Multi-stress accelerated liver cancer progression in rats treated with diethylnitrosamine

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To the Editor: Eighty-four 6-week-old male Wistar rats, weighing from 120 to 180 g, were categorized into three groups: normal control (NC) group, diethylnitrosamin (DEN) group, and multi-stress group. In the NC group, rats were maintained at room temperature with a relative humidity of 60% to 80% and fed with a normal diet and drinking water. In the DEN group, the rats were maintained the same as the NC group, but fed with sterile drinking water supplemented with 0.1 mg/mL DEN daily for 20 weeks. In the multi-stress group, the rats were maintained at 5 to 7°C with a relative humidity of 25% to 32.8%. Then, the rats were exposed to electrical stimulation daily through the food pads (30 min at 35 V for the first week, 35 min at 40 V for the second week, and 45 min at 45 V for the third week). They were also forced to swim in the water at 15 to 25°C for 5 min once a week to establish multi-stress models. The rats were treated with 0.1 mg/mL DEN daily through drinking water for 20 weeks to induce liver cancer. Until the end of the experiment, 22 rats survived in the NC group, 16 rats survived in the DEN group, and ten rats survived in the multi-stress group. The rats were sacrificed by cervical dislocation under anesthetization at 20 weeks after treatment with DEN. This study was approved by the Animal Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University (No. IACUC-20150225-128). The liver tissues were excised and fixed in 10% formaldehyde solution followed by imaging [Supplementary Figure 1, http://links.lww.com/CM9/A544] and staining with hematoxylin and eosin (H&E). Anatomy of the liver and H&E staining demonstrated that the livers of rats in the NC group did not exhibit the cancer symptoms [Figure 1A, upper panels], whereas the rats in the DEN group showed irregular organization, or necrosis of liver cells, distinctive pseudo hepatic lobules, and liver fibrosis [Figure 1A, middle panels]. Interestingly, the rats in the multi-stress group appeared significantly a high incidence of liver cancer compared with NC and DEN groups. All rats ($N = 10$) in the multi-stress group developed hepatocellular carcinoma or bile duct epithelial cancer, which indicated cancer cells and liver tissues were partially transformed into fibrosis.

Signal transducer and activator of transcription 3 (STAT3) as a biomarker of liver cancer was detected using immunohistochemistry and western blotting. The signal of STAT3 protein was obviously observed in both the DEN and multi-stress groups [Figure 1A, lower panels]. The protein level of the multi-stress group exhibited 8.2 times increase compared with the NC group and 2.3 times increase compared with the DEN group [Figure 1B], which suggested that multi-stress significantly promoted the STAT3 expression and the incidence of liver cancer.

To uncover the potential role of regulatory T cell (T_{regs}) in liver cancer progression induced by multi-stress conditions, the number of T_{regs} in blood samples from each group was counted after DEN treatment by flow cytometry. Blood samples were collected under anesthetization after DEN treatment for 20 weeks. Peripheral blood mononuclear cells were isolated using differential centrifugation over Ficoll. The cells with a quantity of 3 to 5×10^5 were incubated with anti-CD4 conjugated with fluorescein isothiocyanate (FITC) and anti-CD25 conjugated with phycoerythrin (PE). The stained cells were resuspended in fluorescence activated cell sorter (FACS) staining buffer and analyzed using FACS. The $CD4^+$ cells were gated and then the percentage of both CD25- and FoxP3-positive cells was determined as T_{reg}. The rats under multi-stress conditions showed a significantly higher frequency of peripheral $CD4^+$ T_{regs} (mean, 7.36%) than DEN rats (mean, 5.23%, $P < 0.01$) and NC rats (mean, 2.79%, $P < 0.01$) [Figure 1C]. Moreover, the frequency of $CD4^+CD25^+$ T_{reg} in total $CD4^+$ was determined by measuring the $CD4^+CD25^+$ population. Our results

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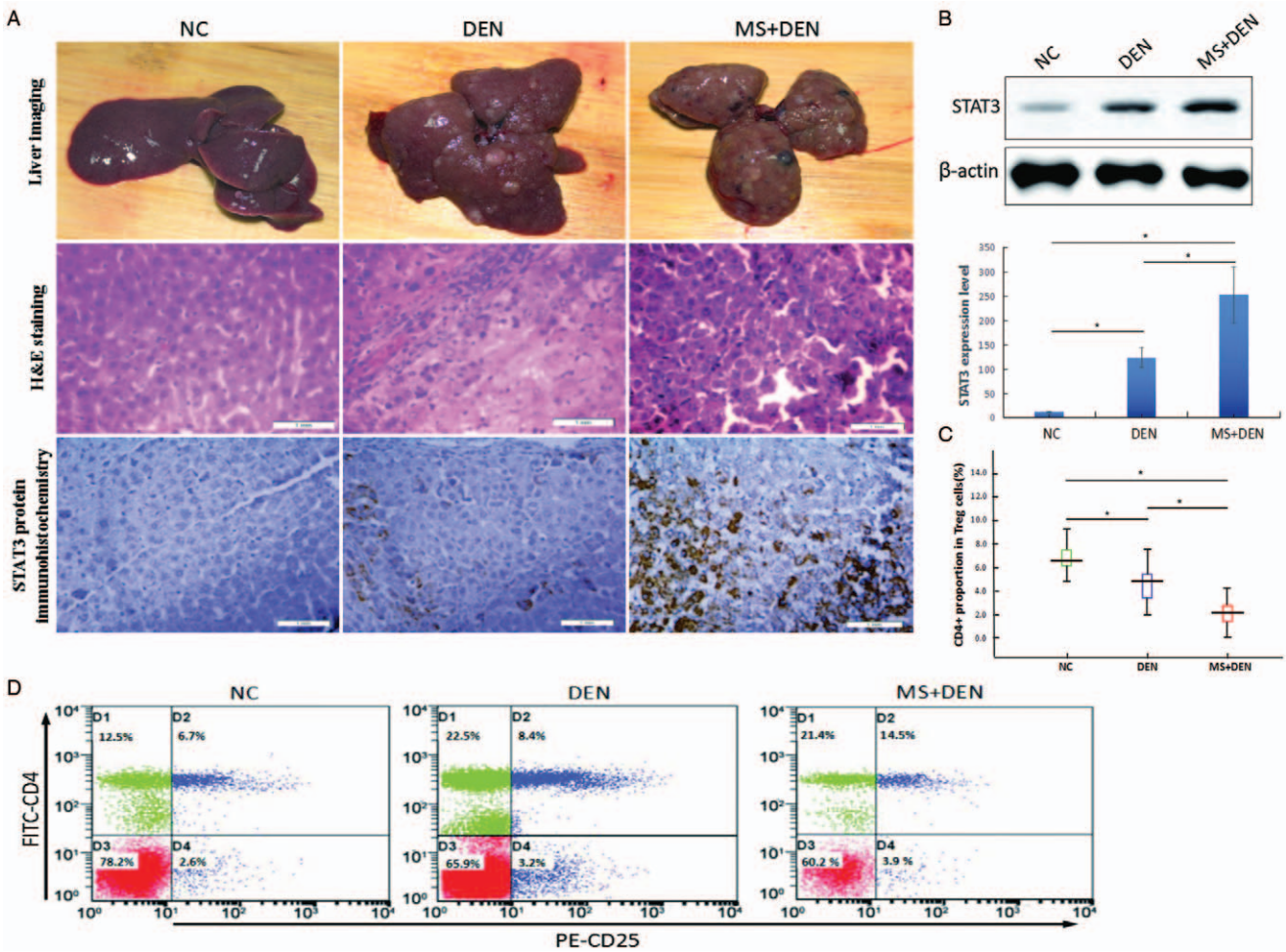

Figure 1: Multi-stress significantly affects the homeostasis of host immunity by regulating the function of T_{regs} to promote the incidence of liver cancer. (A) Multi-stress promotes the cancer development induced by DEN. Upper panels, liver imaging for NC, DEN, and MS + DEN group rats. Middle panels, H&E staining to observe the morphology of liver cancer. Lower panels, immunohistochemistry analysis of STAT3 expression (×400 magnification). (B) STAT3 expression was significantly evaluated under multi-stress conditions. Upper panel, Western blotting detection of STAT3 protein, (β-actin, the reference gene). Lower panel, statistical analysis of STAT3 protein expression in all groups. (C) $CD4^+$ T_{regs} in blood samples from each group was counted. (D) The number of $CD4^+CD25^+FoxP3^+$ T_{regs} in blood samples from each group was counted by flow cytometry. NC rats ($N = 22$). DEN-treated rats ($N = 16$). MS + DEN, multi-stress rats treated using DEN ($N = 10$). Data are shown in mean ± standard deviation. $^*P < 0.01$. DEN: Diethylnitrosamine; H&E: Hematoxylin and eosin; NC: Normal control; MS+DEN: Multi-stress group treated using DEN; STAT3: Signal transducer and activator of transcription 3.

indicated that the multi-stress group showed an obviously higher proportion of $CD4^+CD25^+$ T_{regs} (mean, 14.52%) than DEN rats (mean, 8.42%, $P < 0.01$) and NC rats (mean, 6.7%, $P < 0.01$) [Figure 1D]. These results suggested that the multi-stress further increased the number of $CD4^+CD25^+$ T_{regs} in liver tissues, which eventually resulted in the progression of hepatocellular carcinoma.

Blood samples were collected from all groups at the 20th week for measuring the levels of interleukin (IL)-6, tumor necrosis factor (TNF)-α, and IL-1β by using enzyme-linked immunosorbent assay (ELISA) assay. The detection limits of the ELISA kits were set as 10 pg/mL for IL-6, 20 pg/mL for TNF-α, and 15 pg/mL for IL-1β. As shown in Supplementary Table 1, http://links.lww.com/CM9/A544, multi-stress conditions resulted in significant chronic inflammatory reactions in the rats. All three inflammatory cytokines in the multi-stress group were more significantly elevated compared with the rats in NC and DEN groups, which suggest that multi-stress condition promotes chronic inflammation and even cancer progression.

Traditional Chinese Medicine (TCM) plays a critical role in the prevention and treatment of diseases throughout the history of central Asia. According to the theory of TCM, dynamic homeostasis is the physiological basis for health associated with the incidence of complex diseases.[1] Previous studies have demonstrated that single-stress is closely correlated with the progression of complex diseases, including carcinogenesis and breast cancer.[2-3] DEN treatment under single-stress conditions promoted hepatocellular carcinoma growth and suppressed the anti-tumor immunity of tumor-bearing mice.[4-5] However, multi-stress predisposes the patients to multiple malignant diseases, especially liver cancer, which remain to be elucidated.

In this study, rat models with multi-stress were established by treating with DEN. Our results demonstrate that multi-stress significantly promotes the incidence of liver cancer and affects the homeostasis of host immunity by regulating the function of T_{regs} and inflammatory cytokines to promote cancer development.

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Conflicts of interest

None.

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Corrigendum

Corrigendum: A single-operator technique in ultrasound-guided regional anesthesia

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In the article "A single-operator technique in ultrasound-guided regional anesthesia" which appeared in vol. 134, issue 6, page 735 of *Chinese Medical Journal*,[1] the work was supported by "the Fundamental Research Funds for the Central Universities (No. 3331019031)", which should be corrected as "the Fundamental Research Funds for the Central Universities (No. 3332019031)".

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1. Tang S, Li JL, Zhang YG, Huang YG. A single-operator technique in ultrasound-guided regional anesthesia. Chinese Medical Journal 2021;134:734–735. doi: 10.1097/CM9.0000000000001186.