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Effects of nanocurcumin on ovarian damage induced by emerging contaminants: histomorphometrical and immunohistochemical analysis

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Abstract. *Introduction*. This study aimed to investigate the effects of nanocurcumin on ovarian damage induced by chronic exposure to an environmentally relevant mixture of pharmaceuticals and pesticides.
Materials and methods. Ovarian damage was induced in 28 female rats by daily exposure to environmentally relevant mixture of pharmaceuticals and pesticides containing ibuprofen, diclofenac, naproxen, oxytetracycline, ofloxacin, albendazole, cypermethrin, thiacloprid, and trifloxystrobin via drinking water for 4 months. Animals were randomly assigned to four equal groups: control, ovarian damage, ovarian damage + nanocurcumin, and nanocurcumin alone. Nanocurcumin was administered interperitoneally at 100 mg/kg body weight/day for 15 days. We evaluated ovarian follicle populations, antioxidant status, TNF-α, AMH, and the immunohistochemical expression of p53 and Ki-67.
Results. Nanocurcumin treatment significantly increased the numbers of secondary and antral follicles, as well as AMH, SOD, and TAS levels. Meanwhile, nanocurcumin substantially reduced p53 expression, TNF-α levels, and follicular atresia. However, nanocurcumin did not affect Ki-67 expression or primordial and primary follicle populations.
Conclusion. Nanocurcumin partially attenuated ovarian damage induced by a mixture of pharmaceuticals and pesticides, as evidenced by preservation of growing follicle populations, increased AMH levels, and enhanced ovarian antioxidant status.

Keywords: nanocurcumin, environmentally relevant mixture, ovarian reserve, histology, antioxidant, apoptosis, immunohistochemistry

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Влияние нанокуркумина на поражение яичников, индуцированное загрязнителями нового типа: гистоморфометрический и иммуногистохимический анализ

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Резюме. *Введение.* Работа направлена на изучение терапевтического влияния нанокуркумина на повреждение яичников, развивающееся при хроническом воздействии смеси лекарственных препаратов и пестицидов, присутствующих в окружающей среде.

Материалы и методы. Повреждение яичников у крыс моделировали путем ежедневного поступления смеси лекарственных препаратов и пестицидов, включающей ибупрофен, диклофенак, напроксен, окситетрациклин, офлоксацин, альбендазол, циперметрин, тиаклоприд и трифлоксистробин, с питьевой водой в течение 4 месяцев. Животные были случайным образом разделены на четыре группы: контрольная, группа с повреждением яичников, группа «повреждение яичников + нанокуркумин» и группа, получавшая только нанокуркумин. Нанокуркумин вводили внутрибрюшинно в дозе 100 мг/кг массы тела в сутки в течение 15 дней. Оценивали количество овариальных фолликулов, антиоксидантный статус, а также уровни TNF-α и антимюллерова гормона, p53 и Ki67.
Результаты. Применение нанокуркумина достоверно увеличило число вторичных и антральных фолликулов, уровни антимюллерова гормона, супероксиддисмутазы и общей антиоксидантной способности. Одновременно было выявлено значительное снижение реакции на p53. Кроме того, нанокуркумин не оказывал влияния на уровень Ki67 и количество примордиальных и первичных фолликулов.
Заключение. Нанокуркумин частично ослабил повреждение яичников, вызванное смесью лекарственных препаратов и пестицидов, о чем свидетельствуют сохранение популяций растущих фолликулов, повышение уровней антимюллерова гормона и усиление антиоксидантного статуса яичников.

Ключевые слова: нанокуркумин, экологически релевантная смесь, овариальный резерв, гистология, антиоксидант, апоптоз, иммуногистохимия

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Introduction

Emerging contaminants (ECs) are compounds that are present in the environment at very low concentrations (ng/L to µg/L) and often characterized by high environmental persistence. This term includes both newly identified substances with previously unknown effects and known compounds for whose short-term exposure has raised concerns regarding human health. Among these contaminants, pesticides and pharmaceutical compounds stand out owing to their widespread use and broad range of diverse biological effects [1, 2]. As the number of precursor compounds and their transformation products continues to increase, the list of residual pollutants is steadily expanding. Moreover, the toxicity of these contaminants is often amplified when they occur as mixtures, leading to synergetic interactions that can exert more severe effects than exposure to individual compounds [3].

Curcuminoids, the primary bioactive compounds derived from turmeric (*Curcuma longa*), have attracted considerable attention because of their wide range of biological effects, including anti-inflammatory, antioxidant, and antibacterial, which may contribute to the prevention of chronic diseases [4, 5]. However, their therapeutic potential is substantially limited by poor bioavailability, stability, and solubility [6]. To address these limitations, numerous nanoformulation delivery techniques have been designed. Recent studies have demonstrated that, compared to free curcumin, nanoencapsulated curcumin (like liposomes or micelles) exhibits an improved pharmacokinetic profile, enhanced cellular uptake, prolonged circulation time, and greater stability [6].

Although nanocurcumin exhibits a wide range of biological activities, its effects on the female reproductive system remain poorly understood, especially its potential to mitigate ovarian reserve depletion induced by emerging contaminants [5, 7]. Based on our preliminary unpublished findings, chronic exposure to a mixture of nine emerging contaminants—ibuprofen, diclofenac, naproxen, ofloxacin, oxytetracycline, albendazole, thiacloprid, trifloxystrobin, and cypermethrin—disrupts ovarian function and folliculogenesis, thereby causing diminished ovarian reserve (DOR). Therefore, this research aimed to analyze the therapeutic potential of nanocurcumin in a rat model of DOR induced by pharmaceutical and pesticide mixture (PPM).

Materials and methods

The pharmaceuticals and pesticides used in this study were obtained from commercial suppliers in Batna, Algeria. The concentrations administered in the rats' drinking water samples were based on levels reported in environmental raw water samples and corresponded to estimated daily exposures of diclofenac sodium (130.96 µg/kg body weight/day), ibuprofen (25.18 µg/kg body weight/day), naproxen sodium (21.15 µg/kg body weight/day), ofloxacin

(24.71 µg/kg body weight/day), oxytetracycline (6.70 µg/kg body weight/day), and cypermethrin (17.58 µg/kg body weight/day) [8–14]. The concentrations of albendazole (50 µg/kg body weight/day), trifloxystrobin (40 µg/kg body weight/day), and thiacloprid (10 µg/kg body weight/day) were determined according to their acceptable daily intake values. A stock solution containing PPM in equal proportions was prepared weekly in drinking water. Liposomal nanocurcumin (1400 mg/3 mL; particle size: 1 nm) was purchased locally. The dose of nanocurcumin (100 mg/kg body weight/day) and the treatment regimen were selected based on findings reported in previous research [5] and the results of our pilot experiment.

Experimental design

All animal procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, 10th edition (National Research Council, 2015). The experimental protocol was approved by the Consultative Ethics Committee of the Biotechnology Research Center, Constantine, Algeria (Decision No. 27-BS-05/2024). Twenty-eight female Wistar albino rats (10 weeks old; 180–220 g) were received from the Pasteur Institute (Algiers, Algeria). Animals were housed under controlled conditions (22 ± 2°C, 12-h light/12-h dark cycle) with ad libitum access to standard rodent chow and water. After a 15-day acclimatization period, subjects were randomly assigned to four experimental groups (n=7 per group): control group, untreated rats; ovarian damage (OD) group, rats receiving PPM daily in drinking water for 4 months ad libitum; OD + nanocurcumin (NCU) group, PPM-exposed rats treated with nanocurcumin 100 mg/kg body weight/day; and NCU group, untreated rats receiving nanocurcumin (100 mg/kg body weight/day). Nanocurcumin was administered intraperitoneally during the final 15 days of the experiment. Following a one-week recovery period, all rats were anesthetized and euthanized by cervical dislocation.

Immediately after euthanasia ovaries were excised and rinsed in PBS (pH 7.3). For histological and immunohistochemical studies, the right ovaries were fixed in 5% buffered acid acetic for 24 h and then transferred to 70% ethanol for storage. The left ovaries, designed for oxidative stress assessments, were frozen at –80°C. Serum samples were kept in cryotubes and stored at –80°C.

Histopathological and immunohistochemical findings

Fixed ovaries were subjected to a routine histological processing, embedded in paraffin, and sectioned at a 5-µm thickness. Serial ovarian sections were mounted on Superfrost slides at 10-section intervals, deparaffinized, and stained with hematoxylin and eosin. We analyzed 10 sections per animal for ovarian follicle quantification. Follicle counts were calculated using a semi-automated morphometric approach based on a machine learning tool in the Trainable Weka Segmentation plugin (WEKA, version 3.7.6). Ovarian follicle populations were independently annotated by two trained pathologists who were blinded to the experimental groups.

For immunohistochemical analysis, ovarian sections were mounted on Superfrost Plus slides and incubated with primary antibodies against p53 (AB-2206626, Mouse monoclonal; Dako; dilution 1:50) and Ki-67 (AB-302459, Rabbit monoclonal [SP6]; Abcam; dilution 1:3000). Thereafter, anti-rabbit and anti-mouse antibodies (diluted at 1:200 and 1:100, respectively) were used as secondary antibodies to assess the immune complexes. Finally, sections were counterstained with hematoxylin.

The intensity score was calculated based on the method described by K. Cizkova et al. [15] using Fiji software. DAB staining was converted into pixel intensity values within selected regions of interest. Based on this, the staining intensity was categorized as follows: 0 (negative), 181–255 pixels; 1 (weak), 121–180 pixels; 2 (moderate): 61–120 pixels; and 3 (strong), 0–60 pixels. For each animal, five to eight microscopic fields from two ovarian sections were analyzed.

Biochemical findings

Anti-Müllerian hormone (AMH) levels were measured with electrochemiluminescence using commercial kits compatible with Elecsys automated analyzer (Roche, Germany). Serum tumor necrosis factor-α (TNF-α) was determined using ELISA kits according to the manufacturer's instructions. Ovarian tissue superoxide dismutase (SOD) activity and total antioxidant status (TAS) were measured with the methods described by I. Engelbrecht et al. [16] and M.N. Alam et al. [17], respectively. All results were given in proportion to the total protein content of the ovarian homogenate.

Statistical analysis

An a priori power analysis was conducted using G*Power software (version 3.1.9.7; Düsseldorf, Germany) to determine the minimum sample size required to detect differences among the experimental groups. Based on an effect size (f) of 0.75, a significance level (α) of 0.05, and a statistical power (1–β) of 0.80, a sample size of 7 rats per group was considered sufficient. All statistical analyses were performed using GraphPad Prism software (version 9.0). Data normality was assessed using the Shapiro–Wilk test. Group comparisons were performed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. A p-value <0.05 was considered statistically significant. All analyses included data from every animal in each experimental group.

Results

Histopathological findings

Histological examination revealed a significant decrease in the number of follicles at all developmental stages in the OD group compared to the control group ($p<0.01$). Treatment with nanocurcumin resulted in significant recovery of secondary and antral follicle populations (p=0.0251

and p=0.049, respectively). However, no statistical differeces were detected between OD and OD + NCU groups in primordial follicles (PF; p=0.63) and primary follicles (PrF; p=0.19). Conversely, the number of atretic follicles reduced significantly following treatment with nanocurcumin (Table 1).

Ovarian markers

Strong nuclear Ki-67 immunoreaction was observed in granulosa cells of secondary and antral follicles in all experimental groups (Fig. 1). Compared to the OD group, Ki-67 expression was significantly lower in both the control and NCU groups (p=0.00008 and p=0.0014, respectively). Figure 2 shows p53 immunoreactivity in the granulosa and theca cells of secondary and antral follicles. Compared to the OD group, a weak immune-staining in p53 was observed in both NCU and control groups (p<0.01 and p= 0.0057, respectively). Nanocurcumin treatment restored p53 expression (p<0.01), while no significant effect on Ki-67 expression was detected (p=0.26). Exposure to PPM led to a significant decrease in SOD (p<0.0001) and TAS (p=0.0007) activities. Treatment with nanocurcumin markedly restored both SOD (p<0.0001) and TAS (p=0.0037) levels (Table 2).

Serum biochemical findings

Table 3 presents the immunohistochemical scores for proliferative and apoptotic markers. In the OD group, we detected a significant decrease in serum AMH levels (p<0.0001), associated with a significant increase in TNF-α

Table 1 | Таблица 1

Effect of nanocurcumin treatment on ovarian follicle numbers in rats with ovarian damage induced by an environmentally relevant mixture (Mean ± SEM) | Влияние нанокуркумина на количество фолликулов яичника при повреждении яичников, индуцированном экологически релевантной смесью (среднее значение ± стандартная ошибка среднего)

Follicle type | Вид фолликулов	Control | Контрольная группа	OD | Группа с ПЯ	OD + NCU | ПЯ + НКУ	NCU | Группа, получавшая только НКУ
PF | ПмФ	139.9±1.96****	103.7±5.72	111.0±5.70	28.00±1.57***
PrF | ПвФ	5.67±0.53****	9.43±0.64	6.98±0.62	5.50±0.53***
SF | ВФ	37.71±1.28***	25.00±1.81	34.14±1.58**	37.86±2.26****
AF | АФ	26.57±1.34**	18.86±1.1	23.29±1.19*	28.00±1.57***
AtrF | АтФ	5.67±0.53**	9.43±0.64	6.98±0.62*	5.50±0.53***

PF – primordial follicle, PrF – primary follicle, SF – secondary follicle, AF – antral follicle, AtrF – atretic follicle. Significant difference compared to the OD group: *p<0.05, **p<0.01, ***p<0.001; ****p<0.0001 | ПмФ – примордиальный фолликул, ПвФ – первичный фолликул, ВФ – вторичный фолликул, АФ – антральный фолликул, АтФ – атретический фолликул, НКУ – нанокуркумин. Статистически значимые различия по сравнению с группой ПЯ (повреждение яичников): *p<0,05, **p<0,01, ***p<0,001, ****p<0,0001

Table 2 | Таблица 2

Effect of nanocurcumin treatment on ovarian biomarkers in rats with ovarian damage induced by an environmentally relevant mixture (Mean ± SEM) | Влияние нанокуркумина на овариальные биомаркеры при повреждении яичников, индуцированном экологически релевантной смесью (среднее значение ± стандартная ошибка среднего)

Parameter | Показатель	Control | Контрольная группа	OD | Группа с ПЯ	OD + NCU | ПЯ + НКУ	NCU | Группа, получавшая только НКУ
TAS, nmol/mg protein | ОАС, нмоль/мг белка	1.01±0.04	0.58±0.05	0.93±0.040	1.38±0.15
SOD, U/mg protein | СОД, ед/мг белка	72.15±3.14	42.22±4.70	59.15±4.19	73.81±3.93
Ki-67, IS | ИО	1.88±0.12****	1.12±0.10	1.44±0.13	1.82±0.10**
p53, IS | ИО	0.42±0.14****	1.45±0.11	0.61±0.13**	0.44±0.16***

TAS – total antioxidant status, SOD – superoxide dismutase activity, IS – immunostaining intensity score. Significant difference compared to the OD group: **p<0.01, ***p<0.001; ****p<0.0001 | ОАС – общий антиоксидантный статус, СОД – супероксиддисмутаза, ИО – оценка интенсивности иммунного окрашивания, НКУ – нанокуркумин. Статистически значимые различия по сравнению с группой с ПЯ (повреждение яичников): **p<0,01, ***p<0,001, ****p<0,0001

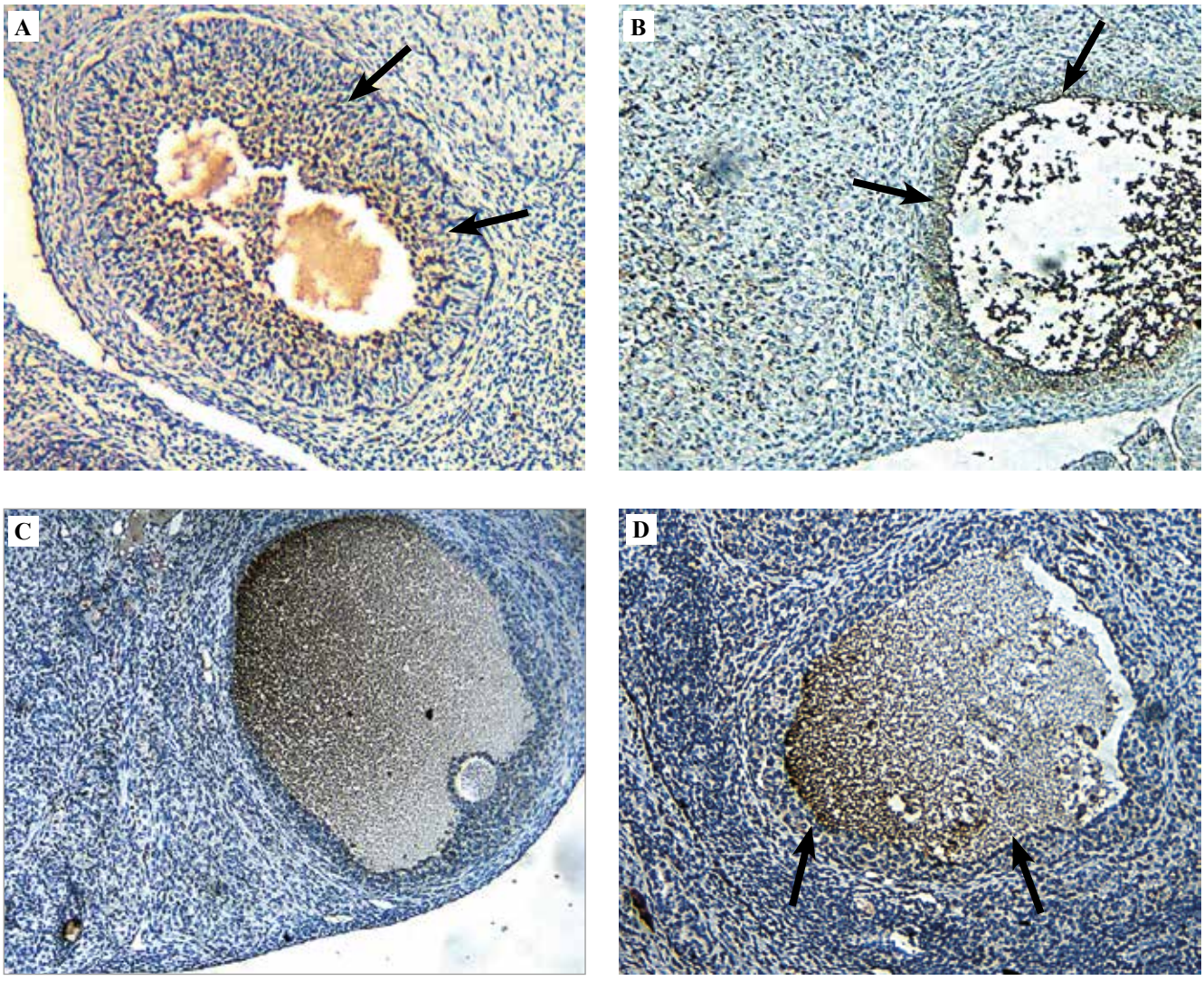


Fig. 1. Photomicrographs showing positive Ki-67 immunostaining (brown) in granulosa cells of ovarian antral follicles (*arrows*).
A – control group, B – NCU group, C – OD group, D – OD + NCU group. IHC, ×400. Scale bar: 250 μm

Рис. 1. Ki67+ (коричневое) окрашивание в гранулезных клетках антральных фолликулов яичника в экспериментальных группах (*стрелки*).
A – контрольная группа, B – группа, получавшая только нанокуркумин, C – группа с повреждением яичников, D – группа «повреждение яичников + нанокуркумин». Иммуногистохимическое окрашивание, ×400. Масштабная линейка 250 мкм

levels ($p<0.01$). Thus, nanocurcumin administration markedly preserved both AMH and TNF-α levels ($p=0.0002$ and $p=0.04$, respectively).

Discussion

This study investigated the effects of nanocurcumin on key indicators of diminished ovarian reserve (DOR) in a rat model in which it was induced by chronic exposure to a mixture of six pharmaceuticals and three pesticides. The contaminants were administered ad libitum via drinking water at environmentally relevant concentrations to stimulate realistic exposure scenarios. Rodents present a well-established experimental model for evaluating the biological effects of environmental contaminants and their implications for human health risk assessment [3]. The main findings indicate that chronic exposure to the pharmaceutical and pesticide mixture (PPM) significantly increased serum TNF-α levels, while serum AMH, healthy follicle counts, SOD, and TAS were significantly decreased. Although the mechanisms underlying PPM-induced DOR remain incompletely understood, chronic inflammation and impaired antioxidant defense of ovarian granulosa cells are likely to play key roles in affecting the ovarian reserve [3, 18–20].

To better understand the mechanisms responsible for the effects of PPM on follicular dynamics, we evaluated the expression of apoptotic marker p53 and proliferation marker Ki-67 in secondary and antral ovarian follicles. PPM exposure was associated with a significant increase in p53 marker accompanied by a decrease in Ki-67 expression.

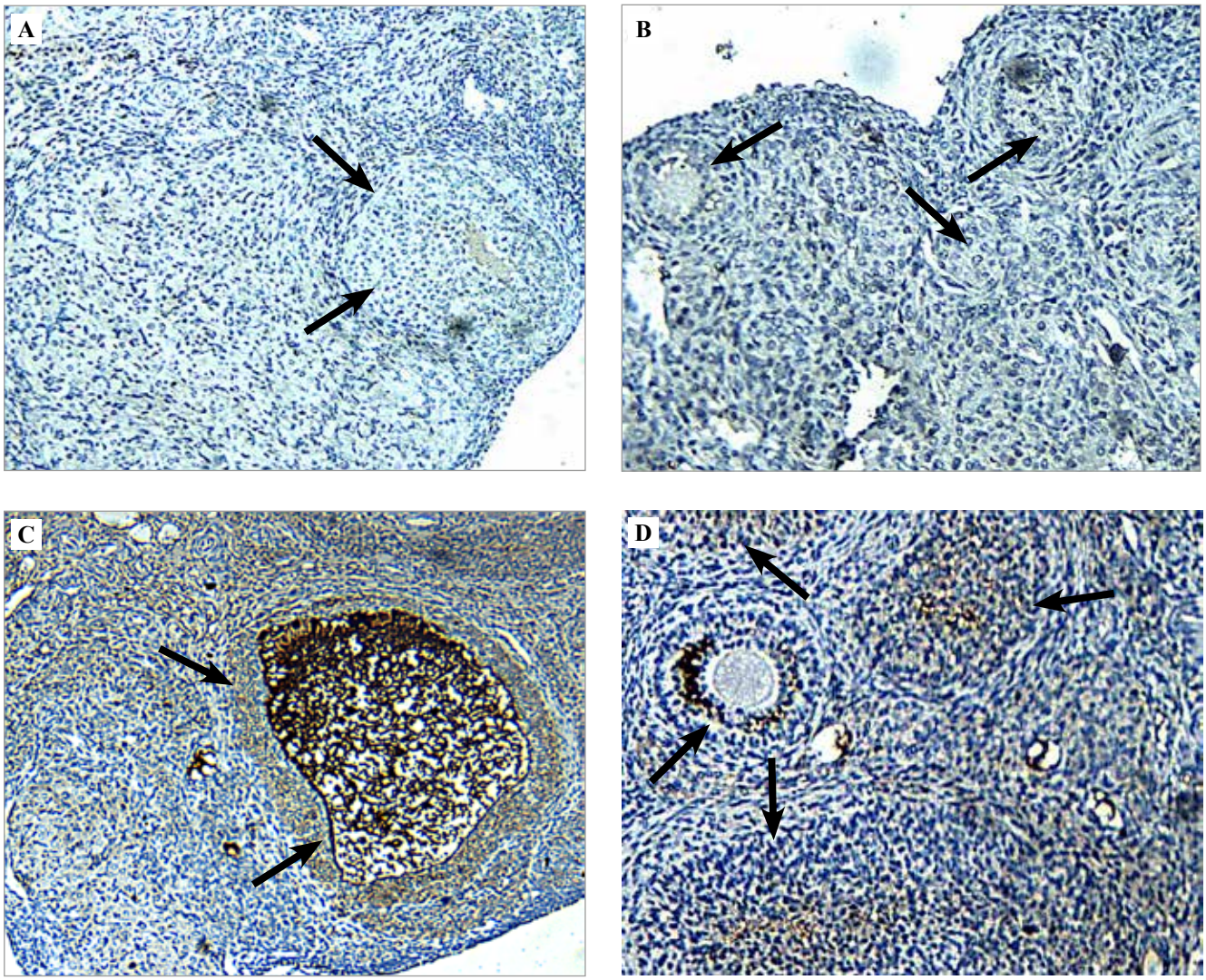


Fig. 2. Photomicrographs showing positive p53 immunostaining (brown) in granulosa and theca cells of ovarian antral follicles (*arrows*).
A – control group, B – NCU group, C – OD group, D – OD + NCU group. IHC, ×400. Scale bar: 250 μm

Рис. 2. p53+ (коричневое) окрашивание в гранулезных и тека-клетках антральных фолликулов яичника в экспериментальных группах (*стрелки*).
A – контрольная группа, B – группа, получавшая только нанокуркумин, C – группа с повреждением яичников, D – группа «повреждение яичников + нанокуркумин». Иммуногистохимическое окрашивание, ×400. Масштабная линейка 250 мкм

Table 3 | Таблица 3

Effect of nanocurcumin treatment on serum biomarkers in rats with ovarian damage induced by an environmentally relevant mixture (Mean ± SEM) | Влияние нанокуркумина на сывороточные биомаркеры при повреждении яичников, индуцированном экологически релевантной смесью (среднее значение ± стандартная ошибка среднего)

Parameter | Показатель	Control | Контрольная группа	OD | Группа с ПЯ	OD + NCU | ПЯ + НКУ	NCU | Группа, получавшая только НКУ
TNF-α, mg/dL | TNF –α, мг/дл	2.99±0.27*	3.87±0.15	3.06±0.13*	2.57±0.29***
AMH, mg/dL | АМГ, мг/дл	9.56±0.35****	5.73±0.58	8.19±0.54*	9.96±0.54****

Significant difference compared to the OD group: *p<0.05, ***p<0.001; ****p<0.0001 | АМГ (мг/дл) – антимюллеров гормон, НКУ – нанокуркумин. Статистически значимые различия по сравнению с группой ПЯ (повреждение яичников): *p<0,05, ***p<0,001, ****p<0,0001

These alterations are consistent with enhanced apoptotic activity and reduced granulosa cell proliferation, which are known to contribute to follicular atresia and depletion of the follicular pool [18, 21].

Collectively, these findings suggest that chronic exposure to PPM and other persistent organic pollutants disrupt ovarian follicular homeostasis by promoting pro-apoptotic signaling and suppressing cell proliferation [22]. Given the central role of granulosa cell proliferation in follicular growth and maturation, impaired proliferative activity may further compromise follicular development and accelerate ovarian reserve depletion [23].

These observations are consistent with previous studies demonstrating that chronic exposure to environmentally relevant mixtures containing ibuprofen, diclofenac, and 17α-ethinyl estradiol induce microscopic ovarian damage and features associated with premature ovarian insufficiency [3].

Nanocurcumin administration improved several biochemical and histological parameters, including increased TAS and SOD activity, AMH levels, and the numbers of secondary and antral follicles. These beneficial effects were accompanied by reduced p53 expression and fewer atretic follicles, suggesting a protection against PPM-induced ovarian damage.

Notably, treatment with nanocurcumin did not significantly affect Ki-67 expression that suggests that nanocurcumin did not restore granulosa cell proliferative activity under the conditions of the experiment. Therefore, the nanocurcumin therapeutic effects appear to be more strongly associated with attenuation of apoptotic processes. These findings are consistent with previous studies reporting the protective effects of nanocurcumin in experimental models of ovarian injury, including ischemia-reperfusion injury and carbon black-induced follicular depletion [5, 24]. The enhanced biological activity of nanocurcumin is generally attributed to its antioxidant and anti-inflammatory properties. Mechanistically, these effects have been linked to modulation of oxidative stress pathways, including regulation of Bax/Bcl-2 balance, inhibition of caspase activation, and upregulation of antioxidant enzymes such as CAT, SOD, and GPx [25].

Despite these improvements, nanocurcumin treatment did not significantly restore primordial and primary follicle populations. This limited responsiveness may reflect severe damage caused by PPM during the early stages of folliculogenesis and the complexity of the molecular signaling pathways in activation of primordial follicles. Indeed, although several therapeutic strategies have been proposed to enhance ovarian reserve by targeting early folliculogenesis, current evidence remains limited and inconsistent, and no approach has yet been established as clinically effective [26].

Taken together, our results highlight a partial protective effect of nanocurcumin against PPM-induced ovarian damage, primarily through reduction of oxidative stress and apoptosis. However, its impact on follicular proliferation and early-stage follicle preservation remains limited. Further studies incorporating longer treatment durations, alternative dosing strategies, and deeper molecular analyses are warranted to better define the therapeutic potential of NCU in the context of diminished ovarian reserve induced by chronic PPM exposure. A notable limitation of the present study is the absence of a vehicle control group receiving empty liposomes. This limitation should be considered when interpreting the results. Future studies incorporating an appropriate empty liposome control group are necessary to more accurately delineate the specific contribution of the active compound versus the delivery system.

Conclusion

The present study demonstrated that nanocurcumin partially mitigates ovarian damage induced by a pharmaceutical and pesticide mixture. Its protective effects were reflected by improvements in oxidative stress markers (SOD, TAS), increased AMH levels, reduced apoptotic activity, and a lower number of atretic follicles. While nanocurcumin was associated with preservation of growing follicle populations, the effects were not consistently observed across all parameters, particularly Ki-67 expression and the numbers of primordial and primary follicles. These findings provide further insight into some mechanisms underlying the detrimental effects by which environmentally relevant mixtures of pharmaceuticals and pesticides potentially contribute to diminished ovarian reserve. They also suggest that nanocurcumin may represent a promising effective strategy for mitigating pollutant-induced ovarian damage on fertility.

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Конфликт интересов. Авторы заявляют об отсутствии конфликта интересов.

Author contributions
Conceived the study and designed the experiment – S. Benbia.
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