Chemoprotective Effect of Syringic Acid on Cyclophosphamide Induced Ovarian Damage via Inflammatory Pathway

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Abstract: Cyclophosphamide (CP) is a well-known anticancer drug and commonly used against various cancers. CP therapy is related to female ovarian cancer and causes female infertility. The ovarian cancer associated with the increase oxidative stress and inflammatory reaction. Syringic acid (SA) is a very well phyto-constituent and already proof antioxidant and anti-inflammatory effects on various diseases. We investigated the chemoprotective impact of SA on CP mediated ovarian damage, and the underlying mechanism. CP (75 mg/kg) was used to cause ovarian damage and rats were randomly divided into separate groups and received a different dose of SA for 14-day. Body weight, food and water intake were determined. Ovarian weight and tumor index was measured. Antioxidant parameters were determined in the serum and ovarian tissue. Pro-inflammatory cytokines, apoptosis parameters and inflammatory mediators were estimated in the serum. Hormonal parameters and Histomorphometry were estimated. Dose dependently treatment of SA significantly ($p < 0.001$) decreased the levels of biochemical parameter such as nitric oxide (NO), myeloperoxidase (MPO) and augmented the antioxidant parameters include catalase (CAT), glutathione (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD) and reduced malondialdehyde (MDA) level in serum and ovarian tissue. SA treatment significantly ($p < 0.001$) suppressed the level of luteinizing hormones (LH), anti-mullerian hormone (AMH), estradiol (E2) and follicle-stimulating hormone (FSH) as well as ovarian follicles. SA significantly ($p < 0.001$) down-regulated cytokines, inflammatory mediator and caspase-3 parameters. Taken altogether, we conclude that SA considerably reduced ovarian damage via reduced oxidative stress and inflammatory reaction.

Key words: syringic acid, cyclophosphamide, ovarian damage, inflammation, oxidative stress

1 Introduction

Being, most common occurring anticancer agents, cyclophosphamide (CP) induce side effects on the tissue of normal body i.e. Ovary, prostate, kidney and liver. Phosphoramid mustard and acrolein are principal active CP metabolites that cause tissue oxidative stress. CP can generate free radicals and hinder endogenous antioxidant enzyme activities for example, SOD, GSH and CAT. The exogenous antioxidants are useful in dropping the toxic substances caused by oxidative stress. The exogenous antioxidants are useful in dropping the toxic substances caused by oxidative stress. On this basis, CP preconditioning has been reported to enhance the dendritic cell numbers that intended for the immune response.

CP, an alkylating agent, is generally associated with a high risk of infertility in female. It is mainly due to inducing toxicity in the ovary and is believed to be associated with high doses of CP. Depreciate of reproductive functions because of quick exhaustion of the reserve of oocyte caused via apoptotic cell death and atrophy in the ovary with the desertion of primordial resting follicles and budding of follicles in females. CP, when used for a longer period in the therapy of cancer, it interrupts the process of insemination by causing side effects on the reproductive system. It also causes apoptosis in ovarian follicle granulosa cells and kills follicles by reducing rates of ovarian glutathione (GSH). Deterioration of ovarian follicles leads to influence reproductive functions and results in lifelong insufficiency of the ovaries. Consequently, after the treatment, young women who seem fine are at risk of producing premature ovarian insufficiency. New technology and alternatives were developed to reduce the toxic effects of
chemotherapy on ovarian functions. The reproductive system contains numerous signaling molecules responsible for oocyte maturation and follicle development. Nuclear protein plays an important role in the cell cycle and is recognized as nuclear cell antigen (PCNA). In strong antral follicles, increase immunoreaction of PCNA suggests the fast development of granulosa and theca cells during follicular enlargement. During the development of follicles, a super family member such as TGF-β, GDF-9 (Growth Differentiation Factor-9) is accounted for granulosa cell growth and differentiation. During follicular growth, they are discharged from the oocytes of mammals. Literature has reported the negative impacts of CP on the tissue of the ovary and no research yet far have explored the effects of syringic acid (SA) on ovaries which was induced by drug CP and evaluating inflammatory pathway.

SA is a benzoic acid in nature, which is obtained from fruits and vegetables and utilized in the management of diabetes as a Ayurvedic Indian traditional medicine. Several studies conducted in vitro and in vivo reported its beneficial function in different cancers and non-communicable diseases. SA has several therapeutic activities for example anti-lipid peroxidative, anti-inflammatory, anti-cancer effects, anti-angiogenesis, antidiabetic, hepatic cancer, and neck cancer effects, anti-angiogenesis, antidiabetic, hepatic cancer via quenching its reactive oxygen species. Earlier research also revealed the inhibition property of SA in the development of exophytic tumor-induced HBPCs in DMBA entirely.

In recent days, the usage of phytochemicals or bioactive compounds to manage diseases in humans has been of extensive community and scientific attention. It has an efficient free radical scavenger and eases the signs of oxidative stress. At positions 3 and 5, SA’s pharmacological property is ascribed via the occurrence of methoxy groups on the aromatic ring. SA’s high antioxidant activity will bestow its valuable effects on the health of human beings. SA can modulate the function of enzymes, the dynamics of proteins and various transcription factors implicated in diabetes, inflammation, cancer and angiogenesis. Experimental evidence and histological studies during in vivo have illustrated the potential underlying mechanisms.

2 Materials and Methods

2.1 Chemical

CP and SA were purchased from Sigma (St. Louis MO, USA). Follicle-stimulating hormone (cat log no.-SL0297Ra), estradiol (cat log no.-SL0268Ra), anti-mullerian hormone (cat log no.-SL0504Ra) and luteinizing hormones (cat log no.-SL1093Ra) were purchased from Sunlong Biotech Co., Ltd., Zhejiang, China. All the chemical and reagent used in the experimental study were analytical grade.

2.2 Animals

In the current experimental protocol, Swiss albino Wistar rats weight 150-180 g sex female used. The rats were collected from the animal laboratory and kept in the single cage under standard experimental conditions (21 ± 3°C temperature and 60% – 70% relative humidity) with maintain the light and dark for 12 h cycle. The rats were free from food and water. The current experimental study was conducted according to the institutional animal protocol. The rats were acclimation for 10 days before the experimental study.

2.3 Preparation of toxicant

CP was used for the induction of ovarian damage. Single intraperitoneal injection of CP (75 mg/kg) was dissolved in the saline and rats were treated once a week for next 3 weeks.

2.4 Experimental protocol

After successfully acclimation of animal (10 days), the rats were grouped as follow and each group contains the 6 rats. Group I: control (receive only saline in the whole experimental period); Group II: received CP (75 mg/kg); Group III: received CP (75 mg/kg) and SA (5 mg/kg); Group IV: received CP (75 mg/kg) and SA (10 mg/kg) and Group V: received CP (75 mg/kg) and SA (20 mg/kg), respectively.

After 14 weeks, the rats were anesthetized using the thiopental sodium and blood samples were withdrawn by puncturing the retro-orbital and centrifuged at 10,000 rpm for 20 min and separate the plasma and serum and stored at the 80°C for analysis of biochemical parameters. After that the rats were scardified using the cervical dislocation and immediately removed the ovarian tissue and processed for further analysis.

2.5 Biochemical assays

For the biochemical assay, ovarian tissue was homogenized using the Teflon glass homogenizer in KCl (150 mM) at a dilution (1:10 w/v). The homogenates were centrifuged for 30 min at 18 k rpm at 4°C.

Catalase (CAT), glutathione (GSH), superoxide dismutase (SOD) and malondialdehyde (MDA) and glutathione peroxidase (GPx) were estimated using ELISA kits.

2.6 Hormonal assays

Hormonal assays such as follicle-stimulating hormone (FSH), estradiol (E2), anti-mullerian hormone (AMH) and luteinizing hormone (LH) were estimated in the serum of experimental rats using enzyme-linked immunosorbent assay (ELISA) kits (Sunlong Biotech Co., Ltd., Zhejiang,
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2.7 Pro-inflammatory cytokines
Cytokines include tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β) were estimated using the ELISA kits (Sunlong Biotech Co., Ltd., Zhejiang, China) using the manufacture instruction.

2.8 Inflammatory and caspase parameters
Inflammatory mediators such as cyclooxygenase-2 (COX-2), prostaglandin E₂ (PGE₂) and caspase parameters such as caspase-3 were estimated using the ELISA kits (Sunlong Biotech Co., Ltd., Zhejiang, China) using the manufacture instruction.

2.9 Histomorphometry
Female rats have a reproductive strategy, which allows ovulating and conceiving within 4–5 days. During the estrous cycle, a cohort of resting primordial follicles starts to expand into primary follicles and this process occurs until the formation of early tertiary follicles. A small number of tertiary follicles enter a preovulatory stage and converted into the Graafian follicle. Following the extrusion of the secondary oocyte from the Graafian follicle, the granulosa and thecal cells of the follicle remnant undergo hypertrophy and hyperplasia. This process is called luteinization that occurs under the influence of luteinising hormone and prolactin. Luteinization results in mature corpus luteum.

2.10 Statistical analysis
For the statistical analysis, GraphPad Prism 5 (USA) was used. Data showed as mean ± standard deviation (SD). All biochemical data were scrutinized using the one way analysis of variance (ANOVA) and Dennett’s test was used. A significant value presented as $p<0.001$.

Fig. 1 The effect of SA on the ovary weight of CP induced ovarian damage in rats. All the data presented ± SEM. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

Fig. 2 The effect of SA on the number of follicles of CP induced ovarian damage in rats. a: Primordial Follicle, b: Follicle, c: Atretic Follicle and d: Corpus Luteum. All the data presented ± SEM. *$p<0.05$; **$p<0.01$; ***$p<0.001$. Where MDA = Malonaldehyde, GSH = Glutathione, SOD = Superoxide dismutase, GPx = Glutathione peroxidase and CAT = Calatase.
3 Result

3.1 Ovarium weight

All rats groups ovary weight are illustrated in Fig. 1. CP-induced group rats showed decreased ovary weight and SA treatment significantly ($p<0.001$) increased the ovary weight as dose dependent manner.

3.2 Follicular population

In Histomorphometrically study, no significant difference was observed among the groups regarding mean no. Of follicle count of normal and atretic size. The results of population of follicles are presented in Fig. 2. No statistically significant difference in the number of primordial or other follicles ($p>0.001$) was found between the rat group. All the groups reported statistically significant differences ($p<0.001$) in the values of corpus lutum and atretic follicles. Corpus lutum and atretic follicles achieved maximum levels in the CP group, whereas the minimum number of corpus lutum and atretic follicles was present in the SA lower dose group.

3.3 Biochemical parameters

Figure 3 illustrates the oxidant profile such as MDA and antioxidant enzymatic profile such as SOD, CAT, GPx, and GSH. CP induced ovarian damage shows up-regulation in the MDA levels and down-regulation in the GSH levels and SOD activity compared to the control group. There was a significant reduction observed in level of MDA and elevation in the level of GSH and SOD activity in the ovarian tissue of the animals treated with SA in dose dependent manner compared to negative controls.

3.4 Hormonal assays

Assessment of the activity of SA on the ovarian reserve markers are displayed in Fig. 4. Significantly reduction was observed in the levels of Serum FSH and LH in SA treated rats in diseases with ovarian damage in a dose dependent way. Although, the level of E2 and AMH in rats treated with SA were remarkably raised contrast with CP induced ovarian damage group. Not more significance difference was observed in the level of serum hormone of the rats group received CP + SA (5 mg/kg bw) and CP + SA (10 mg/kg bw) (Fig. 4).

3.5 Effect on caspase-3 activity

Caspase-3's most strong immunoreactivity was measured by semi-quantitative assay in rats treated with CP when contrast with the other groups. The administration of dif-
3.6 Effects of SA on ovarian NF-κB, TNF-α, IL-1β and IL-6 levels
The levels of NF-κB, TNF-α, IL-1β and IL-6 levels in the ovary of the CP-treated group were significantly elevated as evaluated by the control group. We observed a substantial difference between the CP-treated group and control group in the levels of NF-κB, TNF-α, IL-1β and IL-6. Nevertheless, these levels were significantly modulated by SA treatment (5, 10 and 20 mg/kg) compared with the CP group. Not too much significance difference between the control group rats and in the SA treated group rats (20 mg/kg bw) of ovarian pro-inflammatory cytokine levels NF-κB, TNF-α, IL-1β and IL-6 (Fig. 6).

3.7 Effects of ZO on ovarian and uterine iNOS, PGE₂ and COX-2 activities
In contrast to the control group (p < 0.001), iNOS, PGE₂ and COX-2 behaviors in ovarian damage were observed to be considerably bigger in the CP-treated group. Although, compared to the CP group, treatment with SA (5, 10 and 20 and 50 mg/kg) significantly reduced iNOS, PGE₂ and COX-2 activities in dose dependent manner (Fig. 7).

3.8 Effect of SA on Bcl-2 level
Compared to the control group (p < 0.001), the Bcl-2 level in ovary was significantly decreased in the CP-treated group. Significant elevation was observed in levels of ovarian Bcl-2 after the administration of SA in CP induced ovary damage group (Fig. 8).

4 Discussion
This research examined the potential protecting effect of SA in female rats against ovarian damage induced by CP. Anticancer agents such as CP, cisplatin, methotrexate, are employed in the management of various types of cancer like lung cancer, cancer of testes, ovarian cancer, which is metastatic in nature and in other solid tumors. Con-

![Fig. 4](image)
Fig. 4 The effect of SA on hormonal parameters of CP induced ovarian damage in rats. a: estradiol, b: FSH, c: LH and d: AMH. All the data presented ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001. Where FSH = Follicle stimulating hormone, AMH = Anti-mullerian hormone; LH = Luteinizing hormone.

![Fig. 5](image)
Fig. 5 The effect of SA on caspase-3 activity of CP induced ovarian damage in rats. All the data presented ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001.
Fig. 6  The effect of SA on cytokines and inflammatory mediator of CP induced ovarian damage in rats. a: TNF-α, b: IL-6, c: IL-1β and d: NF-kB. All the data presented ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001. Where TNF-α = Tumor necrosis factor-α, IL-6 = Interleukin-6, IL-1β = Interleukin-1β and NF-kB = Nuclear Kappa B factor.

Fig. 7  The effect of SA on inflammatory mediator of CP induced ovarian damage in rats. a: iNOS, b: COX-2 and c: PGE2. All the data presented ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001. Where iNOS = inducible nitric oxide synthase, COX-2 = Cyclooxygenase-2 and PGE2 = Prostaglandin E2.

Fig. 8  The effect of SA on Bcl-2 activity of CP induced ovarian damage in rats. All the data presented ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001. Where Bcl-2 = B-cell lymphoma-2.
versely, some of these substances lead to cause dysfunction in ovary in females and gonadotoxic and develop an imbalance of hormonal production, injury, cause a infertility, which may be temporary or permanent \(^{24,25}\). Ovarian cancer induced by CP is scrutinized by calculated levels of hormones (FSH, E2, inhibin B and AMH) in plasma \(^{9,26}\).

Cytokines are proteins of low molecular weight mediating the communication between cells. They also control the proliferation, differentiation, cell survival, activation of immune cells, migration of cell and cell apoptosis. NF-κB is most significant transcription factor considered to be oxidative stress prone \(^{7,11}\). This pathway is well known therapeutic objective as it plays a vital role in activating and regulating the genes of transcription factors for example, TNF-α, IL-1β, IL-6, COX-2 and iNOS. Hence, NF-κB inhibition can be helpful in reducing ovarian damage.

Furthermore, tumourigenicity inhibition has been observed in ovarian cancer cells in humans after blocking activity of NF-κB \(^{27}\). iNOS is an enzyme utilized in the formation of nitric oxide (NO) inside the body \(^{28}\). Sulfuriont level of NO reacts to oxidize and nitrate macromolecules such as GSH, proteins, lipids and DNA with a superoxide anion to form the radical peroxynitrite producing damage to cell \(^{29,30}\). Furthermore, intracellular GSH is consumed by extreme NO, thus increased the oxidative stress sensitivity \(^{31}\). In several studies, the critical role of NF-κB and NO in showing the adverse reaction of therapy with CP is well mentioned in literature \(^{31,32}\). Enhanced levels of pro-inflammatory cytokines for example, NF-κB, TNF-α, IL-1β and IL-6, and COX-2 and iNOS activities were found in ovarian cancer induced by CP compared to the control group is recorded in this study. In contrast to the CP-induced group, treatment with SA observed to substantially declined certain values.

The protein family Bcl-2 was also studied for its function in apoptosis \(^{33}\). The family contains anti-apoptotic members (e.g. Bcl-2, Bcl-XL, and Mcl-1) and pro-apoptotic members (e.g., Bax, Bak, Bid, and Bad) \(^{32,33}\). The anti-apoptotic Bcl-2 regulates several primary apoptosis steps involving the ion channel formation mediating cytochrome c release, homeostasis of mitochondria and membrane potential of mitochondria \(^{32}\). This study demonstrated thoughtful effects of CP on caspase-3 and Bcl-2. However, CP increases the expression of Caspase-3 and declined the level of Bcl-2. SA prevents the tissue by raising the Bcl-2 level and reducing the Caspase-3 expression proven the antioxidant properties.

Among all cell lines, SA hindered dose-dependent cell proliferation. The induction of cell apoptosis induction may lead to cell cycle attestation G1/S or G2/M. Yet it is unclear whether other mechanisms exist to explain the induction of apoptosis by SA. In an earlier study, Liu et al. suggested the of MLS-2384 anti-survival effect on different variety of cancer cells such as ovarian cancer \(^{39}\). Yu et al. reported (2Z, 3E)-6-bromoindirubin-3′-oxime inhibition effect on ovarian cancer cell lines regarding proliferation, migration and cell invasion. We performed experiments on 2 separate lineages of ovarian cancer cells in this research. Our findings indicated that SA drastically repressed the viability cell and initiated apoptosis in cancer cells of the ovaries. Mustard phosphoramide lead to apoptosis by cross-linked DNA and acrolein, extremely reactive molecules provoking toxicity and interrupt the function of normal cells. More production of free radicals with DNA binding stimulates caspase-3 signals and facilitates cell death subsequently. This study revealed the presence less quantity of caspase-3 in the SA and CP group as compared CP induced ovarian damage group.

The outcomes of this study reveal the potential of SA strengthened markers of ovarian reserve after CP induced an insufficiency of ovaries. A significant elevation in AMH and E2 levels and substantial decline in levels of FSH and LH were observed in groups treated with different doses of SA as comparative to CP induced ovarian damage in rats. Various research paper has examined the impacts of ovarian harm and antioxidants caused by chemotherapy on markers of the ovarian reserve \(^{9}\). Özcan et al. reported the impact of resveratrol on oxidative damage caused by cisplatin to the ovarian reserve markers in animals. They observed that treatment with resveratrol up-regulated the amount of AMH considerably compared with the control group.

In this study, oxidative stress profile, MDA was observed to be raised and SOD activity was significantly reduced in the CP-induced rat ovary, indicating that administration of CP produces oxidative destruction the ovary component i.e. lipids and proteins \(^{9,34}\). The levels of MDA and SOD activity were reversed after the administration of SA in dose dependent manner and reflecting the protective effects of SA on the side effects of CP. SA declines the generation of peroxynitrite, which is a strong oxidant, formed during the reaction between nitric oxide with superoxide anion by quenching the activity of SOD with superoxide anion. Levels of Catalase were also improved by different doses of SA and it may be estimation of nonselective level of enzyme catalase that utilize hydrogen peroxide. This chemical is not only detached by catalase but some other antioxidant enzymes for example GPx, GSTs are also involved in it \(^{9,34,35}\). According to best knowledge, on the basis of biochemical assays, CP, produce a lipid peroxidation and antioxidant component in the ovary of the rats but administration of SA alleviates the lipid peroxidation induced by a CP and high level of SOD in the ovary of rats, which evidenced the preventive effects of SA in ovarian damage.
5 Conclusion

Collectively, we can say that the SA exhibited the anti-cancer effect against CP induced ovarian damage. SA significantly reduced the antioxidant and biochemical parameters at dose dependent manner. SA significantly reduced the LH, LH, FSH and E2 parameters and ovarian follicles. SA considerably reduced the caspase-3 parameters. We can say that the SA potential pharmacological agents to treat the ovarian cancer.

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