

Atrazine Induces the Cytotoxicity of Ovarian Granulosa Cells via Inhibition of Nrf2-Mediated Defense Response

Xiao-Wei Li

Northeast Agricultural University

Xue-Nan Li

Northeast Agricultural University

Milton Talukder

Northeast Agricultural University

Ying-Xin Zhao

Northeast Agricultural University

Jia-Gen Cui

Northeast Agricultural University

Jin-Long Li (✉ Jinlongli@neau.edu.cn)

Northeast Agricultural University <https://orcid.org/0000-0002-5133-9165>

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Abstract

Atrazine (ATR) is a commercial herbicide, which is widely used worldwide. Diaminochlorotriazine (DACT) is the main metabolite of ATR, which poses potential risks to ecosystem and health. However, the mechanism of ATR and DACT on ovarian granulocyte cells (GCs) is still not clear. To investigate the toxic effect of ATR and DACT in quail ovarian GCs. We established primary ovarian GCs as models. GCs were cultured with ATR (20, 100, and 250 μM) and DACT (20, 100 and 200 μM). Which showed normal morphology and uniform size in GCs of the control group, ovarian GCs are specifically identified, in which cell purity was above 90%. The half-inhibitory concentrations (IC_{50}) of ATR and DACT were 261.20 μM and 214.17 μM , respectively. High doses of ATR and DACT caused changes in the ultrastructure of mitochondria, leading to oxidative stress and changes in apoptosis-related indicators, ATR and DACT activated Nrf2-mediated defense system-related factors. Our research showed that ATR and DACT are cytotoxic to GCs. ATR induced oxidative stress in ovarian GCs and activated Nrf2-mediated antioxidant signaling pathway to alleviate the toxicity of ATR. These results suggested useful evidence to investigate the female reproductive toxicity of ATR and DACT.

Introduction

Atrazine (ATR) was first marketed as an herbicide in 1957 and is widely used in agriculture due to its low price, stable chemical structure and low chemical reactivity. It can accumulate in underground and surface water after long-term application (Ritter, 1990, Solomon, et al. 2013). The influence of ATR on biofilm phospholipids would unbalance the metabolic process of cells and accelerate the bioaccumulation of ATR (Katagi, 2010), which will cause severe harm to human and animal life safety. Diaminochlorotriazine (DACT) (77%) was the main metabolite of ATR exposure (Chevrier, et al. 2014). ATR and DACT were frequently detected in surface water (Komsky-Elbaz, et al. 2019), indicating that ATR and its metabolite DACT pose a very serious threat to ecological environment. In vivo and in vitro experiments, ATR could be used as a hormone stimulator that interferes with the reproductive function of female animals (Breckenridge, et al. 2018, Abarikwu., et al. 2011, Aziz., et al. 2018). ATR could cause the abnormal development of quail ovaries and fallopian tubes (Qin, et al. 2015). When ATR was added to the diet of female SD rats, the rats would show prolonged estrous cycle, early breast cysts and the formation of breast and pituitary tumors (Gryngarten, et al. 2010). ATR promoted the cell proliferation of human intestinal epithelial cells (Greenman, et al. 1997), inhibited the growth of normal human fibroblasts (Powell, et al. 2011). Previous research has investigated the effect of atrazine on lymphocytotoxicity in mice (Chen, et al. 2015). Previous research results published in the Journal of Environmental Pollution showed that exposure to different concentrations of ATR interfered with the hormone regulation of the hypothalamus-pituitary-ovarian axis, caused hormone secretion disorders, led to a gradual reduction in the number and size of ovarian follicles and suppression of oviduct development, ultimately led to abnormal development of quail ovaries and fallopian tubes. Based on the previous study phenomenon, this study further explored the direct effect of ATR on quail ovary granulosa cells (Qin, et al. 2015).

Oxidative damage is mainly caused by increased reactive oxygen species production (ROS). ATR and DACT induced oxidative stress and interfered with endocrine function (Forgacs, et al. 2013, Jin, et al. 2014). Oxidative stress is one of the major toxic mechanisms of ATR in cells, When the balance between ROS production and antioxidant defense is disrupted, it would cause oxidative stress and ultimately lead to cell damage. Cells protect against free radical damage by increasing the binding of glutathione (GSH) and ATR to help detoxify cells. Antioxidant enzymes and small antioxidant molecules can be used as the main antioxidant components to eliminate excessive production of reactive oxygen free radicals. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) are well-known antioxidant enzymes. SOD is mainly responsible for removal of superoxide anion free radicals, whereas CAT is mainly responsible for the decomposition of H_2O_2 , and GSH-PX involves in H_2O_2 and lipid peroxide removal process (Valavanidis, et al. 2006). Studies have shown that ATR treatment-induced oxidative stress and disrupted the antioxidant balance in the quail cerebrum (Lin, et al. 2018). Nrf2 is a transcription factor induced by cell-protective proteins and can be scavenging free radical damage caused by oxidative stress in cells (Huang, et al. 2021). Nrf2 dissociated from the Keap1-Nrf2 complex and transferred to the nucleus, eventually triggering the expression of antioxidant enzymes (Sahin, et al. 2013). A previous study has demonstrated that Nrf2-mediated defense response was activated to attenuate ATR-induced oxidative damage in ovarian tissue (Forgacs, et al. 2013). Activation of the Nrf2/ARE pathway is a pivotal cellular response involved in ROS protection (Lin, et al. 2018). Although toxicity studies of ATR have become an in-depth research area, the mechanism by which ATR induces oxidative stress and then initiates Nrf2 signaling to resist cytotoxicity and adverse cell outcomes are still no clear.

Oxidative stress-induced programmed cell death, such as apoptosis, which regulated cell numbers and removed noxious and potentially dangerous cells as a defense mechanism (Yang, et al. 2019). A characteristic of ATR-induced cellular injury is the generation of oxidative stress (Jin, et al. 2010). In many cases, cells undergo apoptosis when they underwent severe oxidative stress (Kamli, et al. 2021). Certain toxic chemicals induced apoptosis through the involvement of intracellular signaling molecules and transmembrane receptors, which eventually activated caspase-3 (Chu, et al. 2018, Song, et al. 2017). As we all know, the destruction of mitochondrial structures may activate apoptosis through the mitochondrial pathway, which is related to the alteration of gene expression (Bcl-2, Bax, Caspase-3) (Wang, et al. 2018, Liu, et al. 2018). Exogenous signaling pathways are mainly mediated by Fas/FasL, and the receptor protein binds with its ligand FasL to form a death inducing signaling complex, which initiates the caspase-mediated apoptosis pathway (Lettau, et al. 2008).

GCs are the main functional cells of the ovary. Proliferation and differentiation of GCs directly affect the growth initiation, development, ovulation, luteinization and steroid hormone secretion of follicles and other ovarian functions (Zheng, et al. 2017). This study investigated the oxidative damage of ovarian GCs caused by ATR and its metabolite DACT, and the mechanism of apoptosis caused by activation of the Nrf2 pathway against cytotoxicity. This study provides new evidence for ATR-induced reproductive toxicity in females.

In this study, we established the primary quail ovary granulosa cell model and investigated the oxidative damage of ATR and its metabolite DACT on ovarian GCs. A high dose of ATR and DACT induced apoptosis, and Nrf2 antioxidant signaling pathway was activated by oxidative stress produced by ATR to alleviate the cytotoxicity. This study provides new evidence for the reproductive toxicity of ATR in females.

Materials And Methods

Identification of primary ovarian granulosa cell by H & E staining and immunofluorescence

The culture of GCs in the quail ovary is presented in supplementary materials (Supplementary 1). All procedures were in accordance with the Institutional Animal Care and Use Committee of Northeast Agricultural University. The steps of HE staining were as follows: first, fixed with 4% paraformaldehyde for 20 min, then hematoxylin staining for 5 min, eosin staining for 6 min, ethanol gradient dehydration, and finally observed under the fluorescence microscope. Specific expression steps of ovarian GCs, after 48h of cell culture, cells were washed with PBS for 3 times, fixed with paraformaldehyde for 30 min, again washed with PBS for 3 times, permeated with Triton for 10 min, washed for 3 times, and treated at 4 °C. The follicle stimulating hormone receptor antibody FSHR (1:250) was incubated overnight, the secondary antibody (1:3000), DAPI was stained, and the specific binding status was observed under a fluorescence microscope (Motic Company).

Determination of half inhibitory concentration and cellular activity

To determine the half-inhibitory concentration (IC_{50}) of ATR and DACT on quail ovarian GCs, different concentrations at 10, 20, 50, 100, 250, 500, 750, 1000 μ M of ATR and DACT were added into the culture medium of ovarian GCs respectively, after 24 h of culture, OD value measured by CCK-8 method. The GCs of ovary were inoculated in 96-well plates, medium with concentrations of 20 (A20), 100 (A100), 250 (A250) ATR and 20 (D20), 100 (D100), and 200 (D200) DACT were added, and culture was continued for 24 h, CCK-8 operating steps and cell activity calculation was performed according to the instructions (Shanghai Dong ren Chemical Technology Co, Ltd.).

Ultrastructural assessment

A large number of GCs were digested with trypsin from Petri dishes. The sample is processed according to the previous method (Baumgärtner and Krakowka. **1988**) and GCs were observed by transmission electron microscopy (TEM, H-7650, Hitachi Limited, Tokyo, Japan) (n = 5). The damage criteria of cell mitochondria were based on the previous scoring criteria (Schiering, et al. **2017**).

Measurement of oxidative stress

Oxidative stress-related factors, including total antioxidant capacity (T-AOC), total superoxide dismutase (T-SOD), (CAT), (GSH), (GSH-Px) activities, and malondialdehyde (MDA), GSH and ROS were detected

according to the manufacturer's instructions. Determination of ROS in cells was performed by chemical fluorescence method (Nanjing Jian Cheng Institute of Biological Engineering).

Flow cytometry

Ovarian GCs were digested with trypsin and collected by centrifugation at 1000 rpm/min for 5 min. Next, ovarian GCs were washed twice with PBS. $1-10 \times 10^5$ cells/mL were collected and the specific operation steps were based on the previous operation method (Cui, et al. 2021). Indicators of ovarian GCs apoptosis were then analyzed using Flowjo 7.6 software.

RNA extraction and real-time PCR analysis

Total RNA was isolated from ovarian GCs with RNAout reagent (Beijing Tian gen, P.R. China) The mRNA levels of Nrf2 and apoptosis-related factors were analyzed by qRT-PCR according to the previous method (Wei, et al. 2018, Su, et al. 2019). The related gene primers were designed with Oligo 7 software and shown in supplementary materials (Table S1). qRT-PCR was performed by LineGene 9600 (Bioer Technology Co. Ltd). To calculate the mRNA relative expression, the mRNA relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method and normalized to the mean of β -actin1 and 2.

Nrf2 immunofluorescence analysis

The cells were fixed with 4% paraformaldehyde for 30 minutes, 0.1% Triton™X-100 penetrating fixation for 10 min, sealed with 5% skimmed milk for 1 h and incubated overnight with primary anti-Nrf2 (1:2000) (Quail Nrf2 Polyclonal Antibody in Li jin long Laboratory). Petri dishes were washed with PBS 3 times and incubated with Alexa Fluor 488 (1:2000) for 1 h. Finally, Antifade Mounting Medium with DAPI was added and observed under a fluorescence microscope. The DAPI stain (blue) and the secondary Alexa Fluor 488 (red). (Antifade Mounting Medium with DAPI).

Statistical analysis

Our results were statistically analyzed using GraphPad Prism 5.1, Origin 2018, SPSS 22.0 software. All results were applied to analyze the effect of cytotoxicity under ATR and DACT exposure in ovarian GCs. The data were expressed as the mean \pm standard deviation (SD). *P* value of < 0.05 was considered significant.

Results

Identification and activity effect of ovarian GCs

As shown in Fig. 1A and B, H & E staining of ovarian GCs showed complete morphology, uniform in size, sharp edges, which were fusiform or polygonal. The nucleus and cytoplasm stained more evenly, the nucleus was blue in the center of the cell, and the cytoplasm of the cell was reddish and contained many particles. The nucleus was stained with DAPI showed blue fluorescence (Fig. 1C). FSHR was expressed in

the nucleus and cytoplasm of ovarian GCs, showing green fluorescence (Fig. 1D). The visible expression rate under the microscope was > 90% (Fig. 1E). Which proved that the isolated cells were ovarian GCs (Fig. 1F and G). The half inhibitory concentrations of ATR and DACT were 261.20 μ M and 214.17 μ M, respectively and the toxic effect of DACT was 1.2 times that of ATR. The effect of ATR and DACT on the viability of ovarian GCs is shown (Fig. 1H). Compared with the control group, the survival rate of GCs in A20, D20 and A100 groups was not significantly changed, while the ovarian granulosa cell viability of A250, D100 and D200 groups showed significant decrease ($P < 0.001$). It indicated that the primary quail ovarian GCs model was successfully obtained. Meanwhile, the activity of GCs decreased after ATR and DACT treatment, showing a dose-dependent effect.

Ultrastructure observation of ovarian GCs

The control group had intact cell structure, regular shape and clear cell boundary, nearly round nucleus, and abundant mitochondria (ovate mitochondria, with clear cristae body structure). However, A100 and D20 groups showed significant nuclear migration and morphological shrinkage ($P < 0.05$, $P < 0.01$). In A250, D100 and D200 groups, the number of mitochondria decreased, the mitochondrial crest fracture, and the mitochondrial morphology disappeared ($P < 0.001$). The number of mitochondrial lesions in individual GCs was quantified in Fig 2B. These features indicated that atrazine caused damage to the mitochondria.

Expression of ATR and DACT in intracellular ROS

The detection of ROS content in GCs of ovaries by ATR and DACT are shown (Fig 3A). Treatment with ATR and DACT markedly increased ROS levels as compared to control, with the increase of ATR and DACT dose, the green fluorescence intensity of the DCFH-DA probe inserted into quail ovarian GCs increased, and the stronger the fluorescence intensity, the greater the toxicity ($P < 0.001$). The fluorescence intensity is shown in Fig 3B. These data suggested that ATR and DACT induced oxidative stress in quail ovarian GCs.

Expression of ATR and DACT in oxidative stress

The cytotoxicity induced by ATR and DACT was directly related to oxidative damage. To assess the effects of ATR and DACT on cell redox homeostasis, several antioxidant molecules and enzymes were detected. The GSH level was visibly increased in A250, D100 and D200 groups ($P < 0.001$) (Fig. 4A). The MDA expression level of cells in the D20 group was significantly increased ($P < 0.05$) and the highest increased ($P < 0.001$) were observed in the A100, A250, D100 and D200 groups (Fig. 4B). We observed that ATR and DACT exposure markedly decreased the level of GSH-Px, CAT, T-AOC and T-SOD ($P < 0.05$, $P < 0.01$ and $P < 0.001$) (Fig. 4C – F). GSH-Px was markedly decreased in the A250 and D200 groups ($P < 0.01$ and $P < 0.001$) (Fig. 4C). CAT was remarkably decreased in the D100 ($P < 0.05$), A100 ($P < 0.01$) and A250, D200 groups ($P < 0.001$) (Fig. 4D). T-AOC was down-regulated in all treatment groups ($P < 0.001$) (Fig. 4E). T-SOD was markedly decreased in A250 and D200 groups ($P < 0.001$) (Fig. 4F). These results indicated that ATR and DACT exposure resulted in the imbalance of redox steady state.

Effects of ATR and DACT on apoptosis markers

We further used flow cytometry to detect the proportion of early and late apoptosis of ovarian GCs induced by ATR and DACT (Fig. 5A). Compared with the control group, the apoptosis rate of GCs treated with ATR and DACT increased significantly in all treatment groups ($P < 0.001$) (Fig 5B). The effects of ATR and DACT on the apoptosis rate of quail ovarian GCs were dose-dependent.

The apoptosis-related markers (Bcl-2, p53, Bax, Caspase-3, Fas, FasL) were determined and presented in Fig. 5C. The expression of Bcl-2 gene showed significantly downward trend in the A250 and D200 groups ($P < 0.001$). The expression of p53 was increased in the A250 group ($P < 0.05$) and highest significantly increased in the D200 group ($P < 0.001$). Bax was significantly decreased in A250, D100, D200 groups ($P < 0.001$). The mRNA expression level of Caspase-3 was markedly decreased in D100, A250 and D200 groups ($P < 0.01$ and $P < 0.001$). Fas were significantly increased in D200 ($P < 0.05$). The expression of FasL was significantly up-regulated in A250 ($P < 0.05$) and D200 groups ($P < 0.01$), but no significant difference in A20, A100, D20 and D100 groups. Overall trends in apoptosis-related factors are shown in Fig. 5D. ATR and DACT show dose-dependent effects and lead to apoptosis.

Expression of ATR and DACT in Nrf2 signaling pathway

The Nrf2-related markers (Nrf2, SOD3, SOD2, GCLM, HO-1, GCLC, NQO-1, and GST) were determined and shown in Fig 6A, B. We observed that ATR and DACT exposure significantly increased all genes of Nrf2-related pathways. Nrf2 and GCLM were showed significant upward trend in the D200 group ($P < 0.001$). SOD3, SOD2, NQO-1 and GST were showed significant upward trend in the A250 ($P < 0.01$) and D200 groups ($P < 0.001$). HO-1 was showed significantly upward trend in the D100 and D200 groups ($P < 0.001$). GCLC was showed markedly upward trend in the A250 and D200 groups ($P < 0.01$ and $P < 0.001$). The heat map (Fig. 6C) showed the variation trend of Nrf2 pathway-related factors (Fig. 6C). Our data indicated that the Nrf2 pathway was activated to resist cytotoxicity.

Discussion

Groundwater and drinking water supplies are contaminated by several chemicals, including herbicides such as atrazine and its degradants (Barbash, et al. 2001). However, the reproductive effects of atrazine in birds are still largely unknown. Quail is considered to be a better environmental pollution model. Compared with other experimental animals, quail is small in size and has strong adaptability to the environment, so it is suitable as a poultry experimental animal for laboratory research (Kayang, et al. 2002). The ovaries are important reproductive organs of female animals. As an estrogen disruptor, ATR mainly damages the reproductive organs. The DACT, can cross the placental barrier and have been detected in newborn rat pups born to orally exposed dams (Lin, et al. 2013). Therefore, the effect of ATR on the function of GCs in quail ovaries was investigated in this study. Our results showed that ATR and DACT caused oxidative stress in ovarian GCs, resulting in series of changes in cells, prompting the body to activate Nrf2 defense pathway and its downstream related gene expression, and initiating the antioxidant process to resist cytotoxicity.

In this study, we established the primary quail ovary granulosa cell model for the first time and investigated the oxidative damage of ATR and its metabolite DACT on ovarian GCs. A high dose of ATR and DACT induced apoptosis, and Nrf2 antioxidant signaling pathway was activated by oxidative stress produced by ATR to alleviate the cytotoxicity. This study provides new evidence for the reproductive toxicity of ATR in females.

Studies have shown that ATR and other herbicides could promote ROS production (Fatima, et al. 2007). When ROS production exceeds the body's antioxidant capacity, it will affect the normal function of cells and may lead to oxidative damage of proteins, inactivation of enzymes, lipid peroxidation (LPO) and DNA strand break (Dolai, et al. 2011). a number of previous studies have indicated that oxidative stress was the main mechanism in ATR-induced cytotoxicity (Abarikwu and Farombi. **2015**, Figueira, et al. 2017, Gao, et al. 2016, Toughan, et al. 2018). It is ATR that induced excessive overaccumulation of MDA and GSH and inhibited the activities of T-AOC, T-SOD and GSH-Px in ovarian GCs. After treatment with ATR for 16 days, ATR induced down-regulation in GSH and GST activity in rat testis (Abarikwu. **2014**). Bhatti et al. found that the ATR treatment group had significantly increased MDA levels, decreased GSH content, SOD, GSH-PX, and GST. The enzyme activities were significantly higher than the control group (Bhatti, et al. 2011). Exposure to ATR or DACT resulted in delayed puberty in offspring of female rats, and DACT was shown to be consistent with ATR in delaying puberty (Laws, et al. 2003). ROS produced by ATR metabolism can affect the antioxidant defense system, which caused oxidative damage to all cellular components (Monteiro, et al. 2006), It is ATR that directly affected the antioxidant reaction of GCs. Our results are consistent with previous studies. These results indicated that ATR and DACT could promote the production of ROS and lipid peroxides in cells, inhibit the activity of antioxidant enzymes, reduce the total antioxidant capacity, and then go down oxidative stress in quail ovarian GCs.

The Nrf2 signaling pathway is considered to be an important cellular defense mechanism against toxicant-induced oxidative stress (Zhang, et al. 2015). Under normal circumstances, Nrf2 keeps in a quiescent state by interaction with cytoplasmic Keap1. When cells are attacked by ROS or other exogenous toxins, the Nrf2-keap1 complex is destroyed, and Nrf2 is subsequently transported to the nucleus and activated antioxidant transcription (Zou, et al. 2018). Meanwhile, Nrf2 started the transcription of corresponding downstream antioxidant molecules. In this study, we further investigated the effect of Nrf2 signaling pathway by ATR exposure through measuring Nrf2 gene and immunofluorescence expression as well as downstream related regulatory genes of Nrf2 expression. Our results confirmed the positive correlation between Nrf2 and its downstream genes, and the crosstalk between ATR-induced ovarian granulosa cell damage and Nrf2-mediated antioxidant defense.

However, excessive accumulation of lipid peroxides can trigger cell apoptosis (Kensler, et al. 2007). It is Apoptosis that is thought to be a primary process of the majority of physiological responses (Flemming, et al. 2018). There are currently three known apoptotic pathways, they are the perforin/granzyme pathway, the endogenous pathway and the exogenous pathway (Morgan, et al. **2017**). The extrinsic signaling pathway is mainly controlled by Fas/FasL. The endogenous signaling pathways are regulated by the intracellular Bcl-2 family on the mitochondria to initiate apoptosis (Flemming, et al. 2018).

Previous research has proven that ATR could cause apoptosis by dose-dependent up-regulation of Bax and down-regulation of Bcl-2 in rat ventral midbrain (Song, et al. 2015). Caspase-3 is the most well-known apoptotic agent in both endogenous and exogenous apoptotic pathways (Juraver-Geslin and Durand. 2015). When Caspase-3 activity was inhibited, apoptosis was blocked. Furthermore, studies on the toxicity of ATR on mature porcine oocytes revealed an increase in Bax expression in embryos with poor morphology (Yuan, et al. 2017). The crosstalk between endogenous and exogenous apoptotic pathways may have synergistic effects on cell death to aggravate the toxic effect of ATR in GCs of ovary. Therefore, we hypothesized that high-dose ATR and DACT induce oxidative damage to ovarian granulosa cells and regulate the expression of apoptosis-related genes.

Conclusions

In conclusion, we found that exposure to ATR and DACT produced cytotoxins, impaired mitochondrial structure, caused mitochondrial damage, and induced oxidative stress, and excessive accumulation of lipid peroxides induced apoptosis. The Nrf2-Keap1 defense pathway is involved in redox metabolism of cells, and it activated downstream related genes to resist oxidative activation of ovarian granulocytes, thus produced antioxidant effect and alleviated cytotoxic damage. These results are consistent with the results of ATR on quail ovary and fallopian tube development abnormalities *in vivo*, and fully prove the reproductive toxicity of atrazine to female poultry.

Declarations

Supplementary Information

Supplementary Information related to this article can be found online.

Author contribution

J.L: Conceptualization, Methodology, Software X.L1: Data curation, Writing - Original draft preparation. X.L2: Visualization, Investigation. T.M: Supervision. Y.Z: Software, Validation. G.C: Writing - Reviewing and Editing. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Figures

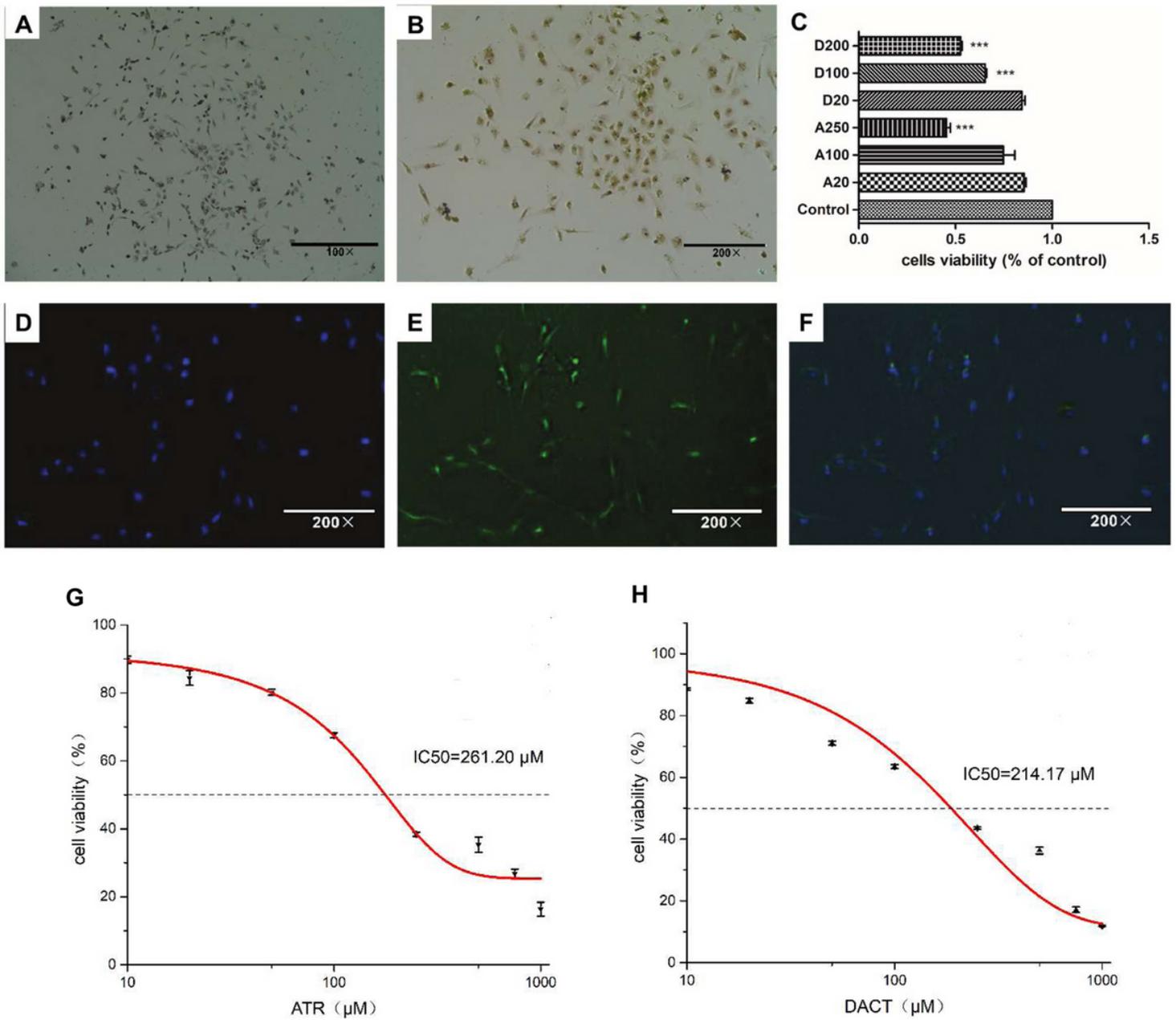


Figure 1

HE staining and identification of ovarian GCs The results of H&E staining in quail ovarian GCs (A: 100 x; B: 200 x); (C) DAPI staining; (D) FSHR staining; (E) Merge of C and D (n = 3); (F and G) the half inhibitory concentrations of ATR and DACT (n = 6) respectively; (H) Cell viability

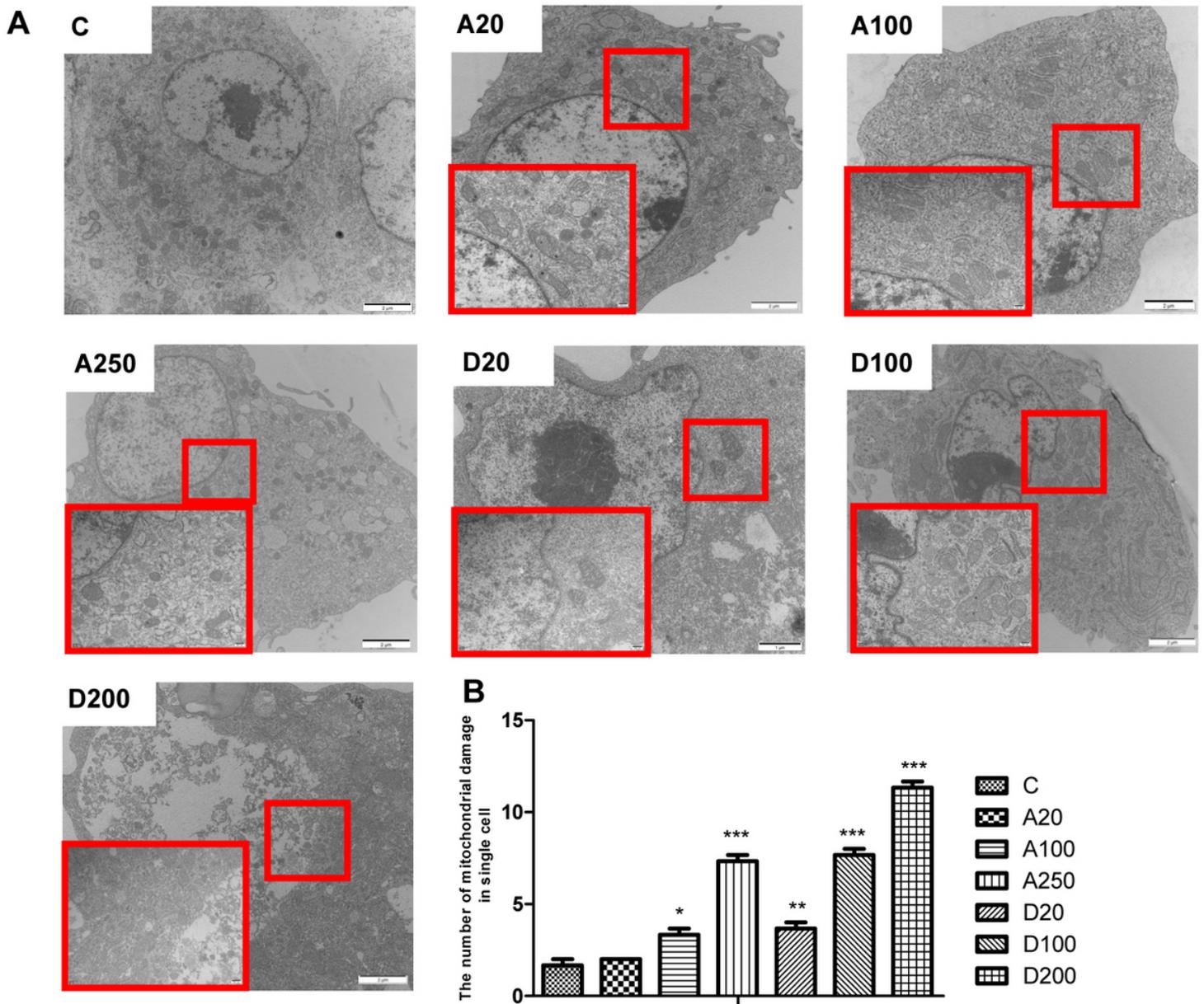


Figure 2

Effects of ATR and DACT on ultrastructure of ovarian GCs (A) Ultrastructural in ovarian granulocyte. The magnification of the microscopic images is 20000 times the original size (n = 3); the red pane represents the location of part of the mitochondrial lesion. (B) a number of damage of mitochondria in a single cell. Data were shown as mean \pm SD. *P < 0.05, **P < 0.01, and ***P < 0.001 vs control

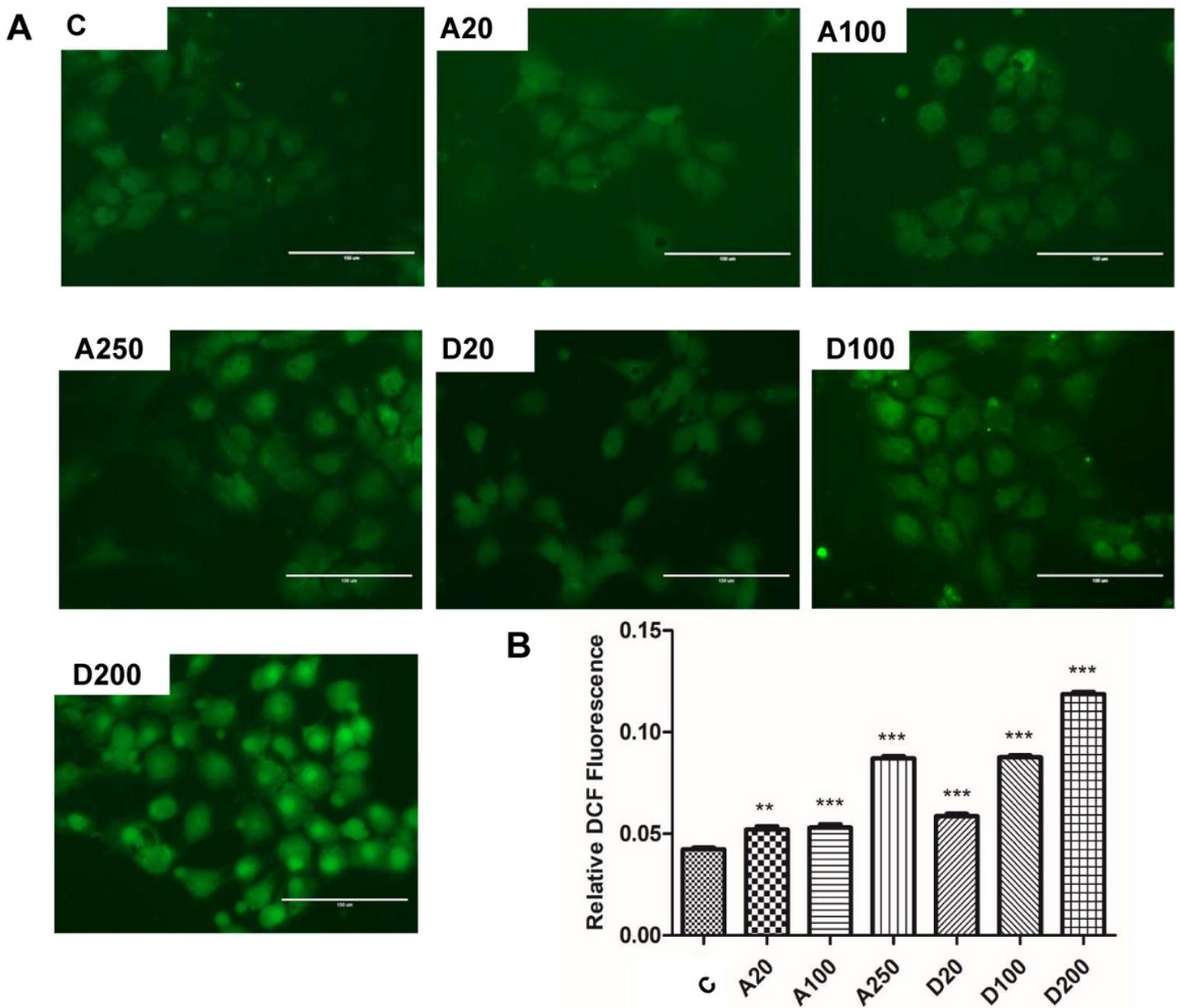


Figure 3

Effects of ATR and DACT on intracellular ROS content Under a fluorescence microscope, (A) the expression of reactive oxygen species (ROS) at different concentrations of ATR and DACT (n = 3). The magnification of the microscopic images is 200-times the original size. (B) ROS expression in different groups. ROS were showed as mean \pm SD. *P < 0.05, **P < 0.01, and ***P < 0.001 vs control

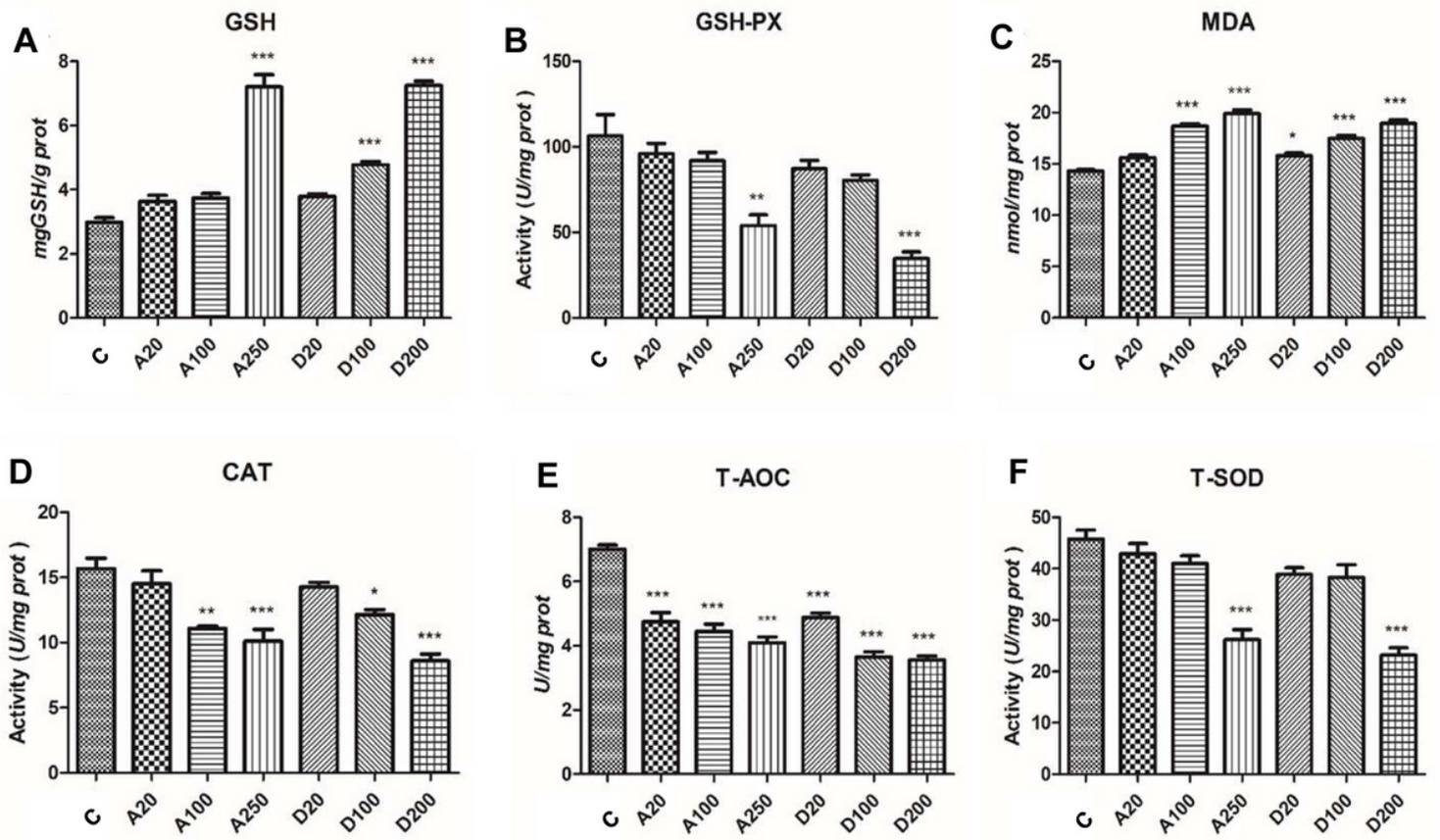


Figure 4

Effects of ATR and DACT exposure on oxidative stress in quail ovarian GCs (A) GSH expression level; (B) GSH-PX expression level; (C) MDA expression level; (D) CAT expression level; (E) T-AOC expression level; (F) T-SOD expression level (n = 6). Gene expression results were showed as mean \pm SD. *P < 0.05, **P < 0.01, and ***P < 0.001 vs control

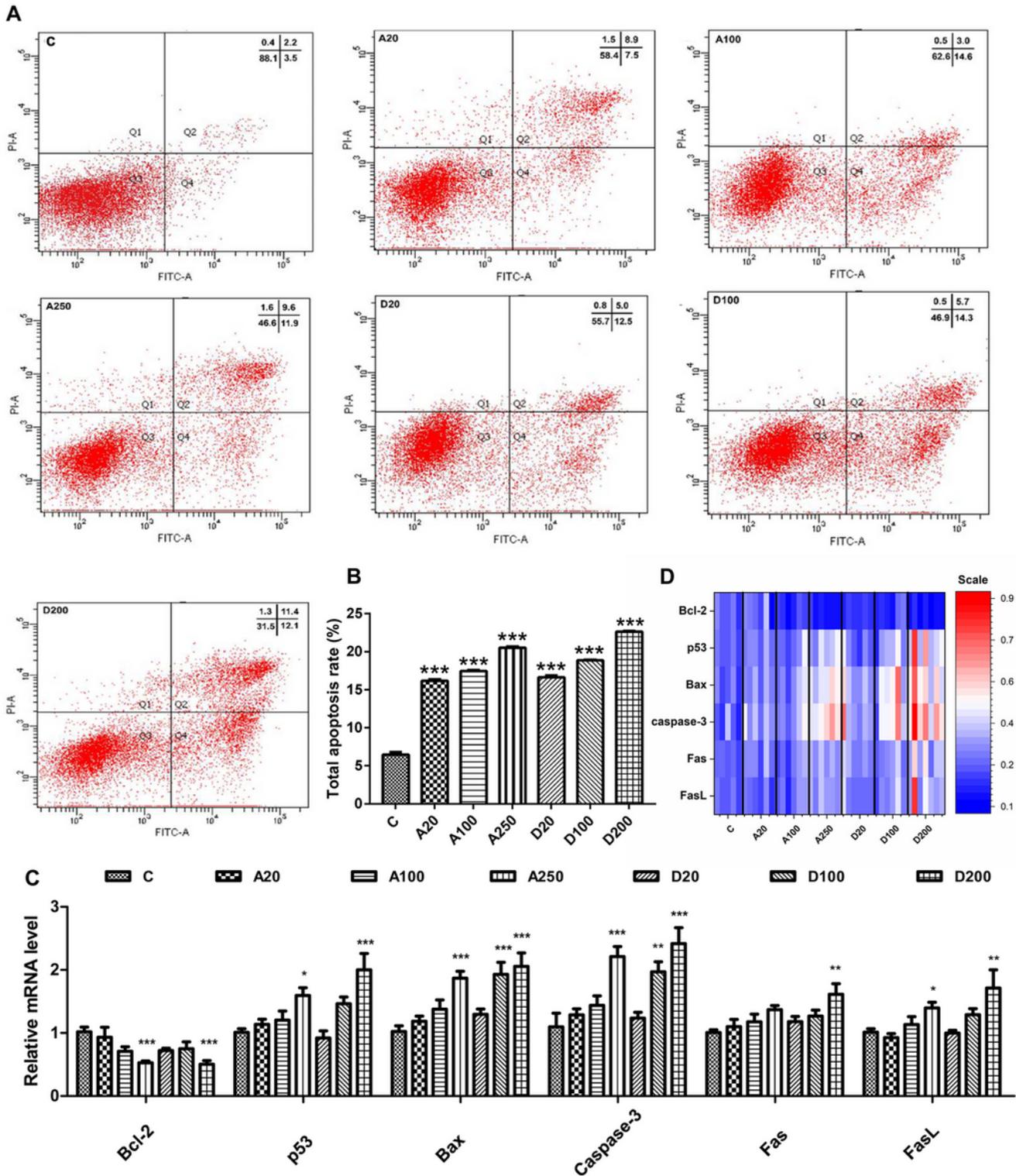


Figure 5

Effects of ATR and DACT on apoptosis related factors (A) Effect of ATR and DACT on quail ovarian GCs apoptosis by flow cytometry analysis (n = 3) (Q1: necrotic cells and cell fragments, Q2: early apoptotic cells, Q3: normal cells, Q4: late apoptotic cells); (B) Represents the expression trend of Apoptosis; (C) Represents the expression level of Apoptosis related factors; (D) Heat map of expression levels of apoptosis genes, RNA gene were detected using the indicated pseudo color scale from blue (0.1) to red

(0.9) relative to values. the different colored squares demonstrate mRNA levels, with blue showing unchanged genes, white and red showing the level of genes significantly increased. Results of apoptotic gene expression were shown as the mean \pm SD. Compared with control: *P < 0.05, **P < 0.01, and ***P < 0.001

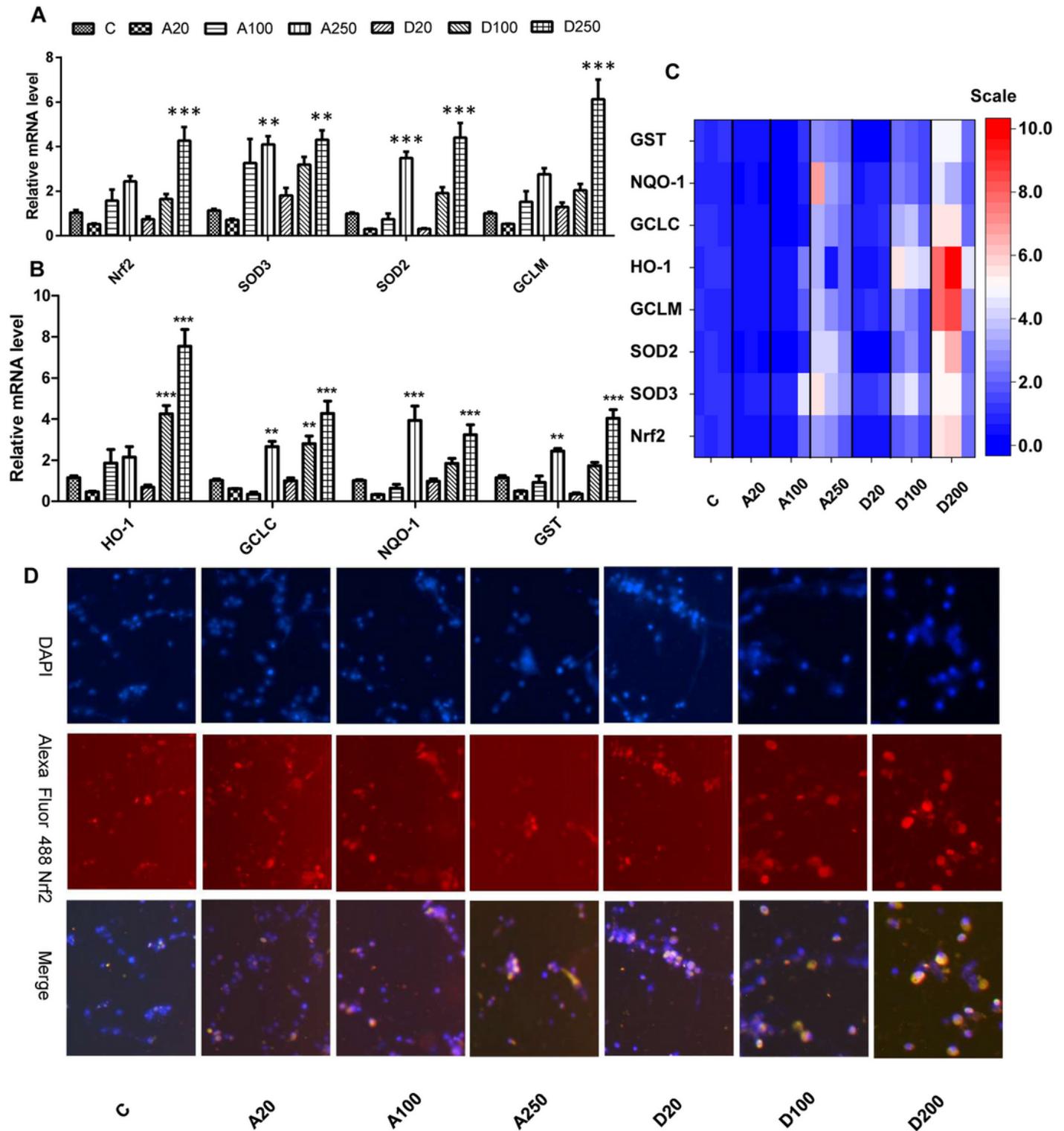


Figure 6

Effects of ATR and DACT on the Nrf2 signaling pathway (A and B) represents the levels of downstream related genes in Nrf2; (C) The thermal map of the downstream gene of Nrf2 relative to mRNA level. Changes in related genes are represented by indicative pseudo color scales from blue (0) to red (10) relative to values, the different colored squares demonstrate relative gene levels, with blue showing unchanged genes, white and red showing increased gene levels; (D) Nrf2 immunofluorescence, the nuclei were detected by DAPI staining (blue), Alexa Fluor 488 (red), the Merge represents the synthesis of DAPI and Alexa Fluor 488. Values are shown as the mean \pm SD. The symbols for the significant of differences between the control and another group are represented as *P < 0.05, **P < 0.01, and ***P < 0.001

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