# The effects of taurochenodeoxycholic acid in preventing pulmonary fibrosis in mice

**Chuan Zhou[1], Youfei Shi[1], Jinlian Li[2]*, Wen Zhang[1], Changqi Fu[1], Yanmin Wang[1], Yan Liu[1] and Jianzhu Liu[1]***

[1]College of Veterinary Medicine, Sino-German Cooperative Research Centre for Zoonoses of Animal Origin of Shandong Province, Shandong Agricultural University, Tai`an, China

[2]Department of biological science and technology, Taishan University Tai`an, China

**Abstract**: The present study prepared the pulmonary fibrosis model in mice by using Bleomycin and carry out the investigations on the effects of taurochenodeoxycholic acid (TCDCA) in preventing pulmonary fibrosis in mice. Expression profiles of the bile acid receptors in the lung of mice FXRα and TGR5 were examined, and pulmonary coefficient, pathohistology as well as expression of TNF-α, MMP-2, MMP-9 and TIMP-2 in pulmonary fibrosis mice. The results showed that FXRα and TGR5 simultaneously expressed in the lung of the mice; TCDCA in dosages of 0.05 and 0.1g/kg can extremely significantly decrease the pulmonary coefficient in the model mice ($P<0.01$), TCDCA in a dosage of 0.2g/kg significantly decreased the pulmonary coefficient in the model mice ($P<0.05$); TCDCA in dosages of 0.05 and 0.1g/kg significantly reduce the pathological damages on their lungs; TCDCA can extremely significantly decrease the expression levels of TNF-α and TIMP-2 in pulmonary tissues in the pulmonary fibrosis mice ($P<0.01$), the expression level of MMP-9 extremely significantly increased ($P<0.01$), while it has no significant effects on MMP2. The results as mentioned above indicated that TCDCA had antagonistic actions on pulmonary fibrosis in mice.

**Keywords**: Taurochenodeoxycholic acid; preventing; pulmonary fibrosis; mice.

## INTRODUCTION

Pulmonary fibrosis is the most common diagnosis among patients presenting with interstitial lung disease and one of the most serious diseases in respiratory system. It is characterized by diffusive alveolitis, structural disturbance in pulmonary alveoli and finally pulmonary interstitial fibrosis. The frequently used drugs in clinical practices include glucocorticoids, immunosuppressive agents, cytotoxic drugs and anti-fibrosis agents. However, treatment options for pulmonary fibrosis are very limited. There is no evidence that any medications can help this condition. Taurochenodeoxycholic acid (TCDCA) is a smell-less conjugated bile acid powder, present in animal bile. Current investigations on the pharmacological actions of TCDCA in China showed that TCDCA had anti-inflammatory (Liu *et al*., 2011), immunore gulatory (Shi *et al*., 2007) and anti-apoptotic (Turner *et al*., 2007) effects. No result has been reported on the effects of TCDCA in preventing pulmonary fibrosis now.

Investigations on bile acid and its active ingredients have appealed intensive attention. Since the discovery of the two receptors for bile acid FXRα (Kawamata *et al*., 2003) and TGR5(Hylemon *et al*., 2009) in 1999 and 2003, bile acid has been considered as a signaling molecule or a kind of hormone (Keitel *et al*., 2008), which play important roles in secretory regulation(Katsuma *et al*., 2005; Watanabe *et al*., 2006), energetic metabolism (Huang *et al*., 2006), liver regeneration promotion (Fiorucci *et al*., 2005), fibrosis prevention and other aspects in organisms. Fiorucci *et al*. (2005) reported that FXRα can decrease the expression of tissue inhibitor of metalloproteinase (TIMP-1) but increase the activity of matrix metalloproteinase in the astrocytes of liver, thus promote fibrolysis in liver and reverse fibrous degeneration. Jiang *et al*. (2007) found that the FXRα excitomotor GW4064 and bile acid can reduce lipid accumulation in kidney and down-regulate the expression levels of SREBP-1, fibrosis-promoting cytokines, inflammatory cytokines and oxidative stress enzymes, significantly improve the symptoms of glomerulosclerosis, tubulointerstitial fibrosis and proteinuria, and protect kidney. Current investigations have confirmed that expression of two kinds of bile acid receptors FXRα and TGR5 simultaneously existed in the lung of human(Hylemon *et al*., 2009; Gharaee-Kermani *et al*., 2005). It has not been reported that whether it also has functions in preventing pulmonary fibrosis. The present study found after tests that FXRα and TGR5 were also simultaneously detected in the lung of mice and thus mice were used as the subjects for preparing the pulmonary fibrosis model. Investigations on the effects of TCDCA in preventing pulmonary fibrosis in mice were carried out, which may provide the basis for developing TCDCA into the drug for preventing pulmonary fibrosis.

## MATERIAL AND METHODS

### *Experimental animals and reagents*

Clean pure Kunming mice weighing 23 ～ 25g were

**Corresponding author:* e-mail: liujz@sdau.edu.cn

provided by the Animal Test Center of Shandong Lukang, female mice and male mice accounted for 50% respectively. The number for the permission certificate: SCXK (Shandong) 20080002. Trizol kit (Cat. No.15596-026, Invitrogen); SYBR Premix EX Taq TM, (DRR041A,L ot BKA703, TaKaRa); Prime Script TM RT reagent Kit, (DRR037A, Lot BK2401, TaKaRa); Bleomycin (No.849007, Zeocin) Prime Script one setp RT-PCR Kit, (DRR055A, BK1501, TaKaRa). Taurocheno eoxycholic acid (TCDCA, purity >98%) were purchased from Shaoxing Kemu Chemical Technology Co., Ltd. (Shaoxing, Zhejiang Province, China, No. 20090510).

***Detection of FXRα and TGR5 expression in the lung of mice***

RT-PCR was carried out to examine the expression of FXRα and TGR5 in hepatic, renal and lung tissues in mice, rats and guinea-pigs. Trizol method was used to extract total RNA from the adrenal gland of mice, Primer Premier5.0 software was used to design some primers for FXRα and TGR5. The forward primer of FXRα5′-GACCACGAAGACCAGATTGCT-3′, the reverse primer: 5′-TCT CCACT GCCTCTCTATCCTT-3′. The forward primer of TGR5: 5′-CTCATCGTCA TCGCCAA CC-3′, the reverse primer: 5′-AGCAGGGAAAGG AAACAAAAG-3′.The PCR products were sent to Beijing Liuhe Huada Gene Scientific and Technological Co., Ltd. for sequencing.

***Establishment and drug administration for pulmonary fibrosis model of mice***

The present study utilized Bleomycin to induce and prepare the pulmonary fibrosis model of mice according to the methods described previously (Kamata *et al*., 2011). The preparation method was as followed: Kunming Mus musculus albus weighing 20±2g were subjected to intramuscular injection with 0.3ml/kg xylidinothiazoline and 0.2ml/kg ketamine for anaesthesia. After that the mice were kept lying on their backs on the plate, the furs in their necks were removed, and conventional skin sterilization was carried out. Incisions of about 1 cm were cut on their necks under sterile conditions and the trachea was gradually separated and exposed. Subsequently the head end of the plate was lifted to keep it in a angle of 35° to the desk top, a syringe of 1 ml was inserted into the trachea at the crotch nearby the trachea and 0.2～0.3ml Bleomycin physiological saline was rapidly injected (Bleomycin was administered in a dosage of 5mg/kg body weight), and the needle was withdrawn immediately. The plate was then held vertically to keep the mice in orthostatic body position, and it was rotated left and right for 1 min in order to distribute the physic liquor uniformly into both lungs as far as possible. The skin was sutured and local sterilization was carried out to prevent infections, and the mice were subjected to conventional rearing in the cages after natural consciousness. The method for preparing the model in the normal control group was the same as mentioned previously, and the only difference was that physiological saline in the same volume was used as the tracheal infusion in stead of Bleomycin.

***Grouping and drug administration***

80 Kunming mice were raised in the laboratory for three days to adapt to the conditions, and they were allowed to take foods and water freely. Subsequently they were randomly divided into five groups, namely the normal group, the pulmonary fibrosis model group as well as the administration groups with TCDCA in three different dosages (high, moderate and low dosages), and the dosages were 0.05g/kg, 0.1/kg and 0.2mg/kg respectively. The mice were subjected to intragastric administration after the model was successfully established, once a day for continuous three weeks and the mice in the model group and the blank group were administered intragastrically with physiological saline.

***Determination of the changes in pulmonary coefficient***

Pulmonary coefficient was calculated according to the formula as below, pulmonary coefficient = weight of lung (g)/body weight (g) ×100%.

***Pathological examinations on pulmonary tissues***

Pulmonary tissues were collected, fixed, embedded and sectioned according to conventional pathological methods. The pathological sections were subjected to HE and Masson staining, and the pathohistological changes were observed under a light microscope.

***Detection of TNF-α, MMP2, MMP9 and TIMP2 expression in pulmonary tissues***

Expression levels of TNF-α, MMP2, MMP9 and TIMP2 in the lung of mice were examined by using real-time fluorescent quantitative PCR. The basic operations included the procedures as below: total RNA was extracted by using Trizol kit and 18S rRNA was used as the internal reference and the primers were synthesized according to previous reports (Vavassori *et al.,* 2009);
F: 5'-TTCGGAACTGAGGCCATGATT-3';
R: 5'-TTTCGCTCTGGTCCGTCTTG-3'.
The primers of TNF-α, MMP2, MMP9 and TIMP2 of mice were designed by using Primer 5.0 software, TNF-α:
F: 5'-GCAGGTCTACTTTGGAGTCATTG-3',
R: 5'-CAGGTCACTGTCCCAGCATCT-3'.
MMP2: F: 5'-GAATGCCATCCCTGATAACCT-3',
R: 5'-GCTTCCAAACTTCACGCTCTT-3'; MMP9:
F: 5'-CACTTACTATGGAAACTCAAATGGT-3',
R: 5'-CCTCAAAGATGAACGGGAACA-3'; TIMP2:
F: 5'-ACCGAGCGAGCAAACGAA-3',
R: 5'-AGCAGCAAGCCCACGGATA-3'; the standard curves of the genes were plotted by using the cDNA samples diluted in gradients. After the reactions, the relatively quantitative analysis was carried out according to the $2\text{-}\Delta^{Ct}$ method, among them Δ Ct=Ct (target gene) –

Ct (internal reference gene); ΔΔCt=ΔCt (the test group) - ΔCt (the mean value of the control group); the relative expression level was represented by using $2^{-\Delta\Delta Ct}$.

***Data processing***

The results from Realtime-PCR detection were analyzed by using ABI 7500 Software SDS v1.31 system; SPSS11.0 statistical software was used for data analysis, the data were represented by $\bar{x} \pm s$, *t*-test was carried out for the comparison in the mean values from two samples, and *F*-test was carried out for the analysis on the mean values of several samples.

## RESULTS

***Detection of FXRα and TGR5 in the lung of mice***

The RT-PCR products for parts of the sequences for FXRα and TGR5 in the lung of mice were shown in *Fig* 1, and the sequencing results for FXRα and TGR5 were subjected to sequence alignments to FXRα sequence (accession number: NM_001163700.1) and TGR5 sequence (accession number: AB089310.1) in GenBank. The homology was very high, which were 98.67% and 94.71%, indicating that FXRα and TGR5 simultaneously expressed in the lung of mice.

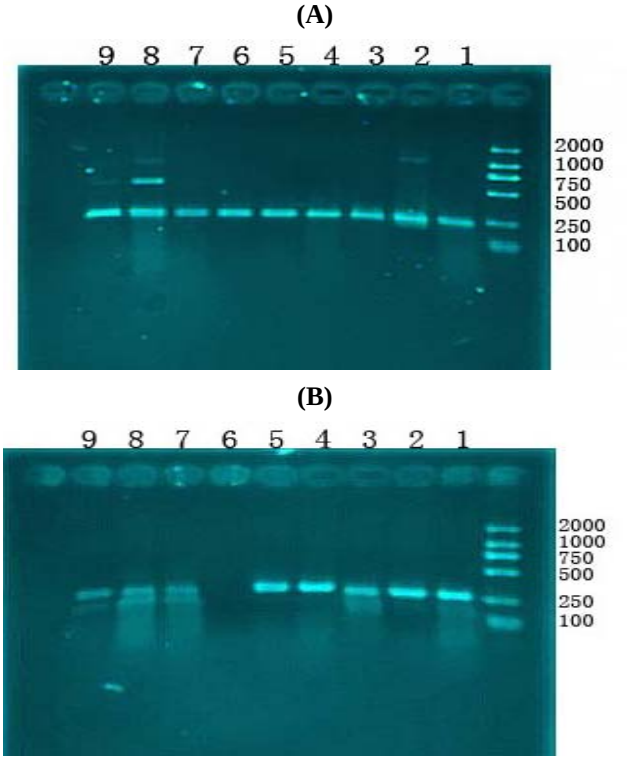


**Fig. 1**: Detection of FXRα and TGR5 expression in the lung of mice by RT-PCR

Line 1, 4, 7, are the liver, line 2, 5, 8 are the kidney, line 3, 6, 9 are the lung in the Kunming mice, wistar mice and guinea pig in A and B, respectively.

***Determination of pulmonary coefficient in the mice***

The results for the pulmonary coefficient were shown in Table 1. It can be found that the pulmonary coefficient in the model group extremely significantly increased in comparison to that in the normal group, while the pulmonary coefficients in the model mice from the low and moderate dosage groups extremely significantly decreased, the high dosage significantly decreased the pulmonary coefficient in the model mice, but the differences were still statistically significant or extremely statistically significant in the comparison between the drug administration group and the normal group, indicating that pulmonary fibrosis in the model mice after drug administration was improved, but it was still not as good as that in normal mice.

**Table 1**: The change of pulmonary coefficient

| Groups | Dose (g/kg) | Pulmonary coefficient |
|---|---|---|
| Normal | 0 | 0.0083±0.0011 |
| Model | 0 | 0.0207±0.0039** |
| Low | 0.05 | 0.0129±0.0026*ΔΔ |
| middle | 0.1 | 0.0153±0.0031**ΔΔ |
| High | 0.2 | 0.0158±0.0027**ΔΔ |

*P<0.05, **P<0.01compare to normal group ; Δ P<0.05 ; ΔΔ P< 0.01, compare to model group。

***Conventional HE and Masson staining and observations for the sections***

The HE and Masson staining results were shown in fig. 2. It can be found that the pulmonary alveoli in the model group and the high dosage group were filled with large amount of inflammatory effusion, the blood vessels in the alveolar wall were significantly stretched and congested, and the pulmonary alveoli underwent consolidation. The alveolar space was filled with large amount of proliferative blood capillaries and fibroblasts, and the normal structures of pulmonary tissues disappeared. Compensatory emphysema in surrounding alveolar spaces was concurrent during the consolidation of some alveolar spaces in the high dosage group. However, though small amount of inflammatory effusion was detected in the moderate and low dosage groups, but it was significantly alleviated in comparison to the model group. The structures and the outline of the pulmonary tissues still existed and their functions were not lost.

***Detection of TNF-α, MMP-2, MMP-9 and TIMP-2 in the pulmonary tissues***

The results from the detection on the effects of TCDCA on TNF-α, MMP2, MMP-9 and TIMP-2 expression in the pulmonary tissues were shown in table 2. The expression levels of TNF-α and TIMP-2 extremely significantly increased in the model group in comparison to those in the normal control, while the expression levels of MMP2

and MMP-9 extremely significantly decreased; the expression levels of TNF-α and TIMP-2 in the model mice of the three dosage groups (low, moderate and high dosage groups) extremely significantly decreased in comparison to those in the model group; the expression level of MMP-9 in the model mice from the low and moderate dosage groups extremely significantly increased in comparison to that in the model group; while statistically significant differences were not detected in MMP2 in the three drug administration groups. The results as mentioned above indicated that TCDCA can improve pulmonary fibers in the mice.

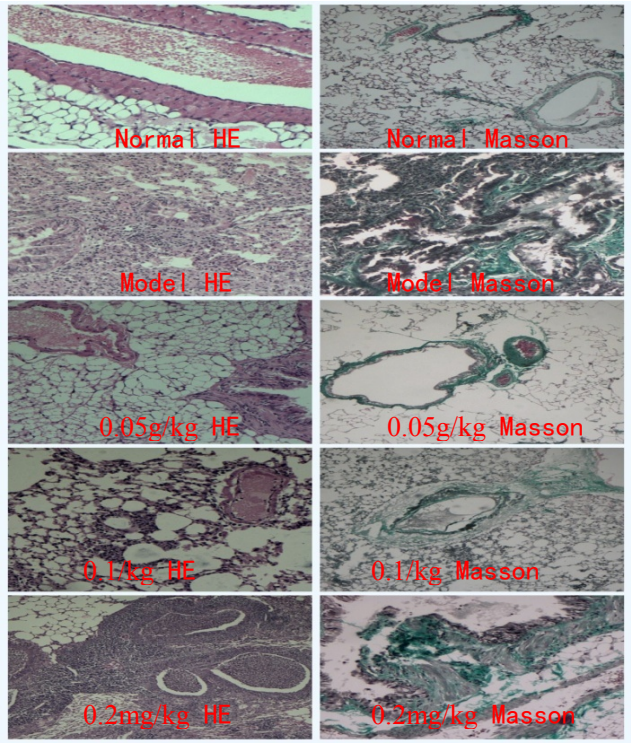


**Fig. 2**: The results of HE and Masson staining in lung tissues

## DICUSSION

Preparation of pulmonary fibrosis model in mice by using Bleomycin is normally composed with two continuous procedures, namely inflammatory reactions in pulmonary alveoli and fibrosis. These two procedures are closely related and can not be separated. During the formation of pulmonary fibrosis, the basic elements as below are involved: damages in pulmonary tissues, involvement of inflammatory mediators' → activation of pulmonary macrophages and other cells → secretion of several kinds of cytokines → stimulation of lung fibroblast migration by cytokines, proliferation, activation and transformation into myofibroblasts → increase in extracellular matrix (ECM) secretion or decreased degradation → formation of pulmonary fibrosis. It can be found that many cytokines play important roles in fibrosis. The present study carried out further analysis on TNF-α. It was found that TNF-α concentration pulmonary tissues after drug administration was extremely significantly inhibited. It has also been confirmed in previous studies that the bile acid receptors FXRα and TGR can both affect the secretion of cytokines. Vavassori *et al* (2008) confirmed that FXRα was an important regulator for natural immunological functions of intestinal tract, and it was found by treating the artificially prepared colonitis model of mice by using FXRα excitomotor that it can inhibit the activation of immunological cells in the intestinal tract, reduce the expression of IL-1β, IL-2, IL-6, TNF-α and IFN-γ, and alleviate the damages in the intestinal tract tissues. Kawamata *et al*.(2003) found that TGR5 receptor was expressed in high level in mononuclear phagocytic system and they confirmed that bile acid can significantly inhibit TNF-α, IL-1α, IL-1β, IL-6 and IL-8 secretion in *in vitro* cultured rabbit alveolar macrophages stimulated by LPS. Moreover, Keitel *et al*. (2008) confirmed that TGR5 distributed in the plasma membrane of Kupffer's cells isolated from the liver of rats and bile acid significantly inhibited IL-1α, IL-1β, IL-6 and TNF-α expression in Kupffer's cells induced by LPS. Therefore, the inhibition of TCDCA on TNF-α expression in the lung of model mice in the present study may be related to its effects on bile acid receptors. Furthermore, pulmonary fibrosis is characterized by its regulation on the accumulation of extracellular matrix (ECM) in pulmonary tissues in large amount, while ECM metabolism was co-regulated by matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinase (TIMPs). In the viewpoint of ECM metabolism, the imbalance in the proportion of MMPs/TIMPs is an influencing factor for pulmonary fibrosis. When the concentrations of MMPs in the pulmonary tissues increase, the concentrations of TIMPs decrease, which can degrade collagen fibers and further reverse fibrosis; on the contrary, collagen fibers are

**Table 2**: Detection of TNF-α, MMP2, MMP9 and TIMP2 expression in pulmonary tissues

| Groups | Dose (g/kg) | TNF-α | MMP2 | MMP9 | TIMP2 |
|---|---|---|---|---|---|
| Normal | 0 | 1.00±0.12 | 1.00±0.12 | 1.00±0.14 | 1.00±0.10 |
| Model | 0 | 1.39±0.15** | 0.58±0.11** | 0.30±0.06** | 2.78±0.46** |
| Low | 0.05 | 0.23±0.08**$^{\Delta\Delta}$ | 0.69±0.14** | 0.57±0.06**$^{\Delta\Delta}$ | 0.39±0.13**$^{\Delta\Delta}$ |
| Middle | 0.1 | 0.16±0.06**$^{\Delta\Delta}$ | 0.57±0.21** | 0.78±0.27*$^{\Delta\Delta}$ | 0.43±0.06**$^{\Delta\Delta}$ |
| High | 0.2 | 0.83±0.10*$^{\Delta\Delta}$ | 0.42±0.08** | 0.16±0.03** | 0.16±0.01**$^{\Delta\Delta}$ |

*P < 0.05, **P < 0.01; compare to normal group ; Δ P < 0.05 ; ΔΔP < 0.01, compare to model group.

produced. The present study carried out detection on the expression levels of MMP-2, MMP-9 and TIMP-2 in the pulmonary tissues. It was found that TCDCA can regulate the imbalance of the proportion between MMPs/TIMPs and thus play roles in preventing pulmonary fibrosis. It can be found from the analysis as mentioned above that TCDCA can inhibit the two continuous procedures during pulmonary fibrosis, namely inflammatory reactions and fibrosis. In other words, it can not only inhibit the production of inflammatory factors, but also regulate the imbalance of the proportion between MMPs/TIMPs, thus alleviate or reverse pulmonary fibrosis. It has been now demonstrated that the bile acid receptor FXRα participates in the regulations on the expression of MMPs and TIMPs, but it has not been reported whether the bile acid receptor TGR5 is also involved in the regulations on MMPs and TIMPs. In conclusion, TCDCA had antagonistic actions on pulmonary fibrosis in mice.

## ACKNOWLEDGMENT

This work was supported by a grant from the Natural Science Foundation of Shandong Province, China (No. ZR2010CQ011).

## REFERENCES

Fiorucci S, Rizzo G, Antonelli E, Renga B, Mencarelli A, Riccardi L, Orlandi S, Pruzanski M, Morelli A and Pellicciari R (2005). A farnesoid x receptor-small heterodimer partner regulatory cascade modulates tissue metalloproteinase inhibitor-1 and matrix metalloprotease expression in hepatic stellate cells and promotes resolution of liver fibrosis. *J. Pharmacol. Exp. Ther.,* **314**(2): 584-595.

Gharaee-Kermani M, Ullenbruch M and Phan SH (2005). Animal models of pulmonary fibrosis. *Methods Mol. Med.,* **117**: 251-259.

Huang W, Ma K, Zhang J, Qatanani M, Cuvillier J, Liu J, Dong B, Huang X and Moore DD (2006). Nuclear receptor-dependent bile acid signaling is required for normal liver *regeneration. Science*, **312**(5771): 233-236.

Hylemon PB, Zhou H, Pandak WM, Ren S, Gil G and Dent P (2009). Bile acids as regulatory molecules. *J. Lipid Res*., **50**(8): 1509-1520.

Jiang T, Wang XX, Scherzer P, Wilson P, Tallman J, Takahashi H, Li J, Iwahashi M, Sutherland E, Arend L and Levi M (2007). Farnesoid X receptor modulates renal lipid metabolism, fibrosis, and diabetic *nephropathy. Diabetes,* **56**(10): 2485-2493.

Kamata H, Tasaka S, Inoue K, Miyamoto K, Nakano Y, Shinoda H, Kimizuka Y, Fujiwara H, Ishii M, Hasegawa N, Takamiya R, Fujishima S, Takano H and Ishizaka A (2011). Carbon black nanoparticles enhance bleomycin-induced lung inflammatory and fibrotic changes in mice. *Exp. Biol. Med.* (Maywood), **236**(3): 315-324.

Katsuma S, Hirasawa A and Tsujimoto G (2005). Bile acids promote glucagon-like peptide-1 secretion through TGR5 in a murine enteroendocrine cell line STC-1. *Biochem. Biophys. Res. Commun.,* **329**(1): 386-390.

Kawamata Y, Fujii R, Hosoya M, Harada M, Yoshida H, Miwa M, Fukusumi S, Habata Y, Itoh T, Shintani Y, Hinuma S, Fujisawa Y and Fujino M (2003). A G protein-coupled receptor responsive to bile acids. *J. Biol. Chem*., **278**(11): 9435-9440.

Keitel V, Donner M, Winandy S, Kubitz R and Häussinger D(2008). Expression and function of the bile acid receptor TGR5 in Kupffer cells. *Biochem. Biophys. Res. Commun*., **372**(1): 78-84.

Keitel V, Kubitz R and Häussinger D (2008). Endocrine and paracrine role of bile acids. *World J. Gastroenterol.,* **14**(37): 5620-5629.

Liu M, Mao W, Guan H, Li L, Wei B and Li P(2011). Effects of taurochenodeoxycholic acid on adjuvant arthritis in rats*. Intl. Immunopharmacol.*, **11**(12): 2150-2158.

Schote AB, Turner JD, Schiltz J and Muller CP (2007). Nuclear receptors in human immune cells: Expression and correlations. *Mol. Immunol.*, **44**(6): 1436-1445.

Shi Y, Li P and Li J (2007). Regulation effect of taurochenodeoxycholic acid immunologic function in mice. *Chin. Pharmacological Bulletin*, **23**(7): 859-865.

Turner DJ, Alaish SM, Zou T, Rao JN, Wang JY and Strauch ED (2007). Bile Salts Induce Resistance to Apoptosis Through NF-κB-mediated XIAP Expression. *Ann. Surg.*, **245**(3): 415-425.

Vavassori P, Mencarelli A, Renga B, Distrutti E and Fiorucci S (2009). The bile acid receptor FXR is a modulator of intestinal innate immunity. *J. Immunol.*, **183**(10): 6251-6261.

Watanabe M, Houten SM, Mataki C, Christoffolete MA, Kim BW, Sato H, Messaddeq N, Harney JW, Ezaki O, Kodama T, Schoonjans K, Bianco AC and Auwerx J (2006). Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. *Nature,* **439**(7075): 484-489.