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Baicalin protects mouse testis from injury induced by heat stress

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ABSTRACT

Heat stress has been documented to reduce reproductive performance of female animals through injury to germ cells, with few studies available in male animals. The objectives of this study were to evaluate protective effects of baicalin on testicular tissue damage of mice subjected to heat stress and its related mechanisms. In this experiment, A total of forty mice were divided into four groups, including control group (C), baicalin group (B), heat stressed group (H) and heat stress with baicalin treatment (H + B) group. Morphological changes, activities of antioxidant enzymes and apoptosis-related parameters in the mice testes tissue were monitored. The results showed that the process of spermatogenesis in mice testis was impaired and the cellular apoptosis increased due to acute heat stress at 41 °C. Interestingly, the tissue damage was alleviated with the significant ($P < 0.05$) increase in the activities of SOD, CAT and GSH-Px enzymes, decrease ($P < 0.05$) in MDA content and number of cellular apoptosis recorded in mice of H + B group compared with those in mice from H group. In addition, the Fas, FasL and P-JNK protein expressions were significantly ($P < 0.05$) increased; and apaf-1, caspase-3, -9 were slightly expressed in the H group, while there was no difference in Bcl-2 expression, compared with C, B and H + B groups. The above results clearly indicate that heat stress induces macroscopic/apoptotic and oxidative changes in the testicular tissue of mice; these changes are alleviated by Baicalin through increasing anti-oxidative enzyme activities and possibly through blocking Fas/FasL pathway.

Organisms have been suffering from serious consequences resulting from rapid global warming. These days, heat stress has become a very serious problem in animal breeding industry, especially due to rapid developments in highly intensive management (Bozaykut et al., 2014). In mammals, the reproduction is the most sensitive process to be damaged by heat stress (Yansen et al., 2013), because the latter affects female animals from egg quality (Xiaonan et al., 2016), follicles (Dash et al., 2016) and embryo (Sakatani, 2017) development to all aspects of pregnancy, while in male animals it reduces sperm production (Xia et al., 2016) and affects semen quality (Xiaotong et al., 2015a, b) through injuring testicular tissue (Chen et al., 2017) and increasing deformed sperms in the ejaculate (Xiaonan et al., 2016) in mice.

A number of studies have demonstrated that baicalin, a flavonoid extracted from and the main active component of *Scutellaria baicalensis* (He et al., 2016), has many pharmacological activities such as antipyretic, and sedative (Zhang et al., 2017), antibacterial and anti-inflammatory (Chen et al., 2014), anti-oxidative (Singh et al., 2017), and anti-cancer. *Scutellaria baicalens* decreases cellular stress and apoptosis through decreasing heat shock protein (HSP) 70, caspase-3 and Bax expressions and increased Bcl-2 expression (Xiaonan et al., 2016). Similarly, Baicalin protects bovine *in vitro*-cultured testicular Sertoli cells from acute injury caused by heat stress (Chen and Riggs, 2011) and prevents lipopolysaccharides (LPS) induced abortion in pregnant mice through regulating the balance of TH1/TH2 cytokines (Xiaodan et al., 2014). Baicalin also has the ability of reducing apoptosis in mice Sertoli cells induced by heat stress through Fas/FasL pathway (Xia et al., 2016). Fas and FasL are two membrane proteins which transmit apoptotic signals and cause apoptosis of Fas antigen-expressing cells when they are cross-linked (Pérez-Crespo et al., 2010). Heat stress causes cell DNA damage (Yao and Somero, 2012) and phosphorylation of C-Jun N-terminal kinase (C-JNK) and stress-activated protein kinase (Lee et al., 1997), which further promotes the cross-linking of Fas and FasL proteins (Pan et al., 2007), activates caspase cascade reaction (Li et al., 2013) and induces cellular apoptosis (Morishima et al., 2001).

It is reasonable to speculate that baicalin would alleviate harmful effects induced by heat stress in animal tissues. However, there is relatively little information available in the existing literature regarding the use of baicalin to prevent apoptosis and/or oxidative damage of animal reproductive organs *in vivo* and also its possible mechanisms. Therefore, the present study was undertaken to explore whether baicalin could protect testicular tissue damage of mice subjected to heat stress and its possible mechanisms.

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1. Materials and methods

1.1. Experimental mice and samples collection

Forty male Kunming mice (6 weeks old) were randomly divided into 4 groups (with 10 mice in each group), including control (C), baicalin (B), heat stress (H) and heat stress with baicalin treatment (H + B) groups. Mice in the groups C and H were intraperitoneally injected with saline and those in the groups B and H + B were injected with 50 mg/kg baicalin (NJJCBIO, Beijing, China) daily for 7 d. The mice in both H and H + B groups were subjected to heat-stress at 41 °C with relative humidity of 80% for 2 h at the eighth day. The mice were killed by cervical dislocation (Zhu et al., 2018) immediately after heat stress and the testes were collected. One testis was stored at −80 °C in a freezer for determination of MDA contents and enzyme activities, while the other testis was fixed in the Boulin's solution for H & E staining and apoptosis analysis.

1.2. Measurements of MDA contents and enzyme activities

The stored testis was comminuted ultrasonically to make 10% tissue homogenate, which was then centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant was separated and activities of SOD (U/mg protein), MDA (nmol/mg protein), CAT (U/mg protein) and GSH-PX (U/mg protein) in the supernatant were determined spectrophotometrically using their corresponding diagnostic reagent kits (NJJCBIO), following the manufacturer's instructions (Jianru et al., 2017). The protein concentrations were determined by using BCA Protein Assay Kit (Thermo, California, USA), as described previously (Almahrouki et al., 2014).

1.3. Testicular tissue processing for histological staining

About 5-μm thick sections of the testicular tissue were prepared for morphology and apoptosis analysis. The hematoxylin and eosin (H & E) staining (ROCHE, Shanghai, China) and the terminal dUTP nick-end labeling (TUNEL) staining (ROCHE) were performed according to the manufacturer's instructions (Breikaa et al., 2013). TUNEL staining was considered to be positive if the nuclei were stained brown when examined under light microscope (OLYMPUS IX-71, Tokyo, Japan).

1.4. Immunohistochemical detection of apoptosis indices

The expression and localization of Bcl-2, Apaf-1, caspase-9 and caspase-3 proteins in the testicular tissue were determined according to the method reported by Xiaofang (Xiaofang et al., 2018). In brief, testicle paraffin sections were deparaffinized and hydrated with xylene and alcohol, respectively, before antigen recapture was performed. The sections were incubated with anti-Bcl-2 (Abcam, USA), anti-Apaf-1 (Abcam), anti-caspase-3 (Abcam) and anti-caspase-9 (Abcam) at the dilution of 1:200 for each protein.

1.5. Western blotting analysis

The total proteins of testicular samples were extracted, and the protein concentrations were determined with BCA Protein Assay Kit (Thermo). Total proteins of each sample were loaded on the 10% SDS polyacrylamide (SDS-PAGE) gel electrophoresis. The gels were transferred to a PVDF membrane at 220 mA for 2 h at 4 °C, and then the PVDF membrane was blocked in 10% Bovine Serum Albumin (BSA) at room temperature for 2 h before incubating overnight at 4 °C with primary antibodies (Abcam) including anti-Fas (1:200), anti-FasL (1:200), anti-JNK (1:1000), anti-P-JNK (1:1000) and anti-Caspase-3 (1:800). It was then incubated with corresponding secondary antibody (1:4000) for 1 h at room temperature. Finally, the Easy See Western Blot Kit was used for chemical illumination in a dark chamber and photographed by UVP gel imaging system (Odyssey CIX, LINCOLN, USA).

1.6. Statistical analysis

All values were expressed as the mean (± SD) of three replicates. Differences between groups were evaluated through one-way ANOVA, followed by Dunnett's *t*-test, using Graphpad Prism 5.01. A difference at P value < 0.05 was considered statistically significant. Western blot images were analysed with Photoshop image J. Immunohistochemical images were analysed under light microscope (OLYMPUS IX-71).

2. Results

2.1. SOD, CAT, GSH-PX activities and MDA contents

The MDA contents in the testis tissue were significantly ($P < 0.01$) higher, while the activities of GSH-Px ($P < 0.01$), CAT ($P < 0.05$) and SOD enzymes were significantly ($P < 0.01$) lower in the mice of group H compared with those in the mice of groups C, B and H + B. However, neither MDA contents nor GSH-Px, CAT or SOD activities in the mice of H + B group showed significant difference compared with those of mice from group C or group B (Table 1).

2.2. Histological changes in the testicular tissue of mice

Histologically, the testicular tissue from mice of C groups appeared quite normal with numerous spermatogenic cell layers (Fig. 1, A). However, in mice of the H group, spermatogenic cells showed abnormalities of various levels, with vacuolated nuclei decreased number of primary spermatocytes and absence of mature spermatozoa (Fig. 1, C). There was no remarkable difference in the histological structures of testicular tissue between mice of the B (Fig. 1, B) and C groups. Interestingly, all the structural abnormalities and cellular changes observed in the testis from mice of H group were absent in the mice of H + B group (Fig. 1, D).

Apoptosis of spermatogenic cells was occasionally observed in the testes of mice from both C (Fig. 2, A) and B (Fig. 2, B) groups, while they were mostly in spermatogonia stage. However, apoptotic cells were higher in number in the testes of mice from H group , while not all testis tubules apoptosed at the same extent, compared with those from C group and these could only be observed in the outside layer of the tubules (Fig. 2, C). This cellular apoptosis was reduced in the testis of mice of H + B group (Fig. 2, D) compared with that in the H group.

Table 1
Mean values (± SD) of MDA contents and GSH-Px, CAT and SOD activities in the testis tissue from mice of four experimental groups.

Groups	MDA/(nmol·mg-1)	GSH-Px/(U·mg^{-1})	CAT/(U·mg^{-1})	SOD/(U·mg^{-1})
C	2.152 ± 01414^{Aa}	48.77 ± 2.091^{Aa}	1.720 ± 0.08590^{Aa}	128.2 ± 5.169^{Aa}
B	2.045 ± 0.1306^{Aa}	41.46 ± 2.145^{Ab}	1.678 ± 0.5830^{Aa}	119.4 ± 3.200^{Aa}
H	3.978 ± 0.2503^{Bb}	27.45 ± 1.873^{Bb}	1.235 ± 0.1365^{Ab}	82.12 ± 2.926^{Bb}
H + B	2.947 ± 0.1030^{Aa}	35.86 ± 1.423^{Ab}	1.596 ± 0.1753^{Aa}	116.0 ± 3.824^{Aa}

Note : In the same column, values with different capital superscripts are significantly different at $P < 0.01$ and values with different lower case superscripts differ significantly at $P < 0.05$.

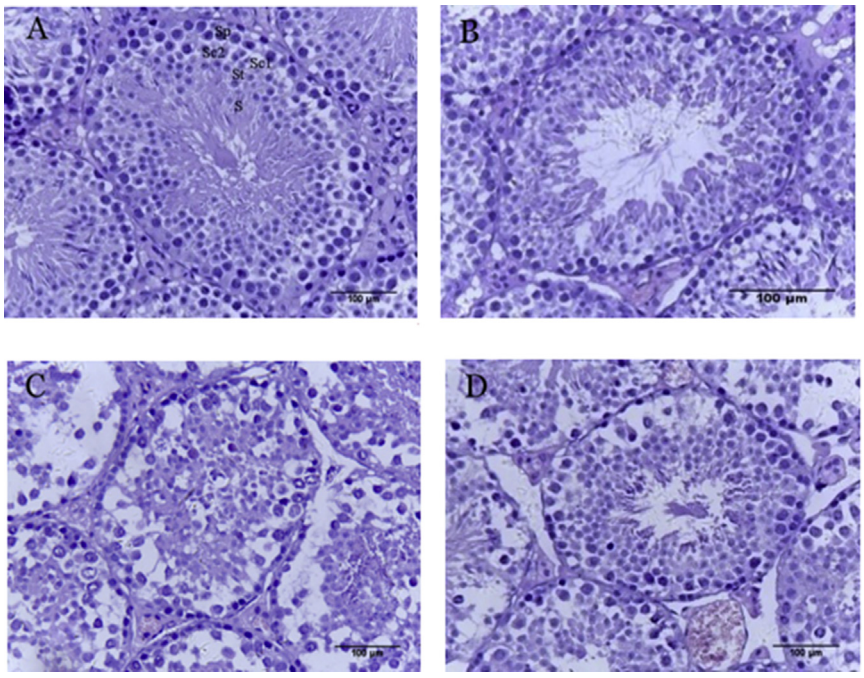

Fig. 1. Histopathological observations of testis tissue from the mice of four groups (H & E, × 100). Sp stands for spermatogonium; Sc1for primary spermatocyte; Sc2 for secondary spermatocyte; St for spermatid and S for spermatozoon (A). Heat stress impaired spermatogenesis (C) and disturbed tissue structure with spermatogenic cells disordered in permutation, vacuolated nuclei, and absence of mature spermatozoa, while baicalin alleviated above effects caused by heat stress (D). Note：A: Control group; B: Baicalin group; C: Heat stress group; D: Heat stress with baicalin treatment group.

2.3. Apoptosis-related indicator expressions

Immunohistochemistry (IHC) results showed that apaf-1 (Fig. 3, g), caspase-9 (Fig. 3, k) and caspase-3 (Fig. 3, o) were positively expressed in the testicular tissue from mice of H group, while neither of them showed any expression in the mice from groups C, B or H + B. Apparently, there was no change in Bcl-2 expression (Fig. 3, a–d) among the mice from four group.

2.4. P-JNK, JNK, FasL, Fas and Caspase-3 protein expressions

Western blot results revealed that the expressions of P-JNK (Fig. 4, A), FasL (Fig. 4, C), Fas (Fig. 4, D) and Caspase-3 (Fig. 4, E) proteins were significantly increased ($P < 0.05$) in the mice of H group compared with the mice of C and H + B Groups. However, JNK protein expression (Fig. 4, B) showed non-significant difference among mice of four groups. Similarly, expressions of four proteins also showed non-significant differences between the mice of C and B groups.

3. Discussion

The temperature and humidity used for induction of heat stress in mice in this experiment were in accordance with the ambient weather conditions which prevail in Qingdao during July to September, when

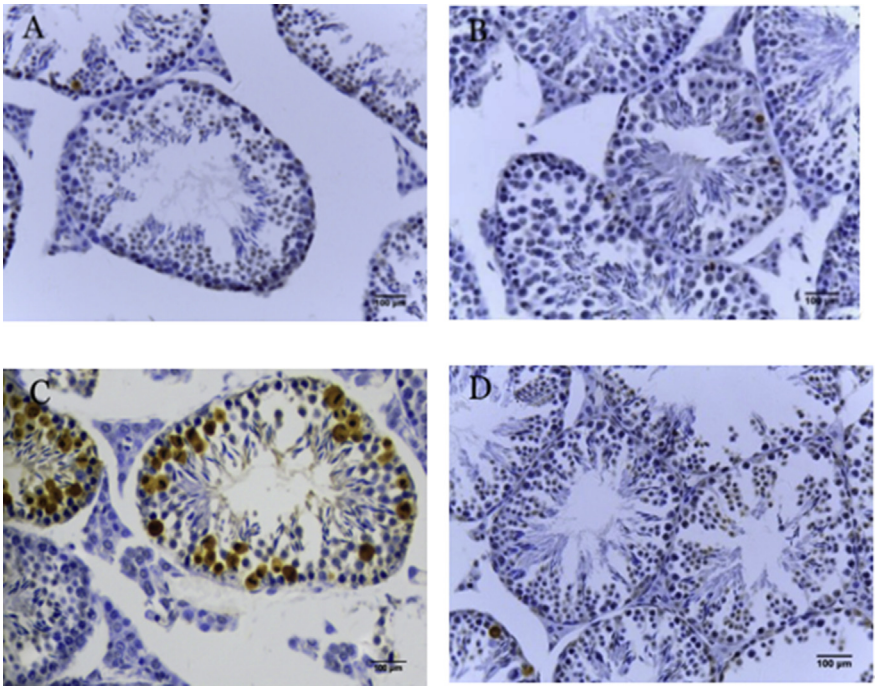

Fig. 2. Apoptosis of spermatogenic cells in the testis tissue of mice from four groups (H & E; × 100). Note：A: Control group; B: Baicalin group; C Heat stress group; D: Heat stress with baicalin treatment group.

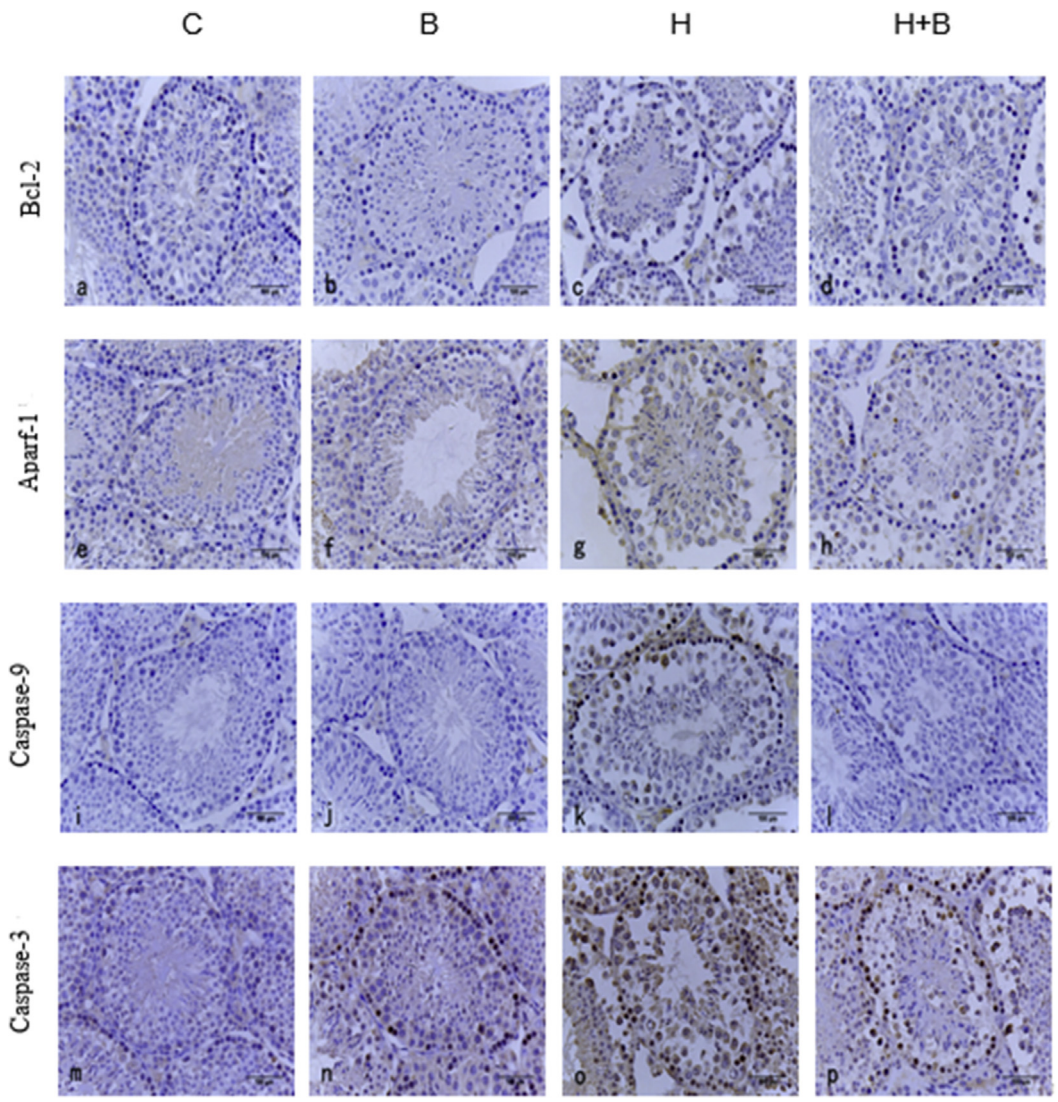

Fig. 3. Immunohistochemical staining of testicular tissue from the mice of four groups (× 100) showing expressions of Bcl-2 (a–d), Apaf-1 (e–h), caspase-9 (i–l) and caspase-3 (m–p) proteins.

the temperature reaches 41 °C with humidity up to 80% (Dong et al., 2013). The results regarding increased MDA contents with simultaneously decreased activities of antioxidant enzymes, namely SOD, CAT and GSH-Px, in the testicular tissue of heat-stressed mice clearly indicate a certain degree of lipid peroxidation and oxidative stress in the testicular tissue of experimental mice. Besides, impaired process of spermatogenesis in the mice by heat stress, as showed in this study through H & E staining, can possibly be one of the reasons for reduced reproductive performance in male animals under heat stress. Lipid peroxides which are normally present in lipoproteins or cell membranes, such as MDA, are known to further induce lipid peroxidation, interfere with the mitochondrial electron transport system, and oxidize sulfhydryl groups of proteins, hence altering its function or otherwise disrupting signal transduction pathways (Rodrigo et al., 2013). MDA is produced by excessive ROS following oxidation of membrane lipids in living organisms. Usually, the extent of membrane lipid peroxidation is determined by detecting MDA contents (Halliwell and Gutteridge, 1984), which can be correlated to the degree of membrane system damage (Qinwen et al., 2016). Hypoxia stress, such as heat stress, causes acute increase of ROS (Hielscher et al., 2015), and is considered to be a kind of strong oxidizer (Bartosz, 2009). Antioxidant enzymes, constituting the most effective defense system that limits the toxicity associated with free radicals/reactive oxygen species (ROS), play a crucial role in protection of tissue against harmful effects of lipid peroxidation. However, antioxidant enzymes, such as SOD (Shadmehr et al., 2017), CAT (Ali et al., 2017) and GSH-Px (Xiaotong et al., 2015a, b), cannot eliminate the excessive ROS in time, which disturbs the balance of redox reaction *in vivo* (Ghosh et al., 2009), leading to accumulation of peroxide products such as MDA and oxidative damage to the tissue (Pei et al., 2017). In this experiment, heat stress induced similar condition in summer, reduced antioxidant capacity and disturbed oxidative/antioxidative balance and produced oxidative damage in mice testis.

Interestingly the results that not all testis tubules undergo cell death to the same extent indicated that the tubules with high apoptotic rate may be representative of specific spermatogenic stages when they are relatively sensitive to heat-induced apoptosis. Positive correlation of Fas and FasL protein expressions with JNK phosphorylation after heat treatments, as found in this experiment, could further activate caspase cascade reaction and lead to cellular apoptosis (Le-Niculescu et al., 1999). Apoptosis of testicular cells plays crucial role in adversely affecting testosterone homeostasis (Thomas et al., 2014) and optimization of sperm production (Gallardo Bolaños et al., 2012). According to Xia (Xia et al., 2017), heat stress induces apoptosis of germ cells in both mice and rats *in vitro*. Moreover, JNK mediated activation of FasL/Fas pathway has been shown to be one of the main causes of neuronal

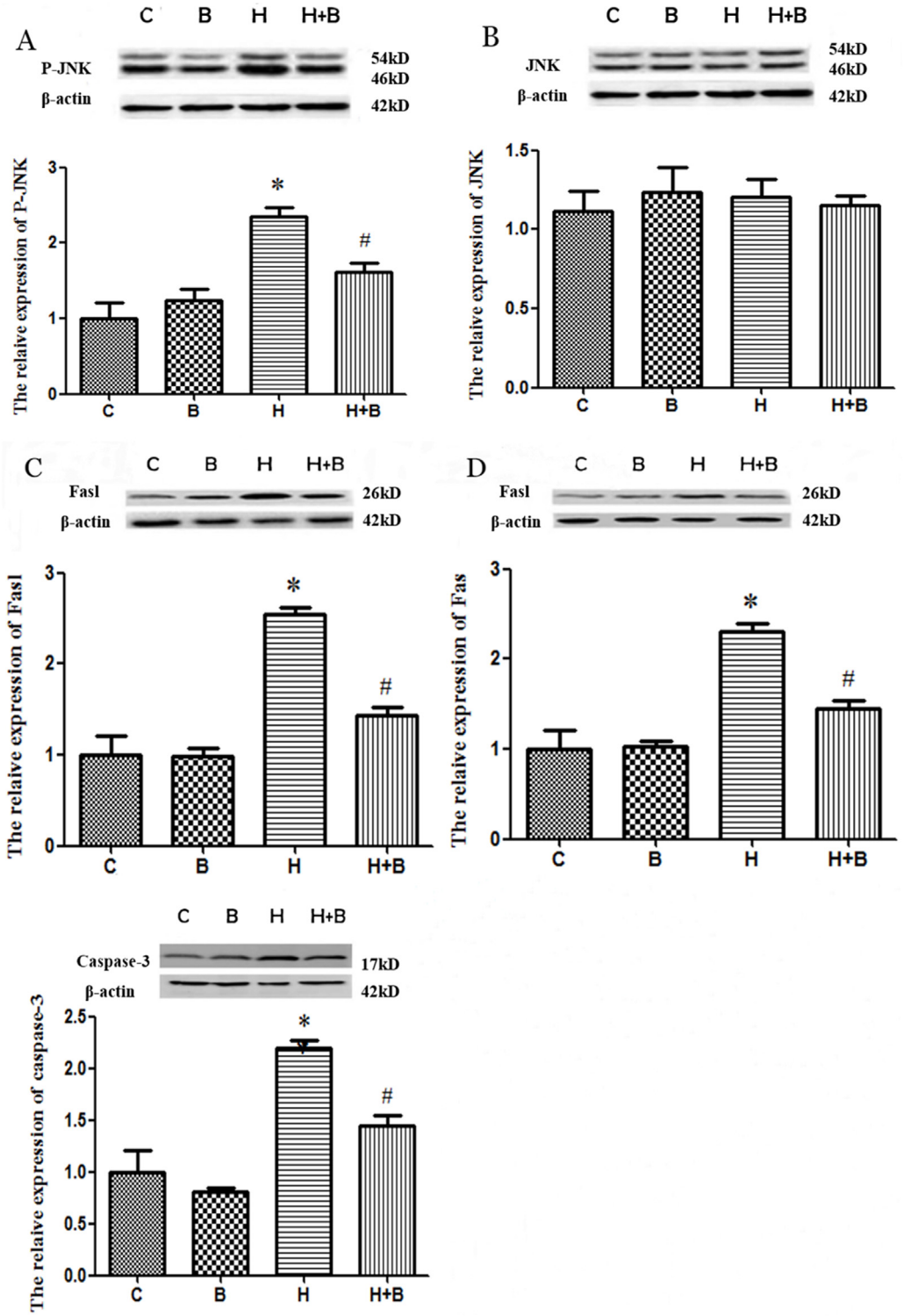

Fig. 4. Expressions of P-JNK (A), JNK (B), FasL(C), Fas (D) and Caspase-3 (E) proteins in the testis tissue of mice from four group. Note : $*P < 0.05$ vs. C group; $\#P < 0.05$ vs. H group.

apoptosis in Alzheimer's disease patient (Le-Niculescu et al., 1999), which coincides with results of our experiments. The down-regulation of Bcl-2 in cells is also related to JNK phosphorylation (Yuanyuan et al., 2015), which further aggravates apoptosis (Bogoyevitch and Kobe, 2006). However, cell apoptosis *in vivo* is much more complicated and can be regulated by various mechanisms through different apoptotic pathways, which can interact with one another.

The results in this experiment that baicalin pretreatment enhanced the activities of antioxidant enzymes effectively and reduced the MDA contents in mice testis could partly explain baicalin's anti-oxidative action *in vivo*. Additionally, baicalin pretreatment reduced the expressions of P-JNK, Fas, FasL, caspase-9, caspase-3 and Apaf-1. Therefore, it can be inferred that baicalin inhibits Fas/FasL apoptosis pathway in the testis of heat-stress mice. Baicalin, as a flavonoid, has been widely used in clinical medicine as a detoxificant (Cui et al., 2017), anti-bacterial (Huaguo et al., 2014) and anti-inflammatory agent. The role of baicalin

in relieving oxidative damage to oviduct in heat stressed mice has also been reported *in vivo* experiments (Mao and Yang, 2001). Moreover, baicalin also alleviated apoptosis of bull calve Sertoli cells induced by heat stress via regulating the Fas/FasL pathway (Xiaotong et al., 2015a, b) and increasing the survival rate of germ cells under heat stress conditions *in vitro* (Gonda et al., 2012). However, damages induced by heat stress can be influenced by various other factors and are regulated by complex signal pathways *in vivo*. The protective effects of baicalin, as well as many other ingredients of Chinese medical herbs, on the male testis under heat stress conditions need further investigations both *in vivo* and *in vitro*.

In summary, heat stress destroys histological structure and increases cellular oxidative damage and apoptosis of testicular tissue in mice. Baicalin alleviates these adverse effects of heat stress *in vivo* through increasing activities of anti-oxidant enzymes or possibly activating the Fas/FasL apoptosis pathway.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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