www.czasopisma.pan.pl PAN POLSKA AKADEMIA NAUK www.journals.pan.pl

Polish Journal of Veterinary Sciences Vol. 23, No. 2 (2020), 235–245

DOI 10.24425/pjvs.2020.133638

Original article

17 beta-estradiol affects proliferation and apoptosis of canine bone marrow mesenchymal stem cells *in vitro*

Z.-H. Zhou[1], C.-W. Gu[1,2], J. Li[1], X.-Y. Huang[1], J.-Q. Deng[1], L.-H. Shen[1], S.-Z. Cao[1], J.-L. Deng[1], Z.-C. Zuo[1], Y. Wang[1], X.-P. Ma[1], Z.-H. Ren[1], S.-M. Yu[1†]

[1] College of Veterinary Medicine, Sichuan Agricultural University, 211 Huimin Road, Chengdu 611130, China
[2] Laboratory Animal Centre, Southwest Medical University, 1 Xianglin Road, Luzhou 646000, China

Abstract

Emerging researches in humans, pigs and mice, highlighted that estrogen plays a pivotal role in self-renewal and differentiation of bone marrow mesenchymal stem cells (BMSCs). The present study aimed at evaluating effects of 17 beta-estradiol (E2) on proliferation and apoptosis of canine-derived bone marrow mesenchymal stem cells (cBMSCs) *in vitro*. The results showed that E2 supplementation at the concentration of 10^{-11} M promoted the proliferation of cBMSCs by CCK-8 assay and RT-qPCR analysis for the proliferation-related genes, with proliferating cell nuclear antigen (*PCNA*), cyclin-D1 (*CCND1*) being up-regulated and cyclin-dependent kinase inhibitor 1B (*CDKN1B*) being down-regulated. Contrarily, analysis of fluorescence-activated cell sorting (FACS) and RT-qPCR demonstrated that E2 supplementation above 10^{-11} M had inhibitory effects on the proliferation of cBMSCs and induced apoptosis. Intriguingly, cBMSCs still possessed the capability to differentiate into osteoblasts and adipocytes with 10^{-11} M E2 addition. Taken together, this study determined the optimal culture condition of cBMSCs *in vitro,* and has important implications for further understanding the regulatory effect of E2 on the self-renewal of cBMSCs, which are helpful for the clinical application of BMSCs.

Key words: apoptosis, bone marrow mesenchymal stem cells (BMSCs), canine, proliferation, 17 beta-estradiol (E2)

Introduction

Mesenchymal stem cells (MSCs) have attracted considerable attention as they have the capacity to be one of the ideal cell sources, they can be harvested from a wide variety of sources such as bone marrow, fat, umbilical cord, cord blood and placenta. Moreover, MSCs have a crucial potential of self-renewal, multiple differentiation and immune modulation (Krause 2002, Grove et al. 2004). After isolated and cultured *in vitro*, MSCs have been used extensively in tissue injury, congenital defects and hereditary diseases, thus the relevant studies that applied MSCs to cell therapy in humans and other species are of intriguing interest (Uccelli et al.

Correspondence to: S.-M. Yu, e-mail: yayushumin@sicau.edu.cn, tel./fax: +86-02886291162
Z.-H. Zhou and C.-W. Gu contributed equally to this work.

2011, Liu et al. 2014, De Becker et al. 2016, Niada et al. 2016). Notably, there is a great deal of current research interest in bone marrow-arrived MSCs (BMSCs), which are considered as one of the future candidates for tissue engineering, cell and gene therapy (Uccelli et al. 2011), which are similar to adipose-derived MSCs in availability. However, various physiological factors including age, origin and the number of generations have been demonstrated to impair their capacities of proliferation and differentiation (Lee et al. 2016, Lee et al. 2017). Moreover, the major obstacle in cell therapy is the poor and insufficient viability as well as the low number of transplanted cells reaching the target tissues that significantly limit their potential of therapeutic effects. Therefore, to meet the clinical needs, *in vitro* culture of BMSCs seems to be a great strategy, and optimizing its culture conditions should be a top priority among all work.

It has been generally accepted that estrogen is one of the critical factors of bone formation and renewal, and the addition of 17 beta-estradiol (E2) *in vitro* can improve the proliferation and differentiation ability of BMSCs in humans and other mammalian species. Previous researchers confirmed that E2 supplements increase telomerase activity in human MSCs during proliferation via the regulation of ER-α, but not ER-β (Cha et al. 2008). Further studies have shown that E2 at the suitable concentrations promotes proliferation and inhibits apoptosis of BMSCs, but there are species and gender differences in E2 effects on BMSCs (Hong et al. 2009, Hong et al. 2011). For example, previous studies have demonstrated the effects of E2 on increasing proliferation of MSCs in rats, yet its effects on human MSCs were controversial (DiSilvio et al. 2006). At present, the research of the effects of the E2 on BMSCs mostly aims at humans, pig and murine. Canine as a companion animal and a kind of crucial experimental model, has obviously unique reproductive properties. Canine-derived BMSCs (cBMSCs) have already been used in treatments of diseases. cBMSCs can promote bone formation significantly, either by systemic injection or transplantation (Liu et al. 2014, Dehghan et al. 2015), which has opened a new chapter in cBMSCs research field.

Nevertheless, there is still a lack of research on the influence of E2 on cBMSCs. Furthermore, the effect of E2 on BMSCs is critical, thus understanding the mechanism of their interaction is vital for obtaining the maximum efficiency from this collaboration. This study evaluated the effects of E2 on proliferation and apoptosis of *in vitro* cultured cBMSCs, to discuss the effects of E2 on cBMSCs *in vitro* for the first time, providing a reliable theoretical basis for clinical application of cBMSCs in tissue engineering and regeneration.

Materials and Methods

Isolation and culture of primary cBMSCs

All animal experiments were conducted in strict accordance with regulations of the animal protection laws of the People's Republic of China (a draft of an animal protection laws in China was released on September 18, 2009) and in compliance with the Sichuan Agricultural University for Laboratory Animal Care recommendations for the care and use of laboratory animals (Ya'an, China; Approval No. 2013-028). In order to ensure that MSCs in bone marrow samples were not affected by estrogen *in vivo*, all bone marrow samples were obtained from male dogs. Under relevant guidelines and regulations, the bone marrow was individually punctured from the femur bone in six sexually mature male beagle dogs after anesthesia with pentobarbital. After being isolated with gradient centrifugation, the nucleated cell fraction was pelleted out by centrifugation at 450g for 25 minutes before being resuspended in PBS, then the sample was centrifuged at 110g for 10 minutes, the supernatant was discarded, and the pellet was plated and cultured in the low--glucose Dulbecco's Modified Eagle's Medium (LG-DMEM; GIBCO, Grand Island, NY, USA) containing 10% FBS (Sigma-Aldrich, St. Louis, Missouri, USA) and penicillin-streptomycin (100 IU and 100 μg/m*l*) at 37°C with 5% humidified CO_2. The medium was changed every 3 days. Flow cytometry findings revealed that these cells were positive for CD29, CD90 and CD105, but negative for CD31 and CD34, indicating that these cells were BMSCs, which have been demonstrated in our previous study (Li et al. 2017). The collected cBMSCs were partly for isolation and culture, and the rest of them were used for characterization, the cells were cultured to 80% confluence and then sub-cultured. Cells of passage 3 to 5 isolated from 6 dogs were used in the following experiments.

Proliferative cBMSCs evaluated by CCK-8 assays

cBMSCs at the exponential growth phase were placed into 96-well culture plates at a density of 4×10^5/well. After incubated for 12h, cells were exposed to the media supplemented with 0, 10^{-7}, 10^{-9}, 10^{-11}, 10^{-13} and 10^{-15} M 17 β-estradiol (Sigma-Aldrich, St. Louis, Missouri, USA) for 5d. The concentrations of E2 were selected based on successful experiments in rats, pigs and humans (Hong et al. 2009, Hong et al. 2011, Lee et al. 2016). Cell samples were collected on day 3 and day 5 separately, cell viability was quantified with the Cell counting kit-8 assay (CCK-8; Dojindo, Kumamoto, Japan), following the manufacturer's pro-

Table 1. List of primers used for reverse transcription-quantitative polymerase chain reactions.

Gene	Primer sequence (5'-3')	PCR product size (bp)	GeneBank accession number
GAPDH	F: TCCCGCCAACATCAAA R: TCACGCCCATCACAAAC	163	NC_006609.3
β-actin	F: CGGAACGCAAGTATTCTGTG R: CGATGGACGATGGAGGG	118	AF021873
PCNA	F: GAGCGGCGTAAACCTG R: GCATTATCTTCAGCCCTTA	191	NC_006606.3
CCND1	F: CCAGTGGCAGAGGAGAACA R: GGGTGGGTTGGAAATGAA	95	NC_006600.3
CDKN1B	F: CGGATGGACGCCAGACA R: ACCTCCTGCCACTCGTATTT	193	NC_006609.3
Bc1-2	F: ATTGTGGCCTTCTTTGAGTTCG R: GCATCCCAGCCTCCGTTGT	151	NC_006583.3
Bax	F: CAAACTGGTGCTCAAGGC R: GCACTCCAGCCACAAAGA	187	NC_006583.3
Caspase-3	F: TCACTTTGTGCGATGC R: TTCTGTTGCGACCTTT	86	NC_006598.3
Caspase-8	F: GCAGATGCGTTGAGTAA R: CATAGATGATGCCCTTGT	174	NM_001048029

tocols, and then absorbance was measured by ultra-microspectrophotometer (Nanodrop 2000, Thermo, Wilmington, DE, USA) at 450 nm.

Apoptotic cBMSCs evaluated by Fluorescence-activated cell sorting (FACS) against Annexin V-FITC/PI double-labeling

cBMSCs were cultured in 6-well plates at a density of 2×10^5/well. The sample collection and treatments were the same as those applied for the CCK-8 assay. After being harvested, the cells were washed twice with PBS before centrifugation. According to the manufacturer's protocols, the cells were filtered before staining, after that, the samples were suspended in 100µl binding buffer with Annexin V-FITC and incubated for 15 min, then after adding 5 µl propidium iodide (PI) (Tianjin Sungene Biotech, Tianjin, China) incubated for 5 min at room temperature in the dark. The samples were analyzed using a BD Accuri™ C6 cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Gene expression analysis by reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

cBMSCs were placed into 24-well culture plates at a density of 1×10^5/well. The sample treatments were the same as those applied for the CCK-8 assay. Gene expression of cell proliferation-related genes (*PCNA*, *CCND1*, *CDKN1B*) and cell apoptosis-related genes (*Bc1-2*, *Bax*, *Caspase3*, *Caspase8*) were analyzed by RT-qPCR (CFX96™ Real-Time PCR Detection System, Bio-Rad, Hercules, CA, USA). In accordance with the manufacturer's instructions, total RNA was extracted from the cBMSCs using the RNAiso Plus (Takara, Shiga, Japan), reverse transcription was performed using the PrimeScript™ RT reagent Kit (Takara, Shiga, Japan), and Real Time PCR was performed using the SYBR® Premix Ex Taq™ II (Takara, Shiga, Japan). Real time PCR reaction conditions were as follows: target genes were amplified in 45 cycles of denaturation 95°C (30 sec), annealing 95°C (5 sec), and elongation 60°C (30 sec), and then melting curve analysis was performed. *Actin* was used as an internal control gene; all mRNA data were normalized to the level of *Actin*. Detailed information regarding each specific primer is presented in Table 1.

In vitro differentiation

According to the above experimental results, cBMSCs were isolated and cultured in the medium supplemented with 10^{-11} M E2. After being subcultured to the 5th passage, cBMSCs were induced to differentiate into adipocytes in adipogenic differentiation medium: 10% FBS (Sigma-Aldrich, St. Louis, Missouri, USA), 100 U/m*l* mycillin (Sigma-Aldrich, St. Louis, Missouri, USA), 1 µM dexamethasone (Sigma-Aldrich, St. Louis, Missouri, USA), 10 mg/L insulin (Sigma-Aldrich, St. Louis, Missouri, USA), 200 µM indometacin (Sigma-Aldrich, St. Louis, Missouri, USA) and 100 mg/L 3-isobutyl-1-methylxanthine (Sigma-Aldrich, St. Louis, Missouri, USA), and

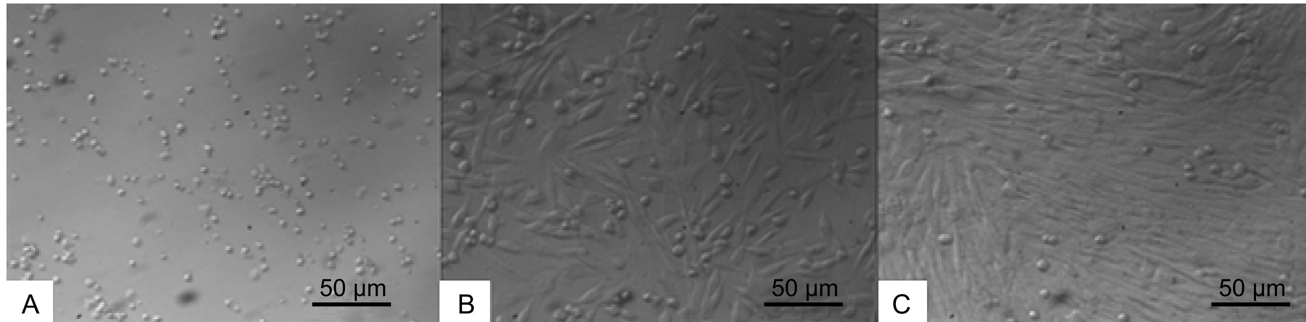


Fig. 1. The morphology of primary cells of canine-derived bone marrow mesenchymal stem cells (cBMSCs) cultured at day 2 (A), day 4 (B) and day 7 (C).

into osteoblasts in osteogenic differentiation medium: 10% FBS (Sigma-Aldrich, St. Louis, Missouri, USA), 100 U/m*l* mycillin (Sigma-Aldrich, St. Louis, Missouri, USA), 10 nM dexamethasone (Sigma-Aldrich, St. Louis, Missouri, USA), 50 μM Vitamin C (Sigma-Aldrich, St. Louis, Missouri, USA), and 10 nM β-glycerolphosphate (Sigma-Aldrich, St. Louis, Missouri, USA) separately. In osteogenic differentiation medium, the alkaline phosphatase (ALP, Sigma-Aldrich, St. Louis, Missouri, USA) activity was evaluated on day 7, the matrix mineralization and calcium deposits were detected by Alizarin Red staining (Sigma-Aldrich, St. Louis, Missouri, USA) on day 21. In adipogenic differentiation medium, on day 21, intracellular red-colored lipid droplets within differentiated adipocytes were identified by Oil Red O staining (Sigma-Aldrich, St. Louis, Missouri, USA). Cells were observed under an inverted microscope (IX70, Olympus, Tokyo, Japan).

Statistical analysis

All data are presented as the mean ± standard deviation. All quantitative data were analyzed by one-way ANOVA after normal distribution testing with the use of SPSS20, p-values less than 0.05 were considered as significant.

Results

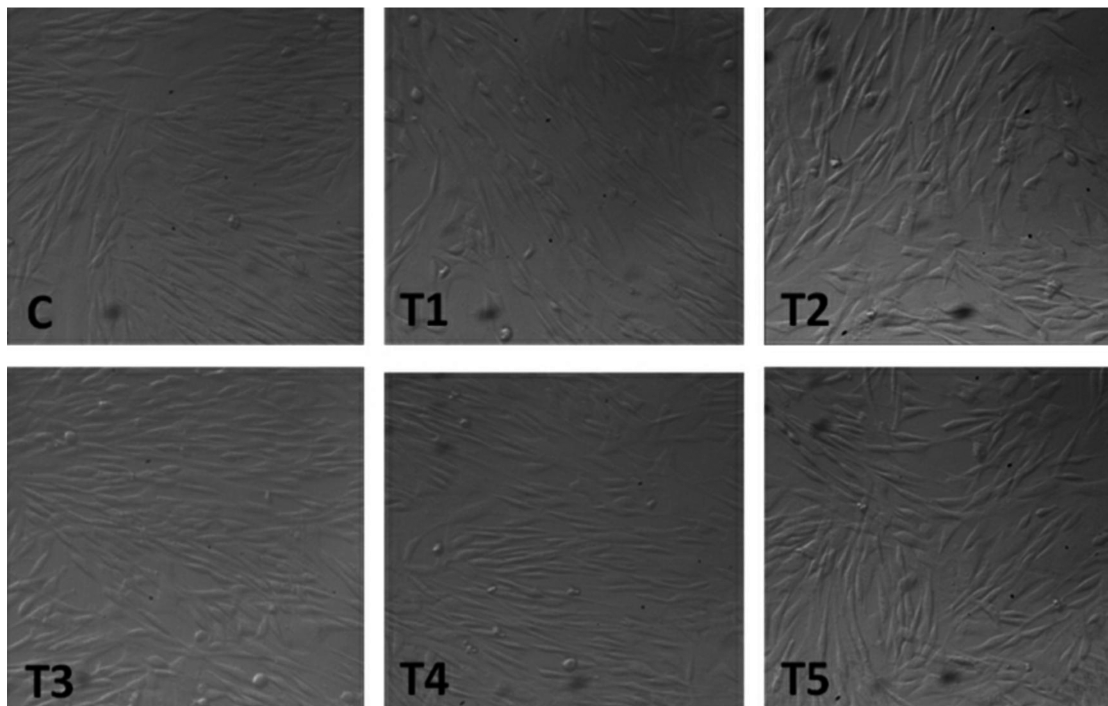
Isolation and culture of primary cBMSCs

The cBMSCs were isolated from the bone marrow of male dogs within six months. As shown in Fig. 1, on day 2, some adherent cells began to exhibit the spindle or polygonal shape (Fig. 1A). On day 4, the adherent cells proliferated significantly and extended to a long spindle shape with fibroblast-like morphology (Fig. 1B). On day 7, the boundaries between cells were not clear, and the arrangement had a spiral shape (Fig. 1C).

Effects of E2 supplementation on the proliferation of cBMSCs

Cells were cultured in the supplementary medium with different concentrations of E2: 0 (C), 10^{-7} M (T1), 10^{-9} M (T2), 10^{-11} M (T3), 10^{-13} M (T4) and 10^{-15} M (T5), and cell morphology was observed under the microscope on day 3 and day 5 respectively. CCK-8 assay showed that on day 3, the proliferation rates in the 10^{-7} M and 10^{-9} M E2 groups were significantly lower compared with such three groups as 0, 10^{-11}, 10^{-13} M of E2 ($p<0.05$). In 10^{-11} M E2 group, the proliferation of cBMSCs was significantly higher when compared with that in 10^{-7} and 10^{-9} M group ($p<0.05$), not significantly compared with that in 0, 10^{-13} and 10^{-15} M group ($p>0.05$). On day 5, there were no significant differences on cBMSCs in all the groups ($p>0.05$). This suggested that E2 above 10^{-11} M had significantly inhibitory effects on cBMSCs proliferation, especially the E2 of 10^{-7} M (Fig. 3A).

PCNA, *CCND1* and *CDKN1B* are often used to detect the proliferative activties of cells (Chu et al. 2008, He et al. 2013). Thus, we evaluated the E2 effects on cBMSC proliferation by RT-qPCR analysis for mRNA expression of the genes *PCNA*, *CCND1* and *CDKN1B*. The expression levels of *PCNA* and *CCND1* were significantly up-regulated on day 3 in cBMSCs compared with all other groups when supplemented with 10^{-11} M E2 ($p<0.05$). Contrarily, expression levels of *PCNA* and *CCND1* were significantly down-regulated in 10^{-7} M E2 group compared with all other groups ($p<0.05$). Furthermore, changes in expression of these two genes in cBMSCs were not significant after supplementation with 10^{-13}, 10^{-15} M E2. Likewise, the expression of *CDKN1B* was significantly down-regulated after supplementation with 10^{-11} M E2 ($p<0.05$), and there were no significant effects observed when supplementation with 10^{-7}, 10^{-9}, 10^{-13},10^{-15} M E2 (Fig. 3B). While on day 5, only the expression of *CCND1* was significantly down-regulated when supplemented with 10^{-7} M E2 ($p<0.05$), and there were no



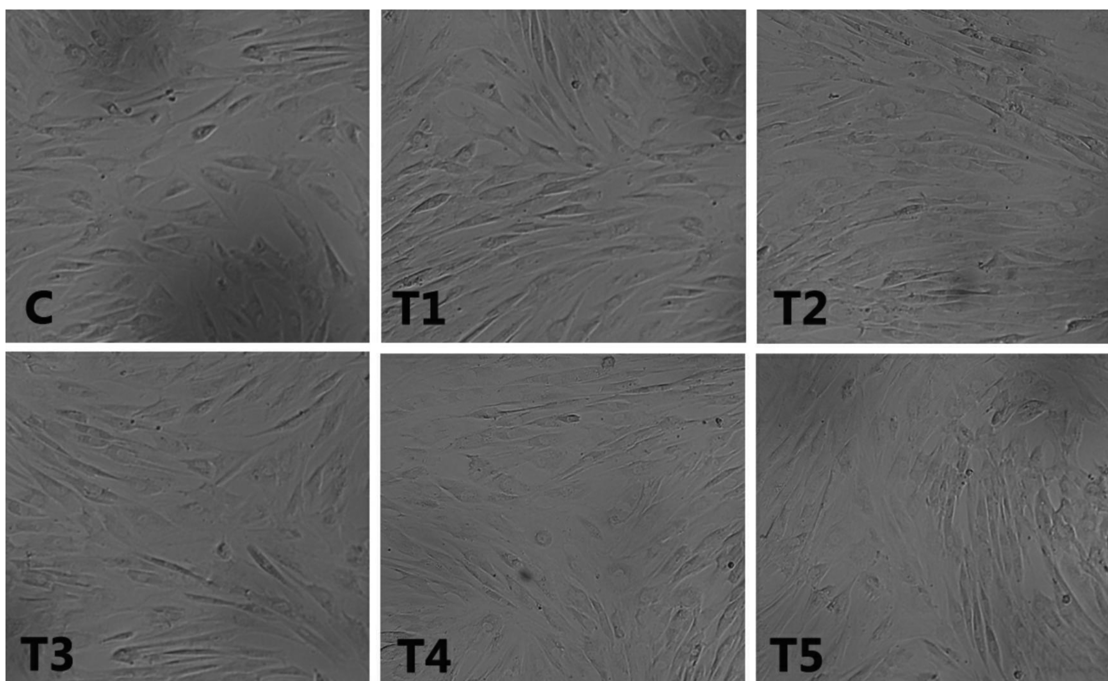


Fig. 2. The morphology of canine bone marrow mesenchymal stem cells (cBMSCs) cultured in media supplemented with different E2 concentrations on day 3 and day 5: 0 (C), 10^{-7}M (T1), 10^{-9}M (T2), 10^{-11}M (T3), 10^{-13}M (T4) and 10^{-15}M (T5) on day 3.

significant differences in the expression of these three genes (*PCNA*, *CCND1*, *CDKN1B*) considering the concentrations of E2 on cBMSCs (Fig. 3C). In conclusion, the above results suggested that E2 at 10^{-11} M promoted cBMSCs proliferation owing to up-regulation of *PCNA* and *CCND1,*and down-regulation of *CDKN1B*.

Effects of E2 supplementation on apoptosis of cBMSCs

FACS for cells stained by Annexin V-FITC and PI, were performed to evaluate apoptosis of cBMSCs cultured in media supplemented with various concentrations of E2 on day 3 and day 5 (Fig. 2). As shown in Fig. 4A and Fig. 5, on day 3, the apoptosis ratios of cBMSCs cultured with the 10^{-7} and 10^{-9} M E2 were significantly

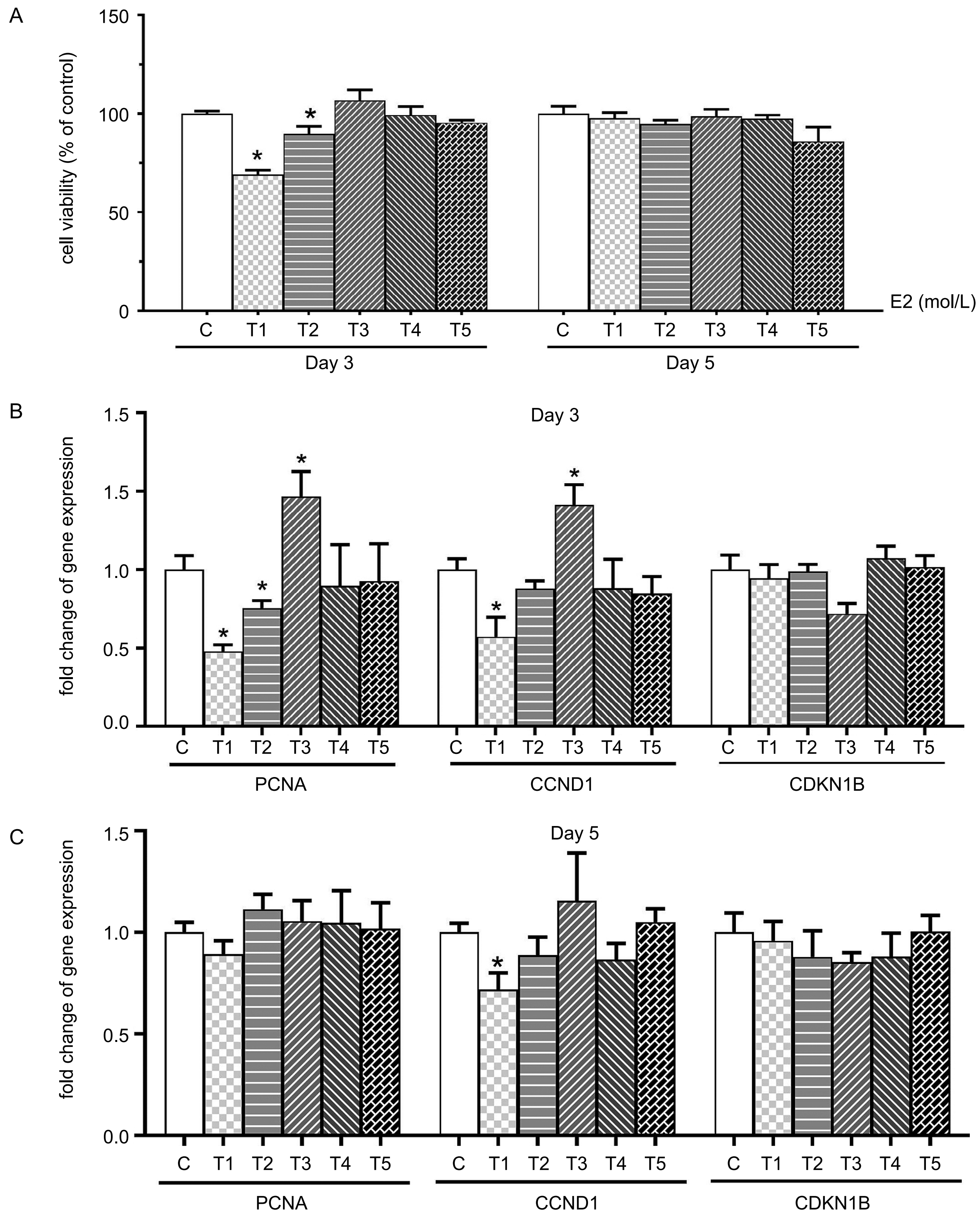


Fig. 3. Analysis of cell viability and proliferation. Cell viability evaluated by CCK-8 assay (A), mRNA relative quantification of the proliferation-related genes, *PCNA*, *CCND1* and *CDKN1B*, was detected by RT-qPCR (B, C), in canine-derived bone marrow mesenchymal stem cells (cBMSCs) cultured in the media supplemented with different E2 concentrations on day 3 and day 5 respectively. C and T1-T5 represent 0, 10^{-7}, 10^{-9}, 10^{-11}, 10^{-13} and 10^{-15}M. Data are presented as means ± SEM, from three independent biological replicates. (*$p<0.05$).

higher than the others ($p<0.05$), while supplemented with 10^{-11} M E2, the apoptosis ratios of cBMSCs were slightly decreased compared with that in such groups as 0, 10^{-13} and 10^{-15} M. As shown in Fig. 4A and Fig. 6, on day 5, the apoptosis ratio of cBMSCs cultured with the 10^{-7} M E2 was significantly higher than that in the

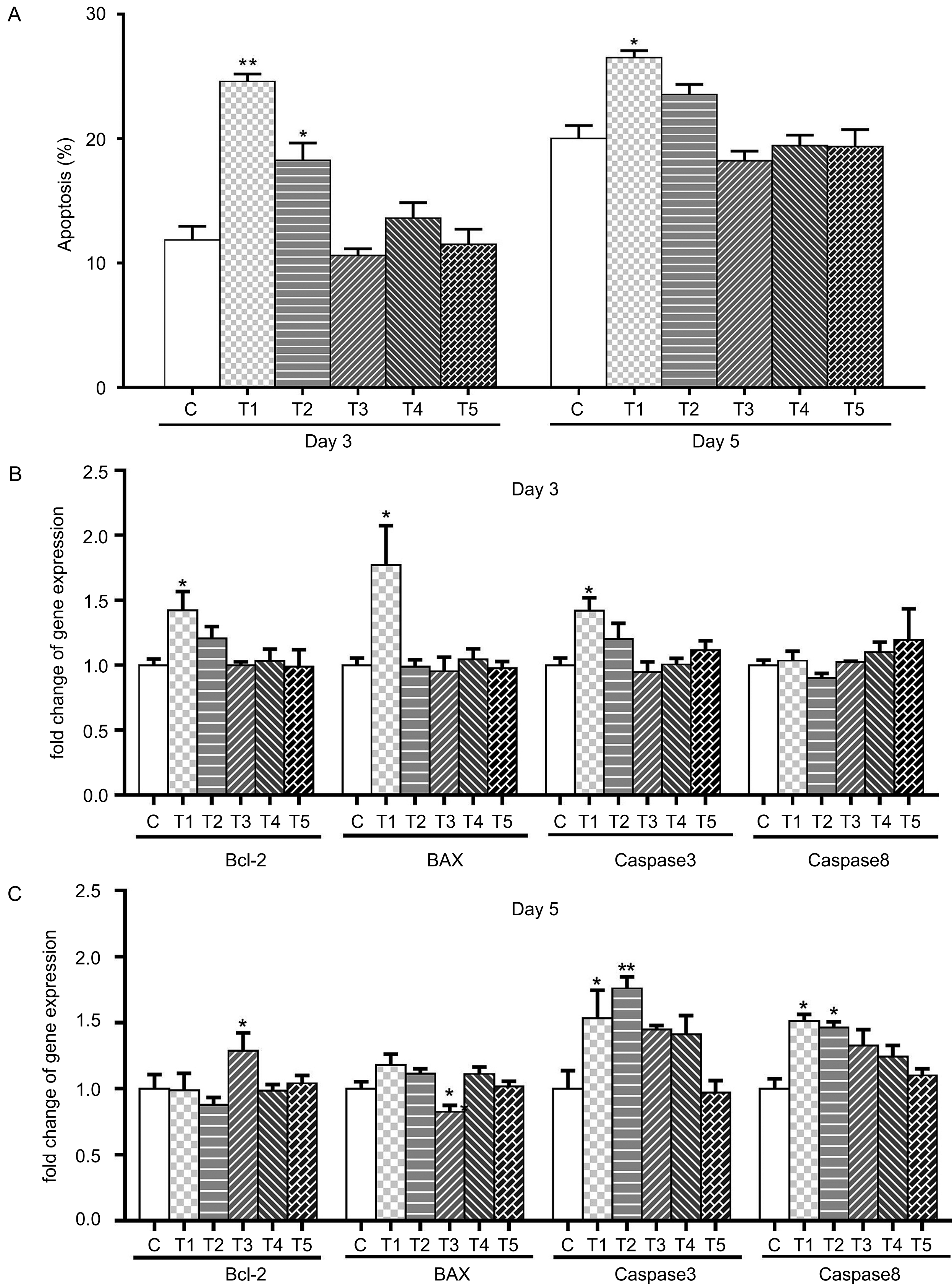


Fig. 4. cBMSCs apoptosis rate and transcription level of apoptosis-related genes. The apoptosis rate of cBMSCs was detected by flow cytometry (A). RT-qPCR was used to detect the mRNA levels of the apoptosis-related genes, *Bcl2*, *Bax*, *Caspase3* and *Caspase8* (B, C). C and T1-T5 represented 0, 10^{-7}, 10^{-9}, 10^{-11}, 10^{-13} and 10^{-15}M. Data are presented as means ± SEM, from three independent biological replicates. (*$p<0.05$).

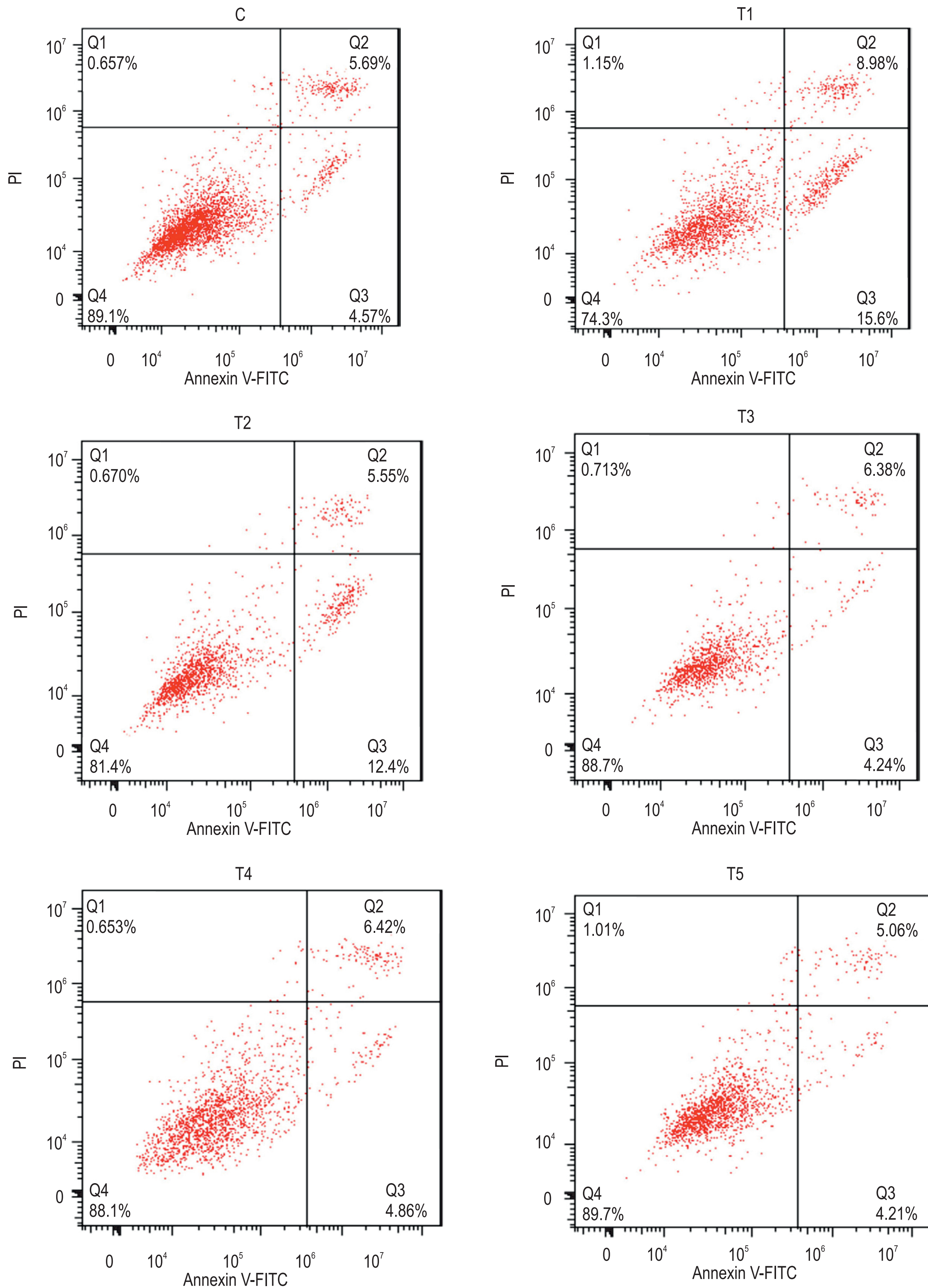


Fig. 5. Apoptotic analysis of canine bone marrow mesenchymal stem cells (cBMSCs) evaluated by FACS against Annexin V-FITC/PI double labeling on day 3. Out of all four, Q4 displays normal cells (FITC-, PI-), Q1 shows dead cells (FITC-,PI+), Q2 shows late apoptotic and necrotic cells (FITC+, PI+), and Q3 represents early apoptosis cells (FITC+, PI−). C and T1-T5 represent 0, 10^{-7}, 10^{-9}, 10^{-11}, 10^{-13} and 10^{-15}M.

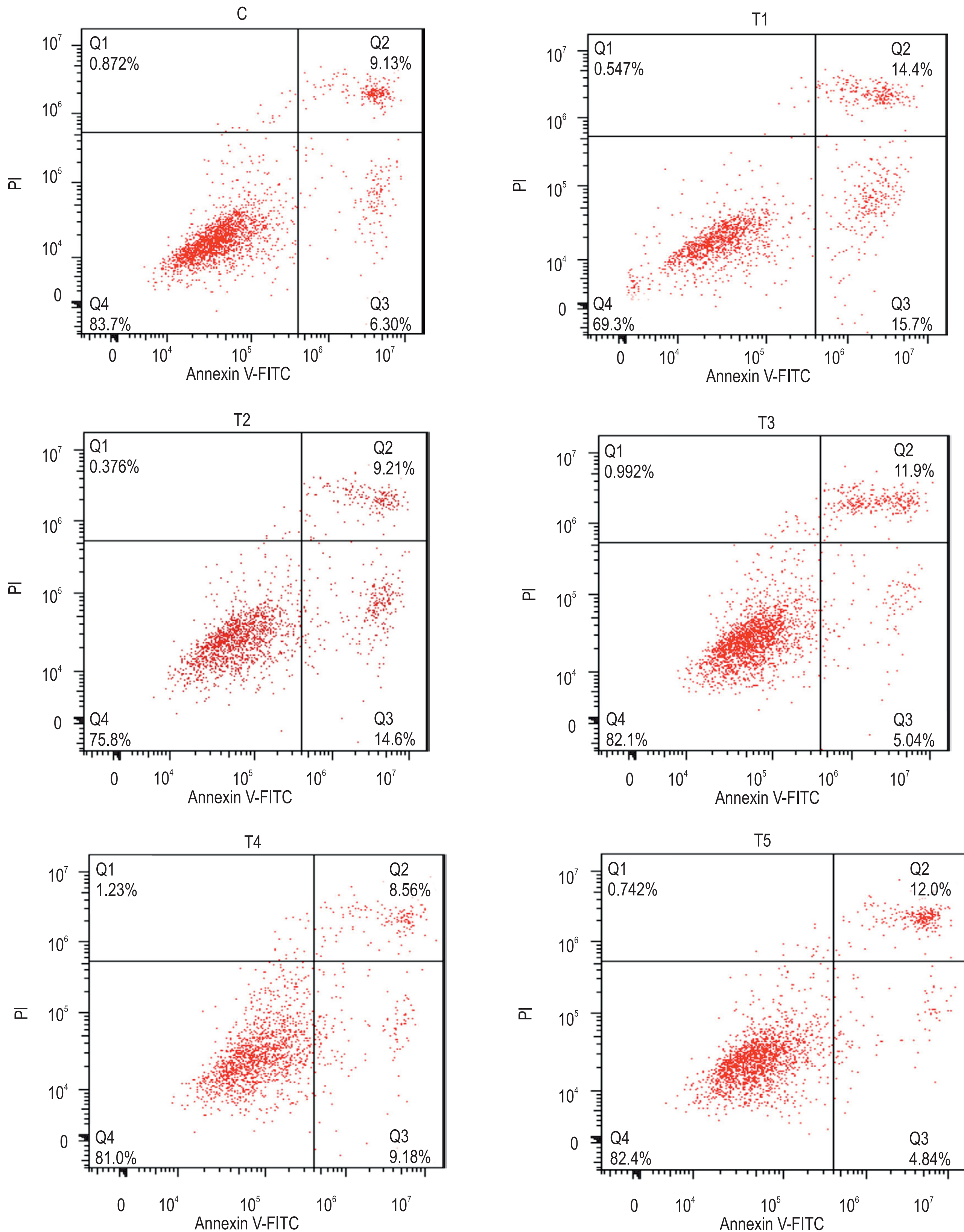


Fig. 6. Apoptotic analysis of canine bone marrow mesenchymal stem cells (cBMSCs) evaluated by FACS against Annexin V-FITC/PI double labeling on day 5. Out of all four, Q4 displays normal cells (FITC-, PI-), Q1 shows dead cells (FITC-,PI+), Q2 shows late apoptotic and necrotic cells (FITC+, PI+), and Q3 represents early apoptosis cells (FITC+, PI−). C and T1-T5 represent 0, 10^{-7}, 10^{-9}, 10^{-11}, 10^{-13} and 10^{-15}M.

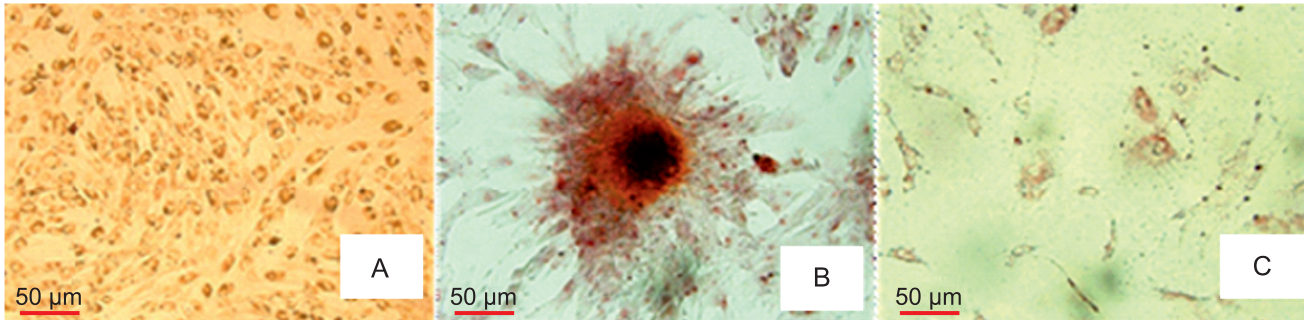


Fig. 7. cBMSCs cultured in the media supplemented with 10^{-11}M E2, could differentiate into osteoblasts stained by alkaline phosphatase (Fig. 4.A) and Alizarin red (Fig. 4B), and adipocytes stained by Oil red (Fig 4.C), ×200.

other groups ($p<0.05$). RT-qPCR was used to detect apoptosis-related genes *Bcl2*, *Bax*, *Caspase3* and *Caspase8*, *Bcl2* and *Bax* are members of the Bcl2 family with different function (Edlich 2018), while *Caspase3* and *Caspase8* are markers of apoptosis (Weng et al. 2015). The results showed that on day 3, the expression of pro-apoptotic gene *Bax*, was significantly up-regulated in BMSCs in the 10^{-7} M E2 group ($p<0.05$), compared with that in the control group, and the expression of *Bcl2*, *Caspase3*, was significantly up-regulated simultaneously (Fig. 4B) ($p<0.05$). On day 5, the expression of *Caspase3* and *Caspase8* was significantly up-regulated in 10^{-7} and 10^{-9} M E2 group compared with that in 0 and 10^{-15} M E2 group ($p<0.05$). Furthermore, the expression of pro-apoptotic gene *Bax* in 10^{-11} M E2 group was down-regulated significantly (Fig. 4C). Above all, this suggests that high concentrations of E2 (10^{-7}, 10^{-9} M) induce apoptosis. Nevertheless, the concentration of 10^{-11} M E2 has an effect of inhibiting apoptosis. So we can conclude that 10^{-11} M is supposed to be the optimal concentration for supplementation.

In vitro differentiation of cBMSCs into osteoblasts and adipocytes

In order to validate the effect of E2 on the differentiation of cBMSCs, cBMSCs cultured in the medium supplemented with 10^{-11} M E2 were isolated and subcultured. Then we examined the osteogenic and adipogenic differentiation ability of cBMSCs at 10^{-11} M E2. For osteogenic differentiation, on day 7, cells were short spindle-shaped and examined by staining with alkaline phosphatase (ALP), which appeared positive (Fig. 7A). On day 21, the differentiated cells were analyzed for the presence of matrix mineralization and calcium deposits (orange-red colored) after staining with Alizarin red (Fig. 7B). For adipogenic differentiation, after staining with 0.5% Oil red O, the cells were long fiber-shaped and became shorter gradually, and intracellular red-colored lipid droplets were observed (Fig. 7C). The results showed that 10^{-11} M E2 had no negative regulation on the differentiation of cBMSCs into osteoblasts and adipocytes.

Discussion

Accumulating evidence in treatments of canine diseases shows that cBMSCs have given good results. Likewise, as an essential steroid hormone, estrogen, plays a crucial role in bone formation and development of human and mammalian species by regulating the self-renewal and differentiation of BMSCs.

However, the range of E2 concentrations for the proliferation of BMSCs varies from species to species and by gender. Liuhong et al. (Hong et al. 2011) confirmed that in humans, E2 promoted the proliferation of MSCs in a dose-dependent manner with the concentration range from 10^{-12} M to 10^{-8} M for males while 10^{-10} M to 10^{-8} M for females. Subsequently, Lee et al. (Lee et al. 2016) demonstrated that supplementation of E2 at 10^{-12} M-10^{-14} M and 10^{-12} M *in vitro*, respectively promoted the proliferation of BMSCs in male and female mini-pigs. Moreover, BMSCs from female rat showed increased cell proliferation in response to 10^{-10} M–10^{-12} M E2, while male-derived BMSCs were not affected by E2 (Hong et al. 2009). In our study, the results of CCK8 assay and RT-qPCR showed that different concentrations of E2 have different effects on the proliferation and apoptosis of cBMSCs. We selected three genes to analyze the mRNA levels of cBMSCs, *PCNA*, *CCND1* and *CDKN1B*, which are closely associated with cell proliferation (Chu et al. 2008, Artaza et al. 2010). The cells subcultured in the medium with the concentration of E2 at 10^{-11} M showed the effect of improving the proliferative activity of cBMSCs, and without affecting its ability to differentiate into adipocytes and osteoblasts. Other researchers also proved that BMSCs differentiation capacity was maintained with E2 supplementation (Zhang et al. 2012, Zamani Mazdeh et al. 2017). However, E2 inhibited proliferation and induced apoptosis at concentrations of 10^{-7}-10^{-9}

M, while had no effect on cBMSCs at 10^{-13}-10^{-15} M. The results revealed that there was no dose-dependent effect, and the concentration range of promoting proliferation was only at 10^{-11} M, which is in line with the previous researches that the optimal concentration of E2 to promote proliferation of BMSCs may vary in different species.

Besides, a remarkable result we found is that high concentrations of E2 strongly inhibited cell proliferation and induced apoptosis. The cause contributing to this situation is closely related to the biphasic effect of steroids, which indicated that steroids could regulate cell proliferation and differentiation at relatively lower concentrations, but inhibit cell proliferation or even lose their function at high concentrations (Hong et al. 2009, Lee et al. 2016).

Generally, apoptosis is an important indicator of cell aging; E2 is critical in inhibiting cell senescence and apoptosis of BMSCs as many studies confirmed. At present, an array of methods are used to analyze cell apoptosis, such as TUNEL, RT-qPCR, β-galactosidase staining and so on. For instance, TUNEL staining and qRT-PCR were performed by Lee et al. (Lee et al. 2016) in the study of the effects of E2 on the aging of BMSCs in mini-pigs. In the present study, we quantitatively analyzed the mRNA levels of Bcl2 family, anti-apoptotic member *Bcl2* and pro-apoptotic member *Bax* (Edlich 2018), *Caspase8* and *Caspase3*. Several studies have demonstrated that the caspase family is able to promote and implement cell apoptosis in mammalian cells (Weng et al. 2015), *Caspase8* plays a central role in executing cell apoptosis in the cell death receptor downstream pathway (Hacker et al. 2007). At first, FasL initiates apoptosis by binding to the Fas receptor, triggering the recruitment of Fas-associated death domain protein (FADD). Secondly, FADD binds to procaspase8 by interacting with the death effector domain. This complex (FasL–Fas–FADD–procaspase8) catalyzes the proteolytic cleavage and transactivation of procaspase8 to generate *Caspase8* (Broughton et al. 2009). The activation of *Caspase8* then promotes the *Caspase3* activations, which hydrolyzes cell-specific proteins and poly-ADP ribose polymerase, thus inducing cell apoptosis (Weng et al. 2015). Therefore, the activation of *Caspase3* is commonly considered as a sign of apoptosis into the irreversible stage (Hacker et al. 2007, Chai et al. 2014). Moreover, two interesting phenomena were observed on the 5th day. One was that the expression of *Caspase3* and *Caspase8* was up-regulated in 10^{-7} M and 10^{-9} M E2 group, which showed that adding high concentrations of E2 directly led to irreversible apoptosis of cells. The other was that on day 5, there was no decrease in viability in CCK-8 test, yet in FACS there was a marked apoptosis at 10^{-7} M. According to the cell growth curve in our recently published paper (Li et al. 2017), we found that cBMSCs were in the plateau stage on day 5, and during this period, the cells were mostly in the state of stopping division, E2 did not exert a significant influence on proliferation, but exerted a significant influence on apoptosis. Indeed, we also found that the expression of pro-apoptotic gene *Bax* was significantly down-regulated in the 10^{-11} M E2 group, which suggested that the appropriate concentration of E2 could inhibit apoptosis in the medium containing 10% FBS. This conclusion is consistent with the results of Mirzamohammadi (Mirzamohammadi et al. 2016), which proved that E2 treatment could decrease serum deprivation-induced elevated ROS concentration and block the elevation of *Bcl2* and *Bax*.

This study provides a foundation for further researches on the regulation of E2 on the self-renewal of cBMSCs and the optimization of the culture conditions of cBMSCs *in vitro*, which is helpful to the clinical application of MSCs. Nevertheless, further investigations are still warranted to better understand other functional effects of cBMSCs due to species specificities.

Acknowledgements

This research was financially supported by grants from the Chang-jiang Scholars and Innovative Research Team for Universities (Grant No. IRT0848).

References

Artaza JN, Sirad F, Ferrini MG, Norris KC (**2010**) 1,25(OH)2vitamin D3 inhibits cell proliferation by promoting cell cycle arrest without inducing apoptosis and modifies cell morphology of mesenchymal multipotent cells. J Steroid Biochem Mol Biol 119: 73-83.

Broughton BR, Reutens DC, Sobey CG (**2009**) Apoptotic mechanisms after cerebral ischemia. Stroke 40: e331-339.

Cha Y, Kwon SJ, Seol W, Park K-S (**2008**) Estrogen receptor-alpha mediates the effects of estradiol on telomerase activity in human mesenchymal stem cells. Mol Cells 26: 454-458.

Chai JJ, Shi YG (**2014**) Apoptosome and Inflammasome: a Caspase-activating Platform. Prog Biochem Biophys 41: 1056-1062.

Chu IM, Hengst L, Slingerland JM (**2008**) The Cdk inhibitor p27 in human cancer: prognostic potential and relevance to anticancer therapy. Nat Rev Cancer 8: 253-267.

De Becker A, Riet IV (**2016**) Homing and migration of mesenchymal stromal cells: How to improve the efficacy of cell therapy? World J Stem Cells 8: 73-87.

Dehghan MM, Baghaban Eslaminejad M, Motallebizadeh N, Ashrafi Halan J, Tagiyar L, Soroori S, Nikmahzar A,