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ESTABLISHMENT AND EVALUATION OF ORTHOTOPIC HEPATOCELLULAR CARCINOMA AND DRUG-INDUCED HEPATOCELLULAR CARCINOMA IN MICE WITH SPLEEN-DEFICIENCY SYNDROME IN TRADITIONAL CHINESE MEDICINE

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Abstract

Background: Spleen-deficiency syndrome (SDS) in Traditional Chinese Medicine (TCM) played pivotal roles on the development of hepatocellular carcinoma (HCC). This study was performed to establish and evaluate HCC model in mice with SDS in TCM.
Material and Methods: A total of 90 C57BL/6 mice were randomized in six groups (n=15 for each group): A, Control group; B, SDS group; C, orthotopic HCC (OHCC) group; D, OHCC based on SDS (SDS-OHCC) group; E, Drug-induced HCC (DHCC) group; F, DHCC based on SDS (SDS-DHCC) group. The SDS model were established by subcutaneous injection of reserpine, followed by the OHCC or DHCC model establishment. The SDS scores, tumor formation rate and survival time were recorded and calculated, as well as the histochemical stain was performed.
Results: The SDS scores of mice in Group B, D, F were 17.57±4.86 ($P<0.05$ vs. Group A), 18.13±4.53 ($P<0.05$ vs. Group A and C) and 23.32±4.94 ($P<0.05$ vs. Group A and E) respectively. The tumor formation rate of mice in Group C, D, E and F were 73.33%, 100%, 60% and 80% respectively. The survival time of mice in Group C, D, E and F were 26.42±5.27, 17.33±4.76 ($P<0.05$ vs. Group C), 35.77±6.12 and 22.61±5.05 ($P<0.05$ vs. Group E) respectively.
Conclusion: The SDS-oriented HCC mice models were simple and easily-operated models for further studies on SDS oriented tumor. Meanwhile, SDS was a pivotal factor for low outcome of hepatic tumor.

Key words: Spleen-deficiency, Hepatocellular carcinoma, Mice model, Evaluation, Traditional Chinese Medicine

Abbreviations: HCC, Hepatocellular carcinoma; OHCC, Orthotopic hepatocellular carcinoma; DHCC, Drug-induced hepatocellular carcinoma; SDS, Spleen-deficiency syndrome; TCM, Traditional Chinese Medicine; SPF, Specific pathogen-free; DEN, Diethylnitrosamine; CCl_4, Carbon tetrachloride; HE, Hematoxylin-eosin; IACUC, Institutional Animal Care and Use Committee.

Introduction

Hepatic tumor is generally known as a malignant tumor the world over. It is the second leading cause of cancer death globally (Petrick et al., 2016). Literature documentation showed that hepatic tumor ranked sixth among morbidity scale of all tumors (Mohammadian et al., 2016). In addition, more than 80% new hepatic tumor cases were reported in Asia and Africa, of which half were Chinese patients. According to previous studies, hepatic tumor mortality increased to the second place among all cancer cases in China (Fang et al., 2015). It was well documented that multiple factors, pathways and steps, including the external environment such as virus, parasite and bacterial, internal environment, contributed to the primary hepatic carcinoma (Clark et al., 2015). Generally, genetic factors could only account for 5% of the tumor pathogenesis, whereas the coefficient of external and internal environment showed its influence on most tumors (Bhattacharjee et al., 2013). The internal environment, considered appropriate for the development of carcinoma, are believed to be one of the most pivotal factors in the formation of tumors (Xu et al., 2013). How genetic factors affected the development of tumors remains unclear, thus it is better to change the external living environment or adjust the internal environment during the prevention and treatment procedure of hepatic tumors.

In the theory of Traditional Chinese Medicine (TCM), pathogenesis of hepatic tumor is total in external and internal factors, the former includes *Liuyin Disease* (six climatic exopathogens) and improper diet while the latter of which includes the deficiency of *Qi* and *Blood* or *Yin* and *Yang*, or accumulation of pathogenic toxin, leading to hepatic mass (Liu and Li, 2012). In the theory of TCM, spleen-deficiency syndrome (SDS) is both organic disorders and functional changes which are widely associated with different systems, organs and tissues. It was proven that changes of most indices or indicators could be attributed to SDS. On the other hand, any of those changes of indices or indicators may not be found in SDS. To sum up, individuals with SDS may suffer from digestive problems such as loose stools, abdominal distension following meals, sallow complexion, and loss of appetite, weight loss, and general

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weakness and/or lower disease resistance. Several TCM researchers demonstrated that the symptoms of the hepatic tumor reflected on the liver while the root of which attributed to spleen, consequently the spleen-deficiency was the basic pathogenesis of hepatic tumor (Li et al., 2014; Sun et al., 2008). Since the treatment for hepatic tumor is limited and unsatisfied, it is necessary for researchers to focus on alternative and complementary therapies such as TCM.

Since SDS played pivotal roles on different kinds of diseases and thereafter became a basis for their pathogenesis, the SDS models were frequently established so as to further explore the corresponding mechanism or treatment. The SDS models had been well established according to the requirement of different studies. In details, the spleen *Yang* deficiency model could be established by single factor methods such as injecting hydrocortisone and gavaging *Sennae Folium* or by compound factors way such as gavaging adenine, improper diet and exhaustion (Pan et al., 2014) whereas the spleen *Qi* deficiency model could be conducted by oral administration of *Radix Rhei* extract as well as the loaded swimming and starvation for 24 hours (Zheng et al., 2014; Tian et al., 2015; Chen et al., 2014a; Liu and Shi, 2015). As for hepatic tumor model, the most common used approaches for orthotopic transplantation HCC model were intravenous, intrasplenic, intrahepatic inoculation of tumor cells and intrahepatic tissue implantation, of which the orthotopic intrahepatic tissue implantation was regarded as the favorable approach (Rao et al., 2016). Even though the spleen deficiency has been well accepted to be an important issue for the development and formation of hepatic tumor, less information concerning the animal model of hepatic tumor based on spleen deficiency was found, thus it is necessary to introduce a new and modified animal model for further investigation in this field.

In this study, an orthotopic liver transplantation model and drug-induced model in mice with SDS was performed according to the theory of TCM, the general state was observed and the SDS model was evaluated by SDS Scale, additionally the tumor formation rate and survival rate were analyzed, as well as the morphological analysis was conducted by histochemical stain, by which the alternative strategies for SDS-oriented hepatic tumor could be further explored prospectively.

Materials and Methods

Animals and cell line

The whole experimental procedure was performed in the Animal Experimental Center of Sun Yat-sen University (Guangzhou, China). In total 90 specific pathogen-free (SPF) C57BL/6 mice (male; age, 6 weeks; weight, 20±2 g; series No.: 44008500006598) and 1 SPF BALB/c nude mice (male; age, 4 weeks; series No.: 44008500003892) were purchased from the Animal Experimental Center of Sun Yat-sen University (Certification No.: SYXK (Guangdong) 2012-0081; Guangzhou, China). The hepal-6 mouse cell line was acquired from the Cell Bank of the Laboratory Center of Sun Yat‑Sen University. All the procedure was monitored strictly by the Institutional Animal Care and Use Committee (IACUC), Sun Yat-sen University according to the "3R" principles with humanism care to the experimental animals (Ethical Approval No.: IACUC-2013-1002).

Study design

A total of 90 mice were randomized into six groups (n=15 per group) in the SPF laboratory as follows: A, Control group, animals received normal food and water; B, Spleen-deficiency syndrome (SDS) group, animal received SDS model establishment; C, Orthotopic Hepatocellular Carcinoma (OHCC) group, animals received OHCC model establishment; D, SDS-OHCC group, animals received OHCC model establishment followed by the SDS model being accomplished successfully; E, Drug-induced Hepatocellular Carcinoma (DHCC) group, animals received DHCC model establishment; F, SDS-DHCC group, animals received DHCC model establishment followed by the SDS model being accomplished successfully. Animals in Group B, C, D, E and F started to receive the corresponding model establishment following 7 days of free feeding for food and water.

Establishment of SDS model

The mice were housed in a dry SPF environment between 24^{o}C to 26^{o}C temperature range at 12-h light/dark cycle with free feeding. The mice in the SDS Group and SDS-OHCC Group received the injection of reserpine (Jinyao Amino Acids Co., Ltd., Tianjin, China) with a dose of 0.1 mg/kg subcutaneously (Zhao et al., 2011; Gao et al., 2009; Ren et al., 2000) while the mice in Control Group and OHCC Group received 0.9% Sodium Chloride Solution (Kelun Pharmacy Co., Ltd., Sichuan, China) at a dosage of 2 ml subcutaneously for 14 days continuously. The body odor, mental state, respiration, hair, feces and food-intake were observed and afterwards the SDS scores were recorded based on the SDS Scale listed in Table 1 in order to evaluate the SDS level. According to the previous study (Sun et al., 2016; Shen and Wang, 1987; Zhang et al., 2014; Chen et al., 2014b), the SDS Score below 7 was defined as "Non-SDS", 7-14 as "Mild-SDS", 15-21 as "Typical-SDS" and above 22 as "Severe-SDS".

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Establishment of HCC models

For the OHCC mice model establishment, at the first stage, 0.2 ml hepal-6 mouse HCC cell lines (1×10^7/ml) was transplanted subcutaneously through the injection in the neck of the nude BALB/c mouse. The tumor was harvested after reaching a diameter of 10 mm at approximately 10 days (Rao et al., 2016). The mouse was anesthetized by an intraperitoneal injection of 10% chloral hydrate (3.5 ml/kg; Sigma-Aldrich Shanghai Trading Co., Ltd., Shanghai, China), afterwards the tumor tissue was carefully transferred to the sterilized Saline solution with the necrotic tissue being removed. Then the tumor was cut into 1-mm^3 pieces and transferred to 4^oC environment prior to being transplanted. 7 days after the establishment of SDS model, the mice in OHCC Group and SDS-OHCC Group were anesthetized through intraperitoneal injection of 10 % chloral hydrate (3.5 ml/kg) in associated with the ether inhalation anesthesia, thereafter while 75% alcohol sterilized, the livers were exposed after a vertical incision under the xiphoid was cut. Subsequently, while the alcohol gauze was hold the liver lobe up, the prepared tumor tissues were implanted into subcapsular of left lobe of liver via the coarse needle (Becton Dickinson Medical Devices Shanghai Co., Ltd., Shanghai, China) with a 20^o intersection angle between the needle and liver. Finally, the hemostasis and penicillin sterilization for abdominal cavities were performed followed by the closure of the abdominal cavities layer by layer using 5# suture (Becton Dickinson Medical Devices Shanghai Co., Ltd., Shanghai, China). The states of animals were strictly monitored till 35 days after model established, the tissues and organs would be harvested for further explorations.

For the DHCC mice model establishment, the whole procedure could be divided into four sections. Firstly, the mice received a single abdominal injection of Diethylnitrosamine (DEN) (Sigma-Aldrich Shanghai Trading Co., Ltd., Shanghai, China) with an initial dose of 0.095 mg/g at the first day; afterwards from the 4th day on, they received the intra-gastric administration of the mix compound of Carbon tetrachloride (CCl_4) (Sigma-Aldrich Shanghai Trading Co., Ltd., Shanghai, China) and Olive oil (Sigma-Aldrich Shanghai Trading Co., Ltd., Shanghai, China) (CCl_4 : Olive oil = 1:4) with a dose of 0.005 ml/g for twice a week; thereafter from the 3rd week on, they received another single abdominal injection of DEN with a reduced dose of 0.05 mg/g as well as the Spirit (Alcohol content, 55%; Red Star Co., Ltd., Beijing, China) was began to administrated intragastrically with the initial dose of 0.05 ml/g; finally from the 4th week on, the mice received the increased dose of both CCl_4 compound and Spirit for 0.008 ml/g and 0.07 ml/g respectively. Unlike the OHCC model, it was at least 42 days that the DHCC model could be established successfully according to our preliminary experiment, therefore all the vital indexes were strictly monitored and recorded until the corresponding organs and tissues were harvested.

Histochemical stain

The hepatic tissue of mice in Control Group and SDS Group and the tumor tissues of mice in the rest Groups were the histochemical stain with the hematoxylin-eosin (HE) (Hematoxylin solution and Eosin Y; Sigma-Aldrich Shanghai Trading Co., Ltd., Shanghai, China). The tissues were conducted the paraffin embedding and section and afterward performed the HE staining according to the instruction. The stained sections were observed by the microscope.

Statistical analysis

The data were analyzed with SPSS software (Version 17.0; SPSS, Inc., Chicago, IL, USA). The analysis of variance was used for the normal distribution measurement data; the chi-square test was used for the continuous measurement data while the Kaplan-Meier survival analysis was used for the survival analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Result

General state of animal models

After injecting the hepal-6 mouse HCC cell lines subcutaneously to the back of the nude BALB/c mouse, the tumor tissue developed gradually. Approximately 10 days later, an obvious 10 mm-diameter tumors raised up from the skin, which indicated that the hepatocellular carcinoma tissue had been formatted successfully (Figure 1A). After receiving the establishment of the SDS models, the mice showed different level of spleen-deficiency syndrome such as fatigue, arched back with trembling, faint especially the brown and erected fur (Figure 1B). For the Control Group, the mice showed no specific situations and survived well for the whole experimental procedure. For the OHCC Group and SDS-OHCC Group, the mice showed typical cachexia symptoms while being implanted the tumor tissues, in brief, the mice got the abdominal distension mostly caused by the ascites, mental fatigue, anorexia, weight loss and some other tumor-related symptoms. The dissection for organ and tissue preparation verified the ascites had been formatted and the HCC tissue could easily be confirmed visibly (Figure 1C). Similarly, the mice in the DHCC Group and SDS-DHCC Group also showed the symptoms as above, however they were not as severe as the mice in OHCC Group and SDS-

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OHCC Group, besides, it took more time to format the tumor which was also with smaller visible volume compared to that of OHCC and SDS-OHCC Group (Figure 1D).

The evaluation of SDS models

The evaluation for SDS models was conducted in all mice including the Control Group, OHCC Group and DHCC Group so as to assess the SDS model and the relationship between spleen-deficiency and hepatocellular carcinoma. By observing towards SDS model establishment procedure, the mice in Control Group showed no spleen-deficiency (4.28±2.72) while the mice in SDS Group had typical spleen-deficiency (17.57±4.86). For the tumor groups, the OHCC mice were similar as Control mice which had no spleen-deficiency (3.98±2.76) while SDS-OHCC mice were similar as SDS mice which also had a typical spleen-deficiency (18.13±4.53); the DHCC mice originally showed a mild spleen-deficiency (8.12±2.33) while SDS-DHCC showed a severe spleen-deficiency (23.32±4.94) (Figure 2).

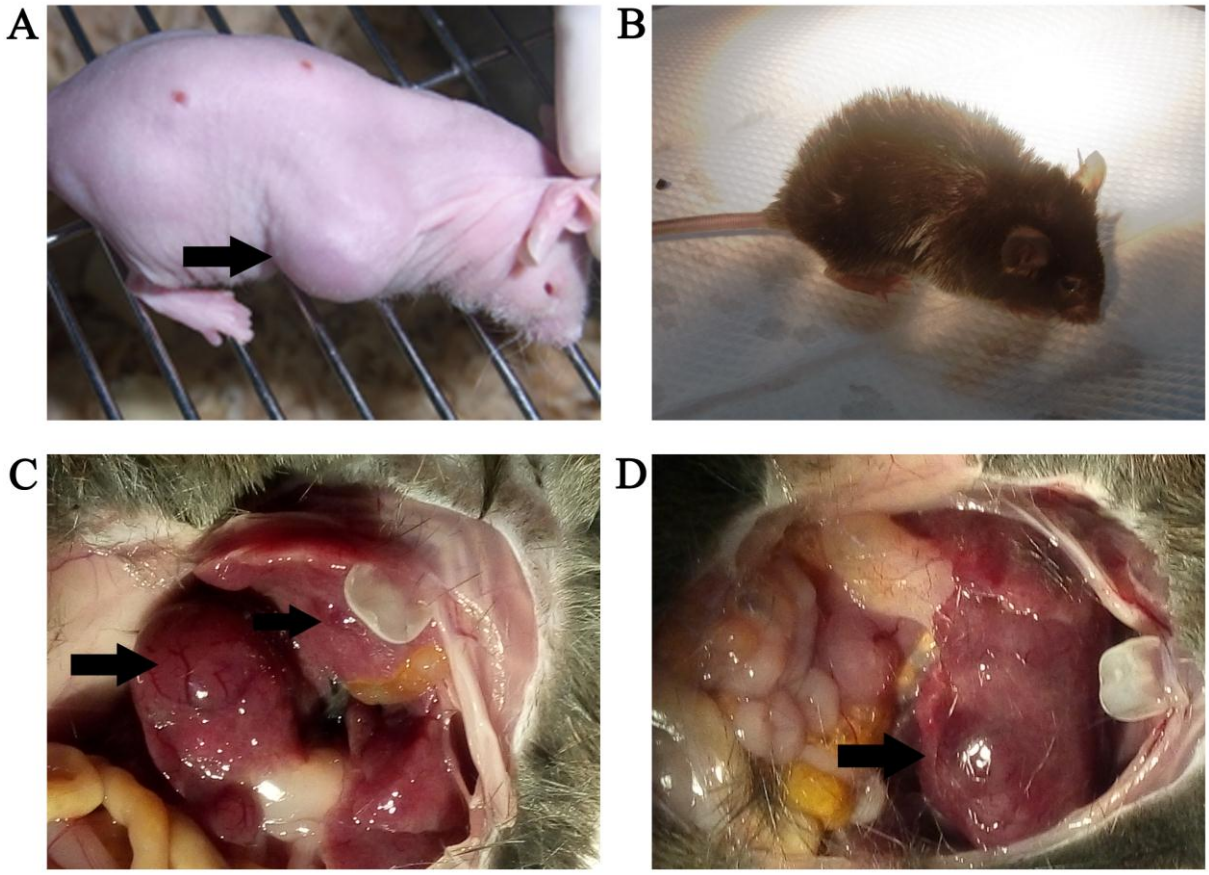

Figure 1: General state of animal models. (A) A visible 10 mm-diameter tumor raised up from the skin of the nude BALB/c mouse while being injected the hepal-6 mouse HCC cell lines subcutaneously to the back; **(B)** The mouse which received the SDS modeling showed typical spleen-deficiency syndrome such as fatigue, arched back with trembling, faint especially the brown and erected fur; **(C, D)** The dissection for tissue preparation showed the tumors had been formatted visibly in the OHCC and DHCC models respectively. *HCC, Hepatocellular carcinoma; SDS, Spleen-deficiency syndrome; OHCC, Orthotopic hepatocellular carcinoma; DHCC, Drug-induced hepatocellular carcinoma.*

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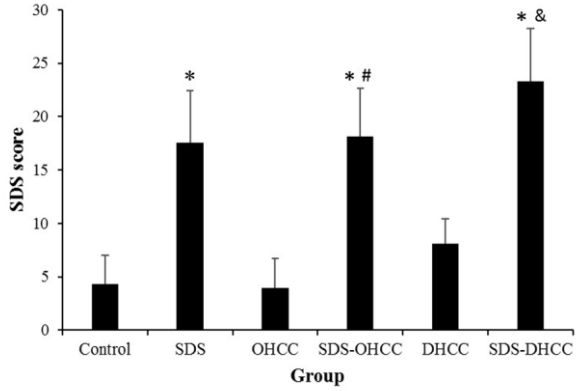

Figure 2: The evaluation of SDS models by SDS scores. **P<0.05 vs. Control group; #P<0.05 vs. OHCC group; & P<0.05 vs. DHCC group. SDS, Spleen-deficiency syndrome; OHCC, Orthotopic hepatocellular carcinoma; DHCC, Drug-induced hepatocellular carcinoma.*

Tumor formation and survival analysis

The tumor formation rate and survival time for HCC model mice were illustrated in Figure 3. For the mice which received OHCC model establishment, 73.33% of which developed the tumor successfully and the mean survival days was 26.42±5.27; while the OHCC mice were SDS-oriented, the tumor formation rate increased to 100% and they survived for the shortest days (17.33±4.76). For the DHCC model, only 9 of 15 mice formatted the tumor tissues (60.00%) and the survival time were the longest (35.77±6.12); in the event of these mice got the SDS in advance, the tumor formation rate raised to 80.00% and they survived shorter (22.61±5.05).

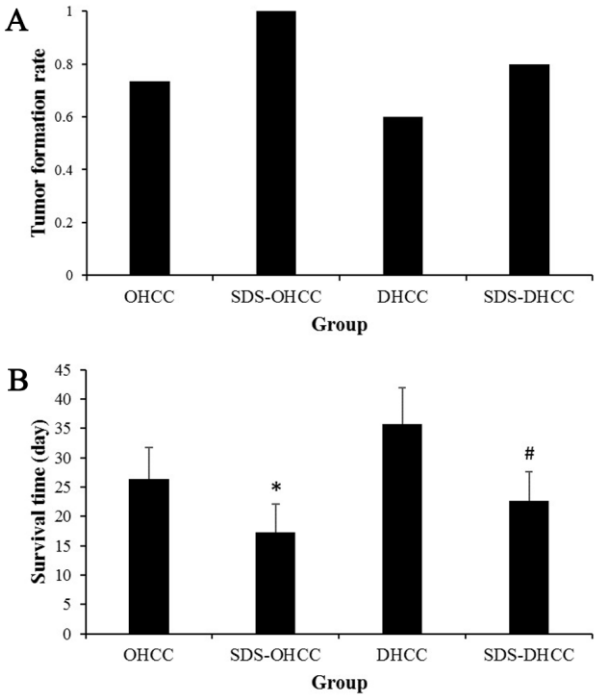

Figure 3: Tumor formation rate and survival time of HCC model mice. (A) Tumor formation rate of the mice in HCC models. **(B)** Survival time of the mice in HCC models. **P<0.05 vs. OHCC group; #P<0.05 vs. DHCC group. HCC, Hepatocellular carcinoma; SDS, Spleen-deficiency syndrome; OHCC, Orthotopic hepatocellular carcinoma; DHCC, Drug-induced hepatocellular carcinoma.*

Histochemical stain

The hematoxylin-eosin (HE) stain results showed that for mice in the Control Group, the constructions of hepatocytes were in normal situation with aligned nuclei, full-constructed hepatic lobes, radially-distributed hepatic cords and apparent hepatic sinusoids (Figure 4A), similarly, the mice in SDS Group had almost the same constructions

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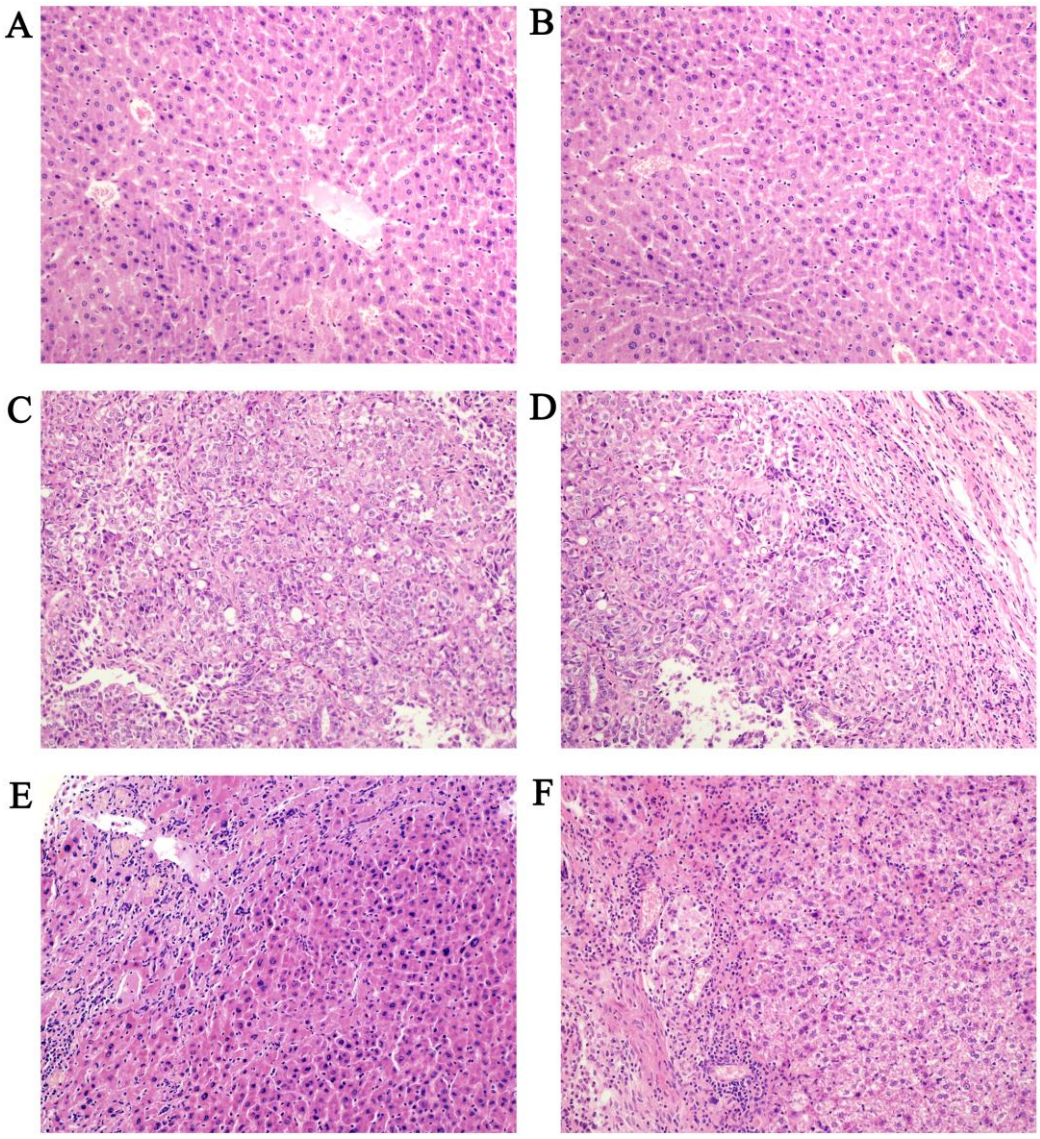
excepted for small part of irregularly distributed hepatocytes (Figure 4B). In contrast, for the mice which received HCC modeling, it showed the different level of dysfunctions and damaged hepatocytes. To be specific, the carcinoma cells were microscopically nest-like distributed and accumulated irregularly without the normal hepatocytes constructions (Figure 4C-F).

Table 1: The evaluation standard of Spleen Deficiency Syndrome Score.

Index/Score	1	2	3	4
Body odor	Odor-free	Mild odor	Medium odor	Severe odor
Mental state	Stable	Irritable	Fatigue	Somnolence
Chill & fever	Normal	Cowered	Chill	Arched back & trembling
Respiration	Normal	Panting	Tachypnea	Faint
Fur	Gloss	Matted	Fluffy & erect	Brown & erect
Feces	Formed	Loose	Wet & loose	Mucous
Appetite	Normal	Reduced to 50%	Reduced to 25%	Not at all



Conclusion

The SDS-oriented OHCC mice model was established successfully, which could be a simple and easily-operated model for the further researches on spleen-deficiency caused tumor in TCM. While the foundation of spleen-deficiency

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existing, the HCC was showed to be developed earlier and rapidly and spleen-deficiency factor in advance was proven to be harmful for surviving.

Discussion

In theory, TCM individuals who are deficiency in spleen and kidney or weak and imbalance in their bodies mostly suffer from abdominal masses disease. Moreover the masses or tumor tissues are developed in the liver as a result of the long-time spleen-deficiency, which lead to heat, stagnation of liquid, extravasation of blood in the liver. In addition, it is reported previously that several HCC patients have already suffered from spleengastric problems for a long time; it is the spleen-deficiency that potentially leads to the HCC (Ni et al., 2012). Likewise, in the views of modern researches, the functions of protection and the source of acquired constitution of the spleen properly reflect that the spleen plays a pivotal role on the body defense, which is in closely related to the immune system including the specific immunity and non-specific immunity such as humoral and cellular immunities (Chen et al., 2014b). In summary, since the spleen-deficiency is regarded as one of the most vital pathophysiological bases, it is necessary to establish the HCC model based on the SDS, by which the pathogenesis in TCM and the molecular basis of hepatic cancer could be well investigated.

Several previous studies had been focused on the OHCC and DHCC animal models. It was reported that the transplanted hepatocellular carcinoma models are characterized by implanting the hepatic tumor tissues from the human or animals, hepatic tumor cell lines or even non-liver-oriented tumor tissues such as breast cancer or colorectal cancer into the bodies of experimental animals (Yan et al., 2013). It is well accepted that the transplanted HCC models can be mainly divided into OHCC model and subcutaneously transplanted HCC, however the OHCC not only maintains the original constructions of tumors, but also the microenvironment in the abdominal cavity and the most biological characteristics compared to the subcutaneously transplanted HCC (Zheng et al., 2000). It is reported by now that the rats are more often used for HCC, nevertheless the mice models are regarded as low-price, easily-housing and simply-operation, which can be utilized for drug screening and large-scale researching projects (Bai et al., 2011). The drug-induced HCC model are generally used for simulating the hepatic tumor which is accumulated by long-time administration of different kinds of medications (Caviglia and Schwabe, 2015), while alcoholic beverages being included, it could also simulate the alcoholic liver cancer additionally (Thompson et al., 2015). For the substances used for inducing the HCC in this study, the DEN is a highly toxic chemical characterized by high risks for carcinogenicity, teratogenicity and genetic mutation, which is often used for induced HCC in mice (Zhao et al., 2015); the CCl_4 is also a common toxic chemical in the fields of chemical engineering and paint which can be a main component of rubber, detergent, pesticide and so on, except for the risks above, furthermore it has chronic toxicity on kidney and liver and could lead to hepatic steatosis, cirrhosis and enlargement of kidney (Cai et al., 2015); the spirit contains high content of alcohol, the 90% of which will be decomposed in liver, thereafter the ethyl alcohol and its metabolite acetaldehyde could directly damage the hepatocytes and probably lead to HCC (Testino et al., 2014).

In this study, the OHCC mice model based on the SDS was established successfully with a 100% tumor formation rate, which indicated that as a most proximate HCC model to the development and formation of hepatic tumor in human beings, the OHCC mice model could be used for exploring the pathogenesis in TCM and new strategies for HCC. On the other hand, the results for DHCC and SDS-DHCC models showed that when mice suffered from spleen-deficiency, the tumor formation rate increased from 60% to 80%, which implied that SDS could be a potential factor of the HCC development. Moreover, the results of SDS scores, tumor formation rate and survival time in both OHCC and DHCC models indicated that while the foundation of spleen-deficiency being existed, the HCC was showed to be developed earlier and rapidly. Certainly, the OHCC model had a higher tumor formation rate but a lower SDS score and survival time compared to that of DHCC models, which could be explained by the facts that as a simulation of primary hepatic carcinoma, HCC model required the tumor tissues should be directly implanted to the mice, which made mice get the worse situation and a shorted life expectancy; on the contrary, due to several medications and substances being used for model establishment, the DHCC model could simulate the chronic hepatic burden such as food, drug and alcohol induced HCC, the symptoms of which were not as severe as that of OHCC, therefore the mice could survive a little longer. Besides, even though the SDS model establishment procedure were terminated while HCC modeling started, the survival time suggested that a spleen-deficiency factor in advance was proven to be harmful for surviving.

On the whole, these two spleen-deficiency oriented hepatocellular carcinoma mice models were successfully according the theory of TCM, which provided the possible approaches to explore the new strategies for hepatic cancer by complementary and alternative medicines. However, certain limitation remained in our study. To begin with, we have just established two HCC models based on the spleen deficiency in advance, however, the certain mechanism underlying the changes of SDS Scale, tumor formation rate and survival rate need to be further confirmed. Furthermore, the spleen deficiency is associated with different systems, organs and tissues which should be stimulated by different factors, therefore, further SDS model using multi-factors should be explored with the HCC models. Finally, the establishment of the models was used for the further studies based on the models, thus several indices should be

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detected so as to clarify the pathogenesis of HCC, by which the complementary and alternative therapies could be found in the futher.

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