, ATGTGATGAAGGAGATGGGA (forward) and ACCAAGGAAAGCAAAGTCTG (reverse). A dissociation curve was run at the end of the experiment to discriminate DNA contamination and PCR product from misannealed primer and thus demonstrate specificity.

Mitochondrial Activity and Biogenesis Tests. For the evaluation of membrane potential, the Tetra Methyl Rhodamine Methyl ester (TMRM) stain (Sigma-Aldrich) was used. For the evaluation of mitochondria biogenesis, Mitotracker Green FM (Invitrogen) was used. Both dyes were used according to the manufacturer's instructions. All experiments were carried out contemporaneously on treated (3 μm resveratrol) and untreated cells. Samples were incubated in DMEM with 30 nM of TMRM and 100 nM of Mitotracker for 20 minutes. At the end of the incubation time, plates were washed with phosphate-buffered saline and analyzed by fluorescent microscopy AxioExaminer (Zeiss) using the fluorescein isothiocyanate filter for Mitotracker green and rhodamine filter for TMRM. Results are expressed as mean normalized intensity variation comparing the two samples (treated vs. untreated) (32, 33).

MTT Assay. COV434 cells were seeded in 96-well plates (Falcon, Corning) with a cell density of 5×10^3. After 24 hours, cells were treated with 3, 10, and 20 μM of resveratrol for 24, 48, and 72 hours. For viability tests, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium reduction) colorimetric assay (Sigma) was used. For the assay, cells were incubated with MTT at a final concentration of 0.5 mg/mL for 3 hours at 37°C. After solubilization, the formazan dye was quantified using a Varian Cary 100 scan spectrophotometer (Agilent). The experimental data were interpreted by equation 2:

$$I_{(|RESV|)} = \frac{A1}{1+\left[\frac{IC_{50}}{(\mathrm{RESV})}\right]^{h_1}} + \frac{A2}{1+\left[\frac{(\mathrm{RESV})}{EC_{50}}\right]^{h_2}} + C$$

where $I_{([Resv])}$ represents the fraction effects, A_1, IC_{50} represent the maximum inhibitory and half-inhibitory effects respectively whereas h_1 represent the Hill's coefficients. A_2 and EC_{50} represent the maximum stimulatory and half-stimulatory effects respectively whereas h_2 and C represent the Hill's coefficients and the no modify fraction respectively.

Electrophysiological Recording. Ion currents in immortalized h-GC line COV434 were recorded by using the patch clamp technique in whole-cell dialyzed configuration (34). Currents were amplified with a HEKA EPC-10 amplifier and analyzed with the PatchMaster and Origin 4.1 software. For online data collection, currents were filtered at 3 kHz and sampled at 40 μs/point. Membrane capacitance measurements were made by using the transient compensation protocol of PatchMaster. The external solution contained 106.5 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM MOPS, 20 mM glucose, and 30 mM Na-gluconate (pH 7.25). The pipette solution contained 57.5 mM K_2SO_4, 55 mM KCl, 5 mM $MgCl_2$, 10 mM MOPS (pH 7.20), and 0.1 mM EGTA-K, with electrical access ranging between 10 and 20 MOhm after cell membrane breaking. The drugs used were dissolved at the final concentration indicated. The cell recording was continually perfused with solution at the rate of about 1 mL/minute. The currents recorded from COV434 cells, in the range of −60 to +50 mV, that are reported in Figure 4 were described with a model composed by a voltage-independent and a voltage-dependent component according to the Boltzmann distribution (equation 3):

$$I_{(V_m)} = \left(V_m - E_0^1\right)\frac{G_{IKUR}}{1+e^{(V_{0.5}-V_m)/K}} + G_{ILEAK}\left(V_m - E_0^2\right)$$

where $I(V_m)$ is the current in fuction of V_m whereas $E_0{}^1$ and $E_0{}^2$ represent the equilibrium potentials of I_{KUR} and I_{LEAK}, respectively. In the voltage-dependent component $V_{0.5}$ and k represent the half-activation voltage and the slope factor for the activation curve, respectively; GI_{KUR} = max conductance (nS) of I_{kur}; GI_{LEAK}= conductance (nS) of leak current; V_m= membrane potential.

The kinetics of the blocking of PAP-1 displayed in Supplemental Figure 1A was fit by the following equation 4:

$$I_{(t)} = A1.e^{(-t+t_2)/\tau} + C$$

where I(t) represents the current in function of PAP-1 application, $A1$ and C represent the blocked and unblocked current by PAP-1 respectively and τ is the time constant of blocking whereas t2 represents a time scaling factor.

Cytosolic Ca2+ Measurements

According to our previous work (35), cells were incubated with FURA-2-AM (3 μM; Sigma-Aldrich) for 45 minutes and extensively washed with external Ringer's solution of the following composition: 140 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM MOPS, and 10 mM glucose, at pH 7.4. Cells were continuously perfused using a gravity-driven perfusion system, focally oriented onto the field of interest. The estimation of intracellular free Ca2+ concentration was reported as the change of the ratio between fluorescence emission at 510 nm obtained with 340 and 380 nm excitation wavelengths (optical filters and dichroic beam splitter were from Lambda DG4, Shutter Instruments). Ratiometric data were acquired every 3 seconds, and fluorescence determinations were

performed using fluorescence microscopy system Zeiss (Axiozoom V16 and Axiocam 502 mono). The intracellular calcium variation after depolarization induced by perfusion of high extracellular potassium concentration solution (40 mM of KCl) was estimated by calculation of 340/380 fluorescence ratio of FURA-2. Extracelluar high potassium concentration solution has the following composition: 102.50 mM NaCl, 40 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM MOPS, 10 mM glucose, at pH 7.4.

Statistical Analysis

The data are reported as the mean ± standard error. The Student's t test was used to evaluate differences, and $P < 0.05$ was considered statistically significant. In Figure 3, a paired t test was applied. Statistical analyses were performed with Origin software.

RESULTS

Low Resveratrol Concentration Increases Cell Viability and Promotes Differentiation in COV434 GCs

Resveratrol was reported to increase viability in COV434 cells at a concentration of 20 μM by using MTT-like assay (25) and to decrease the proliferation rate in rat GCs (36). To verify these effects, we built a growth rate curve by treating COV434 cell cultures with various concentrations (3, 10, and 20 μM) of resveratrol. Culture growth was subsequently evaluated by counting cells throughout the course of treatment (after 24, 48, and 72 hours). At the lowest concentration (3 μM), resveratrol did not modify cell growth relative to control conditions, whereas at higher concentrations (10 and 20 μM), it decreased the number of vital cells (Fig. 1A and Supplemental Table 1). Surprisingly, the evaluation of cell viability through MTT assay displayed a completely different situation. An increase in viability at low resveratrol concentrations and the absence of effects at higher resveratrol concentrations, with a clear bell-shaped dose-dependent relationship (Fig. 1B), was observed. The fitting with equation 2 (see Materials and Methods) shows that the positive increase was associated with a half-maximal activating concentration (EC_{50}) of about 3 μM and the inhibition phase was associated with a half-maximal inhibitory concentration (IC_{50}) of about 10 μM (Supplemental Table 1). Since the MTT assay depends on both the number of vital cells and cellular mitochondrial activity, we normalized the MTT activity for the number of cells to discriminate the effect of the metabolic enhancement only (Fig. 1C). We found an EC_{50} of about 3 μM, in accordance with the bell-shaped equation parameters. Such data indicate that effects of resveratrol are concentration dependent, increasing GC viability at low concentrations and having cytostatic effects at higher concentrations, similar to the effect observed in rat GCs (36). Furthermore, the comparison between cell count and MTT assay in COV434 cells after resveratrol treatment indicates that MTT is not an adequate assay for evaluating the proliferation rate and that caution should be taken to interpret this metabolic assay (37).

Given that the concentration reached in vivo by resveratrol can only be a few micromolars after oral intake, the remainder of the work was conducted to evaluate the effects of 3 μM concentration of resveratrol in GCs after 48 hours of treatment. At the 48-hour mark, 3 μM of resveratrol did not modify BAX expression and increased the expression of BCL2, excluding the involvement of the apoptosis process. Furthermore, we observed the up-regulation of genes involved in differentiation of GCs (Fig. 1D), such as insulin growth factor-1 and epidermal growth factor (38). Altogether, the data indicate that 3 μM of resveratrol increases the metabolic rate associated with the induction of the differentiation program.

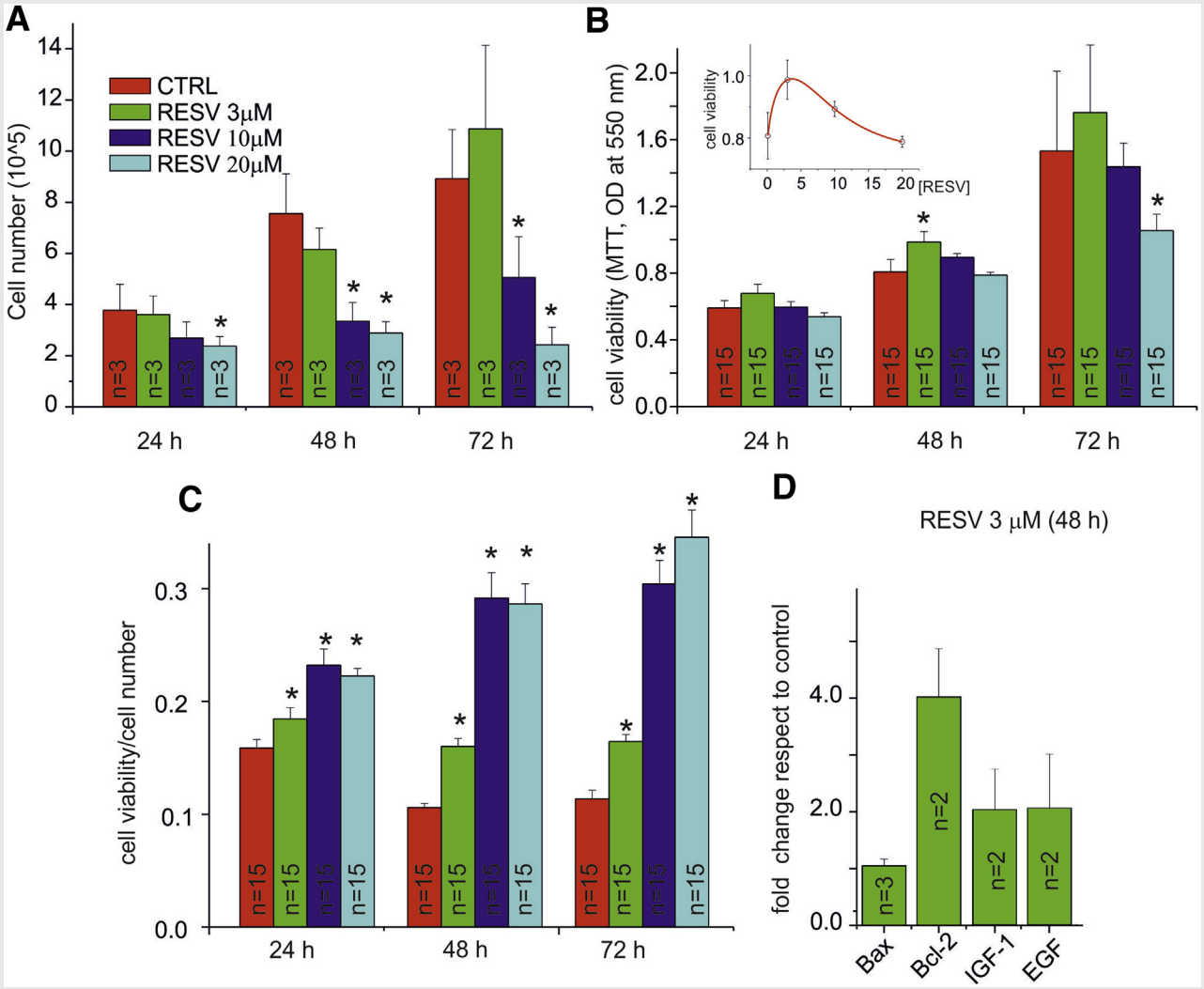
Resveratrol Improves Mitochondrial Function and Increases Mitochondrial Biogenesis in GCs

To investigate the effect of resveratrol on mitochondrial activity, the nernstian dye TMRM, in a no-quenching mode, was used. We observed an accumulation of the dye inside the cells around the nucleus (Fig. 2A). This accumulation was quickly removed by treating the cells with protonophore Carbonil cianuro-p-tri fluoro metossi fenil idrazone according to the dissipation of the mitochondrial membrane potential ($\Delta\Psi_M$, data not shown). After treatment with 3 μM of resveratrol for 48 hours, COV434 GCs showed a significant increase in TMRM accumulation at the single-cell level (Fig. 2B) and intracellular ATP content (Fig. 2C), indicating an increase of the energetic metabolism. To assess the origin of this effect, we evaluated the number of mitochondria. Treatment with resveratrol significantly increased both mtDNA copy number (Fig. 2D) and the incorporation of non-nernstian dye Mitotracker green (Fig. 2E and 2F) compared with control cells, indicating the presence of mitochondrial biogenesis. Mitochondrial biogenesis may underlie the ability of resveratrol to improve the mitochondrial function and energetic metabolism of COV434 GCs.

To confirm the mitochondrial enhancer effect of resveratrol, we subsequently studied h-GCs obtained from infertile patients undergoing assisted reproductive techniques (see Materials and Methods). Seven independent primary cultures were obtained (patient characteristics are shown in Supplemental Table 2) and cultured with 3 μM resveratrol (48 hours). Like the COV434 GC culture, the treatment with resveratrol increased TMRM accumulation in five of the seven primary cultures (Fig. 3A). The p-pair t test performed for all the samples displays a statistically significant increase of dye accumulation (Fig. 3A). In three cell cultures, we were able to perform Mitotracker green staining (Fig. 3B and 3C) and observed a significant increase in the number of mitochondria, indicating again the presence of mitochondrial biogenesis and thus a similar incremental trend. The origin of the individual variability or correlation with any primary indication for IVF needs further investigation due to the low sample size.

Resveratrol Decreases Functional Expression of Voltage-Dependent Potassium Currents and Depolarizes Cell Membrane in COV434 GCs. By using the whole-cell dialyzed configuration of the patch clamp from a Vh of –60 mV, we explored currents evoked upon 100 ms depolarizing pulses from –60 to 80 mV with incremental steps of 10 mV. Typically, voltage-activated outward currents with fast activation

FIGURE 1

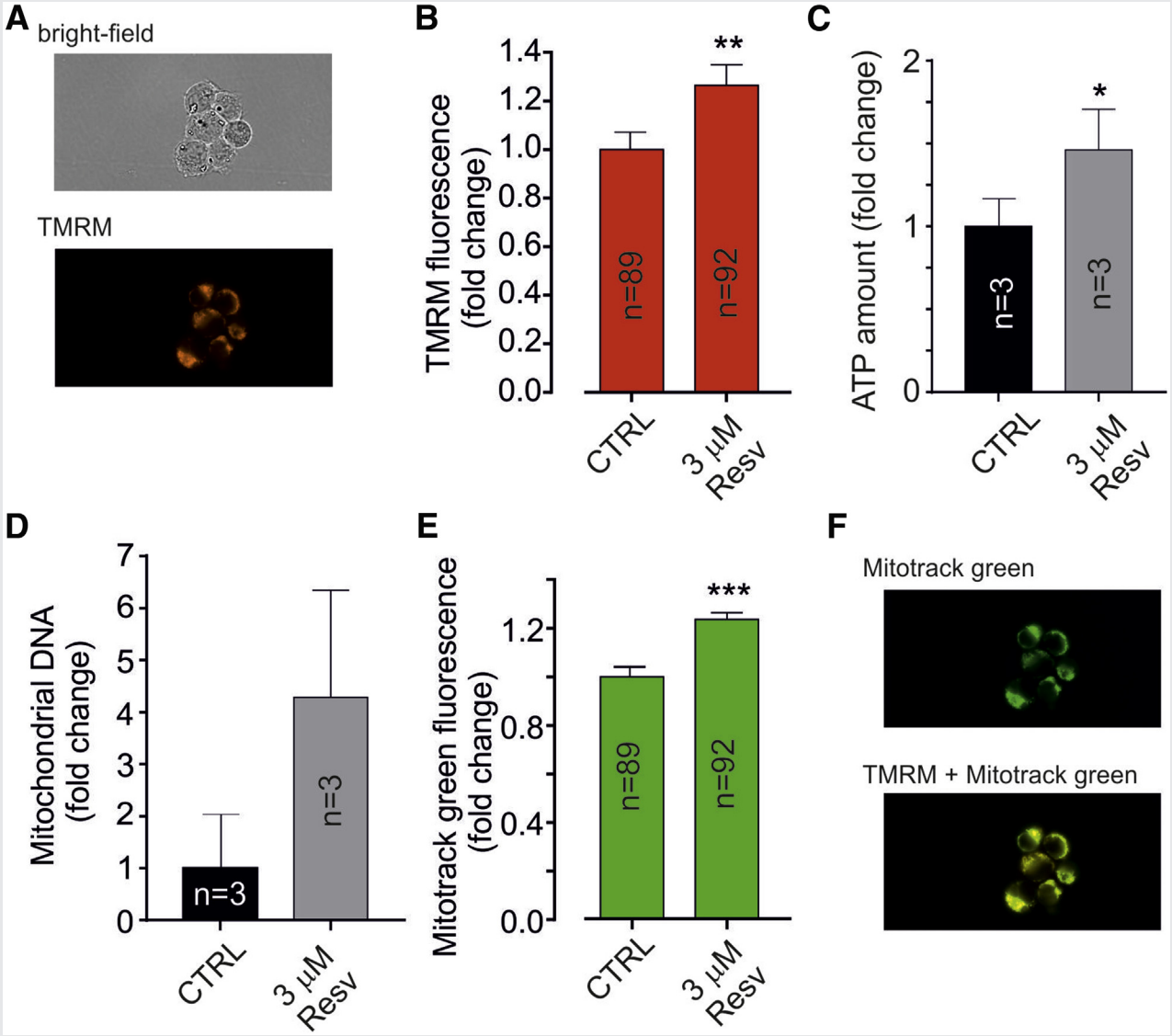


Resveratrol affects the proliferation and metabolism of human granulosa cells in a dose-dependent manner. **A**) Cell counts based on Trypan blue staining of COV434 cells in control condition (CTRL) and in cells treated with resveratrol (RESV 3, 10 and 20 μM) for 24, 48 and 72 hours at 37°C with 5% of CO2. **B**) MTT assay in the same conditions of panel A, note the bell-shape dependences of resveratrol concentration. Inset: fit of data of cell viability at 48 h with equation 2 (see Supplemental Table 1). **C**) Mitochondrial activity (MTT, from panel B) normalized to the number cells (panel A). **D**) BCL-2 and BAX, IGF-1 and EGF expression assessed by RT-PCR in control and 3 μM resveratrol, plotted as fold change in resveratrol with respect to control. Data presented as ±SE. Significant differences between control and treated cells are denoted as * ($P<0.05$).

Ragonese. Resveratrol promotes GC mitochondrial biogenesis. Fertil Steril 2020.

and slow inactivation were observed from voltages more positive than –20 mV (Fig. 4A). The I-V relationship built by plotting the peak currents evoked at the pulse test is displayed in Figure 4B. The single data points were superimposed to a ramp current recording from Vh = –60, with linear gradient from –120 to +120 mV (1.1 s duration). A good superposition is evident in the range from –60 to +50 mV. A clear deviation is evident at membrane potentials higher than +50 mV, which could be related to the inactivation process of the underlying currents (Fig. 4A). Between –60 and –40 mV, the I-V relationship displayed an ohmic behavior, indicating the absence of voltage-activated currents. The fitting of ramp current with a model that involved a leak current (voltage-independent) plus a voltage-dependent current described with Boltzmann's equation described the experimental current recorded in the voltage range between –90 and 50 mV (dotted red line in Fig. 4B). The parameters of voltage-dependent currents obtained by the fitting procedure show a reversal potential (–90 mV) near to the equilibrium potential of the potassium ion (–90 mV, calculated by Nernst's equation) and a steady state of activation characterized by a $V_{0.5}$ of –3 mV and slope factor of 5. Based on the rapid activation and inactivation kinetics, we defined this current as IKur according to the main biophysical properties of potassium currents described in mammalian GCs (16, 18). The treatment with 3 μM (48h) of resveratrol decreased the conductance of

FIGURE 2



Resveratrol increases global mitochondrial membrane potential ($\Delta\Psi_M$) and ATP production as consequences of mitochondrial biogenesis. **A**) Representative COV434 cells stained with TMRM staining (bottom) and in bright field illumination (top). **B**) Accumulation of TMRM of COV434 in control conditions and in resveratrol treatment (3 μM, 48 h): the cell numbers used were obtained by three independent experiments. **C**) Evaluation of intracellular ATP quantity by bioluminescence assay in control condition and in resveratrol treated cells (3 μM, 48 h). **D**) Evaluation of mitoDNA by RT-PCR in COV434 cell line in control conditions and in resveratrol treated cell (3 μM, 48 h). **E**) Mitochondrial membrane staining with Mitotracker green evaluated in the same cells used in panels B. **F**) same field observed with EGFP filter (Mitotracker green, top) and merge with signals obtained from filter for Rhodamine (TMRM, bottom panel A) after merge (yellow, bottom). The acquisition display in A and F origin from same field. Data presented as ±SE. Significant differences between control and treated cells are denoted as * ($p<0.05$), **($p<0.01$), ***($p<0.001$).

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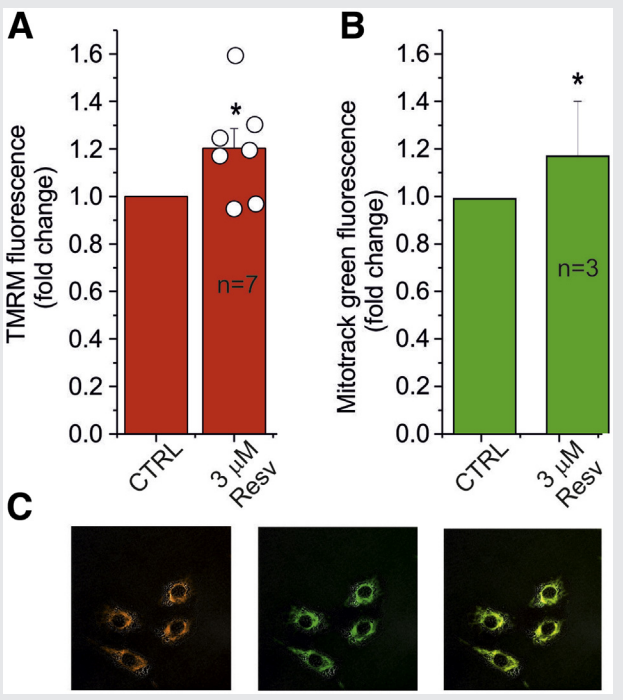
the IKur current by about 45% compared with controls (1.8 and 1.0 nS in control and resveratrol, respectively), without changing its biophysical properties. The mean currents at 40 mV were 311 ± 37 pA (n = 11) and 206 ± 33 pA (n = 12) in control and resveratrol conditions, respectively (Fig. 4D). The $V_{0.5}$ and k were –0.7 mV and 5.6 (n = 11) for control cells, and –3.1 mV and 5.6 in resveratrol-treated cells (n = 12). Any measurable changes of membrane capacitance were observed with 8.4 ± 0.7 pF (n = 17) and 8.3 ± 1 pF (n = 16) in control and resveratrol conditions, respectively. In contrast to the reduction of potassium conductance IKur, no changes were observed in the leak conductance estimated with depolarizing pulse from –60 to –50 mV (2.2 ± 0.2 nS (n = 17) and 2.2 ± 0.2 nS (n = 16) in either control and resveratrol-treated cells (data not shown).

To evaluate the effects of resveratrol-induced IKur reduction on membrane potential, we studied its pharmacological profile. Since IKur is associated with the functional

FIGURE 3



Resveratrol increases global mitochondrial membrane potential ($\Delta\Psi_M$) and mitochondrial biogenesis in human primary granulosa cells from patients undergoing Artificial Reproduction Techniques. **A**) Mean TMRM accumulation normalized with respect to control in human primary granulosa cells following resveratrol treatment (3 μM, 48 h). The single data points represent mean of TMRM fluorescence obtained from 30-90-sample size cells in seven primary granulosa cell cultures. The mean value of the treatment was normalized to parallel cells culture growth in the same condition without resveratrol. Asterisk represents p< to 0.05 in paired t-test. **B**) Mean Mitotracker green accumulation results pooled from three independent primary cell culture experiments. **C**) Representative double staining with TMRM (left) and Mitotracker green (middle) of primary cultures used in the analysis displayed in panels A and B. Right panel represents the merge of TMRM and Mitotracker signals. Data presented as ±SE. Significant differences between control and treated cells are denoted as * (p<0.05).

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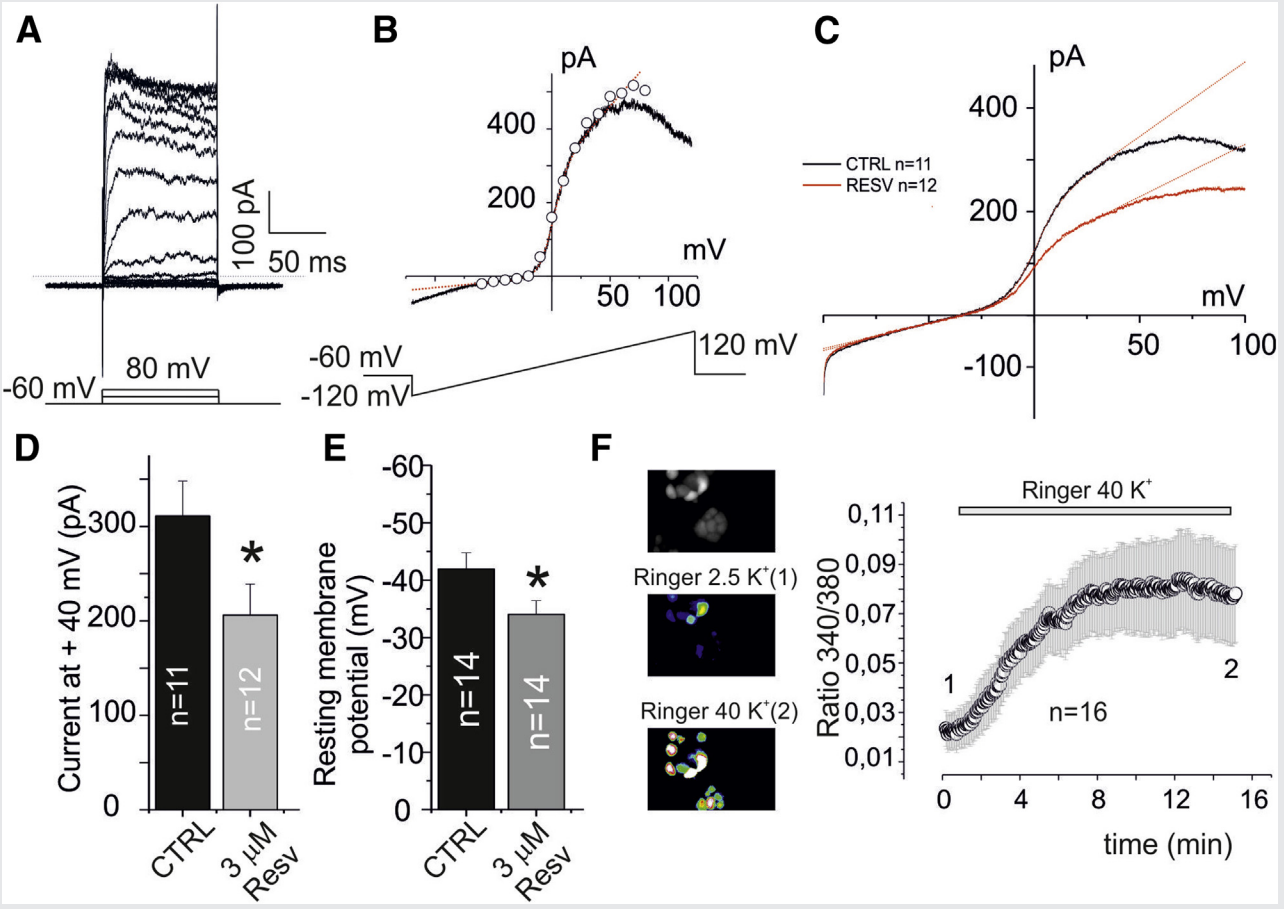
expression of the Kv1 (Shaker) subfamily (18), we performed a pharmacologic characterization by using PAP-1 Kv1 blocker, an inhibitor in the Kv1.x subfamily with particular efficacy on the Kv1.3 subunit (39). Bath application of 1 μM PAP-1 blocked the voltage-dependent potassium currents in COV434 cells with monoexponential temporal profiles (time constant, τ, about 40 s; Supplemental Fig. 1A) almost entirely. Pooled data at varying PAP-1 concentration levels (0.1–3 μM) showed a dose-dependent inhibition curve, fitted with Hill's equation (equation 1; see Materials and Methods), resulting in an IC_{50} of about 400 nM and h coefficient of about 1.8 (Supplemental Fig. 1B). The blocking variability at 1 μM PAP-1 concentration (Supplemental Fig. 1B) indicates a complex molecular nature, suggesting that the voltage-dependent potassium current in COV434 is a mix of voltage-dependent channels of the Kv1 (Shaker) subfamily as described in mammalian GCs (18). With 3 μM PAP-1 application, a full blocking of the current at +40 mV was observed (n = 4; Supplemental Fig. 1B). Real-time PCR confirms the presence of several members of the Kv1 (Shaker) subfamily (18) (data not shown). More interestingly, these experiments show that the PAP-1-sensitive current is important in setting the resting membrane potential (inset Supplemental Fig. 1C). This observation prompted us to investigate the possible impact of resveratrol on the resting membrane potentials in the COV434 cell model. To this end, we measured the resting membrane potential in current clamp mode (currents applied = 0 pA) and found a mean depolarization of about 8 mV in cells treated with resveratrol (3 μM, 48 hours; from a value −41.9 ± 2.8 mV [n = 14] in control to −34.0 ± 2.4 mV [n = 14] in treated cells; Fig. 4E). It is known that h-GCs express L- and T-type voltage-dependent calcium currents and that membrane potential depolarization could promote calcium flux and mitochondrial biogenesis (40, 41). To test whether depolarization observed after resveratrol treatment can promote calcium influx, FURA-2 recording was performed in COV434 after depolarization induced by 40 mM KCl. Before application of 40 mM of KCl, we observed that the intracellular calcium levels were sustained in the same cells (inset of Fig. 4F, middle); however, after applying 40 mM KCl, we observed a general increase of intracellular calcium levels in the majority of cells (Fig. 4F and inset bottom), according to calcium influx associated with voltage-dependent activation of the L-/T-type calcium currents. The data suggest that resting membrane potential controls calcium signaling, which promotes many associated processes, such as cell differentiation and the mitochondrial physiology.

DISCUSSION

The evolution of the early antral follicle depends on the trophic action of the pituitary FSH, which stimulates the proliferation of GCs and follicular growth (3). Under the influence of FSH, the antrum continues to enlarge, resulting in the formation of a preovulatory (antral or late antral) follicle. Before ovulation, GCs begin changing to the luteal phenotype, which is sensitive to LH and characterized by intense progesterone production after corpus luteum formation (42).

The mechanisms responsible for the hormonal effects of FSH/LH on GCs in the promotion of folliculogenesis are not completely known. Even the electrophysiological effects of FSH in GCs are yet to be defined. Luteinizing hormone depolarizes GC culture of about 15 mV, leading to a change in the potential from –45 to –30 mV, in a manner similar to the potential of the oocyte as a whole (14). In contrast, FSH does not have depolarizing effects; rather it is able to preserve cells from the LH depolarizing effects (14). In addition, in luteinized GCs, luteotrophic placental/chorionic gonadotropins can promote depolarization and induce morphological changes characterized by the increase in the mitochondria complex that may be associated with the modulation of sodium ions influx (43, 44). In this context, resveratrol treatment (3μM for 48 hours) promotes membrane potential depolarization of about 8 mV from a resting membrane potential of about –40 mV (Fig. 4). At the same time point,

FIGURE 4



Resveratrol decreases functional expression of voltage dependent potassium currents (IKur) and depolarizes cell membrane in COV434 GCs **A**) Representative family of outward currents recorded in COV434 cells by using patch clamp from a Vh of -60 mV, and applying depolarized pulses from −60 to +80 mV. Note the voltage dependence of activation that increases with depolarization, and the presence of inactivation during the depolarization pulse (100 ms). **B**) I-V relationship between the peak current and the indicated voltage step, superimposed with the current ramp evoked by a linear voltage gradient from −120 to +120 (Vh= −60 mV). The dotted red line represents the best fit with the equation 3. **C**) Averaged current ramps evoked by a linear voltage gradient from −100 to +100 (Vh= -60 mV) obtained from COV434 cells in control condition (n=11) and resveratrol cells (n=12). Note the reduction of currents at positive voltages. The red line represents the best fit with the equation 3. D) Mean current at +40 mV in control (black bar) and in resveratrol treated cells (gray bar). **E**) Mean resting potential recorded in current clamp model from cells in control condition and in cell grown 48 h in 3 μM of resveratrol. Data in figure D and E presented in as ±SE. Significant differences between control and treated cells are denoted as * ($p<0.05$). F) Variation of ratio of fluorescence at 510 nm emission at 340/380 excitation following high potassium solution perfusion-induced depolarization in COV 434 GC indicating intracellular calcium increase. Inset: Image observed with FITC filter (top) and pseudocolours imaging of ratio 340/380 in same cellular field before (middle) and after (bottom) potassium solution perfusion-induced depolarization as indicated in the time course.

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resveratrol increases mitochondrial biogenesis in primary and in immortalized h-GCs (Figs. 2 and 3). We questioned how resveratrol could promote mitochondrial biogenesis. In this regard, depolarization of the membrane is known to increase calcium influx through L- and T-type voltage-dependent calcium channels in GCs (40, 41). An interesting working hypothesis is that intracellular calcium can promote mitochondria biogenesis and altogether can improve the energetic metabolism of the cell. In this context, increased intracellular calcium concentration promoted by ionomycin is known to increase mitochondrial biogenesis at 48 hours in GCs (45). This link is not surprising since after calcium influx, higher intracellular calcium is able to activate Ca^{2+}/calmodulin-dependent protein kinase β/AMPK/SIRT1 pathway, which in turn enhances the expression and decreases deacetylation of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) and mitochondrial biogenesis (46). Our data demonstrated that depolarization of CGs increases intracellular calcium levels to the same extent. Further studies will be needed to define how calcium signaling and SIRT1 pathway activation are orchestrated by resveratrol and the mechanism involved.

Recent publications also suggest that the mitochondrial function of cumulus and mural GCs can directly influence the ability to achieve a successful pregnancy (47–49). These effects on GCs also correlate with current evidence that

suggests resveratrol may have an overall effect on ovarian physiology as well. Interestingly, in vivo treatment with resveratrol affects bovine oocyte maturation and subsequent post-IVF embryonic development by inducing progesterone secretion and providing an antioxidant effect (50). In accordance with our observations, resveratrol increased the ATP content, a finding also demonstrated in pig oocytes via energy homeostasis. Furthermore, this improved the developmental ability of oocytes grown in vitro, and these effects were abolished with the addition of a SIRT1 inhibitor (51). Resveratrol has been also shown to protect the rat ovary against chromium toxicity (20) and irradiation (52) by enhancing endogenous antioxidant enzymes. In the same animal model, resveratrol has been shown to increase the ovarian follicular reserve and prolong ovarian life span (23). Nonetheless, it is known that ovarian aging is associated with impaired regulation of energetic metabolism in the follicle (53).

Conclusion

Resveratrol can exert an important metabolic effect on GCs and thus may also improve ovarian physiology and oocyte development via energy metabolism crosstalk between GCs and the oocyte. This finding means there is a possibility for treatment of infertile women undergoing IVF therapy (54). Based on the sensitivity of the IKur currents described in the COV434 cell line to PAP-1 blocker, we assume a major involvement of the Kv1 (Shaker) channels subfamily members. This aspect is interesting since the blocking of Kv1.3 results in an increase of the effect of FSH-induced proliferation and concomitant differentiation in GCs, such as the ability to increase the production of progesterone (19). Based on the role of IKur in regulatory effects in FSH action, through intracellular calcium flux, resveratrol could regulate the effects of this hormone in CGs (55). In conclusion, the effects of resveratrol on potassium current, calcium flux, and mitochondrial biogenesis in GCs could explain the beneficial effects of this polyphenol on the overall physiology of the female reproductive system and thus have therapeutic implications in the clinical setting.

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El resveratrol despolariza el potencial de membrana de las células de la granulosa humanas y promueve la biogénesis mitocondrial.

Objetivo: Estudiar los efectos biológicos del resveratrol en el crecimiento, electrofisiología y función mitocondrial en las células de la granulosa humana (h-GCs).

Diseño: Estudio preclínico

Entorno: Laboratorio de electrofisiología y unidad de fecundación in vitro

Paciente(s): Este estudio incluye h-GCs de siete mujeres infértiles que recibieron técnicas de reproducción asistida.

Intervención(es): Células de la granulosa tumorales de ovario humano de la línea celular COV434 (GTC) y h-GCs obtenidas después de la aspiración de ovocitos fueron cultivadas en la ausencia o presencia de resveratrol.

Medida(s) de resultado(s) principal(es): Se evaluaron las células de la granulosa por viabilidad celular y actividad mitocondrial. También se realizaron grabaciones electrofisiológicas, evaluación de la corriente de potasio (IKur) y de las concentraciones de Ca_2+.

Resultado(s): El resveratrol indujo actividad mitocondrial en forma de campana, dosis-efecto dependiente. Específicamente, el tratamiento con resveratrol (3 μM, 48 horas) incrementó la producción de ATP, la viabilidad celular y promovió la inducción de la diferenciación celular. Estos cambios biológicos se asociaron con la biogénesis mitocondrial. Las grabaciones electrofisiológicas mostraron que el resveratrol redujo la expresión funcional de una activación ultrarrápida, inactivación lenta y un rectificador retardado de la corriente de potasio (IKur) lo que está asociado a una despolarización de la membrana plasmática y que promueve un incremento del Ca_2+ intracelular.

Conclusión(es): Los efectos del resveratrol en la corriente de potasio y la biogénesis mitocondrial en h-GCs podría explicar los efectos benéficiosos de este polifenol en la fisiología del sistema reproductor femenino. Estos hallazgos sugieren que hay implicaciones terapéuticas del resveratrol en un entorno clínico.