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Effect of boric acid supplementation of ostrich water on the expression of Foxn1 in thymus

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Summary. Foxn1 is essential for thymus development. The relationship between boric acid and thymus development, optimal dose of boric acid in ostrich diets, and the effects of boric acid on the expression of Foxn1 were investigated in the present study. Thirty healthy ostriches were randomly divided into six groups: Group I, II, III, IV, V, VI, and supplemented with boric acid at the concentration of 0 mg/L, 40 mg/L, 80 mg/L, 160 mg/L, 320 mg/L, 640 mg/L, respectively. The histological changes in thymus were observed by HE staining, and the expression of Foxn1 analyzed by immunohistochemistry and western blot. TUNEL method was used to label the apoptotic cells. Ostrich Foxn1 was sequenced by Race method. The results were as following: Apoptosis in ostrich thymus was closely related with boric acid concentrations. Low boric acid concentration inhibited apoptosis in thymus, but high boric acid concentration promoted apoptosis. Foxn1-positive cells were mainly distributed in thymic medulla and rarely in cortex. Foxn1 is closely related to thymus growth and development. The nucleotide sequence and the encoded protein of Foxn1 were 2736 bases and 654 amino acids in length. It is highly conserved as compared with other species. These results demonstrated that the appropriate boric acid supplementation in water would produce positive effects on the growth development of ostrich thymus by promoting Foxn1 expression, especially at 80mg/L, and the microstructure of the thymus of ostrich fed 80 mg/L boric acid was well developed. The supplementation of high dose boron (>320mg/L) damaged the microstructure of thymus and inhibited the immune function by inhibiting Foxn1 expression, particularly at 640mg/L. The optimal dose of boric acid supplementation in ostrich diets is 80 mg/L boric acid. The genomic full-length of African ostrich Foxn1 was cloned for the first time in the study.

Key words: Foxn1, Thymus, Boric acid, African ostrich Chicks

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Introduction

Boron is an essential trace element used in human and animal health. Humans and animals consume boron daily via dietary intake. It is important for bone growth and health (Hunt, 1994; Nielsen, 1997; Basoglu et al., 2002; Adya et al., 2008; Kabu and Civelek, 2012). Also, sodium borate protects against developing a fatty liver (Basoglu et al., 2002; Bobe et al., 2004). The effects of boric acid on animal and human have become the hot spot for its involvement in the functions of the immune system.

It is noteworthy that boric acid can affect the immune response or the inflammatory process (Hunt and Idso, 1999; Armstrong et al., 2001). Bai and Hunt (1996) reported that low boric acid diet increased the serum total antibody concentrations immunized with an antigen in rats. Studies with animals also indicate that dietary boric acid reduces local inflammatory responses. Previous studies indicated that paw swelling in adjuvant-

induced arthritic rats was decreased by supplementing boric acid (Hunt and Idso, 1999; Armstrong et al., 2001; Armstrong and Spears, 2003). Armstrong showed that boric acid can affect serum thyroid hormone concentrations and immune response in gilts (Armstrong et al., 2001). Hunt demonstrated that physiological amounts of dietary boric acid decreased the skinfold thickness after antigen injection in gilts and elevated circulating natural killer cells after adjuvant injection in rats (Hunt et al., 1988; Hunt, 2003).

Verwoerd et al. reported that ostrich chicks had a high mortality rate ranging from 10 to 50% in livestock farms, especially within the first three months of age, which usually resulted from the exposed to various stressors, infections or diseases (Verwoerd et al., 1999). In addition, ostriches are fast-growing birds, and the birds from the same batch often differ greatly in size (Deeming and Ayres, 1993; Deeming et al., 1993), particularly during the first 3 months (Bunter and Cloete, 2004). One of the most important measures of ostrich chicks is growth development, which is usually balanced against the immune function, it is an important survival trait (Bonato et al., 2013). The thymus is the first of the lymphoid organs to be formed and grows considerably immediately after birth in response to postnatal antigen stimulation and is crucially involved in the differentiation of T lymphocytes. It is actually an epithelial organ in which epithelial cells provide a framework containing T cells as well as smaller numbers of other lymphoid cells (Pearse, 2006). The primary function of the thymus is to develop immune competent T cells that will be able to carry out immune functions in the body (Ciofani and Zúñiga-Pflücker, 2007). T cell development is under the tight control of the thymus microenvironment. Thymus microenvironments are mainly composed of thymic epithelial cells, presenting in both the cortex and medulla. Cortical and medullary thymic epithelial cells (cTECs, mTECs) drive T cell differentiation, selection and maturation processes (Van Ewijk, 1988, 1991). Foxn1 is a key regulator of thymic epithelial cell (TEC) differentiation (Nehls et al., 1994). Foxn1 is expressed in all TECs during initial thymus organogenesis, usually during fetal stages (Nehls et al., 1996; Gordon et al., 2001; Itoi et al., 2007), and is also expressed broadly in postnatal TECs (Nehls et al., 1996). Li et al. demonstrated that the consumption of drinking water containing 400mg/L boric acid degenerated the thymus parenchyma, decreased thymocytes, enlarged or increased Hassal's body, and increased the thymus interstitial component in Gushi chickens (Li et al., 2004).

In view of the possible association of boric acid with the development of the thymus, we therefore set out to study the relationship between boric acid and Foxn1 and thymus development in ostrich. Foxn1 is required for the development of both mTECs and cTECs in thymus organogenesis, which are in turn necessary to prevent thymic atrophy (Cheng et al., 2010). On the other hand, high-dose boron supplementation induced lymphocyte depletion and caused immune organ atrophy and degeneration (Jin et al., 2014). Thus, one of the most important objectives of the present study was to confirm the optimal dose of boric acid supplementation by detecting the apoptosis and the expression of Foxn1 induced by boric acid in ostrich thymus. Furthermore, the full genome of Foxn1 in ostrich was also evaluated to determine the genomic characteristics and phylogenetic analysis in the current study. To our knowledge, up to date no study has evaluated Foxn1 expression in the ostrich thymus and its sequence in the African ostrich.

Materials and methods

Animals, feed method, administration procedures and collection of tissues

Thirty-five (one-day-old) African ostriches (Struthio camelus), were obtained from Henan Jinlu special breeding Co., Ltd. (Henan Province, China). After 14 days, 30 healthy ostriches were picked out with similar body size and randomly divided into six groups. The rest of the ostriches were weeded out. During the following 76 days, boric acid was added into the distilled drinking water with different dosages for different groups (Group I, 0 mg/L; Group II, 40 mg/L; Group III, 80 mg/L; Group IV, 160 mg/L; Group V, 320mg/L; Group VI, 640mg/L), Group I was kept as the control group. Apart from the boric acid supplementation in water, the ostriches were fed with a conventional diet. The birds were housed in a temperature- and light- controlled room (12 h light/ 12 h dark). During the first two weeks, ostrich chicks were maintained at a constant temperature of 25°C. The temperature was decreased by 1°C per week until two months old, thereafter, temperature was maintained constant until three months old (Bonato et al. 2013).

At the age of 90 days the thymi were quickly removed from birds by necropsy under deep anesthesia. A portion of the thymic samples were immediately frozen in liquid nitrogen, and then preserved at -80°C for the molecular study. The other potion of the thymi samples were fixed in 10% buffered neutral formalin solution for 24 h, and then routinely dehydrated, and embedded in paraffin wax. Serial sections (4-5 μm thick) were cut and air-dried onto glass slides overnight.

The experiments were conducted according to the rules of the China Management Regulations of Experimental Animals and were licensed by the local authorities. This is in accordance with the International Guiding Principles for Biomedical Research involving animals.

HE staining and immunohistochemistry

After the routine alcohol-xylol process, a portion of the ostrich thymus sections were stained with hematoxylin and eosin (HE staining). The others were

stained by immunohistochemistry. The primary antibodies Rabbit Anti-Foxn1 (1:100, Bioss, China) and Histostain™ - Plus Kit were used (Cat.No: KIHC-5, proteintech™). To confirm that immunostaining was specific, a negative control (no primary antibody control) was also included, and these samples had no detectable staining. All immunohistochemical stainings were evaluated by high-power light microscopic examination (BX51; Olympus, Tokyo, Japan).

The densities of Foxn1-positive products in ostrich thymus were estimated. After taking digital photographs under a light microscope with a digital camera, the mean density of the Foxn1-positive products in each section and the area of the related regions in each section were measured using an Image Pro Plus analysis system. The IPP6.0 macro plug-in (Pathology6@11-13) was used to calculate the mean density by panel detection. Ten fields were selected from each tissue section, data were representative of five tissue sections per ostrich (n=5 per group) for determination of expression values of positive cells.

Apoptosis evaluation

Apoptosis was assessed by an in situ TdT mediated Dutp-biotin nick end labeling (TUNEL) of DNA strand breaks assay (In Situ Cell Death Detection Kit, No. 11684817910). Samples were analyzed in a drop of PBS under a fluorescence microscope. An excitation wavelength in the range of 450-500 nm was used and detected in the range of 515-565 nm (Nikon, ECLIPSE 80i).

Western blot analysis

Frozen tissue preparations were homogenized with sample buffer, centrifuged, and boiled. Total protein concentration of the tissue was quantified using the Bradford method. Protein concentrations were determined using the BioRad protein assay (Bio-Rad, Hercules, CA). Equal amounts of total protein were loaded onto 1% SDS-PAGE and then electrophoretically transferred onto polyvinylidene difluoride membranes (Millipore, IPVH00010). Transferred membranes were blocked using 5% skim milk and incubated overnight with antibodies against Foxn1 (Bioss, China). The same membrane was probed with anti-GAPDH (Xianzhi, China) as house keeping. After being washed with TBST three times, the blots were hybridized with secondary antibodies (1:50000, Boster, China) and conjugated with horseradish peroxidase for 2 h at room temperature. The antibody-specific protein was visualized by ECL detection system.

RACE method

RNA Isolation

The tissue samples were grounded to extract RNA using Trizol reagent, according to the manufacturer's protocol. An RNA sample was dissolved with 20 μL DEPC-treated water and used for reverse transcription immediately. All primers for RACE analysis are listed in Table 1.

PCR amplification

Full-length primers: one set of degenerate primers was designed based on a multiple sequence alignment of entire genome from ostrich thymus. The resulting PCR products were excised from agarose gel and purified using the Axyprep DNA Gel Extraction Kit (AXYGEN, USA). The purified PCR products were ligated into PMD18-T vector (TakaRa, Japan) using T4 DNA ligase (TakaRa, Japan) at 16°C overnight. Recombinant plasmid was transformed into DH5α competent *Escherichia coli* cells (TakaRa, Japan). Plasmids containing the insert fragment were indentified by PCR.

5' RACE

5'RACE was carried out with the 5' RACE System for Rapid Amplification of cDNA Ends, Version 2.0 following the manufacture's instructions. Briefly, annealing of first strand primer GSP1 (CTTTCCTCGT AACTGTA) to mRNA, and copying of mRNA into cDNA with $S_{UPER}S_{CRIPT}$™ II RT was done. The degradation of RNA with RNase Mix and purification of cDNA with G_{LASS}MAX Spin Cartridge was carried out. Purified cDNA was tailed with dCTP and TdT. PCR amplified dC-tailed cDNA using the Abridged Anchor Primer and nested GSP-2 (CTTGGCTTGTAGGGTA TCTTG). Primary PCR product was re-amplified by using AUAP and nested GSP-3 (GGCTGGGGCTGT GAGAAGAC). 5'RACE amplification products were cloned.

Table 1. Degenerate primers for conservative middle segment of Foxn1 and primers used for RACE.

Primer name	Sequence
Degenerate primers for conservative middle segment	
	FONX1F1: TCRGACRSCMGGACAGARAG
	FONX1R1: GGGAYGGRTTVAGAGCRTCGAT
Primers for 5'RACE	
	R907-1(GSP1): CTTTCCTCGTAACTGTA
	R907-2(GSP2): CTTGGCTTGTAGGGTATCTTG
	R907-3(GSP3): GGCTGGGGCTGTGAGAAGAC
Primers for 3'RACE	
	3'566-1: CAACCCAGCATCACCCAGGATTCTC
	3'566-2: CAGCCAGGACAATGCAGGACACACT

Degenerate primers for conservative middle segment of Foxn1 and primers used for RACE. Primer names reflect, for RACE-PCR, the end of the gene being amplified (5' or 3') and the number of the gene specific primer (GSP). All primers were designed manually based on sequences of genes and were custom made by Invitrogen Life Sciences.

3' RACE

The sequence of 3'RACE was carried out with SMARTer™ RACE cDNA Amplification Kit (Clontech, USA) following the supplier's protocol. Total RNA was converted into RACE-Ready first-strand cDNA. First-strand cDNA synthesis was initiated by mixing SMARTScribe™ Reverse Transcriptase and 3'CDS primer A. The first round of amplification was conducted by using the above cDNA and gene specific primer (3'566-1: CAACCCAGCATCACCCAGGA TTCTC; UPM primer). The second round of amplification was conducted by using the first round PCR amplification products diluted 50 times and the specific primers (3'566-2: CAGCCAGGACAATG CAGGACACACT; UPM primer). The second round PCR amplification products were checked by electrophoresis on a 1% agarose gel in 1× tris-borate EDTA (TBE) buffer. The resulting PCR products were excised from agarose gel and purified using the Axyprep DNA Gel Extraction Kit (AXYGEN, USA). The purified PCR products were ligated into PMD18-T vector (TakaRa, Japan). Positive clones, based on PCR identification, were subsequently sequenced. We used a 2-kb DNA ladder to mark target strap. (Life Technologies).

Sequence analysis

Homologous identity comparisons of the sequence of the African ostrich with other species were performed using DNASTAR software (Version 7.1, DNASTAR Inc., Madison, WI, USA). Phylogenetic analysis of the obtained sequence of Foxn1 with other species was analyzed using DNAMAN software (Version 6.0, Lynnon Corp., Pointe-Claire, Quebec, Canada).

Statistical analysis

One-way ANOVA was performed to assess the significance of differences in Mean Density (MD) of the Foxn1 and western blot data. P values <0.05 were

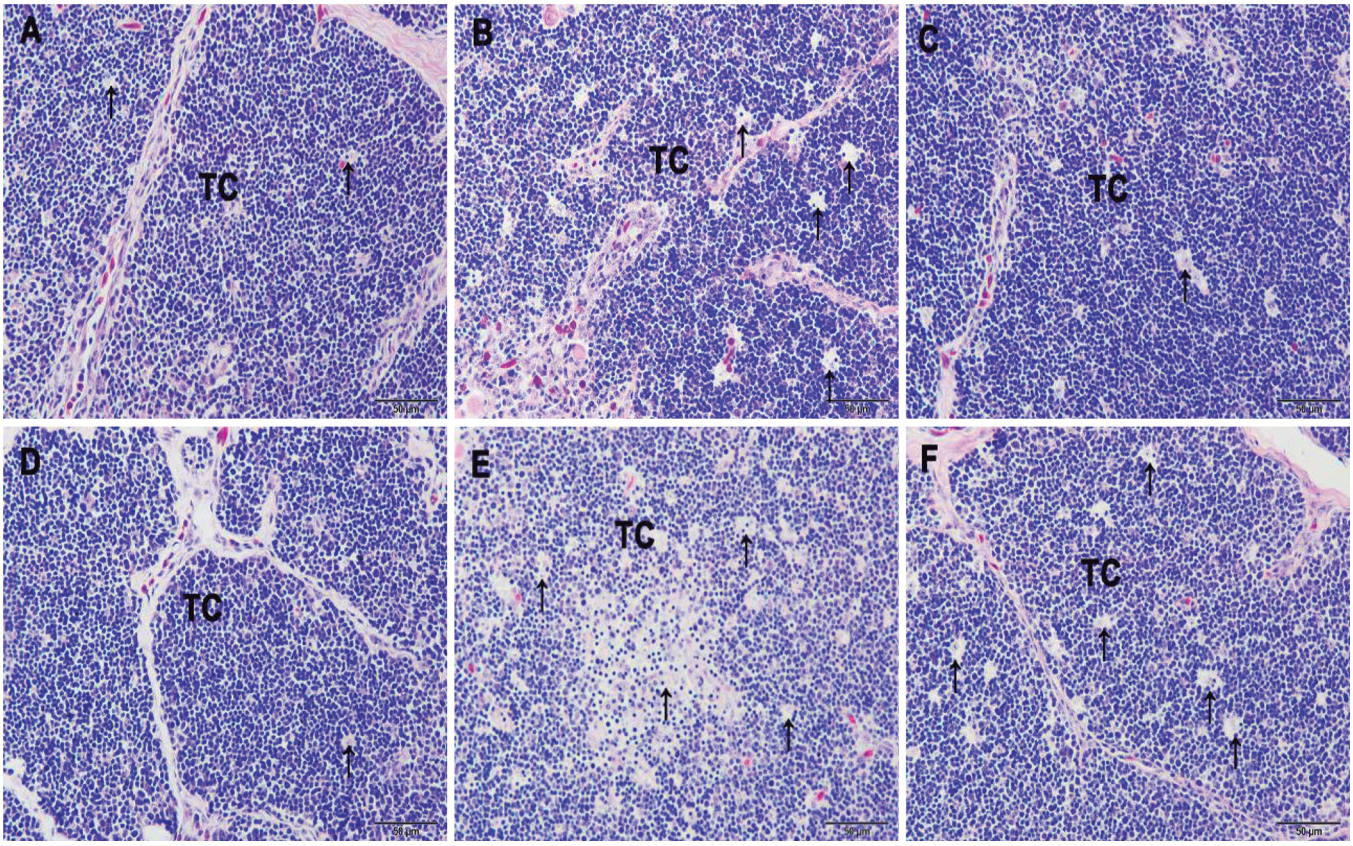

Fig. 1. Effects of boric acid on the microstructure of thymic cortex in Ostriches (stained with hematoxylin-eosin). **A.** Thymus from Group I, 0 mg/L boric acid. **B.** Thymus from Group II, 40 mg/L boric acid. **C.** Thymus from Group III, 80 mg/L boric acid. **D.** Thymus from Group IV, 160 mg/L boric acid. **E.** Thymus from Group V, 320 mg/L boric acid. **F.** Thymus from Group VI, 640 mg/L boric acid. TC: thymic cortex; arrow: "starry-sky" appearance. Bars: 50 μm.

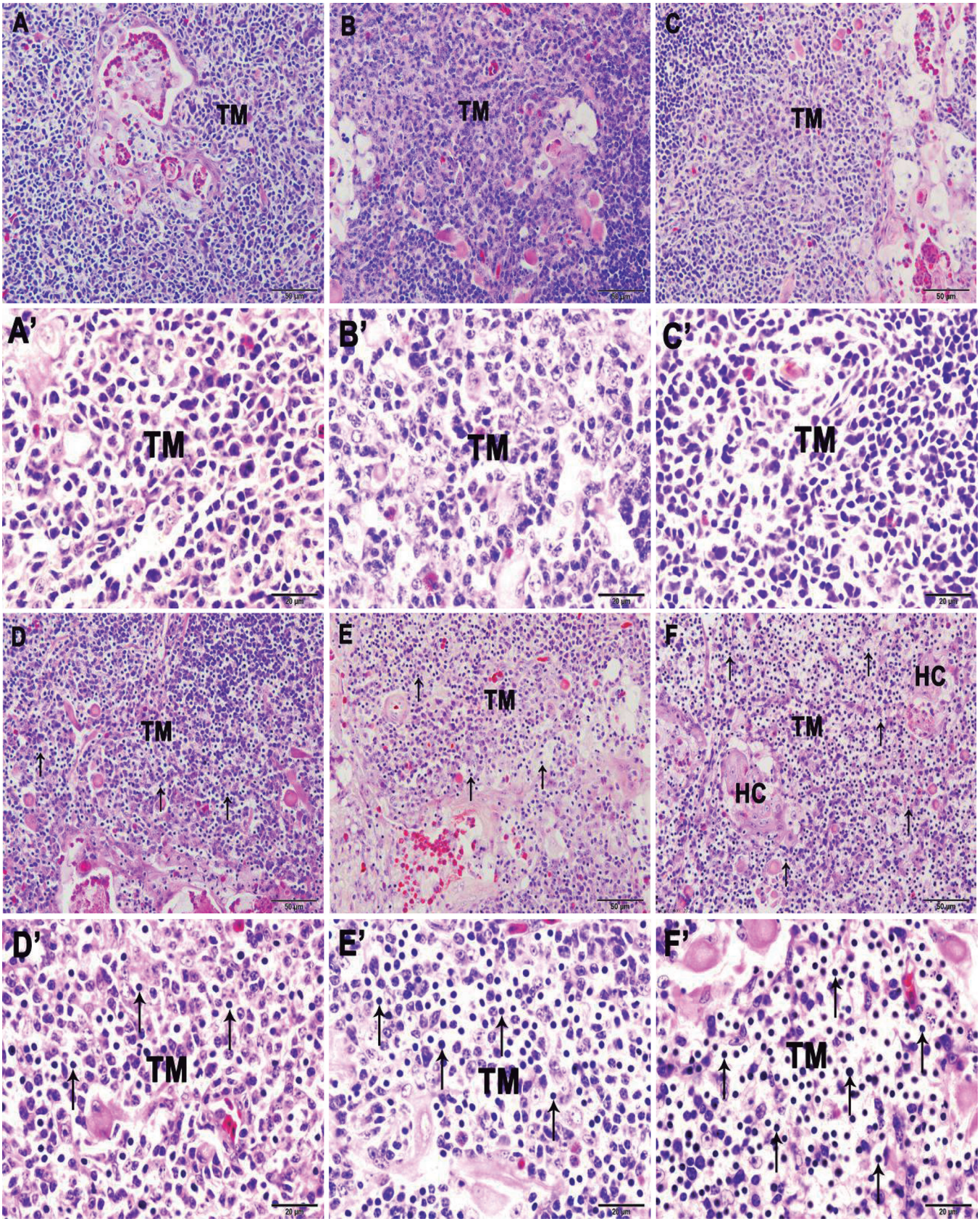

Fig. 2. Effects of boric acid on the microstructure of thymic medulla in Ostriches (stained with hematoxylin-eosin). **A, A'.** Thymus from Group I, 0 mg/L boric acid. **B, B'.** Thymus from Group II, 40 mg/L boric acid. **C, C'.** Thymus from Group III, 80 mg/L boric acid. **D, D'.** Thymus from Group IV, 160 mg/L boric acid. **E, E'.** Thymus from Group V, 320 mg/L boric acid. **F, F'.** Thymus from Group VI, 640 mg/L boric acid. TM: thymus medulla; HC: Hassall's corpuscles; arrows: lymphocytes. A-F, Bars: 50 μm; A'-F', Bars: 20 μm.

considered significantly different.

Results

Effects of boric acid on thymus tissue structure of ostriches

It was observed that the ostrich thymus in control group (group I) was normal. Distinct areas of thymus cortex and medulla were visible in thymus tissue. Thymus was large and lobulated with numerous lobules. The medulla was intact between the adjacent lobules and lightly stained than the cortex. The medulla contained prominent epithelial cells, Hassall's corpuscles, admixed macrophages, lymphocytes and, rarely, myoid cells (Fig. 2A). Compared with the results of control group, the thymus in experimental groups II, III, IV had normal cortico-medullary junction. Obvious histopathological changes were observed in group V and group VI where the thymus had lost morphological cortico-medullary distinctions (Fig. 2E,F).

The cortex was crowded with lymphoid cells and the corticomedullary boundary was clearly defined in control group. Compared with control group, thymic cortex was histologically characterized by karyorrhexis of lymphocytes with active phagocytosis by macrophages, which creates a prominent "starry-sky" appearance of the thymic cortex in boric acid supplemented groups, especially in group V and group VI (Fig. 1E,F). The lymphocytes inside the thymus lobules were reduced and exhausted, and thymus lobules were indistinct, whereas, thymic cortex was normal in group III and group IV as compared with control group (Fig. 1C,D).

The medulla stained paler, less densely cellular than the cortex, and contained more mature T-cells, prominent epithelial cells, Hassall's corpuscles, B lymphocytes and, rarely, myoid cells in control group. Compared with control group, lymphocytes infiltrated in medulla and were condensed (group IV, V; Fig. 2D,D',E,E') or diffused (group VI; Fig. 2F,F'). The number of Hassall's corpuscle was increased and enlarged in all other experimental groups compared to control group. The experimental group VI showed the most significant pathological changes. The structure of thymus was changed, as TEC numbers decreased and the

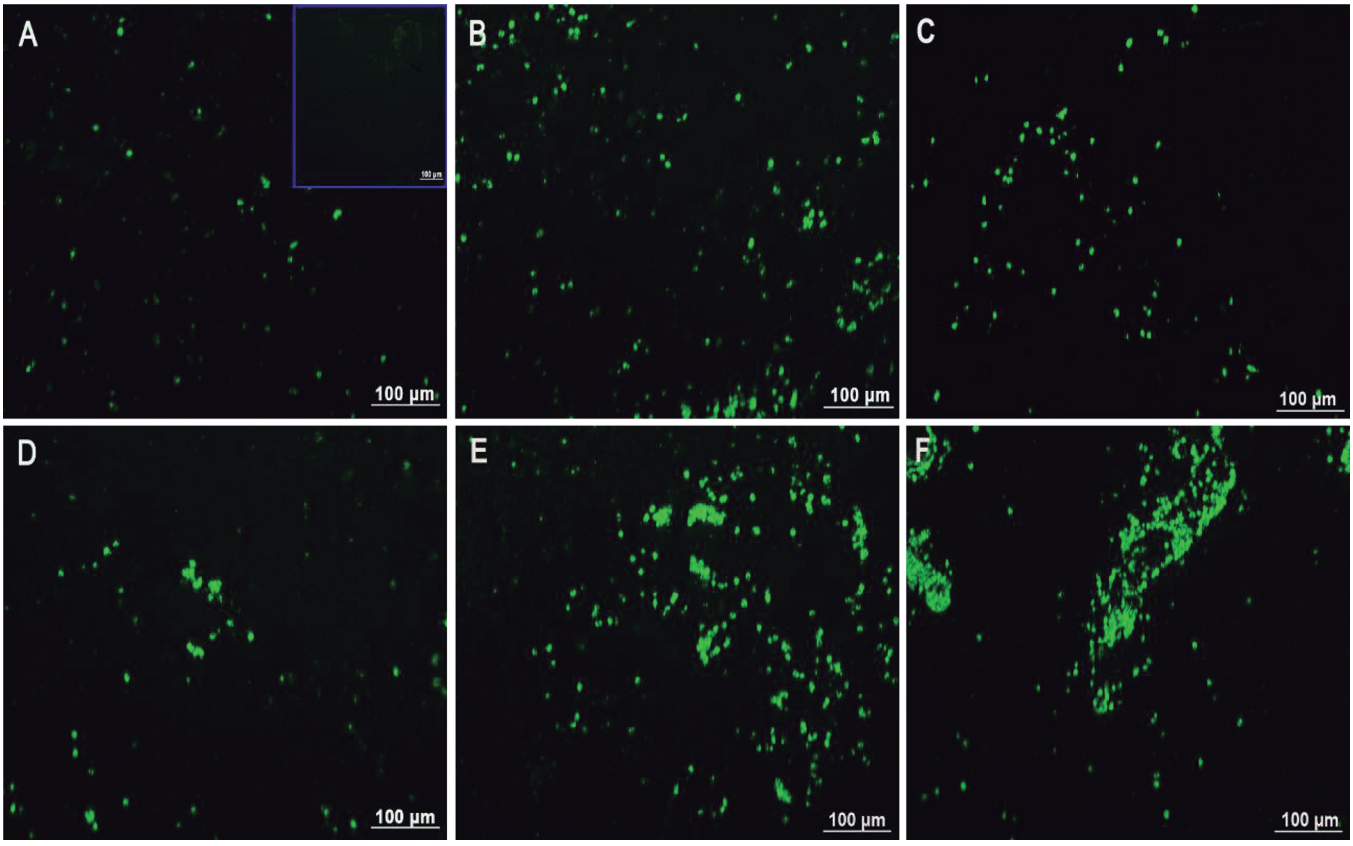

Fig. 3. Effects of boric acid on cell apoptosis in ostrich thymus, detected by TUNEL method. **A.** Group I, the control group. **B.** Group II, 40 mg/L boric acid. **C.** Group III, 80 mg/L boric acid. **D.** Group IV, 160 mg/L boric acid. **E.** Group V, 320 mg/L boric acid. **F.** Group VI, 640 mg/L boric acid. Bars: 100 μm.

cortical and medullary compartments broke down (Fig. 2F).

Apoptosis in ostrich thymus after boric acid supplementation

As shown in Figs. 1, 2, HE staining revealed that apoptosis was obvious in ostrich thymus from group V and group VI, but not in control group and group III. In order to elucidate the effect of boric acid on cell apoptosis in thymus, we detected the apoptosis by TUNEL assay. Examination of TUNEL-stained thymi sections showed that group II, V and VI had high levels of TUNEL-positive cells (Fig. 3B,E,F), but group I, III, and IV had very low levels of TUNEL-positive cells, especially in group I (control group) (Fig. 3A,C,D). As the boric acid concentration increased, increased TUNEL staining in the thymus confirmed cell apoptosis, which was localized mostly in thymic medulla and faintly in thymic cortex. Together with these results, we speculated that low concentrations of boric acid (80 mg/L, 160 mg/L) could inhibit the thymocyte apoptosis, whereas high concentrations of boric acid (320 mg/L, 640 mg/L) could promote the thymocyte apoptosis.

Expression of Foxn1 in the ostrich thymus in different groups

Immunohistochemistry was used to detect the Foxn1 protein expression level in ostrich thymus. Evaluation of Foxn1 expression in ostrich thymus using the antibody indicated a strong labeling of the mTECs (medulla thymic epithelial cells), and weak labeling or negative labeling of the cTECs (cortical thymic epithelial cells). Foxn1 was diffusely expressed in nuclear staining in thymus, whereas the expression was generally focal in thymic epithelial cells of ostrich thymus; they were also present as sporadic cells or clumped in group I (Fig. 4A).

By IPP analysis, compared to group I, increased MD of Foxn1-positive cells were in 40, 80, 160 mg/L boric acid treatment groups when compared to the control group. The MD of Foxn1-positive cells was decreased at the concentration of 320 and 640 mg/L boric acid treatment groups. The MD of Foxn1-positive cells showed a slight increase in group II (40 mg/L boric acid) and group IV (160 mg/L boric acid), respectively, but the

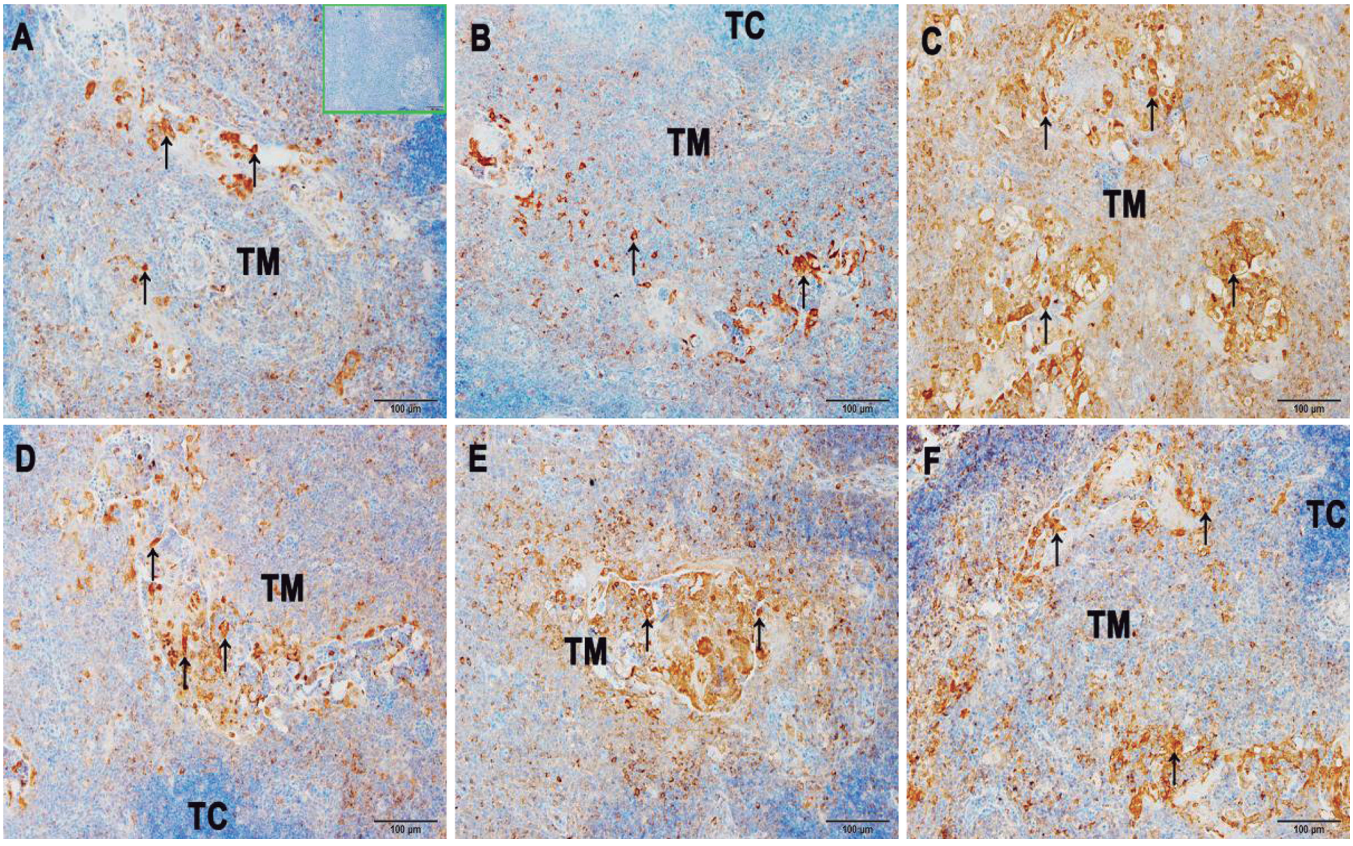

Fig. 4. Distribution patterns of Foxn1-positive cells in the thymus of 90-day-old Ostriches, using the immunoperoxidase method and heamatoxylin countersatin. **A.** Thymus from Group I, 0 mg/L boric acid. **B.** Thymus from Group II, 40 mg/L boric acid. **C.** Thymus from Group III, 80 mg/L boric acid. **D.** Thymus from Group IV, 160 mg/L boric acid. **E.** Thymus from Group V, 320 mg/L boric acid. **F.** Thymus from Group VI, 640 mg/L boric acid. TC: thymus cortex; TM: thymus medulla; arrow: Foxn1-immunopositive cells. Bars: 100 µm.

difference was not statistically significant. Compared to group I, the expression of Foxn-1 positive cells in group III was strong, and the difference was very significant (**p<0.01, Fig. 5C,G). However, the MD of Foxn1-positive cells showed a slight decrease in group V (320 mg/L boric acid), which did not reach statistical significance. A significant decrease of the mean density in group VI (640 mg/L boric acid) was observed as compared with group I (**p<0.01). According to the above data, the expression of Foxn1 changes as the boric acid concentrations changes, which means that there is a correlation between Foxn1 and boric acid.

The trend of Foxn1 expression by western blot analysis was similar to that of immunohistochemistry. As the boric acid concentration increased, the expression of Foxn1 increased, and the expression of Foxn1 reached a peak in group III (80 mg/L). However, as the boric acid concentration went above 160 mg/L, the expression of Foxn1 decreased dynamically, and the expression of Foxn1 was the least in group VI (Fig. 6).

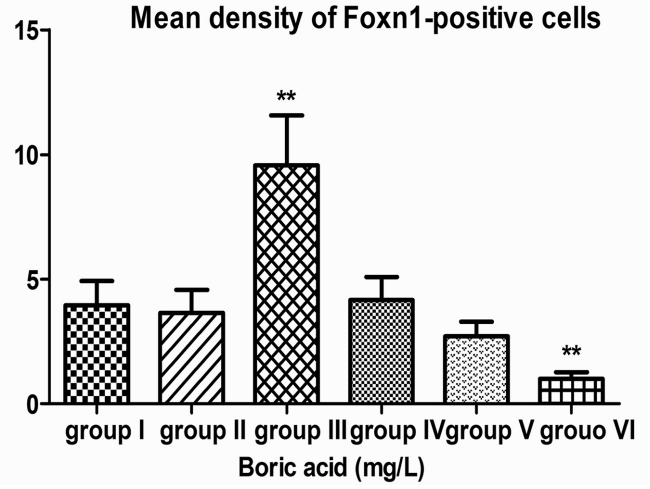

Fig. 5. Mean Density of Foxn1-positive cells; compared with Group I (0 mg/L boric acid), * P<0.05, **P<0.01. Data are representative of five tissue sections per ostrich (n=5 per group).

Cloning and sequence of the Foxn1 gene

Total RNA from the thymus of a ninety-day-old African ostrich was used to amplify Foxn1 gene by RACE method. The sequence of the conservative middle segment of Foxn1 was 1477bp using the degenerate primer by TR-PCR analysis. The sequence of the 5' RACE and 3' RACE of ostrich Foxn1 was 384bp and 1050bp by RACE-PCR analysis, respectively (Fig. 7A). Sequence analysis indicated that the nucleotide sequence and the encoded protein were 2736bp and 654 amino acids in length (Fig. 7B,C). The translation of the nucleotide sequences into amino acids was performed using the DNAMAN software. The nucleotide sequence of the ostrich Foxn1 gene was compared with the corresponding reported sequences of other species. The results suggested that the nucleotide sequence of the Foxn1 gene of the ostrich is highly conserved as compared to those of other species reported. The ostrich Foxn1 gene shared a 92% identity with the peregrine falcon, saker falcon and budgerigar, 91% with the rock pigeon, 88% with mallard and 86% with the chicken, etc (Fig. 8A). To characterize the phylogenetic relationships among Foxn1 gene of these different species, a phylogenetic tree was constructed. The result showed that the ostrich and chicken were placed in the same group and the relationship between the African ostrich Foxn1 and chicken Foxn1 was close (Fig. 8B).

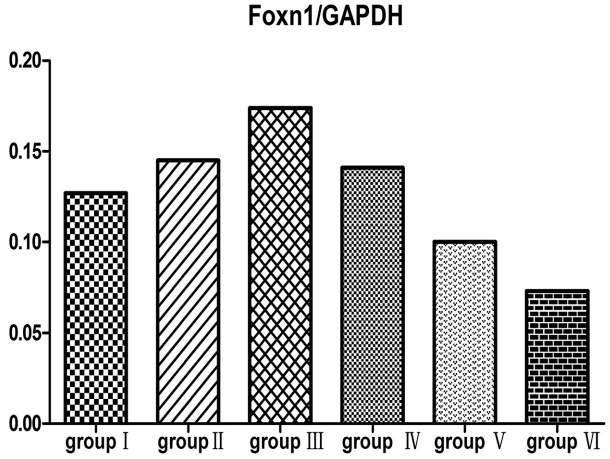

Fig. 6. Western Blot results of the expression of Foxn1 in Ostrich. Foxn1 was analyzed by western blot and relative signal intensities were quantified using BandScan. Protein levels were normalized against the GAPDH level and are shown in the graphs as a fold difference compared with group I. Histogram shows Foxn1/GAPDH arbitrary densitometric units.

Discussion

The thymus is responsible for the provision of T lymphocytes to the entire body, and provides a unique microenvironment in which naïve T cells undergo development and differentiation (Ribatti et al., 2006). The developmental and histological changes of the thymus directly affect the body's immunity and resistance against diseases. Previous studies have shown that boric acid plays an important role in embryonic development, inflammatory response, bone structure and function, and other physiological functions in vivo (Fail

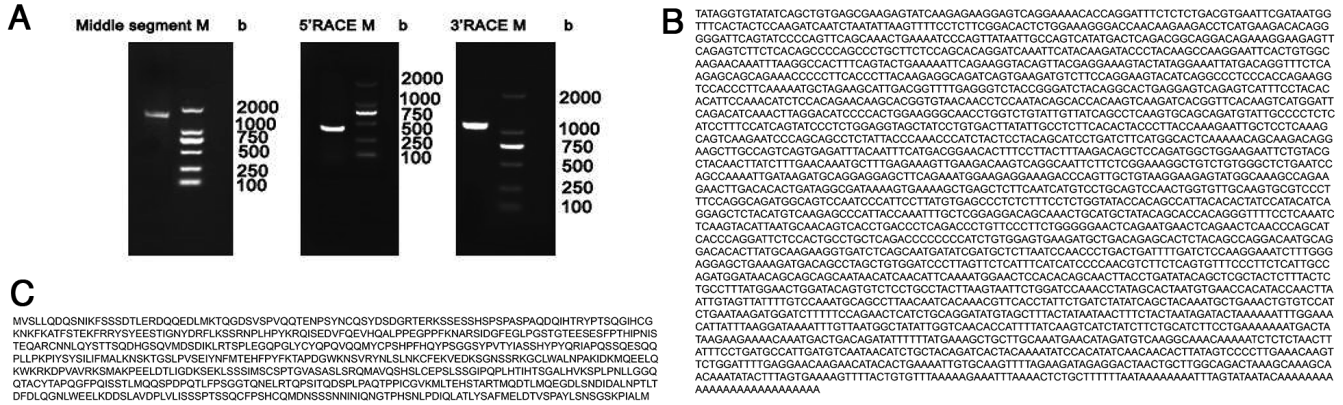

Fig. 7. A. RT-PCR to clone conservative middle segment of Foxn1 using degenerate primers. RACE PCR to clone 5', 3' ends of Foxn1. **B.** Nucleotide sequence of Foxn1 for the African ostrich. The ATG start codon (nt 87-89) and the TGA stop codon (nt 2049-2051) are highlighted by the red font. **C.** Amino acid sequence of Foxn1 for the African ostrich. The amino acid sequence was deduced from the ATG start codon of the nucleotide sequence to the TGA stop codon. The deduced encoded protein sequence of the Foxn1 is highlighted by the shaded box.

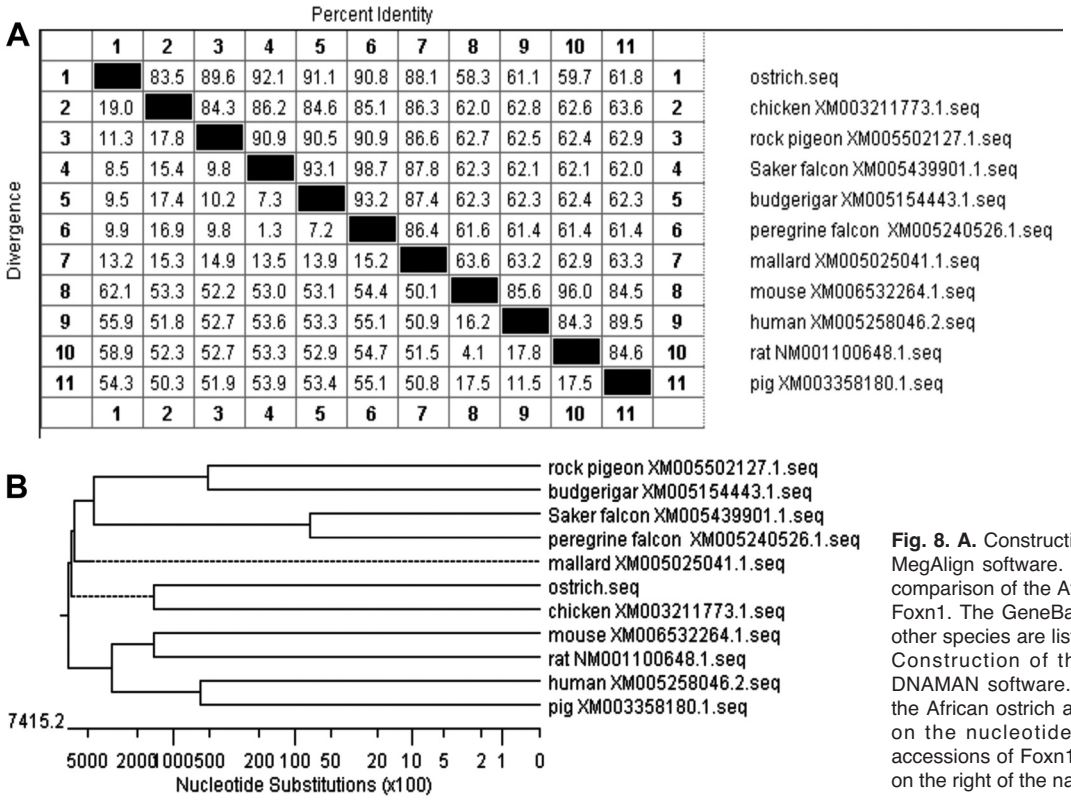

Fig. 8. A. Construction of the homology tree, using MegAlign software. Sequence homologous identity comparison of the African ostrich and other species Foxn1. The GeneBank accessions of Foxn1 of the other species are listed on the right of the figure. **B.** Construction of the Phylogenetic tree, using DNAMAN software. Phylogenetic tree analysis of the African ostrich and other species Foxn1 based on the nucleotide sequences. The GenBank accessions of Foxn1 of the other species are listed on the right of the name of species.

et al., 1998; Hunt, 2003; Kurtoğlu et al., 2005). An appropriate boric acid supplement can promote the development of rat thymus, macrophage Fc receptor expression, and IL-6 secretion; enhance cellular immune function and increase the quantity of circulating NK cells (Armstrong et al., 2001; Li et al., 2009; Ince et al., 2012).

Compared with the thymus tissue structure in the control group, the histological structure of the thymus was well developed in group II, III and IV, while histopathological changes of thymus were observed in group V and VI. In the present study, high doses of boric acid (320, 640 mg/L boric acid) have obviously increased the lymphocytes in thymic medulla, which was usually in response to antigenic stimulation, accompanying inflammation (Elmore, 2006). Also, the thymic epithelial cells were sharply decreased in high dose boric acid groups (320 mg/L, 640 mg/L) and apoptotic cells reached peak in high boric acid concentration groups. This may be due to continuous consumption and accumulation of high concentration of boric acid in the thymus which affect the proliferation and differentiation of thymic epithelial cells and thymocytes and induce the cell apoptosis. Many studies demonstrated that high doses of boric acid had different degrees of toxicity for all the testing organisms (Hunt et al., 1994). In the present study, the "starry sky" appearance was observed in the thymic cortex in high dose boric acid groups, the numbers of lymphocytes were decreased in thymic cortex, leading to decreased cell density, which may be the result of direct thymic lymphocyte toxicity. The high concentration of boric acid that led to direct thymus lymphocyte toxicity may result in increased number of apoptotic lymphocytes. All of the above results indicated that high boric acid supplementation in drinking water seriously damaged the histological structure and induced apoptosis in ostrich thymus. Thus, appropriate boric acid supplementation may have a positive role in the development of thymus, while excessive boric acid may inhibit or even have a toxic effect on the development of thymus.

The thymus is the primary source of T cells. The differentiation of cortical and medullary TEC subpopulations is dependent on T cell lineage commitment, and the maturation of T cells, in turn, is dependent on TEC. Foxn1 regulates development, differentiation, and function of TECs, both in the prenatal (Su et al., 2003; Shakib et al., 2009) and postnatal thymus (Chen et al., 2009; Cheng et al., 2010). In the current report, we showed that Foxn1-positive cells are mainly distributed in mTECs, but rarely in cTECs. These results are consistent with previous studies (Cheng et al., 2010; Guo et al., 2012). Although Foxn1 was required for the development of both mTECs and cTECs in thymus organogenesis, it was essential for the maintenance of steady-state mTECs micro-environment to prevent acute atrophy through regulating apoptosis and promoting epithelial differentiation in the thymus. As the boric acid concentration increased, the MD of Foxn1-positive cells was dynamically increased. In group III (80 mg/L boric acid), the MD of Foxn1-positive cells was highest as compared with other groups. When the concentration of boric acid was beyond 320 mg/L, the MD of Foxn1-positive cells was dynamically decreased. The MD of Foxn1-positive cells was the lowest in group VI (640 mg/L) compared to other groups. The above results showed that the intrathymic changes due to aging led to less expression of Foxn1 in group VI, and resulted in the marked reductions in the quantity of naïve T cells (Yager et al., 2008). This in turn limited the capacity of T-cells to respond to inflammation or infection, resulting in more severe inflammation. In the present study, the reduction of Foxn1 expression postnatally causes premature thymic involution characterized by loss of thymic epithelial cells, and this finding was similar to the previous studies (Chen et al., 2009, Cheng et al., 2010; Corbeaux et al., 2010). Therefore, the appropriate boric acid supplementation (80 mg/L) promoted the Foxn1 expression, which facilitated the thymus development. High boric acid concentration (640 mg/L) inhibited the Foxn1 expression in thymus, which inhibited the thymus development. Taken together, these results suggest that Foxn1 is required to maintain the thymic micro-environment and it may also be needed in thymic epithelial cells from initiation of differentiation throughout the cell's lifetime.

Foxn1 belongs to the Fox transcription factor family implicated in a variety of biochemical and cellular processes, including development, metabolism and aging (Kaufmann and Knöchel, 1996; Schorpp et al., 1997). Foxn1 is selectively expressed only in thymus and skin, and regulates the organ's growth and differentiation (Brissette et al., 1996; Mecklenburg et al., 2005). It is clear that the activity of Foxn1 is required for productive thymic epithelial differentiation (Kindred, 1971).The genome of ostrich Foxn1 had not been reported till now. Moreover, cloning full-length genome of Foxn1 is an efficient way to analyze molecule characteristics. Therefore, the genomic full-length of ostrich Foxn1 gene was cloned and the DNA sequence determined in the present study. Sequence comparison with other species selected from GeneBank revealed that the ostrich Foxn1 gene shared a 92% identity with saker falcon, 91.1% with budgerigar, 90.8% with peregrine falcon, 89.6% with rock pigeon and 83.5% identity with gallus gallus. The full genome of the ostrich Foxn1 gene was 2736 nucleotides in length and a phylogenetic tree was constructed in this study. Ostrich Foxn1 has a close resemblance with chicken Foxn1 and they were included in the same group (Fig. 8A). Furthermore, this is the first report on the gene sequence of ostrich Foxn1.

The translation of the genomic sequence into amino acids was performed using DNAMAN software. The deduced encoded protein was composed of 645 amino acids. Foxn1 was highly conserved among species, which is the reason why a positive signal could be

achieved in the thymus of ostrich in the present study, although primary antibody used in immunohistochemistry was rabbit-anti-chick Foxn1 antibody.

In conclusion, apoptosis and the Foxn1 expression in ostrich thymus were closely related with boric acid concentration. Low concentrations of boric acid could inhibit apoptosis, whereas high concentrations of boric acid could promote apoptosis in ostrich thymus. Similiarly, low concentration of boric acid (80mg/L) promoted the Foxn1 expression, followed by facilitation of thymus development. High boric acid concentration (640 mg/L) inhibited Foxn1 expression in thymus, which inhibited the thymus development. We demonstrated that the optimal dose of boric acid supplementation in ostrich drinking water was 80 mg/L boric acid. Moreover, the genomic full-length of African ostrich Foxn1 was cloned for the first time in the present study.

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