Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;50:2060-2070

DOI: 10.1159/000495051
Published online: 10 November 2018
Accepted: 2 November 2018

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Original Paper

Niacin Inhibits Apoptosis and Rescues Premature Ovarian Failure

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Key Words
Niacin • Premature ovarian failure • Follicle growth • Apoptosis

Abstract
Background/Aims: Over 99% of mouse and human ovarian follicles will undergo specialized cell death including atresia and apoptosis. Reduction of apoptosis may help reduce infertility and maintain the reproductive ability in women. ***Methods:*** 3-day B6D2F1 mice were used to culture small follicle and ovary tissue with niacin and 18-day mice were intraperitoneal injected with niacin to determine its effect on follicle development**.** Then establish 8-weeks POF animal model with cytoxan (CTX) or radiation. Treatment group was given 0.1 mL of 100 mM niacin by an intraperitoneal injection twice before ovulation. The ovaries were collected and the follicles were counted and categorized, and ovarian histologic sections were stained for TUNEL. Ovarian function was then evaluated by monitoring ovulation. Microarray analyses, Western blot, immunofluorescence and real-time quantitative PCR were used to assess the mechanism of ovarian injury and repair. ***Results:*** We found that niacin promotes follicle growth in the immature oocyte and it increased the levels of a germ-line cell marker DDX4, and a cell proliferation marker PCNA in the ovary. Addition of niacin to the cell culture reduced oocyte apoptosis *in vitro*. Administration of niacin to treat premature ovarian failure (POF) in mouse models showed inhibition of follicular apoptosis under harmful conditions, such as radiation and chemotherapy damage, by markedly reducing cumulus cell apoptosis. Additionally, the number of developing follicles increased after administration of niacin. ***Conclusion:*** Niacin may have an important function in treating POF by reducing apoptosis in clinical applications.

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Introduction

In humans, premature ovarian failure (POF) is a disease in which mature oocytes do not develop and can affect life quality in some women. It is classically defined as 4-6 months of amenorrhea in women under age 40 and is associated with menopausal levels of serum gonadotropins (FSH > 40 IU/L) and hypoestrogenism [1, 2]. POF prevents women from being able to conceive and may also be associated with other medical problems, such as blood clots, osteoporosis and heart disease. POF has many causes, including autoimmune disease, chemotherapy and some gene mutations [3]. POF diseases may also be caused by over activated apoptosis or no healthy follicle development during ovulation [4], [5]. Studies have shown that stem cell transplantation or ovary cryopreservation and transplantation can rescue some POF [6-8].

In women, most oocytes will die before ovulation. The ovary exhibits profound cell loss shortly after birth [9]. Preserved primordial follicles are dormant for subsequent decades. After reaching sexual maturity, most germ cells are lost to an atretic pathway and are not selected to grow or ovulate [10]. Women usually have 400-500 mature oocytes throughout their life, but they have millions of oocytes before birth. More than 99% of mouse ovarian germ cells are lost through atresia, and few reach ovulation too [11]. In some chemotherapy or radiation therapies, ovarian cell apoptosis or death are more severe due to DNA damage [12, 13].

In addition to atresia and apoptosis, ovarian function is also controlled by the balance of follicle arrest and primordial follicle activation (PFA), which is a metered process by which primordial follicles are selected from the reserve pool into the growing follicle pool. Many genes are involved in initiating primordial follicle growth and balancing apoptosis. Typically, the PTEN/AKT pathway and FOXO3 control dormant follicle activation [14]. Evidence indicates that a PTEN/PIK3/PDK1/AKT signaling pathway deficiency can affect primordial follicle activation [15, 16]. Kit signaling via PI3K promotes ovarian follicle maturation, but is dispensable for primordial follicle activation [17]. This general model was supported by the discovery of the fork head transcription factor, FOXO3, as a key regulator of PFA within oocytes [18]. The balance of follicle arrest or growth, death or ovulation is critical for ovary function.

Niacin is a vitamin that is changed to NAD+ by the enzyme niacin adenylyltransferase and participates in metabolizing cellular energy, directly impacting normal physiology. Niacin is reported to protect against apoptosis in neurons under oxidative stress [19, 20]. For decades, niacin has been a common drug for treating pellagra disease, which is associated with diarrhea, dermatitis, dementia and death due to niacin deficiency [21]. Niacin can be changed into nicotinamide *in vivo*, which inhibits sirt1 and parp1, and can regulate cellular acetylation and ribosylation [22].

In this study, we found that the niacin inhibited ovarian cell apoptosis and promoted follicle growth. It also cured POF caused by chemotherapy and radiation therapy.

Materials and Methods

Experimental animals

3-day-old, 18-day-old and eight-week-old female B6D2F1 (C57BL/63DBA/2) mice were used in experiments. Mice were purchased from Vital River Laboratories (Beijing, China). All animal procedures were conducted in accordance with the Animal Research: Reporting *in vivo* Experiments (ARRIVE) guidelines for reporting animal studies. Ethical approval was obtained from the Ethics Committee of Military Medical Sciences (Beijing, China) for this study.

Small follicle culture in vitro

Small follicles were collected from 3 day B6D2F1 female mice. The bilateral ovaries were removed under aseptic conditions. They were washed in PBS three times under a stereo microscope, and a 1-ml syringe needle punctured the follicular granulosa cells to release oocytes. Small follicles with oocytes were cultured in Waymouth medium supplemented with 10% fetal bovine serum and 0.23 mM pyruvic acid at 37°C under 5% CO_2 with or without niacin. Granulosa cells we cultured in DMEM with 10% Fetal Bovine Serum (FBS).

Niacin treatment in vivo

We treated 18-day postnatal female B6D2F1 mice with 0.05 mL of 100 mM niacin by an intraperitoneal injection. Forty-eight hours later, female mice were superovulated by an intraperitoneal injection of 2.5 IU of pregnant mare serum gonadotropin. Next, we collected and detected the ovaries after 48 hours.

Short-term in vitro ovary tissue incubation

Small follicles were collected from 3 day B6D2F1 female mice. Paired ovaries from B6D2F1 mice were excised and washed three times in PBS. Ovaries were cultured in waymouth medium supplemented with 0.23 mM pyruvic acid, 50 mg/L of streptomycin sulfate, 75 mg/L of penicillin G., 10% FBS, and 0.03 IU/mL FSH (NV Organon). Media (400 μL) were placed below the membrane insert to cover ovaries with a thin layer. In the apoptosis assay, cultured without FBS.

POF Animal model establishment

To establish the POF model of chemotherapy-induced ovarian damage, adult female B6D2F1 mice were administered CTX (50 mg/kg) for 14 days. They were then randomly divided into two groups, and one group was given 0.1 mL of 100 mM niacin by an intraperitoneal injection twice before ovulation and then given 5 IU of pregnant mare serum gonadotropin(PMSG). Forty-eight hours later, the ovaries were collected. The chemotherapy POF group (CTX group) only received 5 IU of PMSG before being sacrificed. The same aged wild-type controls were only given PMSG, without CTX and niacin.

Eight-week-old mice were exposed to 5 Gy of radiation to make the radiation POF model. The niacin group was administered 0.1 mL of 100 mM niacin by an intraperitoneal injection for two days and then given 5 IU of PMSG on the third day. Negative-control radiation and normal mice were only given PMSG on the third day.

Apoptosis TUNEL assay

An in situ Cell Death Detection Kit, POD (Roche, Germany), was used to detect DNA fragmentation in the mouse ovaries as per the manufacturer's instructions. Next, nuclei were dyed with Hoechst 33324, and the sections were observed using fluorescence microscopy (Olympus, Japan). Granulosa cells showing DNA fragmentation in the ovary were stained green, and five random fields from each sample were counted [33].

Drug treatment and H&E staining of the ovaries

The ovaries were collected after treatment, and the follicles were detected and classified. The ovaries were removed and fixed in 4% paraformaldehyde for at least 24 hours. After fixation, the ovaries were dehydrated, paraffin-embedded, serially sectioned at 5 μm, and mounted on glass microscope slides. Routine hematoxylin and eosin (H&E) staining was performed for histologic examination with a light microscope. PCNA(Cell Signaling Technology, Cat # 13110).

Western blot and Immunofluorescence

To investigate the protein expression level in ovary from different treatments, proteins from the ovary were collected in SDS sample buffer and boiled at 100°C for 5 min. After cooling on ice and centrifuging at 12, 000 g for 5 min, the samples were stored at -80°C until use. Total proteins were separated by SDS-PAGE and electrophoretically transferred to the polyvinylidene fluoride (PVDF) membrane at 4°C. The transferred PVDF membrane was blocked in TBST buffer containing 5% nonfat milk at 4°C overnight. The blocked PVDF membrane was incubated with primary antibody overnight at 4°C. After three washes with TBST (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05% Tween-20), the membrane was incubated with second antibody for 2 h at room temperature. The membrane was extensively washed with TBST three times and processed

with the ECL detection system (Amersham). Anti-FOXO3A antibody (Cell Signaling, #2497), Anti-sirt1 (Invitrogen, Cat # PA5-17232), Anti-acetyl Lysine antibody (Abcam, ab80178). Anti-DDX4 / MVH antibody (Abcam, ab13840). β-tubulin antibody (Invitrogen, Cat # PA5-30380). Goat Anti-Rabbit IgG H&L (HRP) (Abcam, ab6721).

Immunofluorescent staining, confocal microscopy and adobe Photoshop analysis. In brief, the samples were permeabilized with 0.5% triton X-100 and blocked with 5% normal horse serum for 2 h. The primary antibodies were incubated overnight at 4°C. The secondary antibodies were incubated for 1 h at room temperature. Samples were further counterstained with 100 ng/ml of DAPI. Images were obtained with an Olympus IX 71 microscope (Olympus, Japan) equipped with a CCD camera (DVC) or LSM510 Meta confocal microscope (Zeiss). The staining intensity was analyzed by adobe Photoshop and histogram signal intensity. All primary antibodies against epigenetic markers were used at the recommended concentration. Anti-FOXO3A antibody (Cell Signaling, #2497), Anti-DDX4 antibody (Abcam, ab13840). Goat anti-Rabbit (Invitrogen, Cat # A27040).

RNA Microarray analyses

RNA was isolated from the mouse ovaries using Trizol reagent (Invitrogen) by standard methods. Labeling and hybridization were performed at the CapitalBio Company as per the protocols described in the 32 K mouse genome arrays user manual. The data were analyzed using LuxScan 3.0 image analysis software (CapitalBio Company, China).

Real-time PCR

Real-time quantitative PCR reactions were performed in triplicate using the SYBR Green Real-time PCR Master Mix (Applied Biosystems, US) and run on a Bio-Rad CFX96 (US). The PCR primers were designed based on cDNA sequences in the NCBI database. All of the gene expression levels were normalized to the internal standard gene, Gapdh. For expression analysis, data from three replicates were analyzed by using the $2^{-\Delta\Delta Ct}$ method.

Statistical analyses

Statistical analyses were performed using SPSS 14. Student's t-tests were used to determine the significance between two groups. The means ± SD of the data were calculated. One-way analysis of variance (ANOVA) with least significant difference (LSD) tests were used to determine significant differences between three groups. The means ± SEM of the data were calculated. A P value < 0.05 was considered statistically significant.

Results

Niacin promotes primary follicle development both in vitro and in vivo.

We collected the small ovarian follicle from 3-day postnatal female B6D2F1 mice and cultured single small follicles to determine the drug's effect on follicle development. We found that after 48 hours' treatment with 10 mM niacin, the shell outside the oocyte became fluffy, the granulosa cells became multilayered, and the follicle grew (Fig. 1A). To determine whether niacin promotes granulosa cells growth or only causes granulosa cells to disconnect from each other, we tested the effect of niacin on cultured granulosa cells alone. Granulosa cells were plated and cultured for 2 days either with 5 mM or without niacin supplementation. Niacin-treated cumulus cells maintained their proliferation ability for longer time than the control group.

Next, we detected whether niacin promoted follicle growth *in vivo*. We treated 18-day postnatal female B6D2F1 mice with 0.05 mL of 100 mM niacin by an intraperitoneal injection. Forty-eight hours later, female mice were superovulated by an intraperitoneal injection of 2.5 IU of pregnant mare serum gonadotropin. Next, we collected and detected the ovaries after 48 hours. We found that more small follicles grew in the niacin group (151.67±14.72, n=6) than in the control group (91.67±28.40 ,n=6) (*p=0.002) (Fig. 1B).

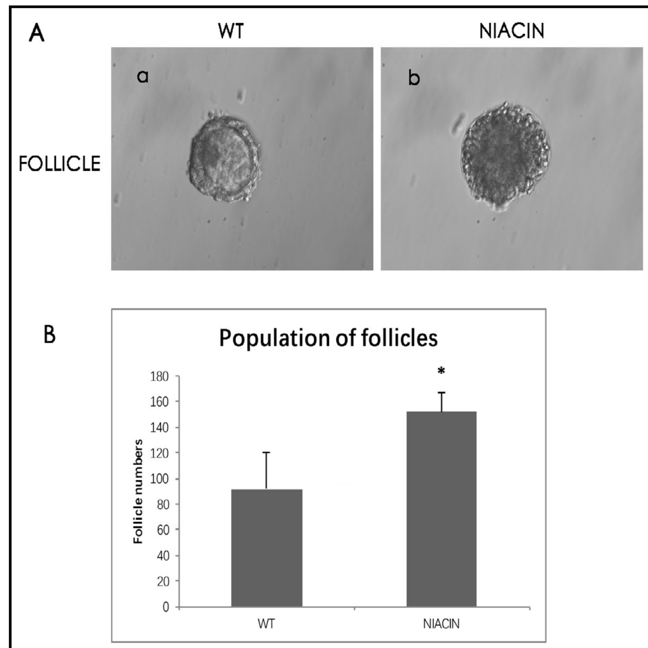

Fig. 1. Niacin promotes granulosa cell growth and follicle development in immature mice. A Top: Cultured follicles with niacin grew in vitro compared with the untreated group. Niacin initiated granulosa cell growth. B: Niacin promoted follicle growth in the immature ovary on postnatal day 18. ($P<0.05$)

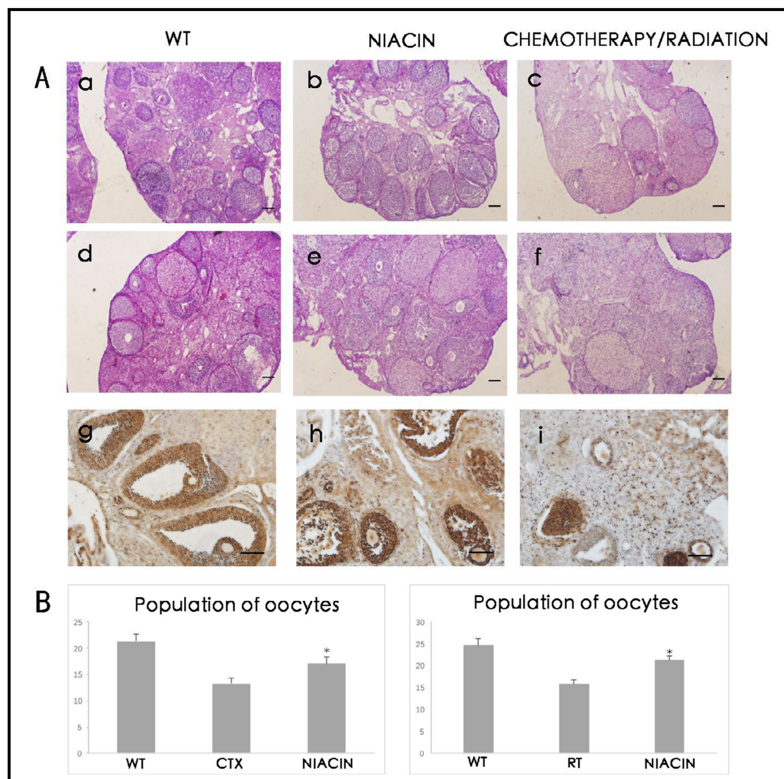

Fig. 2. Niacin rescues both chemotherapy and radiation damage in mouse ovaries. A: Ovarian pathology of the wild-type (WT) group, niacin therapy POF group and POF group. The WT ovaries contained many follicles at all developmental stages, whereas atrophied ovaries of the POF mice after 2 weeks CTX treatment (line 1, right) or 5 Gy of radiation (line 2, right) were predominantly composed of interstitial cells in a fibrous matrix with fewer follicles at each stage and more collapsed oocytes. In the niacin therapy group, there were more normal develop follicles. The third line shows the cell proliferation marker PCNA in the ovaries of the three groups. The WT and niacin-treated POF groups expressed more PCNA in the granulosa cells than the radiation-POF model. B (Left): In chemotherapy group, follicle count revealed significantly more normal follicles in the ovaries of niacin-therapy POF group mice than in the ovaries of POF group mice, and more follicles in WT than niacin-treated POF mice and POF mice. (Right) In radiation induced POF mode, follicle count revealed significantly more normal follicles in the ovaries of niacin-therapy POF group mice than in the ovaries of POF group, and more follicles in WT than niacin-treated POF mice and POF mice. The scale bar is 100 µm.

Niacin recovers POF caused by chemotherapy or radiation in mouse models

To test whether niacin also inhibited cell reduction in the POF model, we constructed two POF models using the chemotherapy drug and radiation methods.

Female mice that were administered nonlethal doses of Cytoxan (CTX) (50 mg/kg) for 14 days exhibited premature ovarian failure. They were then randomly divided into two groups, and one group was given 0.1 mL of 100 mM niacin by an intraperitoneal injection twice before ovulation and then given 5 IU of pregnant mare serum gonadotropin (PMSG). Forty-eight hours later, the ovaries were collected. The chemotherapy POF group only received 5 IU of PMSG before being sacrificed. The same aged wild-type controls were only given PMSG, without CTX or niacin. Fewer follicles developed in the POF disease ovaries than in the wild-type mice. Ovarian weight increased slightly in mice receiving niacin than POF group and ovaries in mice receiving niacin after chemotherapy possessed more oocyte-containing follicles at various developmental stages than the POF group (Fig. 2A, line one). The wild-type mouse ovaries possessed 21.36 ± 1.38 (n=15) follicles, the POF mouse ovaries possessed 13.27 ± 1.07 (n=15) follicles, and the niacin group ovaries possessed 17.09 ± 1.35 (n=15) follicles (*p=0.043) (Fig. 2B, left).

Next, we tested the POF model using radiation. Eight-week-old female mice were exposed to 5 Gy of radiation. The niacin group was administered 0.1 mL of 100 mM of niacin by an intraperitoneal injection for two days and then given 5 IU of PMSG on the third day. Negative-control radiation and normal mice were only given PMSG on the third day. The niacin group has more development follicle than the radiation POF group (Fig. 2A, line two). The wild-type mouse ovaries possessed 24.75 ± 1.51 (n=15) follicles. The niacin-treated group (21.33±0.93, n=15) recovered and had more follicle development than the radiation group (15.81±0.98, n=15) (*p=0.001) (Fig. 2B, right).

We tested the cell proliferation marker PCNA in the ovaries of the three groups. The cell proliferation marker PCNA was elevated in granulosa cells after niacin treatment (Fig. 2A, line three).

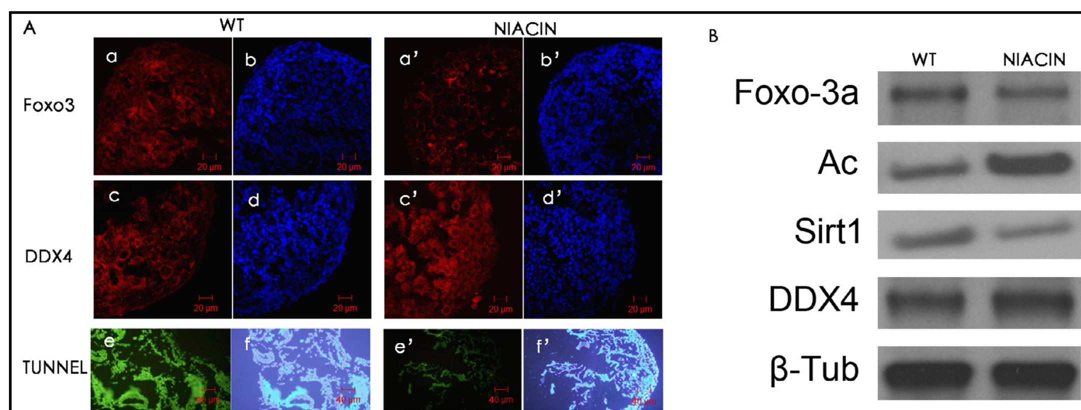

Fig. 3. The niacin affects cell arrest and apoptosis genes. A The cell arrest factor FOXO3 expression in wildtype group and in niacin group. (Red: FOXO3, Blue: DAPI) The DDX4 expression in the mice ovary in wildtype group and in niacin group. (Red: DDX4, Blue: DAPI) The DNA damage marker TUNNEL in wildtype group and in niacin group. (Green: Tunnel, Blue: DAPI) B Western blot analysis of protein expression in the niacin treated group and control group. The FOXO3 expression is slightly decreased and acetylation is increased and the SIRT1 expression is slightly decreased. The oocyte marker DDX4 is increased by niacin too.

Niacin stimulates arrested follicles and inhibits apoptosis

To determine whether niacin is able to wake up dormant follicles, we tested the follicle arrest marker FOXO3 in cultured 3-day mice ovaries *in vitro* cultured with or without 10 Mm niacin in Waymouth medium supplemented with 10% fetal bovine serum and 0.23 mM pyruvic acid. The FOXO3 expression level was decreased after 48 hours' culture with10 mM niacin (Fig. 3A and 3B). Also, we found the oocyte marker DDX4 is increased in the niacin treatment group (Fig. 3A and 3B). In addition, deacetylase sirt1 expression decreased slightly, while the acetylation level increased after niacin treatment (Fig. 3B).

To check whether niacin could reduce apoptosis, we used a TUNEL kit in the niacin group and control. We cultured the 3-day mice ovaries in a dish with Waymouth medium supplemented without 10% FBS for 72 hours, which showed significant apoptosis in the ovaries; however, the niacin group had fewer apoptosis signals compared with the control group (Fig. 3A and 3B).

The microarray of cultured ovaries from the niacin-treated and control groups.

Because niacin is a former coenzyme and affects cell acetylation, it affects many pathways. To determine which pathways niacin affected in the ovary, we used RNA microarrays. We collected and cultured the ovaries from D3 postnatal BDF1 female mice. One group was cultured with a 10 mM concentration of niacin, while the control group was only cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS and supplemented with 0.23 mM pyruvate. After 48 hours, many smaller follicles grew in the niacin-treated ovaries than in those of the control group. Hundreds of genes were affected by niacin.

Niacin up-regulated 813 genes and down-regulated 880 genes in the mouse ovaries during the *in vitro* treatment (Fig. 4A, 4B, supplementary table - for all supplemental material see www.karger.com/10.1159/000495051/). The most markedly changed genes were rich in G-protein-coupled cell signaling pathways, of which many were cell receptors. Some transcription factors were increased, including Ddit3, Rhox4d, Rhox4a, Q2MDF8_MOUSE, Rhox4g, Rhox4b, Rhox4c, Atf3, and Rhox4e. Niacin down-regulates genes involved in apoptosis, cell cycle arrest, and cell adhesion. Many noncoding RNAs were also decreased by niacin.

Niacin inhibited cell cycle arrest associated with gene expression of Pkd2, Tgfb1, and Inhba and negatively regulated cell proliferation in Timp2, Tgf b2, Tgfb3, Cdh5, Gata3, Ptges, Ptgs2, and Ifitm3, which were inhibited by niacin.

Ptch1and Socs2 are responsible for negatively regulating the body size and were decreased by niacin. Cdk5, a negative regulator of protein export from the nucleus, was also decreased by niacin. Eighteen cell differentiation pathway genes were down-regulated: Serpine2, Twist2, Pappa, Racgap1, Bmp3, Hip1, Gldn, Camk1, Adcyap1r1, Nav1, Wnt5a, Tnfaip2, Slit2, Sema3a, Mmp19, Ndrg2, Frzb, and Sfrp2.

GO:0006917 induction of apoptosis was decreased by niacin in Bok, Nod1, Tgfb1, Tgfb2, Tgfb3, Bnip3, Casp12, and Inhba.

Niacin also increased the response to the oxidative stress pathway in Idh1, Prdx3, Cygb, Ptgs2, and Mmp14, and increased the oxidation reduction pathway in Cyp7b1, Hsd17b1, Dhdh, Prdx3, Impdh2, Aldh1a2, Cyp20a1, Vat1, Hsd17b11, Akr1b7, Hsd11b2, Akr1b1, Adh1, Idh2, Ptgs2, Kcnab1, Kcnab1, Lox, Egln3, Loxl2, Vat1l, Scd1, Akr1c14, and Hsd3b1 (Fig. 4C).

Discussion

POF occurs because of reduced oocyte storage, follicle arrest or uncontrolled apoptosis. Strategies for promoting the development of follicles, maintaining normal follicle after chemotherapy or radiotherapy have been important strategies as future clinical therapies for treating infertility. Stem cells are proved to be useful in recover the ovary damage in the chemotherapy POF disease [6, 8], [23], but stem cell may also cause recurrence of the tumor risk [24].

Cell Physiol Biochem 2018;50:2060-2070
DOI: 10.1159/000495051
Published online: 10 November 2018
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Even healthy women oocytes are sequestered in primordial follicles for decades and then recruited into the growing pool, but most will go through apoptosis or die. Granulosa cells control the oocyte's fate, and follicle growth requires granulosa cell proliferation. Granulosa cell death leads to follicle atresia. And cell death happens naturally when ovary suffers damage. If we can inhibit the cell death of granulosa cells, maybe we can help to reduce the exhausting speed of oocyte pool.

Our results demonstrate that niacin promotes primary follicle development both *in vitro* and *in vivo*. We used 18 days fetal mice which ovary follicles are mainly dormant, we found

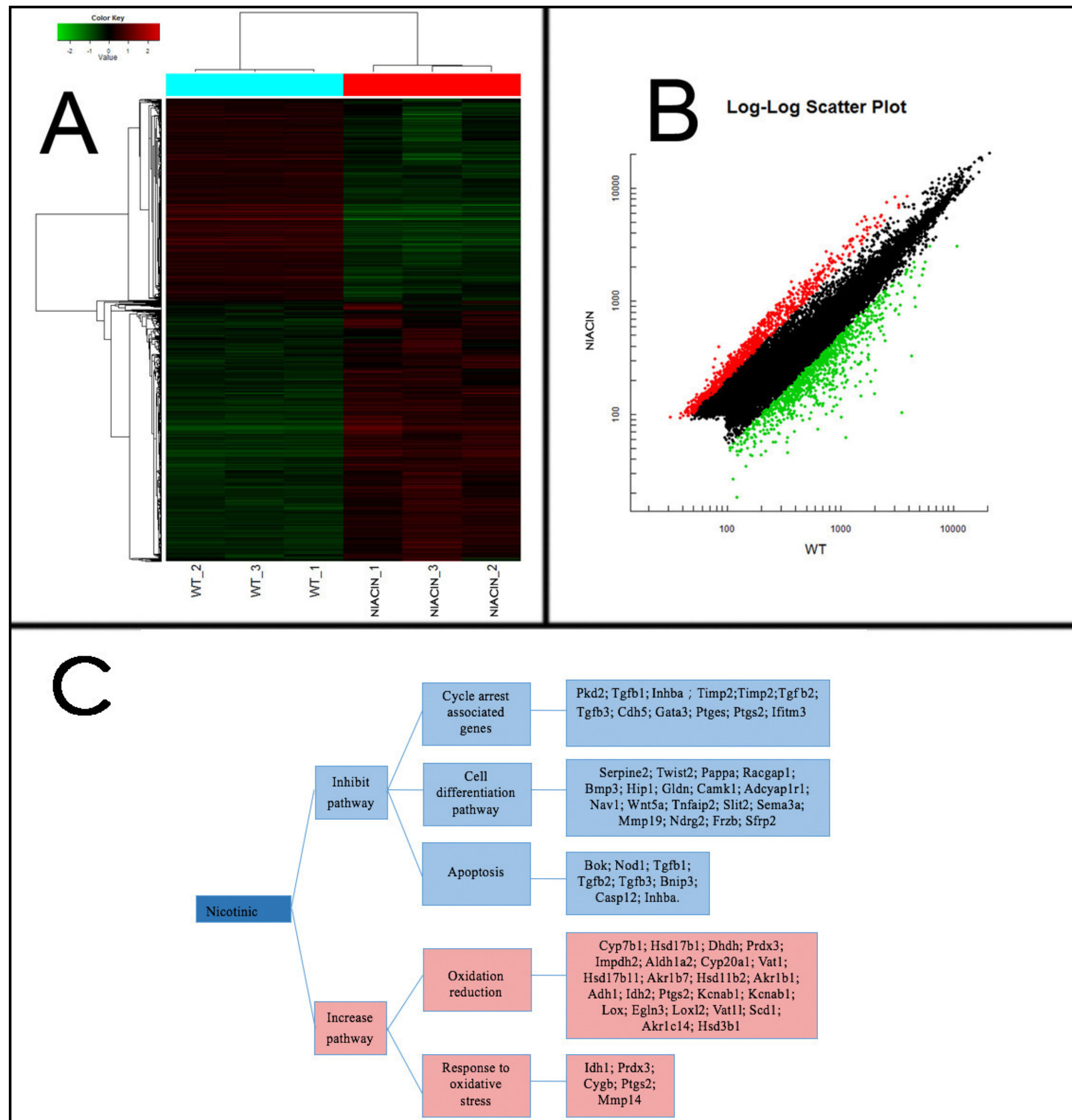

Fig. 4. The RNA microarray data of niacin group and normal control. A RNA microarray data for the 3d-mouse ovaries and niacin-treated 3d-mouse ovaries. The ovaries were collected, cut into several small pieces and cultured with or without niacin for 48 hours before RNA testing. A hierarchical clustering of differentially expressed genes in the ovaries of niacin-treated group and WT group. B Overview of gene expression comparing the WT and the niacin-treated group. The X and Y axes present the intensity of gene transcription in WT and niacin, respectively. The black dots indicate genes with no significant change in transcription level, while the red and green dots indicate up regulated and down regulated genes, respectively. C Genes involved in the cell proliferation and apoptosis pathways that were either up regulated or down regulated in the niacin-treated ovaries.

niacin can promote the granulosa cell and follicles growth. Next, we used 3 days mice which ovary has been observed severe apoptosis when cultured without FBS and we found niacin can reduce granulosa cell apoptosis. We found niacin recovers POF caused by chemotherapy or radiation in mouse models.

We used a microarray to determine the changes in DNA repair, transcription and regulation and found that many genes were affected by niacin. The previous study has shown that FOXO3-deficient mice complete primordial follicle development normally [18], then undergo spontaneous global activation of their primordial follicles immediately after their assembly is complete, leading to premature loss of all follicles and ensuing ovarian failure [17]. FOXO3 promotes p27 expression and inhibits follicle awakening and growth [25]. FOXO3 is only expressed in small follicles [26], and its location is important for cell arrest [27]. We find that niacin slightly affected FOXO3 expression and decreased FOXO3 in the primordial oocyte nucleus. DDX4 oocyte marker expression increased after niacin treatment. The relationship between cell growth and death is dynamic. In the ovary, DDX4 expression is also a sign of cell death reduction.

Niacin also inhibits sirt1 deacetylation. We found that the entire acetylation process was increased in the niacin group. Because acetylation covalently modifies many proteins, it has strong chromatin-modifying potential in DNA repair, transcription and replication. Some evidence has suggested that histone deacetylase (HDAC) inhibitors exert neuroprotective effects against various insults and deficits in the central nervous system [28, 29], and some protein acetylation can help cell survive and resist to oxidative stress [30].

We also found niacin affected cell apoptosis signaling pathway, cell arrest pathway and response to the oxidative stress pathway. These change may also contribute to the cell survive and function recover after radiation or chemotherapy. The niacin acid 5mM that we used did not inhibit ribosylation in the oocytes, possibly for the same reasons discussed in a previous paper, which found that the mouse oocyte numbers increased after treatment with 5-aminoisoquinolinone, a potent inhibitor of poly(ADP-ribosyl)ation [31]. Some study reports that the concerntration over 50mM niacin will cause damage to cell [32], and the concentration of 10mM niacin can protect cells against NaDOC-induced apoptosis [19]. We also find that niacin can cause cell death over certain concentration.

Conclusion

We found that niacin can awaken dormant follicles, inhibit granulosa cell apoptosis, maintain more follicles, and recovers POF caused by chemotherapy or radiation. It is a potential drug candidate that may benefit women with anovulation or POF disease or improve ovarian function in cancer survivors.

Acknowledgements

This work was supported by a grant from the National Natural Science Foundation of China (Grant No. 81473271) and the Chinese Postdoctoral Science Foundation.

S. W. designed the experiment and wrote the main manuscript text, and M. S., L.Y. and Y. W. did the experiment and prepared figures 1–4. All authors contributed to the interpretation of the data and the crafting of the final manuscript. Correspondence to D. W. and Y. Y.

Disclosure Statement

The authors declare no competing interests.

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Cell Physiol Biochem 2018;50:2060-2070
DOI: 10.1159/000495051
Published online: 10 November 2018
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www.karger.com/cpb

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