Oral oyster polypeptides protect ovary against D-galactose-induced premature ovarian failure in C57BL/6 mice

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/jsfa.9997

Abstract

BACKGROUND: Oyster polypeptides have various biofunctions, such as the anti-cancer and the anti-oxidative stress, but whether it has the protective effects to primary ovarian failure (POF) remains poorly understand. To address this issue, daily gavage of oyster polypeptides was performed to investigate their protective effect for POF, basing on D-galactose-reduced POF model in C57BL/6 female mice.

RESULTS: Oyster polypeptides restored the irregular estrous cycles and the abnormal serum FSH, LH and P levels as well as the decreased mRNA expression level of *Amh* that were induced by D-gal. The follicle development of POF mice was improved by increasing the primordial follicle ratio and decreasing the atretic follicle number after oral administration of oyster polypeptides. Moreover, in the oyster polypeptides treated mice, the total SOD (T-SOD) activity was significantly increased, while the MDA levels were significantly decreased. The mRNA expression levels of stress-related genes (*SOD2*, *SIRT1* and *FOXO3a*) were remarkably up-regulated after D-gal induction, but the up-regulation was weakened or disappeared by the gavage of oyster polypeptides. In addition, oyster polypeptides treatment also reduced the apoptosis of the ovarian granulosa cells and down-regulated the mRNA expression levels of apoptosis-related genes (*p53* and *Bad* but not *Bcl-2*).

CONCLUSION: This study reveals that oyster polypeptides may protect ovary against D-gal-induced POF by their anti-oxidative stress activity to rescue D-gal-induced ovarian oxidative damage and therefore to prevent ovarian cells apoptosis, thereby tipping the abnormality trigged by POF to get close to the normal levels.

Keywords: Oyster polypeptides; primary ovarian failure; oxidative stress; apoptosis; follicle

1 Introduction

Primary ovarian failure (POF), also named as primary ovarian insufficiency (POI) or premature menopause, is a common form of hypogonadism in women. In clinic, this hypogonadism is traditionally defined as cessation of menstruation for at least 4 months before the age of 40 years and elevation of follicle stimulating hormone (FSH) levels to menopausal range (> 40 IU/L), and low estradiol (E2) levels (< 50 pg/mL).[1,2] POF leads to infertility as well as complications related to bone and cardiovascular health and emotional distress.[2] It affects approximately 1% of the female population before the age of 40 years and 0.1% before 30 years.[3,4] The etiology of POF is from various sides including genetics, autoimmunity, and iatrogenic disorder, but unfortunately, some patients affected with POF are not yet found the causes.[5,6]

Since galactosemic women eventually develop POF, galactosemia is a conspicuous etiology of POF.[7,8] Classic galactosemia is due to galactose-1-phosphate uridyltransferase (GALT) deficiency induced by *GALT* gene mutation, as the most well-known inborn error of galactose metabolism.[9,10] Newborn with GALT deficiency, without galactose-restricted treatment, presents poor feeding in clinic, such as failure to thrive, jaundice, liver disease, cataracts, *E. coli* sepsis, and neonatal death, described by Waisbren.[7] In women, due to the deficiency of GALT metabolism, the accumulated lactose finally leads to the POF.[11,12] Female animal faced with excess galactose, such as D-galactose (D-gal), is acted as a common model for investigating POF as well as aging.[13,14] Because D-gal is a reducing sugar that can form advanced glycation end-products (AGEs) that can cause excessive reactive oxygen species (ROS) accumulation.[15,16] The ROS-induced oxidative damage and AGEs toxicity gradually lead to the functional attenuation or vanishing of ovary and others tissues that is very similar to the process of aging in human.[15,17]

The traditional therapy of POF is hormone replacement therapy (HRT) that is for supplementing the lack of E2.[18] HRT is mainly administered to postmenopausal women to decrease the incidence rate of osteoporosis and coronary heart disease, but it also elevates the risk of breast cancer during chronic therapy.[19] Melatonin supplementation and immunomodulation therapy have been described as the treatment means for POF, which are to induce ovulation, recover menstruation and even fertility, and prevent menopause-related emotional distress.[18,20-22] Melatonin can not be produced in ovary, but higher concentration of melatonin is detected in the follicular fluid during ovulation.[18] Thus, the mature follicles may accumulate melatonin to regulate the ovulation, which supports the therapeutic strategy of melatonin supplementation. Immunomodulation therapy mainly aim at the POF triggered by autoimmune diseases, but not always suitable for the POF induced by other causes.[18] Apart from that, stem cell transplantation is a new strategy to treat the infertility induced by POF.[23] However, above therapeutic modalities can not entirely reverse POF and complications and avoid the side-effect, preventive action should be considered.

Oyster (*Crassostrea gigas*) is a familiar edible, which was distributed in extensive tropical and subtropical areas. Its bioactive peptides that were usually extracted by enzymolysis attract great interest of researches or food industries, because it has a series of biological functions, such as immunomodulatory,[24] anti-cancer,[25] anti-oxidative,[26] etc. Wang et al. reported that oyster peptides presented the anti-oxidative function base on

their DPPH radical scavenging power.[26] The powerful radical scavenging activity may refer to remit ROS-induced damage in ovary, and present potential functions for protecting ovary against POF. Therefore, the aim of this study is to evaluate the protective effects of oyster polypeptides for ovary against D-gal-induced POF in mice.

2 Materials and methods

2.1 Animal and treatment

A total of 100 C57BL/6 female mice, aged 7-8 weeks and weighted 18-22 g, were purchased from Laboratory Animal Centre at West China Center of Medical Sciences, Sichuan University (Chengdu, China). The mice were housed under natural photoperiod (12L:12D) with suitable temperature (20-25℃) and humidity (40%-70%) in an animal facility. Water and food were freely fed across this study, and the mice were needed to acclimatize for 1-2 weeks. After acclimation, the mice were randomly divided into 5 independent groups (20 mice per group), containing control, POF model, low-dose (low, 250 mg/kg/day), medium-dose (medium, 500 mg/kg/day) and high-dose (high, 1000 mg/kg/day) groups. The mice of POF model and three dose groups were subcutaneously injected daily with D-gal (200 mg/kg/day). Then, daily gavage of the isopyknic saline and different dose oyster polypeptides (provided by Ningbo Bofeng Biological Science and Technology Co., Ltd., Ningbo, China) was respectively performed. The mice of control group were subcutaneously injected daily with the isopyknic saline, and daily gavage of the isopyknic saline was performed. The daily injection was performed at about 9:00 am, while the daily gavage was operated following the injection. All the treatments were started at the same time and sustained for 42 days. The estrous cycles of mice were determined by daily vaginal smear with H&E staining and microscopy for 15 consecutive days before the end of feeding. All the experiments that involve animals were approved by the Animal Protection Committee of Sichuan Agricultural University and were conformity with the National Institutes of Health (NIH) guidelines (No. 85 - 23, revised 1996). At the end of treatments, all the mice were sacrificed at around 7:00 - 9:00 am within their diestrus, under general anesthesia by pentobarbital sodium. The anaesthetized mice were used to collect blood by a heart puncture method. The left and right ovary were respectively removed to biochemical analysis and histological or qRT-PCR assay.

2.2 Sample preparation and biochemical assay

The collected blood was clotted at room temperature for 2 hours and at -4℃ overnight. Then, the blood was centrifuged at 1, 000 xg for 10 min to acquire serum. The serum FSH, luteinizing hormone (LH), progesterone (P) and E2 levels were measured by FSH (ERK R7014) and LH ELISA kit (ERK R7017) from Endocrine Tech (Newark, NJ, USA), P ELISA kit (582601) from Cayman Chemicals (Ann Arbor, MI, USA), and E2 ELISA kit (E-EL-0065) from Elabscience (wuhan, China). The collected ovaries were immediately washed by ice-cold saline and homogenized in 0.1 M Tris–HCl buffer (pH 7.4). The homogenates were

centrifuged at 10, 000 xg for 15 min, and the supernatants were removed to new tubes for further eliminating the insoluble at 100, 000 xg for 60 min. The cytosolic fraction was acquired to determine the enzymatic activity of total superoxide dismutase (T-SOD) and malondialdehyde (MDA) levels. The measure of T-SOD activity and MDA levels was performed following commercially available kits, T-SOD kit (A001-1) and MDA kit (A003-1) from Jiancheng Bioengineering Institute, Nanjing, China.

2.3 H&E staining for primordial follicle counting

Six ovary samples of each group were randomly selected to analyze the primordial follicle ratio. The ovaries were fixed in 4% paraformaldehyde for 12 h, embedded in paraffin, and sectioned into 3-5 μm sections. The sections were mounted into glass slides and then deparaffinized by xylene. One section of each sample was randomly selected to stain with hematoxylin and eosin (H&E). After staining, the sections were observed and imaged by a Motic BA400Digital microscope (Motic China Group, Co., Ltd.). The sections were viewed at one view under 100x magnifcation and at 3 views under 400x magnifcation. All the follicles, including primordial follicles and atretic follicles, were counted according to previous describes.[27] The primordial follicle ratio of each sample was calculated by the number of primordial follicles out of the total follicles, and the number of atretic follicles was also recorded.

2.4 qRT-PCR assay

The total RNA of ten ovary samples of each group were extracted by RNAiso plus reagent (Takara Bio, Co. Ltd., Dalian, China) complying with the manufacturer's instructions. The concentration and purity of acquired RNA was evaluated by Nano-Drop 2000 spectrophotometer (Thermo, USA) and agarose gel electrophoresis, respectively. PrimeScripy® RT reagent kit with gDNA Eraser (Takara Bio, Co. Ltd., Dalian, China) was used to synthesize the cDNA. Then, the qRT-PCR was performed by using SYBR® green II (Takara Bio Co. Ltd., Dalian, China) with the cDNA as template, following conditions: 95℃ for 3 min; 40 cycles of 95℃ for 5s and several annealing temperature for 30s; 95℃ for 10s; melt curve detection of 65℃ for 5s to 95℃ increment 0.5℃. The used primers were listed in Table 1, and the relative mRNA expression levels were calculated by $2^{-\Delta\Delta Ct}$ method, with β-actin as the reference gene for normalization analysis. The gene expression levels were presented as fold-change by normalizing to the expression levels of control group (Fold-change as 1.0).

2.5 In situ TUNEL assay

For the *in situ* TUNEL assay, one section of each sliced sample was randomly selected and used (n = 6). Deparaffinized ovary sections described as above were incubated with proteinase K (20 mg/mL) at 37℃ for 25 min. Then, the TdT-mediated dUTxP nick end labeling (TUNEL) assay was performed by using *in situ* cell

death detection kit (11684817910, Roche). According to the manufacturer's instructions, the sections were incubated with the mixture of enzyme solution and label solution for capturing the fractured DNA of apoptotic cells at 37℃ for 60 min under aphotic and moist conditions. Then, the sections were incubated with converter-POD solution for labeling the fractured DNA by horse-radish peroxidase (HRP), at 37℃ for 30 min under the same conditions. The HRP was colored by diaminobenzidine (DAB) and observed by a Motic BA400Digital microscope (Motic China Group, Co., Ltd.). The sections were integrality viewed at one view under 100x magnifcation and meticulously viewed the apoptosis of the atretic follicles under 400x magnifcation. The ratio of TUNEL-positive cells in antral follicles was analyzed by the Image Pro Plus 6.0 software.

2.6 Statistical analysis

All the data were analyzed and charted by Graphpad Prism 5. The statistical comparisons among the groups were analyzed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test, or two-way ANOVA followed by Bonferroni post-tests, with the significance as $P < 0.05$.

3 Results

3.1 Oyster polypeptides prevented the irregular estrous cycles

D-gal is well-known inducer for POF model that involves in prolonging or stagnating female estrous cycle. In current study, the estrous cycles of all the mice were detected by daily vaginal smear and microscopy. The average of estrous cycles of the control group was 4-5 day, while the estrous cycles of the POF model mice were significantly prolonged, and even stagnated in diestrus more than 15 days, comparing to the control group ($p < 0.001$, Fig. 1A and 1C). The estrous cycles of different dose groups (average of 8 days) were significantly shortened than in the POF model group ($p < 0.001$, Fig. 1C), but the estrous cycles were not recovered to the normal level (4-5 days).

The frequency of proestrus, estrus and metestrus/diestrus was analyzed during the 15 days (Fig. 1B). In the POF model mice, the frequency of proestrus and estrus were remarkably reduced, while the frequency of metestrus/diestrus was significantly increased, comparing to the control group. In different dose groups, the frequency of estrus was significantly increased ($p < 0.001$), while the frequency of metestrus/diestrus were observably reduced ($p < 0.001$) (Fig. 1B).

3.2 Effect of oyster polypeptides on the serum hormone levels and ovarian Amh mRNA expression

In human POF, the elevated FSH levels and the decreased E2 levels were detected.[1,2] In POF model mice, the serum hormone levels that related to HPG axis were disorganized.[28,29] Consistently, after injection with D-gal, the serum FSH and LH levels were significantly increased ($p < 0.05$), and the serum P levels were

remarkably reduced ($p < 0.05$) (Fig. 2). These results reveal that the establishment of the POF model mice is successful. These abnormal hormone levels were restored by oral oyster polypeptides, at least the reverting of FSH and P levels was significant in the low-dose group ($p < 0.05$). The decrease of LH levels was not significant in the different dose groups, but the LH contents were reduced than in the POF model mice. Moreover, without regard to significance, the *Amh* mRNA expression levels were decreased in the POF model group and reverted after the low-dose and the medium-dose treatments. Unfortunately, the serum E2 levels that is an important index for POF model was failed to be detected in this study.

3.3 Effect of oyster polypeptides on follicular distribution

The disturbance of follicular distribution was described in POF mice,[29] which therefore impacts on the reproductive function. Thus, we evaluated the effect of oyster polypeptides on the follicular development by ovarian sections with H&E staining. The section showed the abundant store of primordial follicle in the control group and the change of follicular distribution in other groups (Fig. 3A). In the POF model group, the number of primordial follicles were remarkably reduced ($p < 0.01$), and the number of atretic follicles was also increased (Fig. 3B). Interestingly, the disorder of the primordial follicle number was eliminated by gavage of different doses of oyster polypeptides, especially in low- and medium-dose groups, the number of primordial follicles were significantly increased with the p value less than 0.001. And, the atretic follicles numbers were reduced in different dose groups than in the POF model (Fig. 3B). The primordial follicle ratio was calculated in this study, the results showed that the primordial follicle ratio was significantly decreased in the POF model group than in the control group ($p < 0.01$), but the abnormal ratio was significantly reversed in the different dose groups ($p < 0.05$) (Fig. 3C).

3.4 Effect of oyster polypeptides on ovarian oxidative stress

D-gal-induced POF model also accompanies by aging that relates to oxidative stress that is regarded as the main cause for the occurrence of POF.[15,16] In current study, in order to evaluate the effect of oyster polypeptides on ovarian oxidative stress in POF mice, T-SOD activity, MDA contents and *SOD2* mRNA expression levels were analyzed in ovary. As shown in Fig. 4, T-SOD activity was significantly decreased in the POF model group than in the control group ($p < 0.05$). In the low-dose group, the T-SOD activity was remarkably risen to get close to the level of control group, which reveals the anti-oxidative potential of oyster polypeptides. But the T-SOD activity was not reverted in the medium- and the high-dose groups. The MDA contents was significantly increased in the POF model group ($p < 0.001$), about twice as high as the control group. In the different dose groups, the MDA contents were decreased with different degrees than in the POF model group. These results reveal that MDA accumulation can be induced by D-gal, but the accumulation can be eliminated by additional treatments of different dose of oyster polypeptides. The *SOD2* mRNA expression levels were also analyzed in this study. The expression levels were significantly elevated in the POF model group than in the control group ($p < 0.05$), and the expression levels were decreased in the different dose

groups than in the POF model group. But the significance analysis showed that only low- and high-dose groups presented significance ($p < 0.05$ and $p < 0.01$, respectively), that is, the dose of oyster polypeptides may influence its protective effect for ovary against the oxidative stress in the POF model mice.

3.5 Effect of oyster polypeptides on ovarian cells apoptosis

For analysis of ovarian cells apoptosis, the fractured DNA of apoptotic granulosa cells in the antral follicles were visualized by *in situ* TUNEL assay (Fig. 5). The results showed the ratio of apoptotic granulosa cells was significantly elevated in the POF model group than in the control group ($p < 0.01$). The TUNEL-positive cells ratios were decreased after the additional gavage of different dose of oyster polypeptides, especially in the low-dose group where the TUNEL-positive cells ratio was close to the level of the control group. This result reveals that the low-dose of oyster polypeptides may be considered as the best dose for remitting D-gal-induced ovarian cells apoptosis.

3.6 Effect of oyster polypeptides on ovarian P53, Bad, Bcl-2, SIRT1 and FOXO3a mRNA expression

The mRNA expression levels of anti-apoptosis-related genes (*P53, Bad* and *Bcl-2*) and antioxidant-related genes (*SIRT1* and *FOXO3a*) were analyzed in ovary. The expression levels of *P53* and *Bad* were significantly increased in the POF model group ($p < 0.001$, Fig. 6A and 6B), revealing that D-gal has induced the cell apoptosis in the ovary. Interestingly, their expression levels were remarkably decreased in the different dose groups than in the POF model group. And, their expression levels in the high-dose group were extremely low, even obviously lower than in the control group. However, *Bcl-2* is a classic anti-apoptosis gene, which had no significant expression change among all the groups (Fig. 6C).

Moreover, the mRNA expression levels of silent information regulator 2 homolog (*SIRT1*) and forkhead box O3 (*FOXO3a*) were obviously up-regulated in the D-gal-induced POF model, whereas the expression levels were significantly decreased after gavage of low-dose of oyster polypeptides (Fig. 6D and 6E). In the medium- and high-dose groups, the expression levels of *SIRT1* and *FOXO3a* were not significantly changed than in the POF model group (Fig. 6D and 6E).

Discussion

In this study, POF model mice were successfully established by the D-gal induction. In the model, the stagnated estrous cycles, the increased FSH and LH levels, the decreased P level, the reduced primordial follicle ratio, the elevated atretic follicle number, and the triggered ovarian oxidative stress and granulosa cells apoptosis were observed. Gavage of a suitable dose of oyster polypeptides could reverse these abnormalities. The mechanism of the protective effects of oyster polypeptides may be involved in reducing the cell apoptosis by the suppression of the mRNA expression of *P53* and *Bad*, and inhibiting the oxidative stress by SIRT1/FOXO3a/SOD2 pathway.

Clinical POF presents abnormal estrous cycles, high FSH levels to menopausal range, and low E2 levels.[1,2]

Consistently, previous studies demonstrated that D-gal-induced POF showed the same features in mice.[28-30] In current study, the estrous cycles of the D-gal-induced POF model mice were disordered, the estrous cycles were prolonged, even stagnated in diestrus for more than 15 days (Fig. 1B). In the D-gal-induced POF model mice, the serum FSH and LH levels were significantly increased, while the serum P level was significantly decreased (Fig. 2). Unfortunately, perhaps the serum E2 levels were lower than the detection threshold (25 pg/mL), we failed to detect the serum E2 levels in the POF model mice (data not shown). Even so, the increased FSH and LH levels and the decreased P level could be regarded as the valuable evidences for reflecting the success of establishment of the POF model, which was consistent with previous studies.[28,29] Amh is a marker of ovarian reserve, which reflects the number of preantral follicles.[31,32] In human female, serum Amh level was steadily decreased with increasing age,[33] meaning that Amh of low levels relates to aging as well as ovarian failure. After D-gal induction, the low Amh level was indirectly presented by low mRNA expression level of *Amh* in ovary, meaning that ovarian failure has occurred in the model mice. Taken together, the POF model mice was successfully induced by D-gal in this study.

Mouse estrous cycle is divided into four stages, containing proestrus, estrus, metestrus, and diestrus, and one cycle is maintained for 4 to 5 days unless pregnancy, pseudopregnancy, or anestrus.[34] In this study, D-gal-induced POF mice showed the irregular estrous cycles, but in the dose groups, oyster polypeptides positively regulated the irregular estrous cycles to get close to the level of the control group. This result uncovers the regulatory function of oyster polypeptides for estrous cycle during POF. Both the gonadotrophin FSH and LH are necessary for ovarian follicular maturation and the production of E2 and P.[35] POF is characterized by deficiency of E2 and elevation of FSH.[36] Monitoring in the serum FSH and E2 levels, as well as the serum LH and P levels, is a good way for checking POF which also relates to the abnormality of the follicular maturation.[18] Small primordial follicle is the initial unit of ovarian follicular maturation or folliculogenesis that is a highly organized and complex process.[37] The initating store of primordial follicle in individual is determined when giving birth, and the store of primordial follicles is not renewable and serves the entire reproductive life span of the adult.[38] The primordial follicle consists of an oocyte undergo a set of developmental stages (primordial, primary, secondary and mature stages) for releasing oocyte to the reproduction of offspring. But the majority of primordial follicles finally undergo atresia, only a few can be destined to reach maturity.[39] In current study, we found that D-gal induction leaded to elevate the serum FSH and LH levels (Fig. 2), down-regulate the serum P level (Fig. 2), decrease the primordial follicle ratio and increase the atretic follicle number (Fig. 3B and 3C), comparing to the control group. Interestingly, the disorder was rescued by gavage of different dose of oyster polypeptides. These results reveal that oyster polypeptides have a biofunction to save abnormal ovary function that relates to HPG axis and the follicular development, that is, oyster polypeptides may delay the ovarian recession and can be considered as daily healthcare product for preventing the ovarian dysfunction.

T-SOD plays a key role in anti-oxidative response, which consists of three types of SOD: intracellular copper-zinc superoxide dismutase (Cu,ZnSOD or SOD1), intracellular manganese superoxide dismutase

(MnSOD or SOD2), and extracellular Cu,ZnSOD (ecSOD or SOD3). Among all SOD, SOD2 is an important antioxidant enzyme in body, and its mRNA expression level is increased following oxidative stress.[40] In this study, the T-SOD activity was significantly decreased and the *SOD2* mRNA expression level was significantly increased after D-gal induction (Fig. 4). These results accord with previous study,[29] meaning that the strong oxidative stress was induced by D-gal. Considering that oyster polypeptides have anti-oxidative stress function reported in previous,[26] we guess that D-gal-induced oxidative stress could be rescued by gavage of oyster polypeptides. Consistently, the rescue was predominately occurred in the low-dose group, revealing that oyster polypeptides exert its antioxidative function to protect ovary against the oxidative stress, but this effect may depend on its dose. MDA is a product of lipid peroxidation which has been reported to occur in animal tissues under the condition of anti-oxidative deficiency.[41,42] Thus, the elevation of MDA content in the D-gal group is well-deserved, consistent to the SOD results, oyster polypeptides also reduced ovarian damage shown by the low levels of MDA in the different dose groups than in the D-gal model group (Fig. 4).

SIRT1 was identified as a cell survival factor against DNA damage and can be regarded as a key regulator of cell defense and survival under oxidative stress condition.[43-45] SIRT1 can attenuate oxidative damage by increasing FOXO3a's ability to resist oxidative stress.[45] FOXO3a is a transcription factor that can regulate the expression of scavengers of ROS, such as SOD2.[46] Research in mouse oocytes under oxidative stress has demonstrated that *FOXO3a* and *SOD2* transcripts were increased upon oxidative stress with the same kinetics as *SIRT1* transcripts, and the increased expression of *SOD2* was prevented by oocytes treatment with SIRT1-specific inhibitor, indicating that SIRT1 acts upstream to the FOXO3a-SOD2 axis.[47] In this study, the mRNA expression levels of *SIRT1*, *FOXO3a* and *SOD2* were significantly increased by D-gal induction but significantly decreased by additional gavage of oyster polypeptides (Fig. 6D and 6E, Fig. 4). These results reveal that oyster polypeptides may protect ovary against the oxidative stress via SIRT1-FOXO3a-SOD2 axis. However, the protective effect may dependent on the dose of oyster polypeptides, lower dose of oyster polypeptides seems like more effective to protect ovary against the oxidative damage in POF. In addition to FOXO3a, studies with mice have shown that FOXO3a is a suppressor of primordial follicle activation, and knockout of FOXO3a exhibits a distinctive ovarian phenotype of global follicular activation that was followed by rapid depletion of follicles.[48-50] Activation in FOXO3a leads to infertility in mice duo to the retardation of oocyte growth and follicular development.[50] The up-regulation of *FOXO3a* expression shown in POF model group may relate to the retardation of oocyte growth and follicular development which was evidenced in the prolongation of estrous cycle (Fig. 1). Given that FOXO3a is expressed mainly in the nuclei of oocytes of primordial follicles and early primary follicles,[50] and the up-regulation induced by D-gal did not increase the reserve of primordial follicles (Fig. 3). Thus, we speculate that D-gal-induced the ovarian oxidative damage could be partly rescue by oyster polypeptides.

We also found that oyster polypeptides had the anti-apoptosis function to protect ovary against D-gal-induced POF, by *in situ* TUNEL assay and the mRNA expression analysis of apoptosis-related genes (*p53*, *Bad* and *Bcl-2*) (Fig. 5A and 5B, Fig. 6A - C). There are two distinct pathways to apoptosis in

mammalian cells: the so-called BCL-2-regulated pathway activated by stress conditions and the so-called death receptor pathway activated by ligation of members of the tumour necrosis factor receptor (TNFR) family.[51,52] In the BCL-2-regulated pathway, cell apoptosis is initiated by up-regulation of the pro-apoptotic BH3-only members of the BCL-2 protein family, such as Bad and Bcl-2. Bad can bind and inhibit the anti-apoptotic BCL-2 proteins, such as Bcl-2, to promote cell apoptosis.[53,54] In addition, p53 can induce cell apoptosis, which can be blocked by anti-apoptotic protein Bcl-2.[55,56] In this context, the analysis in the mRNA expression change of *p53*, *Bad* and *Bcl-2* genes is a good way to evaluate the effect of oyster polypeptides that may function as anti-apoptosis to protect ovary against POF. As the results showed (Fig. 6A and 6B), the distinct up-regulation of the mRNA expression levels of *p53* and *Bad*, induced by D-gal, was significantly down-regulated in oyster polypeptides treatment groups. Interestingly, the mRNA expression levels of *Bcl-2* were not distinctly changed neither in D-gal group nor oyster polypeptides treatment groups. It implies that the anti-apoptotic ability of oyster polypeptides may be exerted by the transcriptional down-regulation of the pro-apoptotic genes, such as *p53* and *Bad*. In addition, D-gal-induced apoptosis in the antral follicles was detected by *in situ* TUNEL assay. The TUNEL-positive cells represented the apoptotic granulosa cells. In the D-gal group, approximately 30% granulosa cells in the antral follicles were induced to apoptosis, but the ratio was reduced to get close to the control group by gavage of low-dose of oyster polypeptides. This result emphasized the anti-apoptotic ability of oyster polypeptides again, and the ability was partial to the low-dose.

Given that the anti-oxidative stress activity of oyster polypeptides has been reported,[26] in our knowledge, there is no direct evidence that can prove the anti-apoptotic activity oyster polypeptides have. This study is first evidence to prove its anti-apoptotic activity presented by inhibiting D-gal-induced ovarian cell apoptosis. However, considering in the experimental design, daily gavage of oyster polypeptides was performed following to the daily D-gal injection, and both treatments were maintained in the same days (42 days). D-gal used in this study was to induce the POF, but the aging that relates to strong oxidative stress was also shown. Indeed, D-gal-induced POF depends on the production of oxidative damage in ovary.[13] In the activation of apoptotic processes, stress-induced damage can trigger an apoptotic signaling program that leads to cell death.[57] In this context, we infer that oyster polypeptides may protect ovary again D-gal-induced POF according to its anti-oxidative stress activity. However, whether oyster polypeptides have the anti-apoptotic activity remains to be explored.

The identification of the effective component(s) of oyster polypeptides will be of great importance and significance for questing new treatment or prevention strategies for human POF. According to previous studies, some anti-oxidative peptides were identified from oyster, such as Leu-Lys-Gln-Glu-Leu-Glu-Asp-Leu-Leu-Glu-Lys-Gln-Glu, Pro-Val-Met-Gly-Asp and Glu-His-Gly-Val.[26,58] These anti-oxidative peptides may play an important role in preventing the POF as well as aging which relate to the oxidative damage in ovary. However, whether they exist other effective components remains to be identified by multiple chemical and physical methods, such as sephadex, HPLC and mass spectrometry. In addition, the researches of the mechanism of oyster polypeptides against POF are encouraged to use more strategies (such as gut microbiome

analysis in animal model) in the future study [59-63]. The in-depth studies will comprehensively reveal the protective mechanism of oyster polypeptides against POF and promote it to be applied as a daily health-care product or a clinical medicine.

Acknowledgements

This work was supported in part by Two Sides Supporting Plan in Sichuan Agriculture University (00770103), Public welfare project in Zhejiang Province (LGN18C200003), Technology enriches the people project in Ningbo city (2016C10030) and key research and development project in Zhejiang Province (2019C02071).

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Figure Legends

Fig. 1. Effect of oyster polypeptides on estrous cycles. Four stages (proestrus, estrus, metestrus and diestrus) of the estrous cycle were determined by daily vaginal smear with H&E staining and microscopy at 7:00 – 9:00 am. A: Representative examples of the cyclicity of control, POF model and three dose groups. B: Frequency of occurrence of cycle stages during the 15 days (n = 20). The statistical analyses were performed using two-way ANOVA followed by Bonferroni post-tests. Data are shown as the mean ± S.D. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs the control group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs the POF model group. C: The length of the estrous cycle in different treatment groups (n = 20) during the 15-days administration period before the end of feeding. When the number of estrous cycles ≥ 2, the mean value is presented. Bars and error bars are means and S.D, respectively. The statistical analyses were performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test.

Fig. 2. Effect of oyster polypeptides on the HPG axis and ovarian AMH. The serum FSH, LH and P levels were tested in different treatment groups, and AMH mRNA expression in the ovarian tissues was also tested. All data are shown as the mean ± SEM (n = 10). The statistical analyses were performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test. $^{*}p < 0.05$ and ns not significant.

Fig. 3. Effect of oyster polypeptides on the development of follicles. A: Follicles were presented after H&E staining. The follicle classification was based on the characteristics proposed by Hirshfield and Midgley. B: The statistics of the number of different follicles. The statistical analyses were performed using two-way ANOVA followed by Bonferroni post-tests. Data are shown as the mean ± S.D (n = 6). $^{**}p < 0.01$ vs the control group; $^{\#\#\#}p < 0.001$ vs the POF model group. C: Primordial follicle ratio counting of ovarian section was performed in different treatment groups. The primordial follicle ratios were calculated as the percentage of the primordial follicle number to the total follicle number. Data are shown as the mean ± S.D (n = 6). The statistical analyses were performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test. $^{*}p < 0.05$ and $^{**}p < 0.01$.

Fig. 4. Effect of oyster polypeptides on oxidative stress. T-SOD activity and MDA level were measured in the ovarian tissues. *SOD2* mRNA expression was tested using qRT-PCR. All data are shown as the mean ± SEM (n = 10). The statistical analyses were performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and ns not significant.

Fig. 5. Effect of oyster polypeptides on D-gal-induced apoptosis of ovarian cells. A: Apoptosis was analyzed by *in situ* TUNEL. In the *in situ* TUNEL assay, the fractured DNA of ovarian cells were labeled by horse-radish peroxidase (HRP) that was subsequently coloured by a response with its substrate

diaminobenzidine (DAB). B: The TUNEL-positive cells ratios in different treatment groups were calculated as the TUNEL-positive granulosa cells out of all the granulosa cells in ovarian antral follicles. The data are shown as the mean ± S.D (n = 6). The statistical analyses were performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test. $^{**}p < 0.01$ and ns not significant.

Fig. 6. Effect of oyster polypeptides on the mRNA expression levels of apoptosis- and stress-related genes. The mRNA expression levels of ovarian *P53* (A), *Bad* (B), *Bcl-2* (C), *SIRT1* (D) and *FOXO3a* (E) genes were analyzed using qRT-PCR in different treatment groups. All data are shown as the mean ± SEM (n = 10). The statistical analyses were performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test. $^{**}p < 0.01$, $^{***}p < 0.001$ and ns not significant.

Figure 1

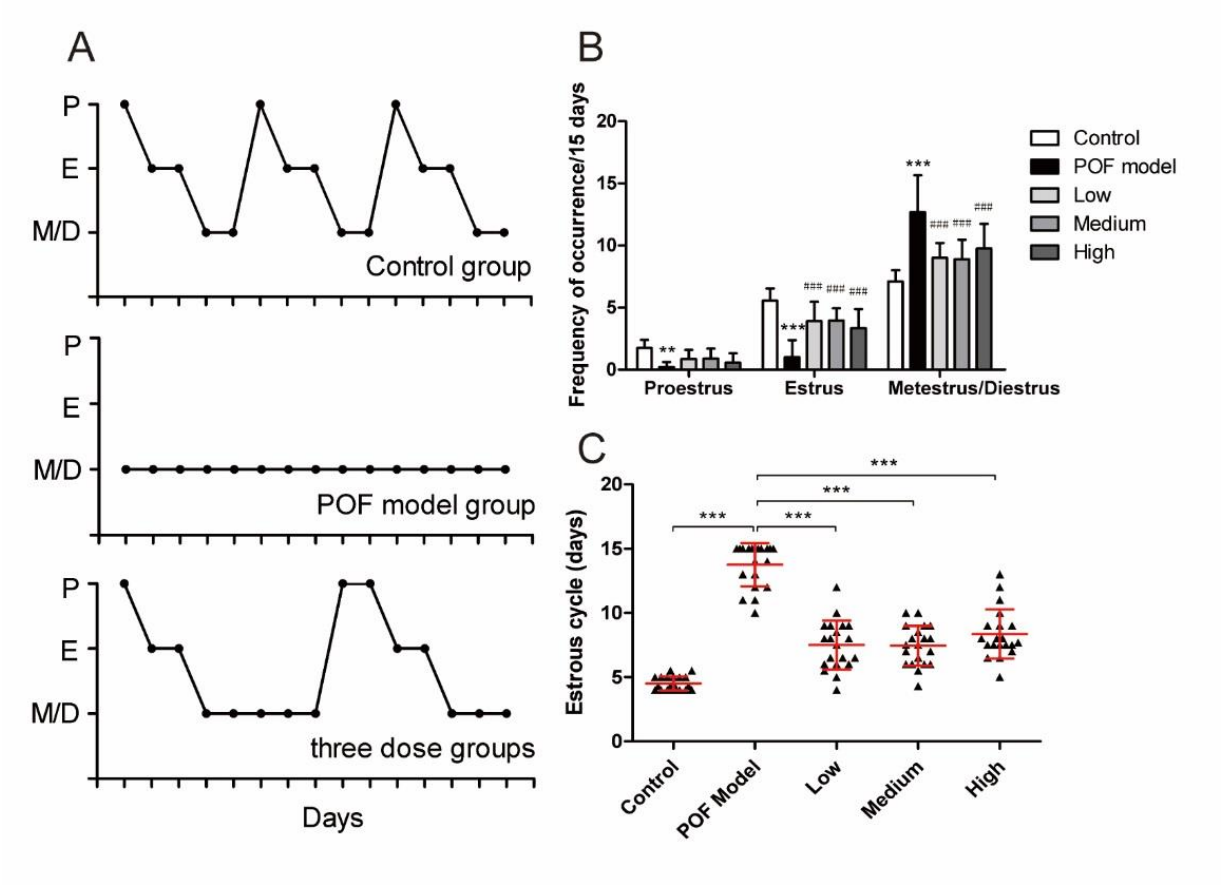

Figure 2

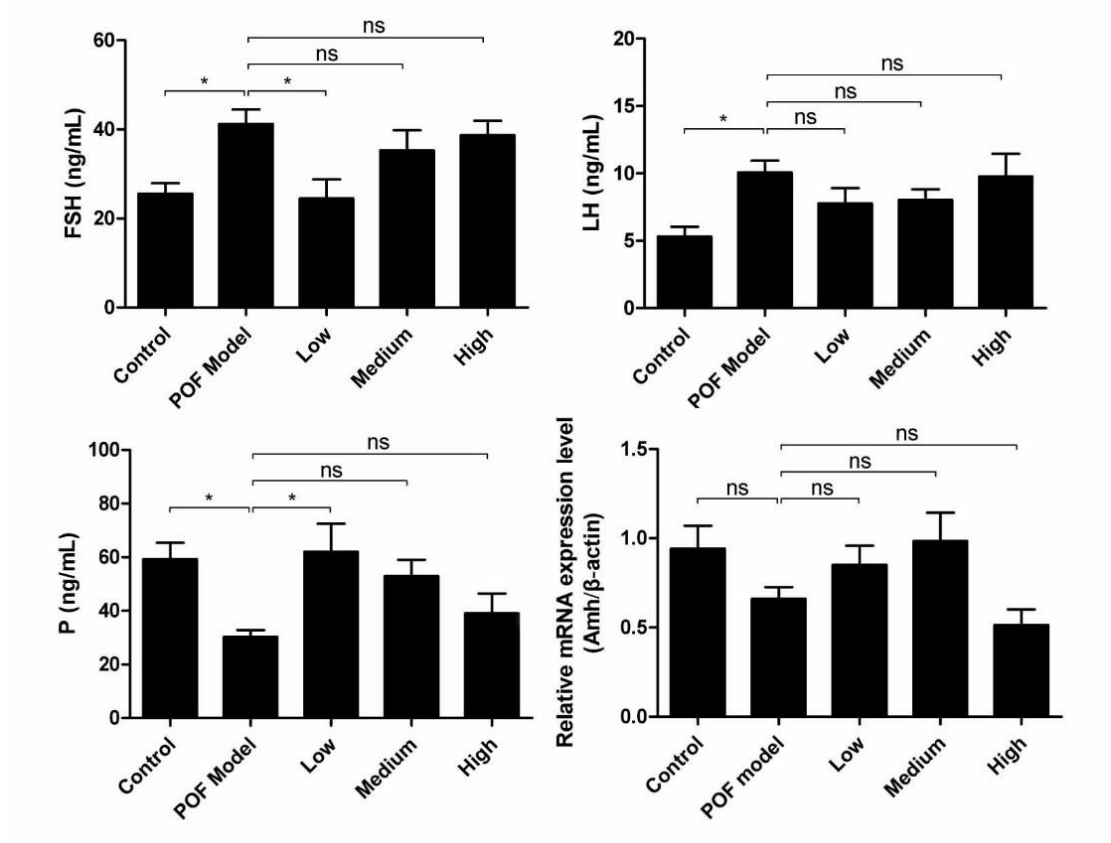

Figure 3

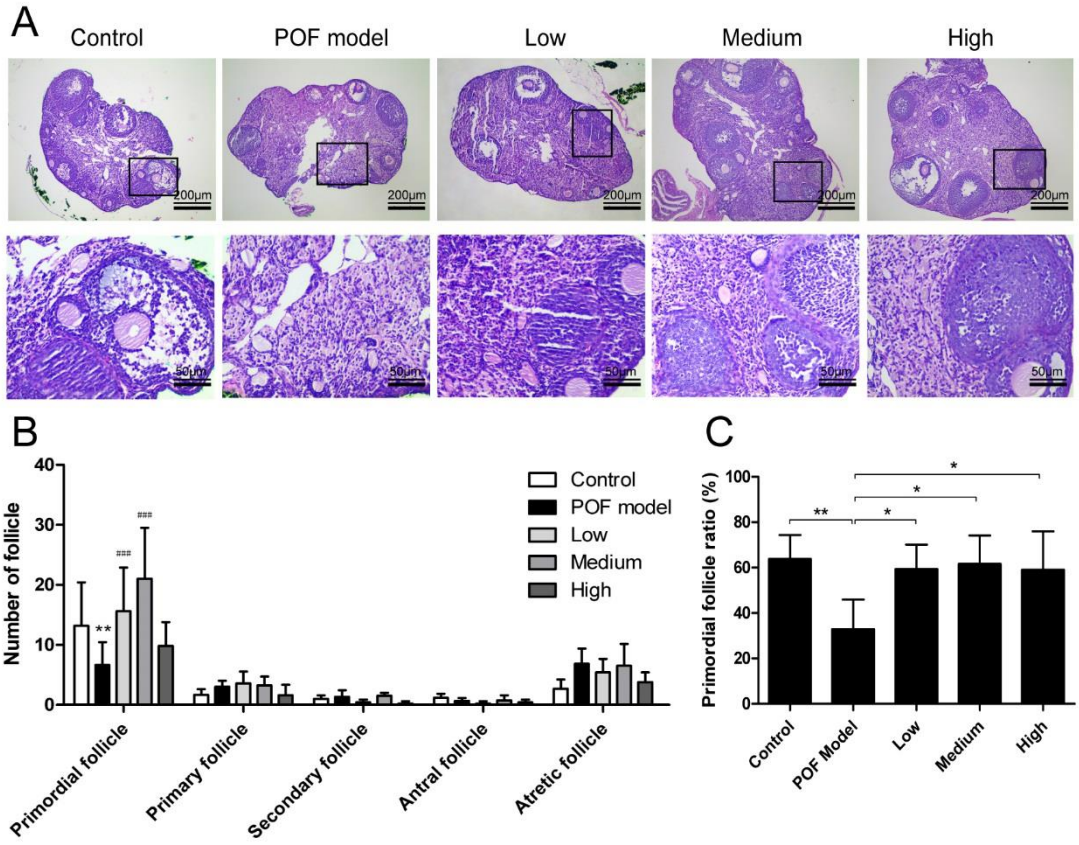

Figure 4

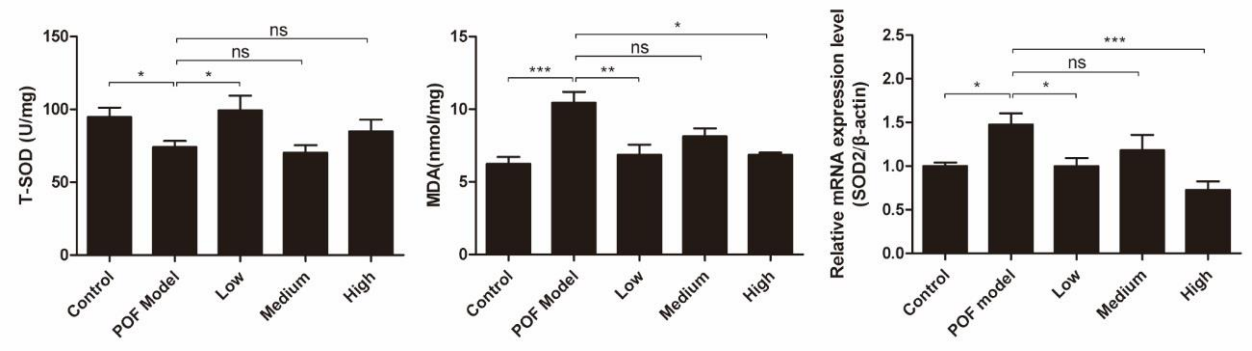

Figure 5

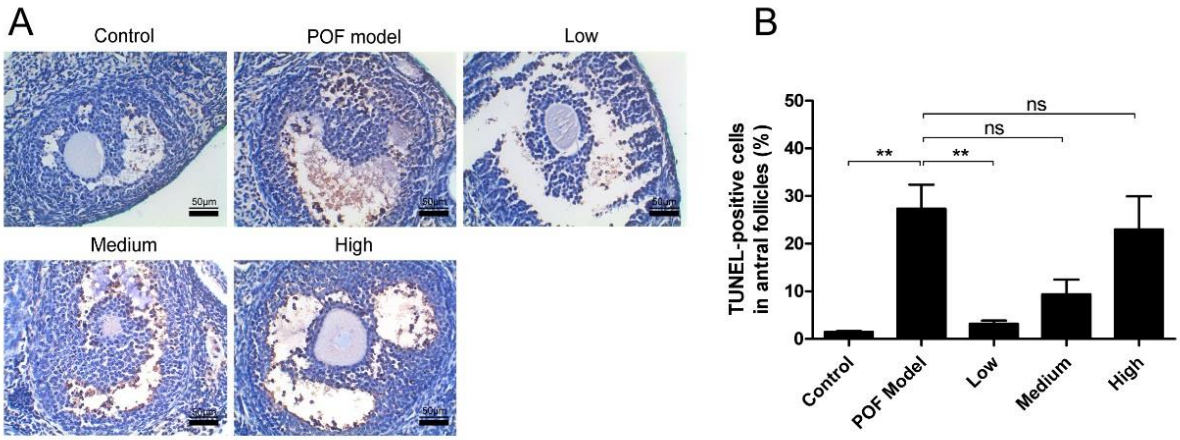

Figure 6

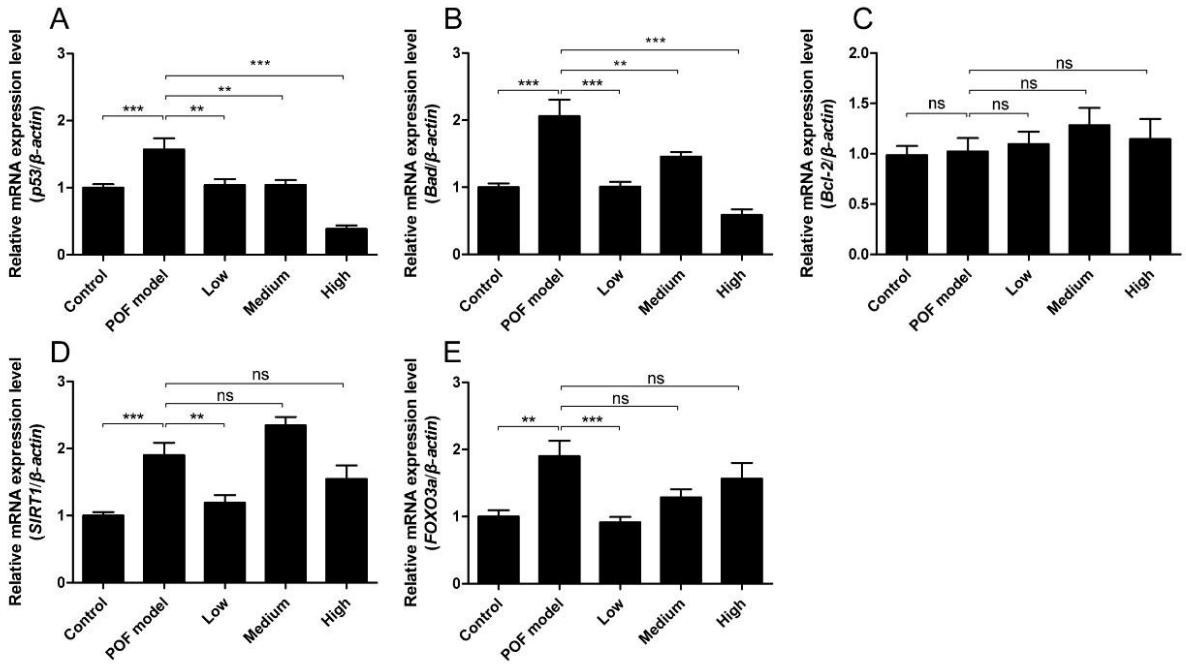

Table 1 Primers used in this study.

Genes		Primer Sequence(5'to 3')	Accession number	Length of product (bp)
β-actin	Forward	AAGTGTGACGTTGACATCCG	NM_007393.5	94
	Reverse	TCTGCATCCTGTCAGCAATG		
Amh	Forward	TCCTACATCTGGCTGAAGTGATATG	NM_007445.2	166
	Reverse	CAGGTGGAGGCTCTTGGAACT		
SOD2	Forward	GTGAACAATCTCAACGCCA	NM_013671.3	189
	Reverse	GATAGCCTCCAGCAACTCT		
FOXO3a	Forward	TTTGTCCCAGATCTACGAGTGG	NM_019740.2	139
	Reverse	ATTCTGAACGCGCATGAAGC		
SIRT1	Forward	ACGCCCACAAAAGGAATTGG	NM_019812.3	81
	Reverse	TCAGGTGAACTTGAGTCTTCCG		
p53	Forward	TGAGCCAGGAGACATTTTCAGG	NM_011640.3	85
	Reverse	AACAGATCGTCCATGCAGTG		
Bad	Forward	AGGATGAGCGATGAGTTTGAGG	NM_007522.3	81
	Reverse	TTGTCGCATCTGTGTTGCAG		
Bcl-2	Forward	TTCGCAGAGATGTCCAGTCAG	NM_009741.5	134
	Reverse	ACCCCACCGAACTCAAAGAAG		