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The therapeutic role of *Aronia melanocarpa* on cyclophosphamide-induced premature ovarian failure

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ABSTRACT: The purpose of the study was to evaluate the effects of *Aronia melanocarpa* (*A. melanocarpa*) extract on ovarian cell apoptosis, vascularization, and follicle reserve in the premature ovarian failure (POF) induced by cyclophosphamide (CYC). After creating a premature ovarian failure model with 100 mg/kg CYC, *A. melanocarpa* was given to Wistar albino female rats by gavage 15 times every other day (200 mg/kg).Vaginal smears were taken to evaluate the estrus cycles before, during and at the end of the experiment. Histological ovarian follicle count was performed by examining the ovaries with Hematoxylin&Eosin staining. Immunohistochemical evaluation of apoptosis markers of caspase-3 (cas-3), caspase-9 (cas-9) and vascular endothelial growth factor (VEGF) was performed. *A. melanocarpa* reregulated the estrus cycles in a similar way to the controls by reducing cas-3 and cas-9 in ovarian cells and increasing the number of ovarian cells. While CYC disrupted ovarian vascularization, *A. melanocarpa* contributed to ovarian reserve by increasing VEGF immunopositivity. In chemotherapy-induced POF, *A. melanocarpa* extract increases ovarian reserve by providing vascularization, decreasing apoptosis and providing cycle control in female rats.

KEYWORDS: Aronia melanocarpa; premature ovarian failure; cyclophosphamide; VEGF; apoptosis.

1. INTRODUCTION

Premature ovarian failure (POF) [1] is a reproductive endocrine dysfunction that causes female infertility before the age of 40 [2]. Amenorrhea is characterized by low estrogen levels, high gonadotropin levels, infertility and the absence of mature follicles [3]. By reducing the ovarian reserve, POF leads to such negative pathologies as osteoporosis, cardiovascular disease and sexual dysfunction. It is known that genetic and autoimmune diseases, environmental factors, radiotherapy and chemotherapy play a role in its etiology [4].

Chemotherapy is one of the most important reasons for the appearance of POF in society. In people receiving chemotherapy with alkylating agents such as CYC [5], apoptosis is triggered in primordial follicles [6,7], and menstrual irregularities and ovarian dysfunction occur by decreasing the reserve in avascular and inflammatory ovaries [8]. While many herbal, chemical and molecular methods are being tried against the ovaritoxicity caused by CYC [9], it is still unknown what the treatment for preventing ovarian cell apoptosis is.

Aronia melanocarpa is a food native to North America [10], newly approved by the Chinese National Health Council in 2018 [11]. *A. melanocarpa*, which is used as an antihypertensive and antiatherosclerotic drug in Russia and Eastern European countries [12], is rich in flavonoids, polyphenols, polysaccharides, organic acids, and dietary fiber [13]. It is known that *A. melanocarpa*, which has antitumor, anti-inflammatory, antiviral and antioxidant properties, reduces free radicals, affects telomere length, regulates the immune system, increases apoptosis in cancerous cells [14], and decreases it in healthy cells. It is also

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argued that *A. melanocarpa* extract has many protective properties such as cardioprotection [15], neuroprotection [16], hepatoprotection [17] and it improves health by providing its protective properties through apoptosis [18] and vascularization [19].

In our study, after creating an experimental POF model with CYC, a chemotherapeutic drug, the effect of *A. melanocarpa*, which has a high anthocyanin content, on ovarian reserve, ovarian apoptotic pathway, and vascular endothelial growth factor was investigated. Thus, it was aimed to develop a new non-invasive medical treatment to reduce the apoptotic and vascular side effects of gonadotoxic chemotherapy on the ovaries and to preserve fertility.

2. RESULTS

2.1. *A. melanocarpa* Treatment Regulated Abnormal Estrous Cycles In CYC-Induced POF

Ovarian weight and diameters did not differ between the groups after *A. melanocarpa* application ($p>0.05$) (Table 1).

Table 1. Ovary weights and diameters between groups.

	CONTROL	POF	POF+ARONIA	p
Weight (mg)	1088.60 ± 180.79	1137.61 ± 185.98	1035.38 ± 180.65	>0.05
Diameter (cm)	0.5 (0.5-0.6)	0.5 (0.5-0.6)	0.6 (0.4-0.6)	>0.05

Estrus cycles of the rats were evaluated, and the presence of healthy cycles was observed in all animals before the experiment. After the premature ovarian failure model was created, healthy cycles continued consecutively in the control group, while cycles were impaired in the POF and POF+ARONIA groups, and leukocyte cells specific for anestrus and diestrus were observed. In the post-treatment smears, normal estrus cycles were observed in CONTROL and POF+ARONIA groups, while the cycles were disrupted in the POF group (Figure 1). This proved that the administration of *A. melanocarpa* significantly ameliorated abnormal estrus cycles in CYC-induced POF rats.

2.2. *A. melanocarpa* Improved the Ovarian Reserve in a CYC‑Induced POF Rat Model

In the histopathological examination, there were many follicles in the ovarian tissues of the control group, including functional follicles (primordial follicles ($p<0.002$), primary follicles ($p<0.001$), secondary follicles ($p<0.001$), Graafian follicles ($p<0.001$)) and atresia follicles. In contrast, an abnormal histology with inflammatory cell infiltration ($p<0.001$) and hemorrhage due to vascular damage ($p<0.001$), cystic structures with severe fibrotic formation and fewer functional follicles were observed in the ovaries of POF rats, the number of atresia follicles in POF rats was significantly increased compared to the control group ($p<0.001$) (Figure 2).

After *A. melanocarpa* application, improvement in the morphology and structure of the ovarian tissues was observed in all samples (n=6) as an increased number of functional follicles. There were no differences between the CONTROL group and the POF+Aronia group in the number of the primordial follicle ($p=0.517$), the secondary follicle ($p=0.671$), the tertiary follicle ($p=0.395$), the atretic follicle ($p=0.132$) and ovarian hemorrhage ($p=0.067$). The numbers of the primordial follicle ($p<0.039$), the secondary follicle ($p<0.002$) and the tertiary follicle ($p<0.004$) were statistically and significantly higher in the POF+Aronia group compared to the POF group, and atretic follicle number was lower ($p<0.006$). According to the results, it was observed that *A. melanocarpa* contributed positively to the development of follicles in the ovary (Figure 2).

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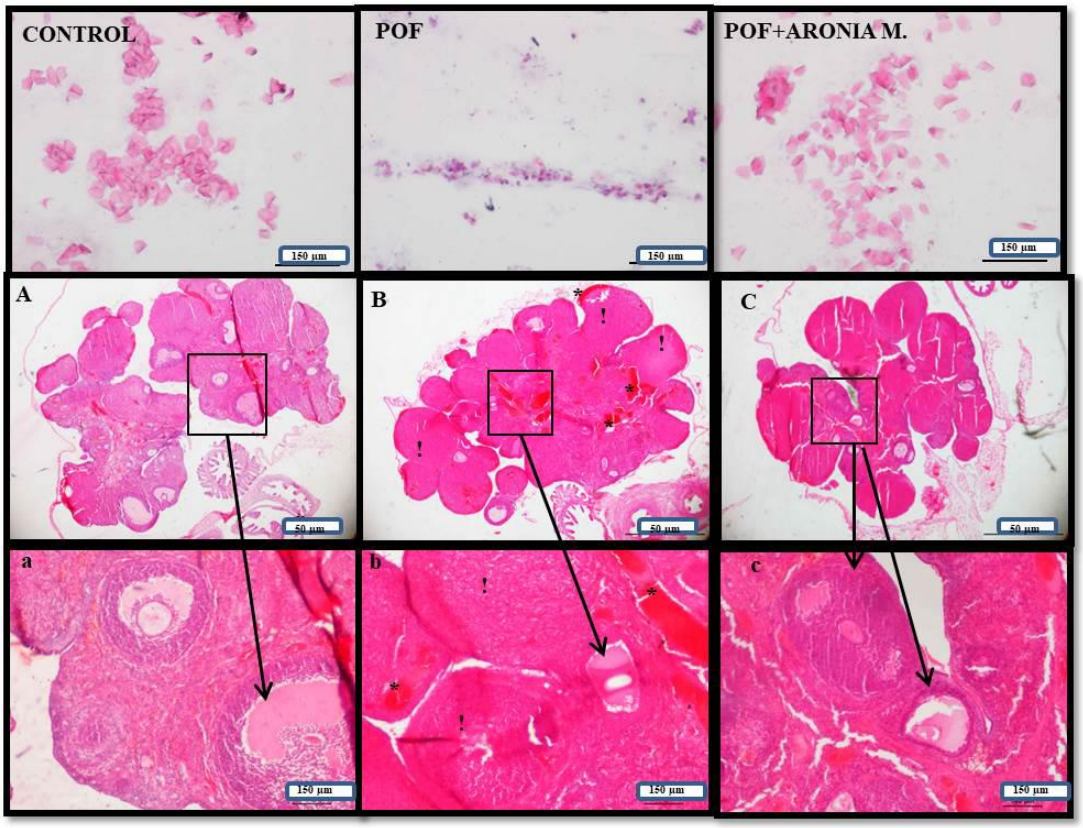

Figure 1. Comparison of intergroup estrus phase in rat estrus cycle changes after A. *melanocarpa* treatment (150 µm). CONTROL and POF+ARONIA groups normal estrus phase, POF group CYC-induced impaired diestrus phase. **A)** Control group ovarian cortex and medulla. **a)** Graafian follicle in the cortex of the control group **B)** POF group ovarian cortex and medulla. **b)** POF ovarian group atresia follicle **C)** POF+Aronia group ovarian cortex and medulla. **c)** POF+Aronia group secondary follicle. Cystic follicles, (*) hemorrhage.

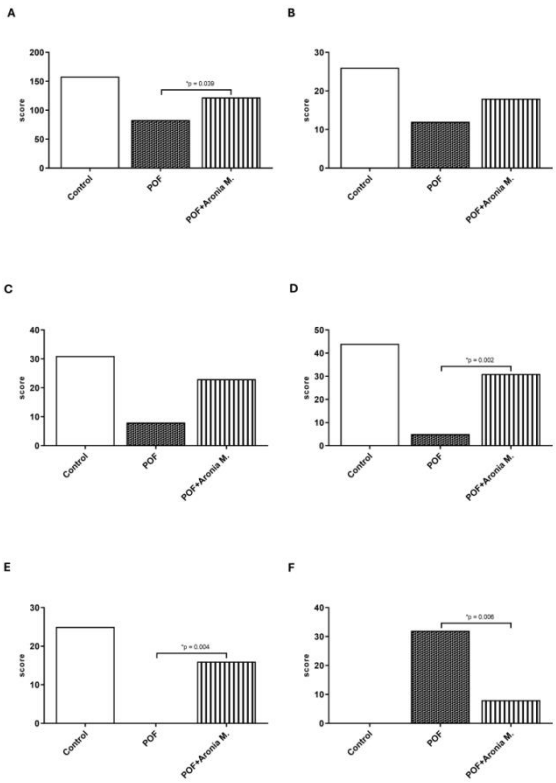

Figure 2. Comparison of ovarian follicle numbers between groups. There were no differences between the CONTROL group and the POF+Aronia group in the number of the primordial follicle (A), the secondary follicle (D), the tertiary follicle (E), the atretic follicle (F) and ovarian hemorrhage (p=0.067). No difference was found between the groups in terms of unilaminar (B) and multilaminar follicles (C).

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2.3. IHC Results of Ovarian Vascularization and Apoptotic Proteins After *A. melanocarpa* Administration in CYC-Induced POF Rats

As shown in Figure 4, IHC staining results of VEGF protein in the ovary showed that the positive intensities of VEGF were significantly higher in the control and POF+Aronia group compared to the POF group ($p<0.001$, $p<0.001$) (Table 2). No difference was found between the control and *A. melanocarpa* groups ($p=0.097$). In addition, in the IHC results of Cas-3 and Cas-9 apoptosis proteins in the ovary, immunopositivity was observed more intensely in the POF group than in the control ($p<0.001$) and POF+Aronia group ($p<0.001$) (Table 2), whereas in the control and POF+Aronia groups, similarly, positivity was less and no statistically significant difference was observed ($p=0.482$) (Figure 3). This confirmed that treatment with *A. melanocarpa* after CYC induction significantly promoted proliferation in ovarian cells and follicles.

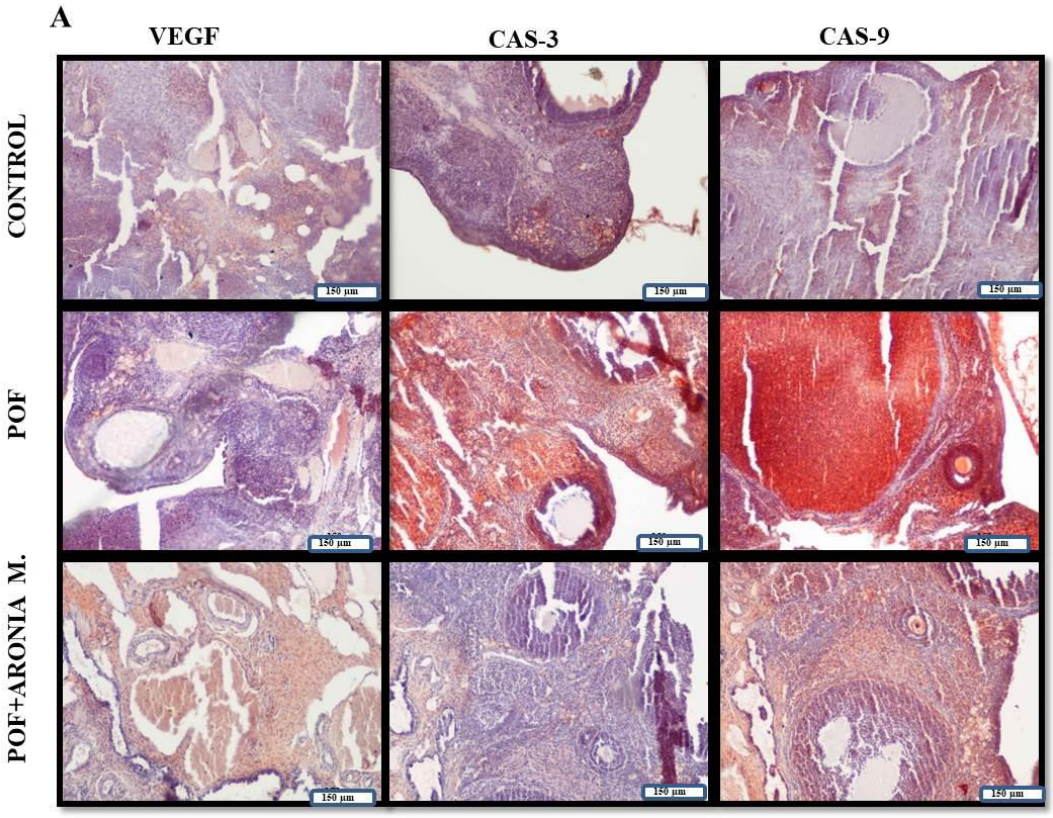

Figure 3. The Immunohistochemical staining results of ovaries from CYC-induced POF rats after *A. melanocarpa* administration. The representative Immunohistochemical staining of VEGF, Cas-3 and Cas-9 of ovaries from CYC-induced POF rats in different groups after *A. melanocarpa* administration. Scale bar/Magnification=150 µm. The relative expression levels of VEGF, Cas-3 and Cas-9 of ovaries from CYC-induced POF rats in different groups after *A. melanocarpa* administration.

Table 2. Immunopositivity assessment in ovarian tissue between groups

Ovary / *Immunpositivity*	*CONTROL*	*POF*	*POF+ARONIA*
VEGF	+++[a]	+[b]	+++[a]
Cas-3	+[a]	+++[c]	+[a]
Cas-9	++[a]	++++[c]	+++[a]

a:No difference was found between CONTROL and POF+ARONIA groups.

b: VEGF were significantly higher in the CONTROL and POF+ARONIA group compared to the POF group.

c: Immunopositivity of Cas-3 and Cas-9 apoptosis proteins was observed higher in the POF group than in the CONTROL and POF+ARONIA group.

3. DISCUSSION

This study supplies an original result to observe the curative effect of *A. melanocarpa* treatment on the protection of fertility with CYC-related ovarian damage in rats. Significant improving in the estrus cycle stages, which were disrupted by CYC application in rats, increasing the number of primary, secondary and tertiary follicles, increasing the number of corpus luteum, suppression of apoptosis in the ovaries, and increasing VEGF were observed by *A. melanocarpa* treatments.

In reproductive endocrine dysfunction, premature ovarian failure, embryo freezing, cryopreservation, endocrine and antioxidant drugs are used to protect the reproductive function of the ovaries, especially in the case of chemotherapy, and the search for new methods continues [20]. *A. melanocarpa*, which is known to be good for many chronic and metabolic diseases, is an important source of polyphenols rich in anthocyanins, flavonoids, and procyanidins, and is the subject of many *in vivo* and *in vitro* studies [21] with its antioxidant [22], anti-inflammatories [23], anti-cancer [24], effects. However, although it has been studied in many systems, there are limited studies on women's health.

Chemotherapeutic alkylating agents, such as CYC, trigger apoptotic molecular pathways by forming DNA double-strand breaks [25]. Apoptosis is the basic cause of ovarian dysfunction [26]. At this stage, ovarian damage induced by experimental CYC constitutes a successful model for the experimental premature ovarian failure. Because, CYC causes atresia in primordial follicles by triggering apoptosis in granulosa and theca cells, and disrupts the estrus cycle [27]. It was observed that Ki-67 increased TUNNEL positivity in granulosa cells (150 mg/kg CYC) [28], Cas-3 in primordial follicles (100 mg/kg CYC) [29], Cas-3 immunopositivity of granulosa and theca cells in multilaminar primary, secondary and Graaf follicles [30]. Similarly, in our study, Cas-3 and Cas-9 immunopositivity was observed to increase in the ovarian sections of the POF group, and incompatible with this finding, an increase in the number of atretic follicles and a decrease in the number of primary, secondary and tertiary follicles was observed in the H&E sections. Many studies are showing that *A. melanocarpa* extract used by us to prevent ovarian dysfunction caused by CYC, which is used in the treatment of different pediatric and adult solid tumors [31], improves health in many tissues such as the heart [32], liver [33] and kidney [34]. It is known that *A. melanocarpa* exerts neuroprotective effects by suppressing Cas-3, Cas-9, Bcl-2 and Bax genes in neurotoxicity induced by amyloid in the SH-SY5Y cell line, which has been modeled for Alzheimer's Disease [34]. *A. melanocarpa* has been shown to have protective effects against kidney apoptosis by reducing Cas-9 in renal tissue in renal ischemia-reperfusion injury [35]. In addition, it was observed that the application of *A. melanocarpa* in rats which is induced by experimental polycystic ovary syndrome increased the primary follicle, antral follicle, primordial follicle and antral follicle [36]. In a similar study, 28 days of A. melanocarpa administered to rats with polycystic ovary syndrome improved reproductive and metabolic changes by reducing oxidative stress parameters; increased serum progesterone [37].In our study results, it was also found that premature ovarian failure was caused by CYC, *A. melanocarpa* suppressed apoptosis by reducing Cas-3 and Cas-9 immunopositivity in ovaries, contributing to increasing the number of primordial, primary, secondary and tertiary follicles. In accordance with this information, *A. melanocarpa*, probably like in many other diseases, exerts an antiapoptoic effect on the pathophysiology of ovarian cell apoptosis in POF.

Estradiol and progesterone released from granulosa and theca cells during the folliculogenesis stage are closely related to the sequencing and regularity of the estrus cycles [38]. Apoptosis triggered by CYC in follicles also disrupts estrus cycles, contributing to the infertility picture of premature ovarian failure [39]. Consistent with other studies in which CYC disrupts estrus cycles, *A. melanocarpa* extract provided cycle regulation in the repeat diestrus and anoestrus cycles model and improved ovarian health.

Angiogenesis has a great importance in the ovulatory cycle [40], ovarian follicle development [36,37], corpus luteum and endometrium formation [41] in the female reproductive system. At this stage, VEGF, an angiogenic factor, plays a role in blood vessel development [42]. It is said that one of the mechanisms of CYC destruction of primordial follicles is by disrupting microvascularization [43]. In our study, CYC decreased vascularization and impaired ovarian blood supply by causing hemorrhage; on the other hand, it triggered follicle damage by decreasing VEGF. *A. melanocarpa* treatment increased VEGF in the ovaries and contributed significantly to the follicle reserve by providing angiogenesis. Consistent with our study, it was observed that *A. melanocarpa* extract increased ovarian VEGF in rats with experimental polycystic ovary syndrome [36].

When the effects of *A. melanocarpa* are evaluated clearly, it is thought that it may have shown an effect on estradiol, and more detailed studies are needed to explain apoptotic physiology in this regard. In our study, the fact that the interaction of ovarian and pituitary hormones and the apoptotic pathways with these hormones were not evaluated, and infertile or fertile conditions were not observed when the animals in the groups were mated restricts our study results. However, being the first study to evaluate the effects of *A. melanocarpa* on ovarian damage caused by CYC constitutes our superiority and originality.

4. CONCLUSION

Our study foresees that *A. melanocarpa* extract can be used as a supportive treatment for infertility and assisted reproductive techniques by guiding future experimental and clinical studies. The protective feature of *A. melanocarpa* extract, which has no known side effects, can be used after chemotherapy to protect against future complications.

5. MATERIALS AND METHODS

5.1. Animals

Ethics committee approval of the study was obtained from Bolu Abant Izzet Baysal University (BAIBU) Experimental Animals Local Ethics Committee with the number 2021/13. In the study, 8-10 weeks old Wistar albino female rats with a body weight of 200-250 gr were purchased from BAIBU Experimental Animals Application and Research Center and food and water were given ad libitum under 19±2 ◦C temperature and 55-60% relative humidity. In the study, 3 groups were designed as the control(n=10), the premature ovarian failure group (POF) induced by CYC (n=10) and POF+Aronia (n=10).

5.2.Creating POF Model and *A. melanocarpa* Administration

A total of 100 mg/kg of CYC was dissolved in 0.2 cc of saline and administered intraperitoneally (i.p.) to the animals [20]. Then, after 30 days and following the formation of a POF model, estradiol was withdrawn from the body in sufficient quantity [44]. At the end of the 30th day, an animal was sacrificed to confirm the formation of the model, and the ovaries were histologically checked. Serum estradiol value was measured, but the confirmed animal was not included in the experiment. After the model formation was confirmed, 200 mg/kg *A. melanocarpa* [45] was given to the rats in gavage form 15 times every other day [46] (Figure 4).

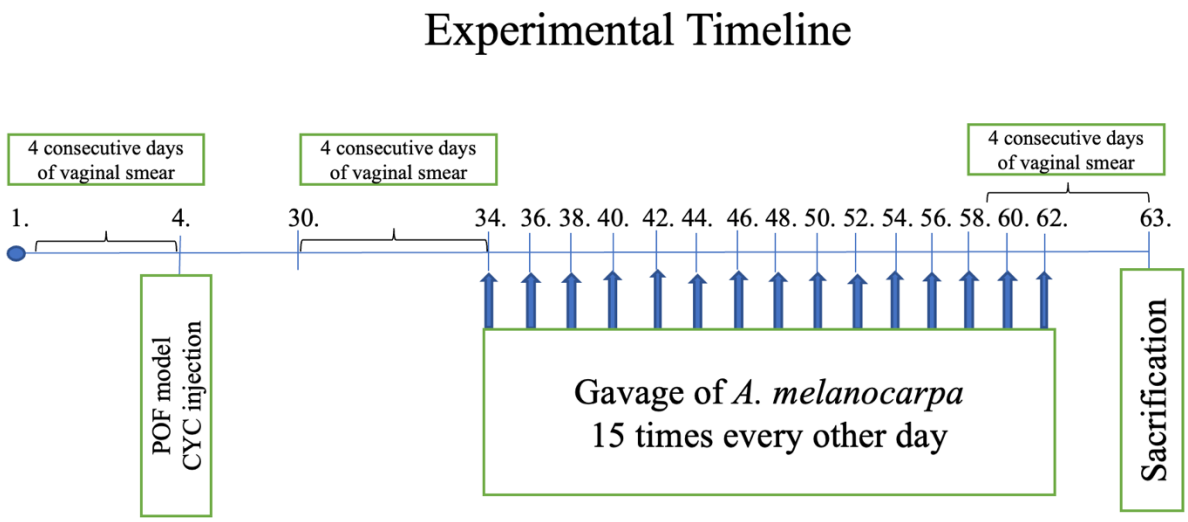

Figure 4. Experimental timeline.

5.3. Estrous Cycle Assessment

To observe the estrus cycle, the cytological examination was performed with a vaginal smear on the 1st day of the experiment, on the 30th day after CYC injection, and on the 45th day after *A. melanocarpa* treatment, in the same morning hours, consecutively in all experimental groups. The rat vagina was swabbed gently with a cotton swab washed with normal saline and spread on slides. After air drying, it was fixed with methanol and stained with Heametoxylin & Eosin. The experiment was started after the healthy cycle of the rats was determined. On the 1st, 30th, and 45th days of the experiment (after all smears were examined for 4 consecutive days), vaginal smears were evaluated according to their cytology under a light microscope (Nikon Eclipse 80i Melville U.S.A.) separately [47].

5.4. Sacrification

After 63 days, rats were sacrificed, ovaries were removed from the animals under anaesthesia (i.p., 100 mg/kg ketamine (Ketalar, Pfizer) and 15 mg/kg Xylasine (Xylanzinbio, Bioveta)). Then an incision of abdominal median were made longitidunal from xiphoid to pubic region and abdomen was opened. The intestines carefully removed and the ovaries were reached. Some of the excised ovarian tissues were deposited in a 10% formalin solution for histological analysis, while the remaining tissues were heated to -80 degrees for the biochemical process.

5.5. Measuring Ovary Weight and Diameter

After we sacrified the animals, we measured ovary weight with a ruler and height with a precision scales. We compared mean POF+ARONIA and POF groups versus control at the end of the experiment.

5.6. Histopathologic Examinations

Primordial follicles are the first follicle cells to develop in the female reproductive system during the fetal period. They are located beneath the germinal epithelium, which covers the outer surface of the ovary, in millions. Single-layered squamous epithelium follicle cells are found around the primary oocyte. These squamous epithelium cells develop to form the structure of the primary follicle. Between the granulosa cells surrounding the primary follicle, follicular fluid accumulates, creating the antrum structure. This new structure is called a secondary follicle or antral follicle.

The antrum grows and develops, pushing the oocyte towards the periphery of the follicle. The oocyte is surrounded by a glycoprotein layer called the zona pellucida, and the cells facing the follicular fluid are known as cumulus oophorus cells. The granulosa cells surround the cumulus oophorus cells. The outermost layer consists of single-layered cuboidal cells called theca cells. This resulting structure is called a mature follicle or Graafian follicle.

If the oocyte within the follicle degenerates, an atretic follicle is formed. These atretic follicles are usually follicles that have regressed or are held in reserve.

These processes are important for the normal development of the female reproductive system and the process of ovulation [48].

After recording the weights of the ovaries and uterus for H&E staining, the ovaries of each sacrificed rat (n=10) were fixed in 10% formalin for histological evaluation under a light microscope. They were embedded in paraffin wax and sectioned into 5 µm slices using a rotary microtome. Following dehydration, the sections were stained with H&E.

Subsequently, the number of follicles at different stages (primordial, secondary, antral, and atretic follicles) in six sections of each rat was recorded using a random numbering system. To ensure the accuracy of follicle stages and counts in different groups, periodic evaluations were performed. Five different areas were blindly counted using a Nikon Eclipse 80i microscope in Melville, U.S.A.

5.7.Immunohistochemistry Staining

Immunohistochemistry (IHC) staining was performed in 5 µm serial sections (n=6) from *A.melanocarpa*-treated and CYC-induced POF rats to detect the expression levels of VEGF, Caspase-3 (Cas-3), and Caspase-9 (Cas-9) proteins to evaluate the effect on the proliferative abilities of follicles. After sectioning,

gradient rehydration, and antigen retrieval, sections were blocked with 5% bovine serum albumin (BSA, A8010, Solarbio, Beijing, China) for 30 minutes at 37°C. The sections were then incubated overnight at +4 °C with rabbit anti-VEGF, anti-Cas-3 and anti-Cas-9 primary antibodies (1:200 diluted concentrations, Thermo Scientific Lot:1212B, Lot: 1510C, Cat: PA5-22252). After overnight incubation, serial sections were rinsed three times with PBS solution (5 minutes for each time) and then incubated with goat anti-rabbit HRP secondary antibody (1:200 diluted concentrations, Thermo Scientific HRP kit biotinylated secondary antibody Lot: PBN1-4111E) for 2 hours at 25 °C, respectively. The serial sections were incubated for 3 minutes at 25°C with a freshly prepared 3'-diaminobenzidine (DAB) substrate chromogen solution (DA1010, Solarbio, Beijing, China) for color reaction**.** The ground staining was performed for 5 minutes with Mayer's hematoxylin solution. IHC-stained sections were examined by light microscope (Nikon Eclipse 80i Melville U.S.A.) and were blindly analyzed according to cell positivity in 100 different areas from 6 different samples.

5.8. Immunohistochemical Scoring

VEGF, Cas-3, and Cas-9 immune positivity were evaluated by H-Score analysis and were scored using a semi-quantitative scoring system for staining intensity as previously described. Firstly, positive cells were scored according to staining intensity, colorless to 0, light yellow to 1 point, brown to 2 points, and brown to 3 points (staining depth should be compared with background). The percentage of positive cells was then scored as 0–1%=0, 1–10%=1, 11–50%=2, 51–80%=3, 81–100%=4 [49].

5.9.Statistical Analysis

The IBM SPSS Statistics 25.0 (IBM Corp., Armonk, New York, USA) statistical package program was used to evaluate the data. Descriptive statistics were given as the number of units (n), percentage (%), mean±standard deviation (x±SD), and median (Q1-Q3) values. The normal distribution of the data of the numerical variables was evaluated with the Shapiro-Wilk Test of normality and Q-Q graphs. Comparisons between the groups were performed by one-way analysis of variance in normally distributed variables and by Kruskal-Wallis analysis in non-normally distributed variables. As a multiple comparison tests, the Tukey HSD test was used for normal distributed variables, and the Mann Whitney U test with Bonferroni correction was used for normal distributed variables. The p value of <0.05 was considered statistically significant.

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