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Research article

Optimized platelet rich plasma releasate (O-rPRP) repairs galactosemia-induced ovarian follicular loss in rats by activating mTOR signaling and inhibiting apoptosis

Wesam El Bakly [a], Marwa Medhat [b], Mohamed Shafei [c,d], Reham Tash [e,f], Mohamed Elrefai [e,g], Yousef Shoukry [e], Nesreen Nabil Omar [h,*]

[a] Department of Pharmacology, Faculty of Medicine, Ain Shams University, Cairo, Egypt
[b] Department of Pharmacology, National Center for Social & Criminological Research, Egypt
[c] Obstetrician and Gynecologist at Sidnawy Health Insurance Hospital, Cairo, Egypt
[d] Dar Alshifa Hospital, Kuwait
[e] Department of Anatomy and Embryology, Faculty of Medicine, Ain Shams University, Cairo, Egypt
[f] Department of Anatomy and Embryology, Faculty of Medicine in Rabigh, King Abdulaziz University, Saudi Arabia
[g] Basic Medical Science Department, Faculty of Medicine, Hashemite University, Alzarqa, Jordan
[h] Department of Biochemistry, Faculty of Pharmacy, Modern University for Technology and Information, 11 Saudia Buildings, Nozha Street, 11371, Cairo, Egypt

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ABSTRACT

Platelet rich plasma contains a collection of growth factors, and an optimal formulation, named O-rPRP, contains the highest possible concentration of growth factors.

Purpose: Challenging the healing power of O-rPRP in a high-galactose diet-induced premature ovarian insufficiency (POI) experimental rat model.

Methods: Rats were divided into four groups of ten rats each and treated for four week as follows; 1) the control group, fed with normal diet and received intraperitoneal (i.p.) injection of PBS once/week; 2) the POI group, fed with galactose diet (50%) and received PBS (i.p.) once/week; 3) the POI/O-rPRP group, fed a 50% galactose diet and received O-rPRP (i.p.) once/week; 4) the O-rPRP group (negative control), fed with a normal diet and received O-rPRP (i.p.) once/week. The levels of galactose, follicle stimulating hormone, 17 β-estradiol, anti-mullerian hormone and inhibin B were measured in serum samples. Western blotting and quantitative real-time PCR assays were employed to investigate the levels of miR-223, β1 integrin, p70S6k and MCL-1 in ovarian tissues.

Results: After O-rPRP treatment, β1 integrin expression was enhanced, and miR-223 expression was decreased. Unlike the untreated galactose group, in the group treated with O-rPRP, p70S6k and MCL-1 expression levels were increased, indicating that the mTOR growth signaling pathway was active and that apoptosis was inactive. After the introduction of O-rPRP, the number of follicles and the follicular maturation improved, which was consistent with the improvement of inhibin B levels and subsequent inhibition of FSH.

Conclusion: O-rPRP inhibited galactose-induced excessive atresia and provided an overall protective effect on the ovarian follicles.

1. Introduction

Premature ovarian insufficiency (POI) is a state of ovarian inefficiency in women less than 40 years old; it is characterized by hypergonadotropism and hypoestrogenism, and it affects 1% of women in the general population [1]. Importantly, in assessing adults with galactosemia, POI was found to be a common complication in female subjects in numerous studies [2, 3]. Galactosemia is a potentially lethal disorder marked by genetic deficiency of galactose-1-uridyltransferase (type I) [4], galactokinase (type II) and UDP galactose-4-epimerase (type III). POI syndrome not only impacts fertility but also affects bone health and neurological function, which has a significant impact on the

* Corresponding author.
E-mail address: Dr.n.nabil@gmail.com (N.N. Omar).

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quality of life, and it impacts cardiovascular health, which is associated with a decrease in life expectancy [5].

POI is caused by wide variety of factors including chromosomal and genetic defects, autoimmune processes, chemotherapy, radiation, infection, and surgery; however, the causes remain largely unclear [6]. A large number of POI animal models have been developed, and it has been found that a galactose-fed rat model proceeds through the same dichotomous pathways of genetic and toxin exposure that is observed in clinical disease origin [7]. Feeding rats a high galactose diet is thought to produce an innate model for the study of POI and to discover potential therapies [8]. Several reports chronicled that POI is the ultimate outcome in women with galactosaemia [9]. The causative relationship between galactosemia and POI is not clear. However, it has been suggested that the toxic effects of galactose and its metabolites cause premature depletion of ovarian follicles [10].

In the current work, we tested the ability of galactose to act as a stressor that impedes ovarian functions by blocking the signaling of growth peptides namely, activin and inhibin, whose endocrine signals regulate the production of pituitary follicle stimulating hormone (FSH) [11]. We posit that the initiating mechanism in galactose-induced ovarian toxicity is a miR-223 related event that interferes with mTOR signaling and cells survival.

Several stressors, such as tobacco, alcohol and diet, have been found to upregulate the expression of miR-223 [12,13,14]. miR-223 has been shown to target the gene encoding transmembrane receptor β1 integrin [15], resulting in its downregulation. β1 integrin is a prerequisite for oocyte integrity, follicular development and granulosa cell proliferation [16].

Currently there is no approved and effective therapy for POI syndrome, and oocyte donation is an alternative [17]. However, platelet-rich plasma (PRP) therapies are being increasingly used in clinical practice [18], despite the lack of evidence-based data on the efficacy of PRP and the exact mechanism of action [19]. PRP is found to elicit a major regenerative response in different types of wounds by delivering autologous growth factors that are thought to drive the body's own tissue-healing mechanisms [20]. Moreover, PRP has the advantage of being safe with no concerns that it promotes tumorigenesis [21], yet the use of PRP in the treatment of organ failure is still under investigation. Importantly, the PRP therapy is based on its preparation, which must therefore be standardized to obtain PRP products with uniform composition [22].

Because it is a rich pool of growth factors, PRP regulates cell growth and proliferation by binding to membrane-bound tyrosine kinase receptor (RTK) [23]. RTK activation requires integrin receptor competence [24]. Mainly, β1 integrin has been shown to impact cell cycle progression by mediating adhesion to the extracellular matrix while potentiating the transmission of growth factor receptor signaling in response to its cognate ligands [24]. In addition, activation of growth factor receptors can lead to preferential enhancement of RTK subsets that bind to integrin receptors [25]. The signals from growth factors are incorporated by mammalian target of rapamycin (mTOR), which activates downstream serine/threonine kinase p70S6k to facilitate protein translation and cell growth [26]. Significantly, activation of the mTOR growth signaling pathway inhibits degradation of Mcl-1 and suppresses apoptosis [26].

In this regard, the present study aims to explore the protective effect of PRP on the ovarian insufficiency in experimental rat model induced by high galactose diet. In this work, we prepared optimized PRP releasate (O-rPRP) to improve growth factor outcomes and to reach a consensus on the reproducibility of the method [27].Furthermore, the mechanism of O-rPRP action was investigated; mainly, its impact on the ovarian reserve, apoptosis, and mTOR growth signaling.

2. Methods

2.1. Animals and study design

Eighty adult female Sprague-Dawley rats (40 animals were used to evaluate the best O-rPRP regimen and 40 animals were used to study O-rPRP mechanism of action) were obtained from the Nile Co. for Pharmaceuticals and Chemical industries, Cairo, Egypt. All rats were 35 days of age. Fifteen female Sprague-Dawley rats (70 days of age) were also obtained for PRP preparation and optimization. The study was conducted in the Laboratory Animals Research Center in Faculty of Medicine, Ain Shams University. This research protocol was approved by the Research Ethics Committee of the Faculty of Medicine, Ain Shams University (FMASU-REC) with the approval number "FMASU R49/2018". All animal procedures and care were carried out according to the general guidelines of the FMASU-REC, and they conformed to the guiding principles of the ARRIVE guidelines and were in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines; EU Directive 2010/63/EU for animal experiments. The rats were maintained under controlled temperature and 12 h light/12 h dark conditions for one week before the start of the experiments. They were allowed ad libitum access to standard laboratory feed and tap water. At the start of the experiments, pubertal female rats were 42 days of age (35days +7 days acclimation period), since Sprague Dawley females have their first estrus and vaginal opening at the age of 35 days [28]. Only females with a regular 4-day cycle were retained in this study, their selection was based on vaginal smears according to the methods and criteria described previously [29].

2.1.1. Study 1

To determine which dose of O-rPRP was more protective against toxic galactose, two doses of rPRP (0.25 and 0.5 ml/kg) [30, 31] were tested. Upon administration, rPRP was diluted with PBS at 1:3 (0.25 and 0.5 ml/kg rPRP were added into 0.5 and 1 ml of PBS respectively) and mixed thoroughly. After adjusting to the average weight of the animals (130–140 mg), the volumes of O-rPRP were 0.1 and 0.2 ml.

Forty female rats were partitioned into the following 8 groups of 5animals each: (1) normal-diet fed rats (control); (2) rats fed with 50% galactose diet (El-Nasr Chemical Co, Egypt); (3, 4, 5) rats were fed the 50% galactose diet concurrently with intraperitoneal injection of 0.1 ml of O-rPRP once per week, once every two weeks, and once every four weeks respectively; (6,7,8) rats were fed 50% galactose diet concurrently with intraperitoneal injection of 0.2 ml of O-rPRP once per week, once every two weeks, and once every four weeks respectively. Hormones were assessed, and based on the results obtained from this experiment, the appropriate O-rPRP dose was chosen and used for further mechanistic studies. The O-rPRP volume of 0.2 ml once per week provided better hormonal results and therefore was selected.

2.1.2. Study 2

Mature female rats were randomly assigned to four groups (n = 10) and were treated for four weeks as follows; Group I was the control group, and the members of the group were given standard laboratory feed and received an intraperitoneal injection of PBS (0.2 ml) once per week for four weeks; group II was the POI group, and the group members were fed a 50% galactose diet and received an intraperitoneal injection of PBS (0.2 ml) once per week for four weeks; group III was the POI/O-rPRP group, and the group members were fed a 50% galactose diet and received an intraperitoneal injection [19, 32] of O-rPRP (0.2 ml) once per week for four weeks; and group IV was the O-rPRP group (negative control), and the group members were fed with a normal diet and received an intraperitoneal injection of O-rPRP (0.2 ml) once per week and for four weeks. At the end of the four weeks, pregnant mare's serum gonadotropin (PMSG) 150 IU/kg was used to stimulate follicle growth; then, 48 h later, human chorionic gonadotropin (hCG) 75 IU/kg was used to induce ovulation, and 13 h later the rats were sacrificed [33]. Induction of ovulation by PMSG and hCG can be done at any stage of estrus cycle in adult female rats to bring about typical ovulation in normal conditions. This guarantees that all female rats are at the same ovulation stage upon sacrifice and eliminates variability in the estrus cycle phases among different female rats in the same group and between groups [33]. At the end of the experiment, female rats aged 10 weeks were fully

reproductive, were anaesthetized by ether. Then, they were sacrificed by cervical dislocation in line with euthanasia guidelines.

2.2. Preparing platelet-rich plasma

PRP was obtained from the blood of fifteen age-matched healthy female rats by a two-step centrifugation method. To alleviate pain, rats were anesthetized by ether before blood withdrawal. rPRP preparation was repeated three times on three different days (n = 5 rats/day). On three different days (n = 5 rats/day), an equal volume of the whole blood of rats was collected through cardiac puncture and each sample was transferred into a test tubes containing 3.8% sodium citrate. The blood was centrifuged at 180 × *g* for 10 min, and the supernatant was transferred to another tube and was designated PRP. In order to obtain the highest concentration of growth factors, PRP was incubated at 4 °C for 30 min [27]. After incubation, PRP was then coagulated by the addition 10% $CaCl_2$, and clots were allowed to form for 40 min at 37 °C. Following clot removal, the exudate was centrifuged at 890 × *g* for 10 min, and the three tubes of supernatant (prepared in three different days) containing the platelet-derived growth factors (rPRP) were stored at -80 °C until the growth factors concentrations in rPRP were analyzed. Then, the three tubes of rPRP were pooled together, divided into aliquots and frozen.

2.3. Optimization of rPRP

After obtaining rPRP by preconditioning at 4 °C for 30 min (as described above), further optimized rPRP (O-rPRP) was produced by diluting rPRP in a ratio of 1:3 with PBS at the time of administration [27].

2.4. Platelet counts

The initial platelet counts in the whole blood and the average platelet counts in PRP preparations (n = 3) were counted automatically using a hematology analyzer.

2.5. Determination of growth factors and miR-223 concentrations in O-rPRP

Concentrations of TGF-β, IGF-1, PDGF-BB, and VEGF were measured in pools (n = 3) of O-rPRP samples (size of each pool = 5 rats) using commercially available Quantikine ELISA kits (R&D Systems, Minneapolis, MN, USA), according to manufacturer's instructions. Last, miR-223 was measured in O-rPRP to exclude its plasma source.

2.6. Blood and tissue collection and processing

At the end of the four weeks and after induction, blood samples were collected from the retro-orbital plexus and were allowed to clot. Serum was separated by centrifugation at 3000 × *g* for 15 min then was used for the assessment of galactose, FSH, 17 β-estradiol (E2), anti-mullerian hormone (AMH), and inhibin B. When rats were sacrificed, both ovaries from each animal were dissected (both ovaries exhibit the same morphological changes throughout the cycle and ovulate—bilaterally—at the same time); the ovaries were washed with ice-cold saline and weighed. One ovary from each animal was fixed in 10% buffered formalin and processed for routine histology staining and the remaining ovary was kept frozen at -80 °C until processing for RNA and protein extraction.

2.7. Body and ovary weights

Animal body weights were measured every week until the time of sacrifice. Ovaries were weighed after dissection, and then ovarian index was calculated as an organ to body weight ratio.

2.8. Histopathological examination

Ovaries tissues were fixed in 10% buffered formalin overnight and then were embedded in paraffin block. Serial 4 μm-thick sections were stained with hematoxylin and eosin for light microscopic histological examination. Following H&E staining, ovarian follicles were counted using the fractionator and nucleator principles, as previously described [34]. A stratified sample, consisting of every fifth section, was used to estimate total numbers of primordial, preantral, antral, and atretic follicles and corpora lutea in every ovary. Depending on their follicular development, follicles were classified as primordial if they contained an oocyte surrounded by a single layer of spindle-shaped granulosa cells; preantral follicles were defined as containing an oocyte with a visible nucleolus, more than one layer and fewer than five layers of granulosa cells but lacked an antral space; and antral follicles were defined as containing an oocyte with a visible nucleolus, more than five layers of granulosa cells and/or an antral space [35]. Graafian follicles are large follicles that display oocyte and antrum, protrude from the surface of the ovary, while corpus luteum is the remnant of graafian follicle after ovulation. If a follicle shows two or more of the following criteria within a single cross-section, it is classified as atretic: there are more than two pyknotic nuclei in the granulosa cell layer, granulosa cells and cell debris within the antral cavity, oocyte and granulosa cells fragmented and pulled apart from the basement membrane [36].

To obtain an estimate of the total number of follicles per ovary, the number of different types of follicles present in the marked sections was multiplied by 5 to account for the fact that every fifth section was used in the analysis [34]. Atretic follicles were identified due to the presence of a degenerating oocyte or pyknotic granulosa cells [37].

2.9. Galactose assay

Galactose was measured using galactose colorimetric detection kit (Invitrogen, Catalog number, EIAGALC) as a modification of a protocol described before [38], wherein measured volumes of sera and standards were used instead of dried blood spots and standards. The kit uses galactose oxidase, which reacts with galactose to produce hydrogen peroxide, and, in the presence of horseradish peroxidase, the reaction with a colorless substrate produces a colored product. Galactose standard at concentrations of 0, 6.25, 12.5, 25 and 50 mg/dl were provided to generate a standard curve. The absorbance at 570 nm was monitored. The sensitivity of the assay was 0.493 mg/dl, and the intra-assay coefficient of variation was 4.8%. Concentrations were expressed as mg/dl.

2.10. Serum FSH, E2, AMH and inhibin B

In the first study, we estimated the levels of FSH, E2 and AMH in order to evaluate the best O-rPRP regimen. In the second study, the levels of FSH, E2 and AMH and inhibin B were determined. E2 and AMH serum levels were measured using ELISA kits (Uscn Life Science Inc. Wuhan). Assessment of FSH and inhibin B was performed with an ELISA kit (EIAAB, Inc., and Wuhan and Diagnostic Systems Laboratories, Webster, respectively).

2.11. Total RNA and MicroRNA extraction

Total RNA and miRNA was extracted from cells using a mirVana PARIS kit (Applied Biosystem, Ambion, Austin, TX, USA) according to the manufacturer's instruction. Briefly, cell disruption buffer was added to the cells for lysis and dissociation of any RNA:protein complexes. Acid-phenol:chloroform was added for phase separation. The aqueous phase containing the total RNA was carefully removed, after which total RNA was gained by adding 1.25 volumes of 100% ethanol to the aqueous phase. Then, the sample was eluted in nuclease-free water. miRNA was extracted from the aqueous phase by using 2/3 volumes of 100% ethanol, which was followed by eluting it in nuclease-free water.

Table 1. List of genes selected for RT-PCR validation.

Gene	Forward Primer	Reverse primer	Amplicon size
GAPDH	AGTGCCAGCCTCGTCTCATA	ACCAGCTTCCCATTCTCAGC	223
β1 integrin	ACAAGAGTGCCGTGACAACT	AGCTTGATTCCAAGGGTCCG	325
MCL-1	TCTTTTGGTGCCTTTGTGGC	CAGTCCCGCTTCGTCCTTAC	110

2.12. RT-PCR

For mRNA quantification, total extracted RNA was reverse transcribed using a QuantiTect Reverse Transcription Kit (Qiagen) (cat. No. 205310) and RT Primer Mix (1 μg). The expression of each gene was analyzed by RQ-PCR using a QuantiTect SYBR Green PCR kit (Cat. no. 204141) and an Applied Biosystems StepOnePlus instrument. GAPDH served as an internal control. Selected primer sequences are shown in Table 1.

For mature miR-223 quantification, extracted miRNA was reverse transcribed using a TaqMan reverse transcription kit (Applied Biosystems), which was followed by quantitative PCR with a TaqMan qPCR assay. miRNA specific signals were normalized using U6 RNA level. The RT primer sequence for miR-223-3P was 5′-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACGGGGTA-3′ and the PCR primer pairs for miR-223-3P were 5′-TGTCAGTTTGTCAAA-3' (forward) and 5′-CAGTGCGTGTCGTGGAGT-3' (reverse) [39]. U6 rRNA was used as an internal control. The RT primer sequence for U6 was 5′-CGCTTCACGAATTTGCGTGTCAT-3′ and the PCR primers for U6 were 5′-GCTTCGGCAGCACATATACTAAAAT-3' (forward) and 5′-CGCTTCACGAATTTGCGTGTCAT-3' (reverse). Relative fold change of target gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.13. Western blot

Protein was extracted from tissue using a mirVana PARIS kit, where cell disruption buffer was used to disrupt samples, and the resulting lysate was split for both RNA isolation and protein analysis. The portion of lysate used in protein analysis was placed on ice for 5–10 min and centrifuged at 4 °C for 1–2 min. Protein concentrations were estimated by the Bradford method. Equal amounts of protein per lane were separated on 10% gels via SDS polyacrylamide gel electrophoresis and were electrophoretically transferred to polyvinylidenene difluoride (PVDF) membranes. Membranes were then incubated at room temperature for 2 h with blocking solution comprised of 5% nonfat dried milk in 10 mM Tris-Cl, Ph 7.5, 100 Mm NaCl, and 0.1% Tween 20. Membranes were incubated overnight at 4 °C with the indicated primary antibodies against GAPDH, β1 integrin (Santa Cruz Biotechnology), MCL-1 (Cell Signaling Technology) and p70S6k (Proteintech); then, the membranes were incubated with a mouse anti-rabbit secondary monoclonal antibody conjugated to horseradish peroxidase at room temperature for 2 h. After each incubation, the membranes were washed four times with 10 mM Tris-Cl, Ph 7.5, 100 Mm NaCl, and 0.1% Tween 20 at room temperature. Chemiluminescence detection was performed with an Amersham detection kit according to the manufacturer's protocol. The amount of the protein of interest was quantified by densitometric analysis using BioRad software, USA. Results were expressed as arbitrary units after normalization to GAPDH protein expression.

2.14. Statistics

Data were analyzed using a one-way ANOVA (Prism 5, GraphPad), which were followed by Bonferroni-corrected post hoc tests when appropriate. Pearson's correlation was utilized to examine potential associations between continuous variables. All results are presented as the means ± SEM. A threshold of $P < 0.05$ was considered statistically significant.

3. Results

3.1. O-rPRP characterization

3.1.1. Count of cells and platelets

The autoanalyzer detected almost no white blood cells (fewer than $1 \times 10^2/mm^3$) or red blood cells (fewer than $1 \times 10^3/mm^3$). The initial platelet count was $550 \pm 175 \times 10^3/mm^3$. The average platelet count in PRP was $2150 \pm 350 \times 10^3/mm^3$.

3.1.2. Concentrations of growth factors in O-rPRP

At 3 different days, concentrations (ng/ml) of growth factors in n = 3 pools of optimized PRP releasate samples (size of each pool = 5 rats) were measured from equal volumes of whole blood; the data are shown in Table 2. There was no significant difference for each type of the four measured growth factors at 3 different days. The % coefficients of variation for TGF-β (transforming growth factor beta), IGF-1 (insulin-like growth factor-1), PDGF-BB, (platelet-derived growth factor isoforms BB), and VEGF (vascular endothelial growth factor) were 2.32, 2.7, 8.3, 5.5 respectively.

3.1.3. Screening of miR-223 in O-rPRP

miR-223 was not detected in O-rPRP.

3.2. Study 1

3.2.1. Serum levels of hormones

The POI group had significant higher levels of FSH and lower levels of E2, AMH, compared to that of the control group ($P < 0.05$). When the galactose rat group received 0.1 ml O-rPRP once per week, once every two weeks, and once every four weeks, their FSH, E2 and AMH levels did not change significantly. In addition, administration of 0.2 ml O-rPRP to the galactose rat group once every two weeks, and once every four weeks, did not produce significant change in the levels of these hormones. However, when the galactose rat group received 0.2 ml O-rPRP once per

Table 2. Concentration of growth factors in O-rPRP.

Growth factors concentrations (ng/ml)	Day 1	Day 2	Day 3	Mean	SD	%CV
TGF-β	110	105	107	107.3	2.5	2.34
IGF-1	55	58	56	56.3	1.5	2.7
PDGF	22	24	26	24	2	8.3
VEGF	8.5	9	9.5	9	0.5	5.5

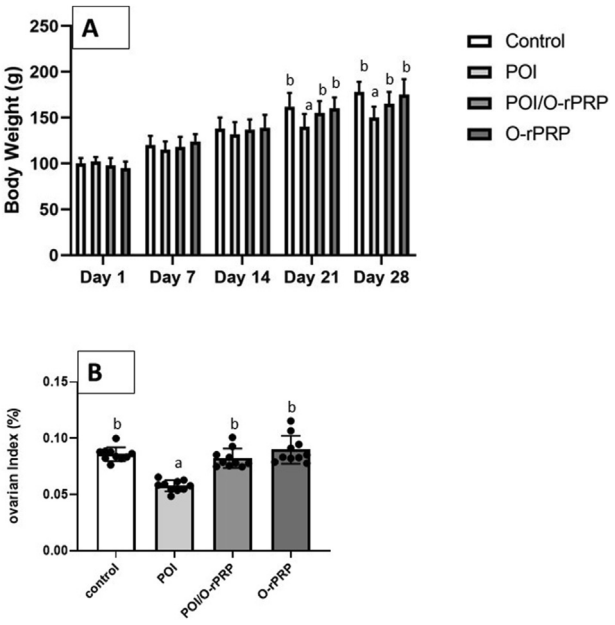


Figure 1. Body weight and ovarian index in different groups. (A): Rat body weight (g) at 1, 7, 14, 21 and 28 days in different groups. (B): Ovarian index (%) in different groups of rats that were injected with PMSG and killed at 13 h post hCG injection. Data are presented as the means ± SD. a, significant ($P < 0.05$) versus control; b, significant ($P < 0.05$) versus POI group; data were analyzed with one-way ANOVA followed by Bonferroni-corrected post hoc tests. POI, premature ovarian insufficiency; O-rPRP, optimized PRP releasate. Individual data are provided as Supplementary Table 1.

week, E2, AMH levels increased significantly, while FSH levels decreased significantly as compared to POI group ($P < 0.05$).

3.3. Study 2

3.3.1. Body and ovarian weight

The body weights for control, POI, POI/O-rPRP, and O-rPRP rats are shown in Figure 1A. At the beginning of the experiment and up to day 14, there was no significant difference in body weight between the different groups. At 21 and 28 days, body weight in the POI group was significantly lower ($P < 0.05$) than it was in the control group. At 21 and 28 days, the group that was administered O-rPRP had significantly increased body weight compared to that of the POI group ($P < 0.05$). At the end of the study period, ovarian weight was determined, and ovarian indices were calculated. Ovarian index = ovarian weight/body weight. Compared with the ovaries from control rats, ovaries of POI rats displayed a significant decrease in the ovarian index ($P < 0.001$), indicating severe atrophy. However, the ovarian indices in the POI/O-rPRP and O-rPRP groups were significantly greater they were in the POI rats, suggesting that O-rPRP reduces ovarian atrophy and development of POI (Figure 1B).

3.3.2. Ovarian histology

Ovarian sections of control and O-rPRP groups (Figures 2A, 2D) showed the presence of follicles at different stages of maturation and the corpora lutea (CL).

The POI group (Figure 2B) had few primordial, antral and Graafian follicles, but it exhibited an abundance of both preantral follicles with pyknotic granulosa cells and atretic follicles. The POI/O-rPRP group showed recovery of follicles at various stage of growing in addition to intact granulosa cells (Figure 2C). The O-rPRP group had significantly higher number of follicles at various stage of growing compared to that of the other groups, and they also exhibited a significant increase in primordial follicles and a significant decrease in atretic follicles (Figure 2D).

Morphometric analysis showed that the distribution of different types of follicles in the POI/O-rPRP group was the same as that of the control group. In addition, compared with the POI group, the number of primordial follicles and corpora lutea in the POI/O-rPRP group increased significantly, while the number of atretic follicles decreased (Figure 2E).

3.3.3. Serum levels of galactose and hormone

The galactose levels of the POF and the POF/O-rPRP groups were significantly increased (6.9 ± 0.4 and 6.7 ± 0.5 mg/dl, respectively), while the O-rPRP treated and the control groups both showed significantly lower levels of galactose (<0.493 mg/dl) (Figure 3A). Meanwhile, the POF group had a significant increase in FSH (by 73%) and a decrease in E2, AMH and inhibin B (by 33, 54, and 47%, respectively), compared to that of the control group ($P < 0.05$).

O-rPRP administration to the galactose rat group yielded a significant decrease in FSH levels (by 37%) and an increase in the levels of E2, AMH and inhibin B ($P < 0.05$) (by 35, 60, and 77.7%, respectively), compared to that of the POI group (Figures 3B, 3C, 3D, 3E).

3.3.4. miR-223 and mRNA expression of β1 integrin and MCL-1

The level of miR-223 in the POI group was significantly higher than it was in the control group (1.5 times) ($P < 0.05$). In the POI/O-rPRP and O-rPRP groups, the miR-223 levels (1.12 and 0.98 folds) were significantly lower than they were in the POI group ($P < 0.05$), and there were no significant difference from the control group (Figure 4A).

The mRNA level of β1 integrin was studied in the POI group (0.6 fold) and was found to be significantly downregulated (by a factor of 1.6) when compared to the levels in the control group ($P < 0.05$). However, the level of β1 integrin mRNA was significantly upregulated in the POI/O-rPRP and O-rPRP groups (0.9- and 1.1-fold) compared to that of the POI group ($P < 0.05$) (Figure 4B).

As shown in Figure 4C, the POI group showed a 2-fold decrease in mRNA level of MCL-1 (0.5-fold) with respect to the control group ($P < 0.05$). The levels of MCL-1 in the POI/O-rPRP and O-rPRP groups (0.8- and1.1-fold, respectively) were 1.6 and 2.2 times higher than they were in the POI group ($P < 0.05$).

3.3.5. Protein expression of β1 integrin, MCL-1 and p70S6k

Western blot analysis demonstrated that the protein expression of β1 integrin in the POI group (0.2 fold) was significantly lower than it was in the control group (0.65-fold), which was a 3.25-fold difference ($P < 0.05$). The protein expression of β1 integrin in the POI/O-rPRP and O-rPRP groups (0.55-, 0.58-fold) was significantly higher than it was in the POI group ($P < 0.05$) (by factors of 2.75 and 2.9, respectively) (Figure 5A).

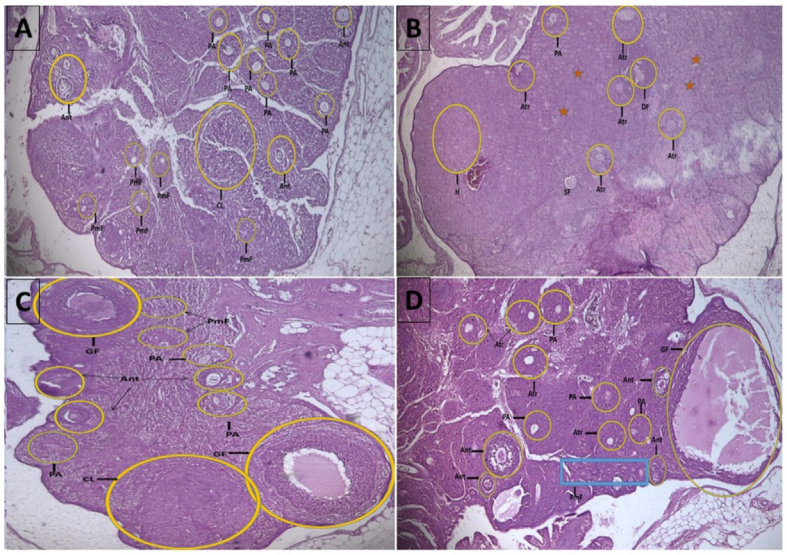
Additionally, the expression of MCL-1 protein in the POI group (0.4-fold) was significantly lower than it was in the control group (1.18-fold) at $P < 0.05$. In the POI/O-rPRP and O-rPRP groups, MCL-1 protein expression was 0.9- and 1.18–fold respectively, and the differences were significantly higher than it was in the POI group ($P < 0.05$) (Figure 5B). Moreover, protein expression of p70S6k in the POI group (0.56-fold) was inhibited by 50% compared with that of the control group (0.7-fold) at $P < 0.05$. Furthermore, in the POI/O-rPRP and O-rPRP groups, the protein expression of p70S6k (0.55-, 0.56-fold) were activated by 62 and 63%, respectively, compared to the levels in the POI group ($P < 0.05$) (Figure 5C).

3.3.6. Association between miR-223 levels and other variables

In this study, a significant inverse correlation was found between miR-223 levels and β1 integrin in the POI group ($r = -0.791$, $P = 0.019$) and after intervention ($r = -0.622$, $P = 0.055$).

4. Discussion

A large body of evidence indicates that galactose toxicity can be used as a model for POI [40]. In a large number of studies that assessed female



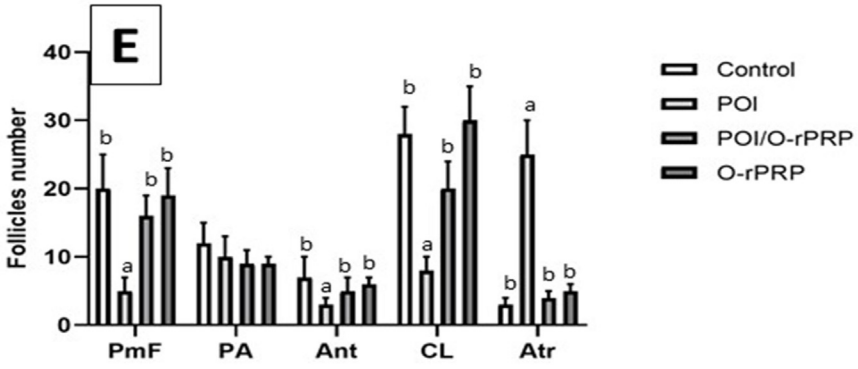


Figure 2. Photomicrographs and stacked bar graph for ovarian sections stained with Hematoxylin- and eosin (H&E x40) in different groups of rats that were injected with PMSG and killed at 13 h post hCG injection. (A): Ovarian sections from control group showing primordial, preantral and antral follicles, and corpora lutea. (B): Histological sections from the POI group showed few preantral follicles, degenerated granulosa cells, many atretic follicles, extensive fibrotic stroma (star) and hemorrhage (H). Only primordial or antral follicles were found. (C): Ovarian sections from the POI/O-rPRP group showing an abundance of follicles at different stages of growth, mainly, primordial, preantral, antral and graafian follicles, in addition to corpora lutea. (D): Ovarian sections from the O-rPRP group showing a pool of primordial follicle and few atretic, primordial, preantral and antral follicles. (E): Stacked bar chart illustrates quantification of the stages of ovarian follicles development in different groups of rats which were injected with PMSG and killed at 13 h post hCG injection. Data are presented as the means ± SD. a, significant ($P < 0.05$) versus control; b, significant ($P < 0.05$) versus POI group; one-way ANOVA followed by Bonferroni-corrected post hoc tests. POI, premature ovarian insufficiency; O-Rprp, optimized PRP releasate; PmF, primordial follicles; PA, preantral follicles; Ant, antral follicles; CL, corpora lutea; Atr, atretic follicles. Individual data are provided as Supplementary Table 2.

patients with galactosemia, POI was found to be a common feature [41]. A possible mechanism of galactose-induced ovarian toxicity is the direct toxicity of galactose and metabolites in mature ovaries, allowing high levels of FSH and low levels of E2 to persist [42]. However, the exact cellular and molecular mechanisms of galactose-induced ovarian toxicity are not fully understood.

This work showed that rats fed with 50% galactose for four weeks had atrophied ovaries, elevated levels of galactose and FSH, and decreased E2 levels in the circulation relative to that of the control group. The current findings represent a state of galactosemia-induced hypergonadotrophin hypoestrogenism. In accord with the current results, experimental galactose toxicity was successfully used as a model for POI [8].

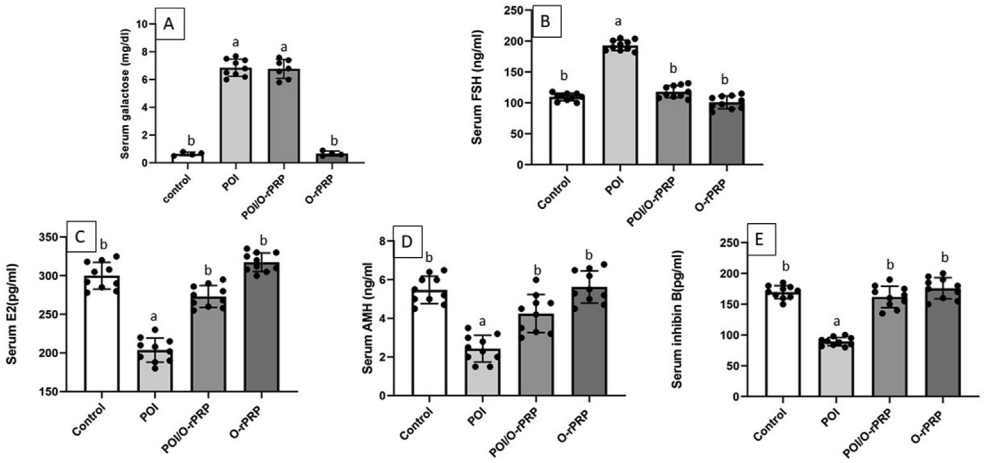


Figure 3. Effect of galactose and O-rPRP on serum galactose (A), FSH (B), E2 (C), AMH (D) and inhibin B (E) as detected by ELISAs performed on different groups of rats that were injected with PMSG and killed at 13 h post hCG injection. Data are presented as the means ± SD. a, significant ($P < 0.05$) versus control; b, significant ($P < 0.05$) versus POI group; one-way ANOVA followed by Bonferroni-corrected post hoc tests were performed. FSH, follicle stimulating hormone; E2, Estradiol; AMH, anti-mullerian hormone.

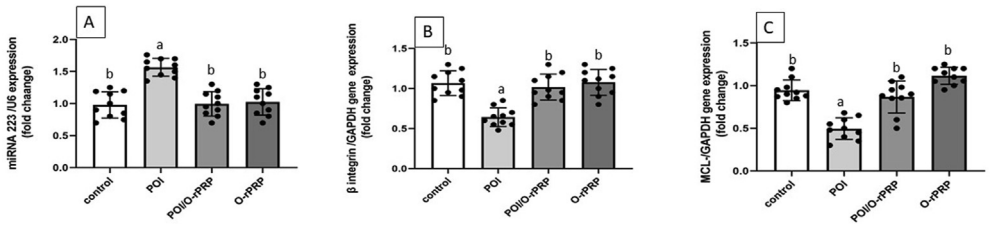


Figure 4. Effect of galactose and O-rPRP on miRNA-223 in ovarian tissue (A), mRNA expression of β1 integrin (B) and MCL-1 (C). Bars represent fold change, and individual values are expressed as the means ± S.D. a, significant ($P < 0.05$) versus control; b, significant ($P < 0.05$) versus POI group; one-way ANOVA followed by Bonferroni-corrected post hoc tests were performed.

Follicular development begins with granulosa cells, which, in addition to expressing FSH receptors, synthesize a number of active peptides that promote their own proliferation [43]. In tandem, AMH is secreted by growing follicles to impede initial primordial follicle activation; therefore, the serum level of AMH is as an indicator of the ovarian reserve and prospective fertility [44]. Ordinarily, fully mature or ovulated follicles secrete the inhibin peptide to inhibit circulating FSH and subsequent growth signals [45]. In contrast, progressive loss of ovarian follicles leads to reduced inhibin production and a loss of restraint on FSH secretion [46]. In this work, a decrease in both AMH and inhibin B serum levels were observed in the POI group with respect to the control group. An earlier study has found that low levels of AMH and inhibin B are good at indicating a diminished ovarian reserve [47].

Further, morphometric analysis of ovaries from the POI group after gonadotrophin induction showed that the ovarian follicles were arrested at the preantral follicles stage, and there was little transition from the preantral to the antral stage. The results observed after induction confirm that ovarian dysfunction in the POI group was not caused by loss of FSH biopotency, but it may be due to defects in receptors signaling [48]. Therefore, we investigated the ovarian mRNA and protein expression of β1 integrin, which is a prerequisite for integrating growth signals.

In this study, β1 integrin receptor mRNA and protein expression were downregulated in the POI group compared to the control group. Previously, β1 integrin expression was inhibited in a rat model of ovarian failure [49]. Recently, miR-223 was found to oppose the expression of β1 integrin [50]. In this study, the POI group showed higher miR-223 expression than the control group. Upregulation of miR-223 expression was found under a variety of stress conditions, where it has an inhibitory effect on proliferation. Multiple mechanisms are proposed for the miR-223 anti-proliferative effect, such as targeting genes IGFR1 [51], ACVRIIa and β1 integrin [42]. In agreement, we found that miR-223 expression is negatively correlated with β1 integrin levels. Consistently, a previous study identified a seed sequence (7 base pairs of complementarity) for miR-223 in the 3′UTR of β1 integrin [15].

Downregulation of β1 integrin is known to attenuate the phosphorylation of growth factor receptors and subsequently results in the phosphorylation of Akt, ultimately leading to inactivation of the mTOR signaling pathway [52]. In this study, expression of p70S6k, a downstream kinase of mTOR, was found to be downregulated in the POI group when compared to that of the control group. Compromised mTOR signaling enhances granulosa cell autophagy and impairs follicular development [53]. Previously, FSH treatment was found to activate the mTOR pathway and inhibit autophagy, but this effect was abolished in the presence of an AKT phosphorylation inhibitor [54]. In a previous report, there was an association between aberrant mTOR/Akt signaling and galactosemia [55]. According to the results reported here, we suggest that galactose stress induced miR-223, which targeted β1 integrin expression and resulted in impairing AKT phosphorylation and the downstream mTOR pathway. Interestingly, miR-223 was deemed to be a tumor suppressor through inhibiting Akt/mTOR, which resulted in the suppression of cell proliferation and activating autophagy [56]. Multiple lines of evidence found that autophagy was involved in follicular atresia during ovarian follicular development [57].

In addition, it has been found that induction of autophagy in granulosa cells is closely related to the occurrence of apoptosis [58]. Previously, the loss of β1 integrin expression preceded follicular apoptosis and ovarian regression [59].

Antiapoptotic MCL-1 has been described as a link between the stressors that initiate apoptosis and the machinery of the intrinsic apoptotic pathway, since loss of MCL-1 results in the release of BIM to

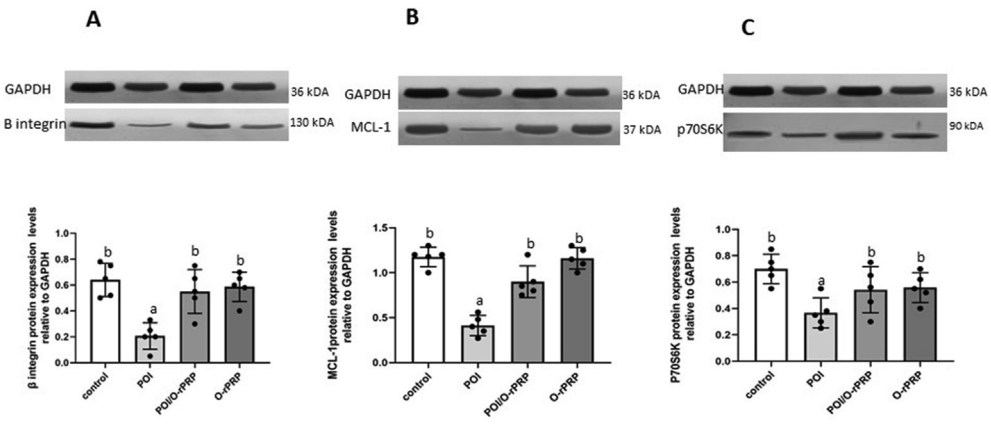


Figure 5. Effect of galactose and O-rPRP on protein expression of β1 integrin (A), MCL-1 (B) and p70S6K (C) in ovarian tissue, as shown by Western blot analysis. Bars represent protein expression levels relative to GAPDH. Values are expressed as the means ± S.D. a, significant ($P < 0.05$) versus control; b, significant ($P < 0.05$) versus POI group; one-way ANOVA followed by Bonferroni-corrected post hoc tests were performed. Full, non-adjusted image for the gel is provided as supplementary Fig. 1.

activate BAX or BAK [60]. BIM is expressed in ovarian cells and was initially termed "the gene Bcl-2 related ovarian death agonist (BOD)" [61].

In this work, we analyzed MCL-1 and found that the POI group had lower expression of MCL-1 than the control, confirming the activation of apoptosis and increased fragmentation.

Altogether, the repression of mTOR signaling and MCL-1 expression found in this work may indicate excessive atresia, which indicates that the immature pool of follicles failed to reach maturation. POI has been long considered to be irreversible; however, it has subsequently been confirmed that POI is different from menopause, and despite the presence of elevated gonadotropins, residual ovarian function may still be present [62]. Pharmacological protection strategies that targeted multiple points in the galactose metabolic pathway could not offer the expected deliverance from POI [63]. Meanwhile, POI with a nongalactosemic etiology showed promising outcomes when undergoing a growth hormone intervention [64, 65]. Therefore, in this study we applied O-rPRP to a POI experimental rat model. The platelet concentration of the PRP formula used in our study ($2150 \pm 350 \times 10^3$ platelets/mm^3) was 4 times greater than that of the baseline. Formerly, the therapeutic efficiency of PRP has been shown to be related to a minimum platelet count of 1000×10^3 platelets/mm^3 [66]. In the present work, we isolated the supernatant rich in growth factors from PRP, which is known as the PRP releasate. Interestingly, this process enabled the elimination of platelets and therefore reduced the variability in growth factor content [67].

In the present work, we modified the experimental conditions and prepared O-rPRP that contained an enriched concentration of growth factors. The method utilized is highly reproducible and yields consistent growth factor concentrations among different preparations [27]. Moreover, we aimed to determine the best O-rPRP regimen that will impact the ovarian reserve hormones, so it can be used to study the influence of O-rPRP on follicle population and the mechanism of action. In this regard, we found that compared with the POI group, once a week administration of 0.2 ml of O-rPRP significantly increased the levels of E2, AMH, and at the same time significantly reduced FSH levels, therefore we applied it to further mechanistic study.

In the second study, the POI/O-rPRP group did not have atrophied ovaries, and it showed nonsignificant difference in serum galactose levels from those of the POI group; however, the serum E2 and AMH levels were elevated, and serum FSH concentrations were reduced relative to those of the galactose group. Additionally, higher inhibin B serum levels were observed and corresponded to a large number of mature follicles in the ovarian tissue sections of the POI/O-rPRP group. Consistently, PRP was found to have a protective effect on cyclophosphamide induced ovarian failure in rats [19].

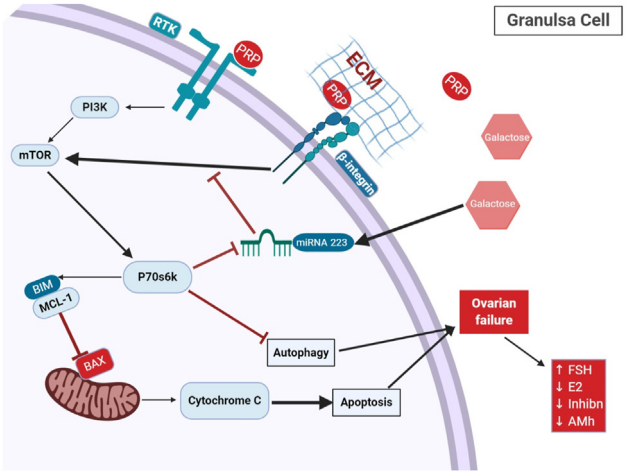


Figure 6. miR-223 regulates ovarian folliculogenesis through its sensitivity to hypergalactosemia by inhibiting integrin and therefore mTOR signaling. Cotreatment with PRP would ameliorate adverse impacts on ovarian function via overriding the effect of galactose on miR-223 target genes.

In the POI/O-rPRP treated group, miR-223 was suppressed. Previously, growth factors, namely, vascular endothelial cell growth factor and basic fibroblast growth factor, decreased miR-223 expression and induced the proliferation of murine endothelial cells [15]. β1 integrin expression in the POI/O-rPRP group was activated. Previously, growth factors were shown to preserve β1 integrin expression and retain active signaling [15]. It is well established that restoring integrin-mediated adhesion to the extracellular matrix increases intracellular growth factor transduction pathways signals, thus converting mechanical signals into chemical signals to activate proliferation [43].

The study showed that O-rPRP administration resulted in increasing the expression of p70S6k relative to that of the POI group. The results demonstrate that O-rPRP confers upstream activation of mTOR. Recent studies have shown that mTOR activator encourages the growth of early secondary follicles to the antral/preovulatory stage, which is a finding that may be the basis for developing infertility treatments [68].

In addition, active mTOR signaling is known to initiate short-lived anti-apoptotic MCL-1 by preventing it from being phosphorylated by glycogen synthase kinase 3 (GSK-3) [69]. Phosphorylation of MCL-1 results in targeting MCL-1 for ubiquitinylation and its subsequent rapid degradation [60]. Accordingly, MCL-1 expression as a survival protein was analyzed and found to be upregulated in the POI/O-rPRP group. MCL-1 is required for the transition from primordial follicles to primary follicles and for sustaining follicle growth [70]. Formerly, several reports have shown that different growth factors are capable of upregulating the transcription of MCL-1 [71].

The current report highlights the inhibitory effect of O-rPRP on miR-223 and the resulting proliferation outcome, which might be a single mechanism among the many pathways by which O-rPRP provides ovarian protection (Figure 6).

The current study has made a significant contribution to the literature as this is the first study to prove that the optimized form of PRP has promising results in addressing POI in experimental animal model. In addition, in clinical trials, PRP is increasingly used for the management of POI [72, 73], but the action and mechanism of the optimized form have not yet been clarified. However, it is important for future research to study whether injection of O-rPRP under a longer of galactose diet will have a similar effect.

On the other hand, this study may have some limitations, which can be resolved in future studies. First, apoptosis was assessed in an indirect way, therefore the direct effect of PRP treatment over ovarian cell apoptosis is questionable. Second, in the current experimental model, the toxic effects of galactose represent some but not most cases of POI. Third, we have performed miRNA, mRNA, and protein analysis in the entire ovarian tissues, so further experiments in isolated granulosa cells will confirm the present findings. A potential weakness of this work is that PRP has been only partially characterized. Additionally, we have not performed inhibition experiments, so the present findings could be partly due to the growth factors, but other factors may play a role as well. As PRP has an ameliorating effect on POI, it is recommended that future experimentation test each growth factor alone or different combinations of the growth factors found in O-rPRP.

In conclusion, this study demonstrates the protective effect of PRP on galactosemia-induced ovarian insufficiency, which might be related to the overexpression of the β1 integrin receptor and MCL-1, activation of mTOR signaling, and downregulation of miR-223, all of which result in the promotion of ovarian follicle growth.

Declarations

Author contribution statement

W. El Bakly and N. Nabil: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

M. Medhat and M. Shafei: Analyzed and interpreted the data.

R. Tash, M. Elrefai, Y. Shoukry: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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