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A New Therapeutic Combination of Pelvic Local Moderate Hyperthermia with HSP70 Peptide Complex Derived from Autologous Invasive Bladder Tumor in Rats

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Abstract

Introduction:

The objective of this study was to explore the mechanism of local moderate pelvic hyperthermia combined with heat shock protein 70 peptide complex (HSP70-PC) in the treatment of bladder tumors.

Material and methods

Rats were randomly divided into four groups: A (control); B (hyperthermia); C (HSP70-PC treatment); and D (combination treatment). The pelvic cavity of rats in groups B and D was heated to 41°C using a Endogenous Field Tumor Hyperthermia System, while those of groups C and D were intraperitoneally injected with HSP70-PC.

Results

More lymphocytes, granulocytes, and macrophages were present in group D tumors, including apoptotic bodies. The bladder weight, tumor staging, and PCNA labeling index in group D were significantly lower than those in groups A–C ($P < 0.01$). The ratios of HSP70-positive cells and S-100 protein-positive cells in tumors were significantly higher in group D than in group C ($P < 0.01$). Moreover, the apoptosis indexes of tumors, the ratio of CD8 protein-positive cells in tumors, the ratio of S-100 protein-positive cells in lymph nodes, the ratios of CD8 protein-positive cells and S-100 protein-positive cells in the spleen, and rat IFN-γ levels in serum were significantly higher in group D than in groups A–C ($P < 0.01$).

Conclusion: Local moderate pelvic hyperthermia may promote tumor cell apoptosis, inhibit tumor cell proliferation, and upregulate the immune status, thereby exerting synergistic and complementary effects with the antitumor immune effect induced by HSP70-PC. The therapeutic efficacy of this combination treatment is higher than that of local moderate pelvic hyperthermia or HSP70-PC alone.

1 Introduction

The incidence rate of bladder cancer is the first in the urinary system tumors, accounting for more than 50% in the urinary system tumors. The recurrence rate of the nonmuscle invasive type bladder cancer within one year after surgery was 60%–70%. The recurrence rate within 5 years can reach 70%–80%. Patients may require repeat surgery and may not respond to bladder perfusion Bacille Calmette–Guérin vaccine therapy or have muscle infiltration. The existing treatment methods are limited and the prognosis is poor. Therefore, there is an urgent need for innovative therapies that are more efficient, are associated with low toxicity, can reduce the recurrence rate, and preserve bladder function in clinical practice. Local moderate pelvic hyperthermia inhibits and kills tumor cells through various mechanisms (1, 2). Moreover, hyperthermia can induce tumor cells to produce heat shock protein 70 (HSP70). Purified HSP70 from tumors acts as a molecular chaperone, exerting antitumor immune effects through multiple pathways (3–5). The mechanism of action of local mild pelvic hyperthermia is similar to that of HSP70, and there is a certain causal relationship.

In a study, recombinant mouse HSP70 and magnetic cationic liposomes were injected into subcutaneous transplanted melanoma tumors in mice and heated to 43°C using a magnetic field for 30 min. It is thought that this combination treatment kills tumor cells due to the direct effect of heat, and exerts a strong antitumor immune effect; in addition, its therapeutic effect is considered better than that of monotherapy (6). Thus, it is hypothesized that the combination of local moderate pelvic hyperthermia and subcutaneous injection of bladder tumor heat shock protein 70 peptide complex (HSP70-PC) from the same strain of rats may exert synergistic and complementary effects on bladder tumors. To date, there have been no studies addressing this hypothesis. Therefore, based on our previous research, in the present study we combined these two methods for the sequential treatment of infiltrating bladder tumors in rats. The purpose of this investigation was to evaluate the efficacy of the combination treatment and elucidate the underlying mechanism.

2 Materials and Methods

2.1 Laboratory Animals

Twenty 6-week-old female inbred F344 rats (each weighing 151.62 ± 6.51 g) were obtained from Beijing Weitong Lihua Laboratory Animal Technology Co., Ltd., Beijing, China (level: specific pathogen-free/virus antigen-free; Beijing Laboratory Animal quality certificate number: 0047634). This study was approved by the Medical Ethics Committee of Shanxi Cancer Hospital (Taiyuan, China).

2.2 Reagents

The reagents used in this study included: N-Methylnitrosourea (MNU) (Sigma–Aldrich, St. Louis, MO, USA); HSP70-PC (100 μg, derived from F344 rat bladder tumor HSP70-PC purified from previous research (3)); mouse anti-HSP70 monoclonal antibody (Wuhan Doctoral Biotechnology Co., Ltd., Wuhan, China); mouse anti-polymerase chain reaction protein monoclonal antibody, mouse anti-S-100 protein monoclonal antibody, and *in situ* cell death kit (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China); rabbit anti-CD8 monoclonal antibody (Lab Vision&NeoMarkers, Freemont, CA, USA); mouse Streptavidin (SP) and rabbit SP immunohistochemistry (IHC) staining kits (ZYMED, South San Francisco, CA, USA); and Rat interferon-gamma (IFN-γ) enzyme-linked immunosorbent assay (ELISA) detection kit (lot number: 3200-07T; Diaclone, Besançon, France).

2.3 Instruments

The instruments utilized in this investigation included: Type NRL002 Endogenous Field Tumor Hyperthermia System (Jilin Maida Technology Co., Ltd., Jilin, China); transmission electron microscope (TECNAI G2 12 model; FEI, Eindhoven, Netherlands); and SUNRISE ELISA (TECAN, Grödig, Austria).

2.4 Experimental Methods

The experimental methods employed in this study strictly followed guidelines stipulated by the Medical Ethics Committee of Shanxi Cancer Hospital. Orthotopic rat bladder tumor model preparation and

grouping were performed as follows. MNU was dissolved in 50 mM citric acid buffer (pH 6.0) prior to use (3), and 1.2% sodium pentobarbital was injected intraperitoneally (30 mg/kg) for anesthesia. After catheterization, 0.2 ml of 1% MNU was infused into the bladder of each rat once every 2 weeks. Following six infusions, the rats were maintained for 8 weeks and randomly divided into four groups (n = 5 per group): A (control); B (hyperthermia); C (HSP70-PC treatment); and D (combination treatment).

2.5 Treatment Methods

Rats in group A received two subcutaneous injections with phosphate-buffered saline (PBS; 0.01 M, pH 7.5) into both lower limbs (200 μl per injection) with a 2-week interval (i.e., a total of 400 μl). In group B, the pelvic cavity of rats was heated using the endogenetic field hyperthermia system from outside the body (1), to increase the rectal temperature to 41°C for 1 h. Hyperthermia was repeated once every 2 weeks for the second time with an interval of 24 h (total of four times), one rat per time. On day 5 following hyperthermia, 400 μl of PBS (0.01 M, pH 7.5) was injected into each rat. The injection method was the same as that described for group A, twice in total. Rats in group C were injected with HSP70-PC 400 μl/rat (5 μg/mouse/time). Prior to treatment, HSP70-PC lyophilized protein was dissolved in PBS (0.01 M, pH 7.5), and filtered through 0.22-μm microporous membrane to remove bacteria. The injection method was the same as that described for group A, twice in total, 10 μg/rat. The treatment method for group D was the same as that described for group B. HSP70-PC was injected on day 5 after hyperthermia, and the method was the same as that described for group C. The animals were sacrificed after 4 weeks of treatment. The sacrifice method involved intraperitoneal injection of 1.2% pentobarbital sodium (60 mg/kg) for excessive anesthesia, causing the rats to lose consciousness.

2.6 Detection

After weighing the bladder containing tumor tissue, perform pathological staging of the tumor. The ultrastructure of tumor cells and the changes of immune cells in tumor tissues were observed by transmission electron microscopy. The apoptosis index (AI) of tumor cells was detected and calculated by the terminal deoxynucleotidyl transferase uridine triphosphate nick-end labeling method. Paraffin sections were permeabilized using the pressure cooker method. Other operations were conducted according to the instructions provided by the kit manufacturers and 3,3'-diaminobenzidine (DAB) color development. The nucleus of apoptotic cells appeared brown. To determine the apoptosis index, the number of positive cells per 100 tumor cells in 10 high-power fields (HPF) was counted. The tumor cell proliferating cell nuclear antigen (PCNA) labeling index (LI) was detected and calculated by IHC. Paraffin sections were repaired with the pressure cooker method. The dilution ratio of the PCNA monoclonal antibody was 1:100. DAB was developed according to the instructions provided in the mouse SP IHC staining kit. The nuclei of PCNA-positive cells appeared brown. To determine the PCNA LI, the number of positive cells per 100 tumor cells in 10 HPF was counted. The expression of HSP70 in tumor cells was detected and calculated by IHC. Paraffin sections were repaired with the pressure cooker method. The dilution ratio of the HSP70 monoclonal antibody was 1:50. DAB was developed according to the procedures indicated in the mouse SP IHC staining kit. The nucleus and cytoplasm of HSP70-positive cells appeared brown. To determine the ratio of HSP70-positive cells, the number of positive cells per

100 cells was counted in 10 HPF. The distribution and morphology of S-100 protein-positive cells were detected by IHC. The ratio of S-100 protein-positive cells in bladder tumor tissue and muscle layer, iliac artery and paraaortic lymph node in the bladder drainage area, and the spleen were calculated. Paraffin sections were repaired with the pressure cooker method. The dilution ratio of the S-100 protein monoclonal antibody was 1:100. DAB was developed according to the procedures described in the mouse SP IHC staining kit. The nucleus and cytoplasm of S-100 protein-positive cells appeared brown. The morphological changes of positive cells were observed after staining. To determine the ratio of S-100 protein-positive cells, the number of positive cells per 100 cells was counted in 10 HPF. The ratio of CD8-positive cells in bladder tumor tissue and muscle layer and in the spleen were detected by IHC. Paraffin sections were repaired with the pressure cooker method. The dilution ratio of the CD8 monoclonal antibody was 1:40. DAB was developed according to the procedure described in the rabbit SP IHC staining kit. The cell membrane of CD8-positive cells appeared brown. To determine the ratio of CD8-positive cells, the number of positive cells per 100 cells was counted in 10 HPF. The detection IFN-γ levels in rat serum was conducted using the ELISA kit according to the instructions provided by the manufacturer. Absorbance was measured at 450 nm wavelength, and the concentration of IFN-γ in rat serum was calculated.

2.7 Statistical Analysis

All statistical analyses were performed using SPSS version 26 (IBM Corp., Armonk, NY, USA). Continuous data are presented as the mean ± standard deviation. The average counts of various indicators between the groups were compared by analysis of variance Q test. Fisher's exact probability test was used to compare the ratio of tumor stage and grade. Statistical significance was set at P-values < 0.05.

3 Results

The results regarding wet weight of the bladder containing tumor tissue, tumor cell apoptosis index, tumor cell PCNA LI, ratio of tumor cell HSP70 expression, ratio of S-100 protein-positive cells (tumor tissue and muscle layer, iliac artery and para aortic lymph node in the bladder drainage area, and the spleen), ratio of CD8-positive cells (tumor tissue and muscle layer, and the spleen), and IFN-γ levels in rat serum are shown in Figs. 1–3. The difference in wet weight of the bladder containing tumor tissue among groups was statistically significant. There were no significant differences in tumor cell PCNA LI, ratio of tumor cell HSP70 expression, and ratio of S-100 protein-positive cells between groups A and C (P = 0.786, 0.741, and 0.545, respectively). Moreover, there was no significant difference in ratio of tumor cell expression of HSP70 and the ratio of S-100 protein-positive cells between groups B and D (P = 0.825 and 0.686, respectively).

3.1 Ultrastructural Changes in Tumor Cells and Changes in Immune Cells of tumor tissues

No apoptotic cells were found in group A, and the tumor nuclei showed obvious heteromorphism and large nucleoli; macrophages were occasionally observed (Fig. 4A). In group B, mitochondrial cristae were swollen, the endoplasmic reticulum was vacuolated, chromatin was aggregated, and apoptotic cells and apoptotic bodies were more common than in other groups; moreover, neutrophils were aggregated and macrophages phagocytosed apoptotic bodies (Fig. 4B). Apoptotic bodies were more common in group C than the other groups. In tumor tissues, there were more macrophages, lymphocytes, and plasma cells, but fewer neutrophils (Fig. 4C). Apoptotic cells and apoptotic bodies were more common in group D than the other groups. In tumor tissues, there were numerous lymphocytes and neutrophils, and macrophages phagocytosed apoptotic bodies (Fig. 4D).

3.2 Distribution and Morphology of S-100 Protein-positive Cells

S-100 protein was expressed in the nucleus and cytoplasm of dendritic cells (DC), but not in bladder tumor cells. Most of the DC in bladder tumor tissues and muscle layers in each group were regular in shape and without pseudopodia. In groups B and D, DC in the iliac artery and paraaortic lymph nodes in the bladder drainage area were clustered in the lymph node cortex, with more pseudopodia and encircling lymphocytes. In groups A and C, DC were scattered in the cortex, with regular morphology and few pseudopodia. DC in the spleen of groups B and D were mostly distributed in the white pulp of the spleen parenchyma, and most of them were regular in shape and without pseudopodia. In group C, DC were mainly distributed in the white pulp of the spleen parenchyma, with more pseudopodia and encircling lymphocytes (Fig. 5).

4 Discussion

When choosing the hyperthermia regimen in this study, we considered that different regimens of local moderate pelvic hyperthermia can induce the heat shock response of orthotopic rat bladder tumors and upregulate the expression of HSP70. The ratio of tumor cell HSP70 expression was not positively correlated with the number of hyperthermia, but the quality of expression was positively correlated with the number of hyperthermia. Hyperthermia sessions with 24-h intervals induce the highest expression of HSP70 in tumor tissues, which leads to the most significant inhibition of PCNA (1). Moreover, they induce the greatest changes in immune cells and result in the best treatment effect. Therefore, this approach was selected for the combination treatment in this study.

Both local moderate pelvic hyperthermia and tumor-derived HSP70-PC exert antitumor effects through multiple pathways, and their mechanisms of action are different (1–5). Previous research showed that, although hyperthermia in the pelvic region did not affect the staging and grading of bladder tumors, it could inhibit tumor proliferation, induce tumor cell apoptosis, and reduce tumor weight within a short period of time. Following long-term hyperthermia, these three effects persist, thus delaying the growth of tumors; additionally, there was no heat tolerance due to the increase of heat shock proteins (1). With the increase of hyperthermia sessions in the pelvic region, the immune cells of rats with tumors redistribute;

this redistribution is conducive to tumor antigen presentation and effector cells attacking tumors. In a study of subcutaneous injection of HSP70-PC in rats, it was observed that DC migrated to the spleen to mature. This effect subsequently promoted the proliferation of splenic $CD8^+$T cells, generated specific $CD8^+$ cytotoxic lymphocyte, and pooled them to the target organ bladder tumor tissue to play an antitumor role. Treatment with HSP70-PC can upregulate the immune status of mice with tumors and promote tumor apoptosis, but has no effect on tumor cell proliferation (3).

The wet weight of bladders and tumors in the combination treatment group was lower than that measured in the other groups, indicating that the comprehensive effect of the combination treatment was greater than that of monotherapy. There was a certain internal relationship between the two treatment methods, and its mechanism could be elucidated through the pathological study of tumor and immune cells.

The PCNA index of tumor cells in the combination treatment group decreased, suggesting that the proportion of tumor cells in the proliferative state decreased and tumor proliferation was inhibited. The lower proportion of tumor cells observed in the hyperthermia group is attributed to the increase in the number of apoptotic cells. Although treatment with HSP70-PC has no effect on the malignant tendency of tumor cells and does not inhibit their proliferation, it leads to an increase in the number of apoptotic cells (3). Its role in promoting apoptosis is consistent with the induction of tumor cell apoptosis by local moderate pelvic hyperthermia, which has a superimposed effect. Therefore, the proliferation rate decreases due to the increase in the rate of apoptosis. Because the expression of HSP70 is non-persistent (1, 2, 6), the expression of HSP70 in tumor cells of the combination treatment group and the hyperthermia group was low. Consequently, with the degradation of HSP70, the PCNA synthesis in these two groups is inhibited and does not fully recover (1), which also reduces the PCNA LI. However, the tumor grade of the combination treatment group remained G3 as that of the monotherapy group, which had no effect on the malignant degree of tumor cells, but only delayed the proliferation rate.

Ultrastructural study revealed that apoptotic cells and apoptotic bodies were more common in the combination treatment group, a greater number of lymphocytes and neutrophils clustered in the tumor tissue, and macrophages phagocytosed apoptotic bodies. However, there were fewer neutrophils in the HSP70-PC treatment group. In recent years, studies have suggested that neutrophils have antitumor effects. The infiltration of neutrophils in tumors is associated with a better prognosis, playing a key role in the induction of tumor rejection by all cytokines. Notably, neutrophils often cooperates with CD8-positive T cells to play an antitumor role through immune surveillance (7, 8). The HSP70 produced by bladder tumors after local moderate pelvic hyperthermia is consistent with the changes in immune cells; that is, with the increase of HSP70, the CD8-positive cells in bladder tumor tissue and muscle layer increase (1). Therefore, the apoptotic index associated with the combination treatment was higher than that of monotherapy.

Local moderate pelvic hyperthermia caused an increase in DC and CD8-positive lymphocytes in the tumor tissue and muscle layer, in the iliac artery and paraaortic lymph nodes in the bladder drainage

area, and in the spleen. Treatment with HSP70-PC increased the number of CD8-positive lymphocytes in tumor tissue and muscle layer, as well as increased the numbers of DC and CD8-positive lymphocytes in the spleen. Therefore, the changes in the number and distribution of DC and CD8-positive lymphocytes in the combination treatment group showed that the effect of local moderate pelvic hyperthermia and treatment with HSP70-PC was enhanced and complementary.

After being taken up by DC, HSP70-PC can present antigens to both CD8-positive and CD4-positive lymphocytes. However, DC can secrete IL-2 and IFN-γ (9). CD4-positive lymphocytes and natural killer cells release IFN-γ, while CD8-positive lymphocytes release IFN-γ after contact with target cells (10). These cells secrete IFN-γ into the tissue microenvironment at different stages. Hyperthermia can enhance the antitumor activity of T lymphocytes, B lymphocytes, and natural killer cells. As the first-line immune response cells, natural killer cells can exert a nonspecific killing effect without activation, rapidly secrete IFN-γ, IFN-α, interleukin-2 (IL-2), granulocyte-macrophage colony-stimulating factor (GM-CSF), etc., and exert an antitumor effect through these cytokines. Therefore, the IFN-γ levels in the serum of rats were higher in the combination treatment group than the other groups.

The results of other studies also showed the advantages of regional hyperthermia combined with HSP70-PC. Wang et al. used hyperthermia combined with HSP70 and HSP110-peptide complex vaccines to further shrink experimental transplanted tumors. The investigators demonstrated that hyperthermia could change the activity of lymphocytes and increase the entry of lymphocytes into tumor tissue (11). Ito et al. injected recombinant mouse HSP70 and magnetic cationic liposomes into subcutaneously transplanted melanoma tumors in mice, and subsequently used a magnetic field to achieve hyperthermia (43°C). The researchers reported that the combination treatment killed tumor cells due to the direct effect of heat, as well as the strong antitumor immune effect. Furthermore, the curative effect of the combination treatment was better than that of monotherapy (12).

Numerous tumor cells remain in the body even after achieving complete remission of malignant tumors. Moreover, tumor radiotherapy and chemotherapy is linked to certain adverse effects, especially damage to immune function (13). Eliminating these residual tumor cells and recovering the immune function of the body to effectively block the recurrence and metastasis of tumors is a major challenge in current cancer treatment. The present experimental study of local moderate pelvic hyperthermia combined with HSP70-PC in the treatment of invasive bladder tumors proposes a new method for the treatment of advanced disease.

The limitation of this study is the short-term treatment (i.e., the animal experiments were terminated after 4 weeks of treatment). Additional studies with prolonged duration of treatment (e.g., 8 weeks) can provide insight into the long-term effects of this combination regimen. This may enhance our understanding of the degree of tumor suppression and tumor regeneration, as well as assist in determining whether the treatment exerts a long-lasting effect on tumor immunity.

5 Conclusions

In summary, local moderate pelvic hyperthermia may promote tumor cell apoptosis, inhibit tumor cell proliferation, and upregulate the immune status, thereby exerting synergistic and complementary effects with the antitumor immune effect induced by HSP70-PC. The new regimen combining local moderate pelvic hyperthermia with HSP70-PC for the treatment of orthotopic rat invasive bladder tumors is more effective than monotherapy.

Abbreviations

HSP70
heat shock protein 70
HSP70-PC
heat shock protein 70 peptide complex
MNU
N-Methylnitrosourea
AI
apoptosis index
SP
Streptavidin
IHC
immunohistochemistry
IFN-γ
interferon-gamma
ELISA
enzyme-linked immunosorbent assay
PBS
phosphate-buffered saline
DAB
3,3'-diaminobenzidine
HPF
high-power fields
PCNA LI
proliferating cell nuclear antigen labeling index
DC
dendritic cells.

Declarations

Competing Interests

The authors declare that there are no competing interests.

Fundings

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Author Contribution

Study concept and design: Xu Zhang. Acquisition, analysis, and interpretation of data: Xu Zhang, Xiaozhen Li, Shaolin Han, Xingfu He. Statistical analysis: Xu Zhang. Drafting of the manuscript: Xu Zhang. Revision of the manuscript: Xu Zhang.The authors have declared that no competing interest exists.All authors reviewed the manuscript.These four authors contributed equally to this work and share first authorship.

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Data Availability

The experimental data and statistical analyses results of the current study are available from the corresponding author on reasonable request. All data were available in the manuscript.

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Figures

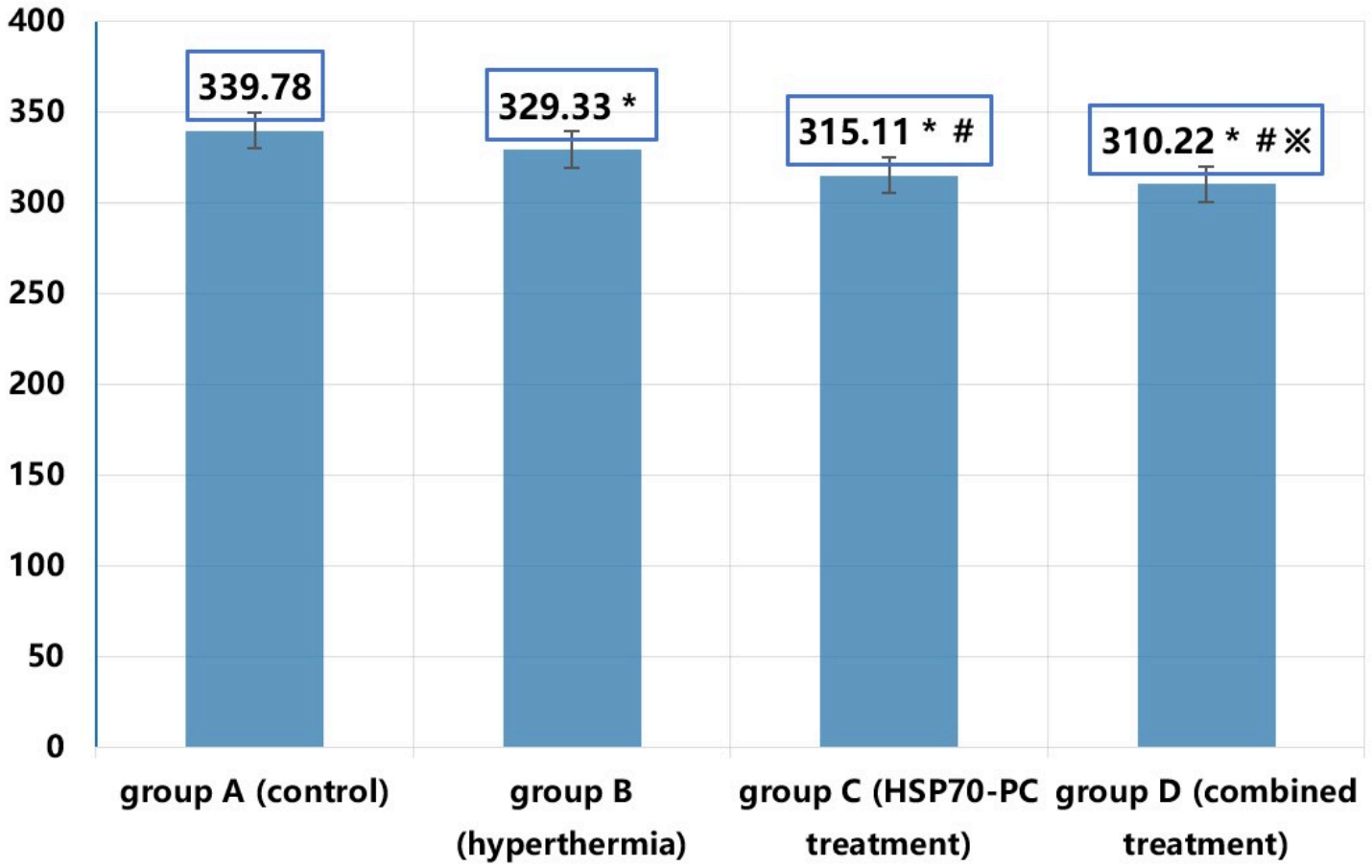


Figure 1

Wet weight of the bladder containing tumor tissue (mg). The mark □ indicates a statistically significant difference compared to group A (P<0.05). The mark □indicates a statistically significant difference compared to group B (P<0.05). The mark ※ indicates a statistically significant difference compared to group C (P<0.05).

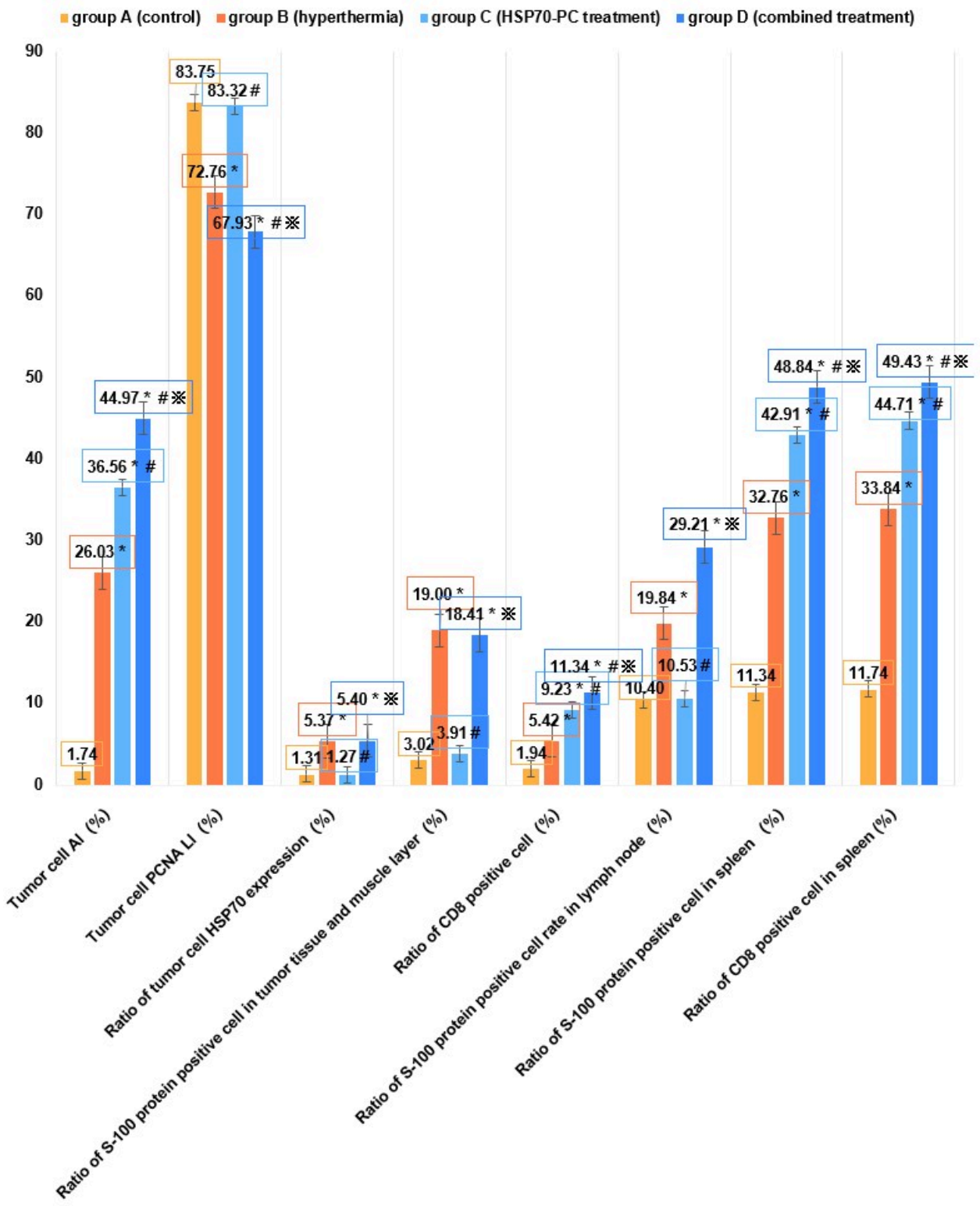


Figure 2

pathological changes and immune changes of orthotopic rat bladder tumor after treatment. The mark indicates a statistically significant difference compared to group A (P<0.05). The mark indicates a statistically significant difference compared to group B (P<0.05). The mark ※indicates a statistically significant difference compared to group C (P<0.05).

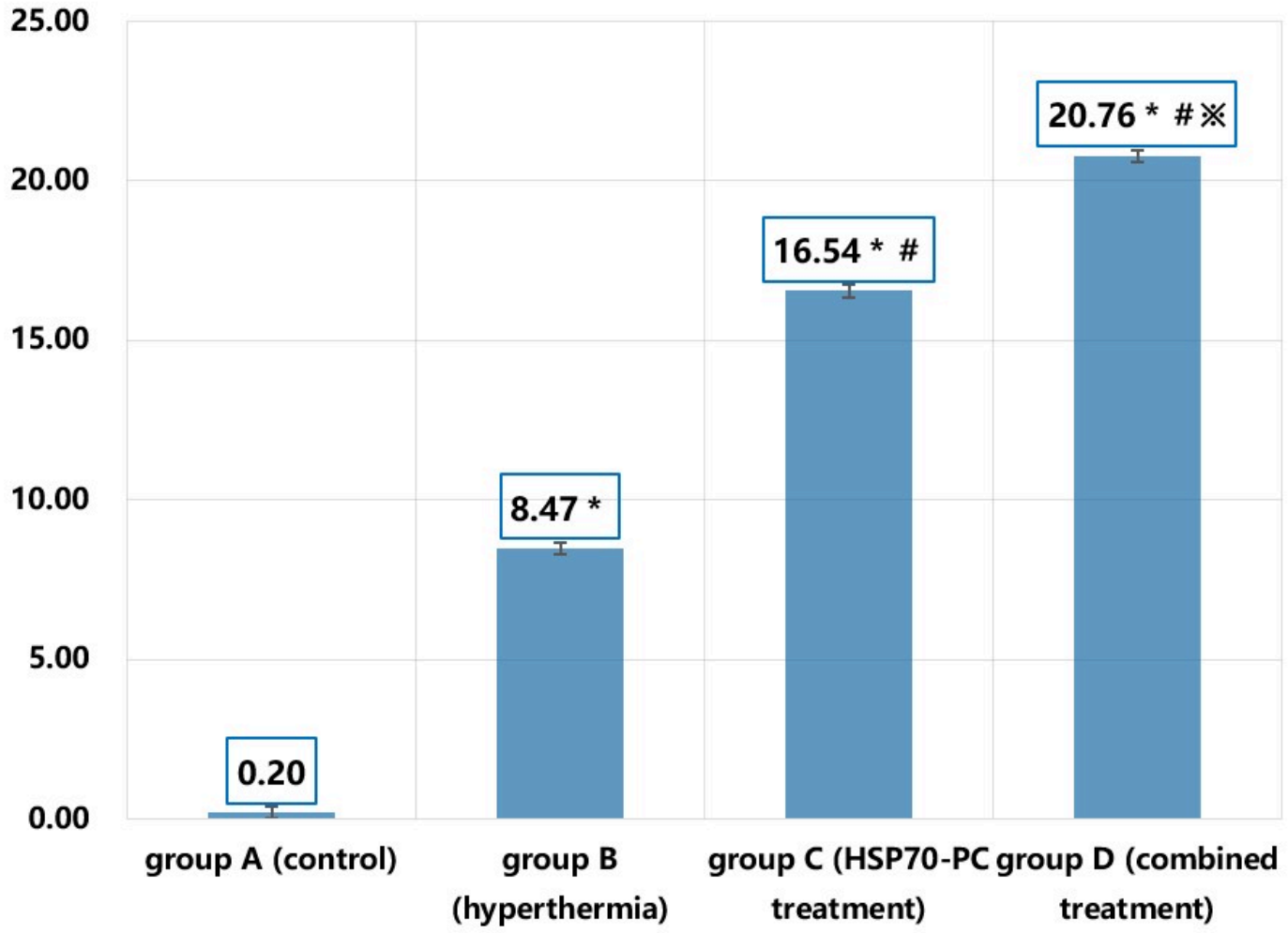


Figure 3

Rat IFN-γ in serum(pg/ml). The mark ▯ indicates a statistically significant difference compared to group A (P<0.05). The mark ▯ indicates a statistically significant difference compared to group B (P<0.05). The mark ※ indicates a statistically significant difference compared to group C (P<0.05).

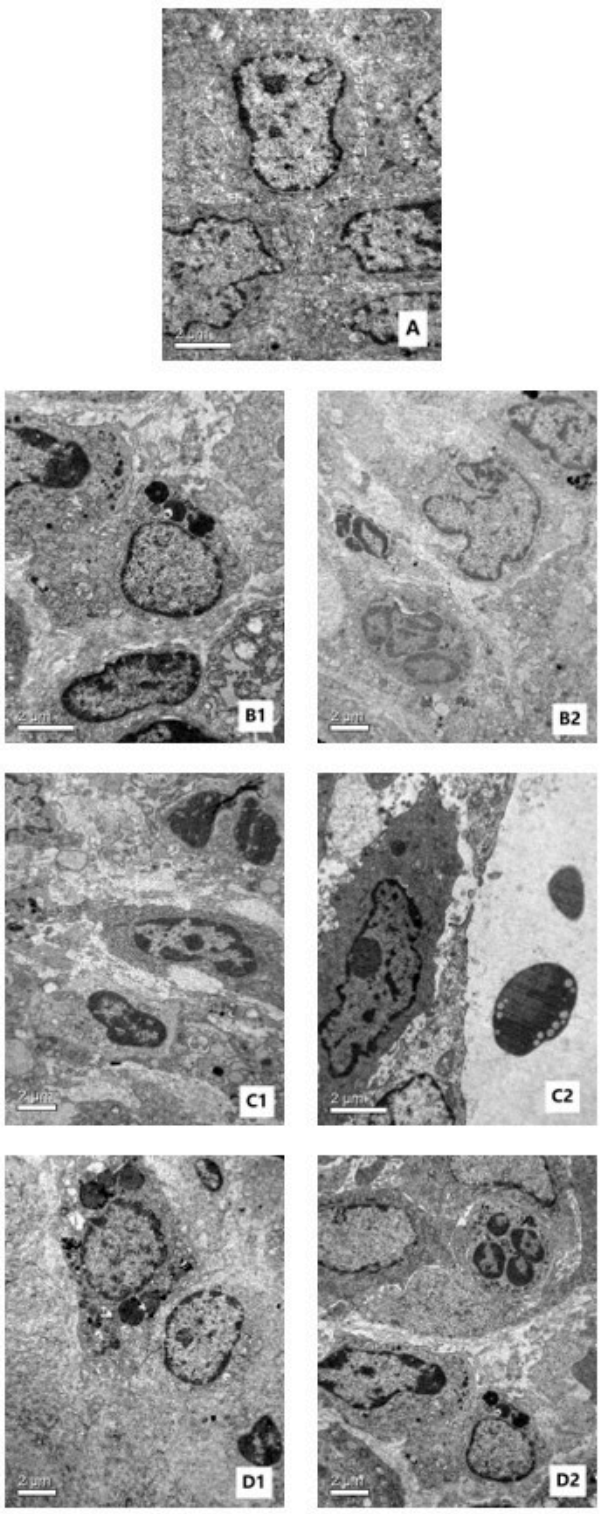


Figure 4

Ultrastructure of bladder tumor cells. Transmission electron microscopy (×25000). A shows that no apoptotic cells were found in the control group, and the tumor nuclei showed obvious heteromorphism and large nucleoli. B1 showed swelling of mitochondrial cristae, vacuolization of endoplasmic reticulum, chromatin aggregation, and phagocytosis of apoptotic bodies by macrophages in the hyperthermia group. B2 is the aggregation of neutrophils in the hyperthermia group. C1 is that there are more

lymphocytes and plasma cells in the tumor tissue of HSP70-PC treatment group. C2 is macrophage and apoptotic body in HSP70-PC treatment group. D1 is that apoptotic cells and apoptotic bodies are more common in the combined treatment group, with more lymphocytes. D2 shows that neutrophils in the tumor tissue of the combined treatment group aggregated and macrophages phagocytosed apoptotic bodies.

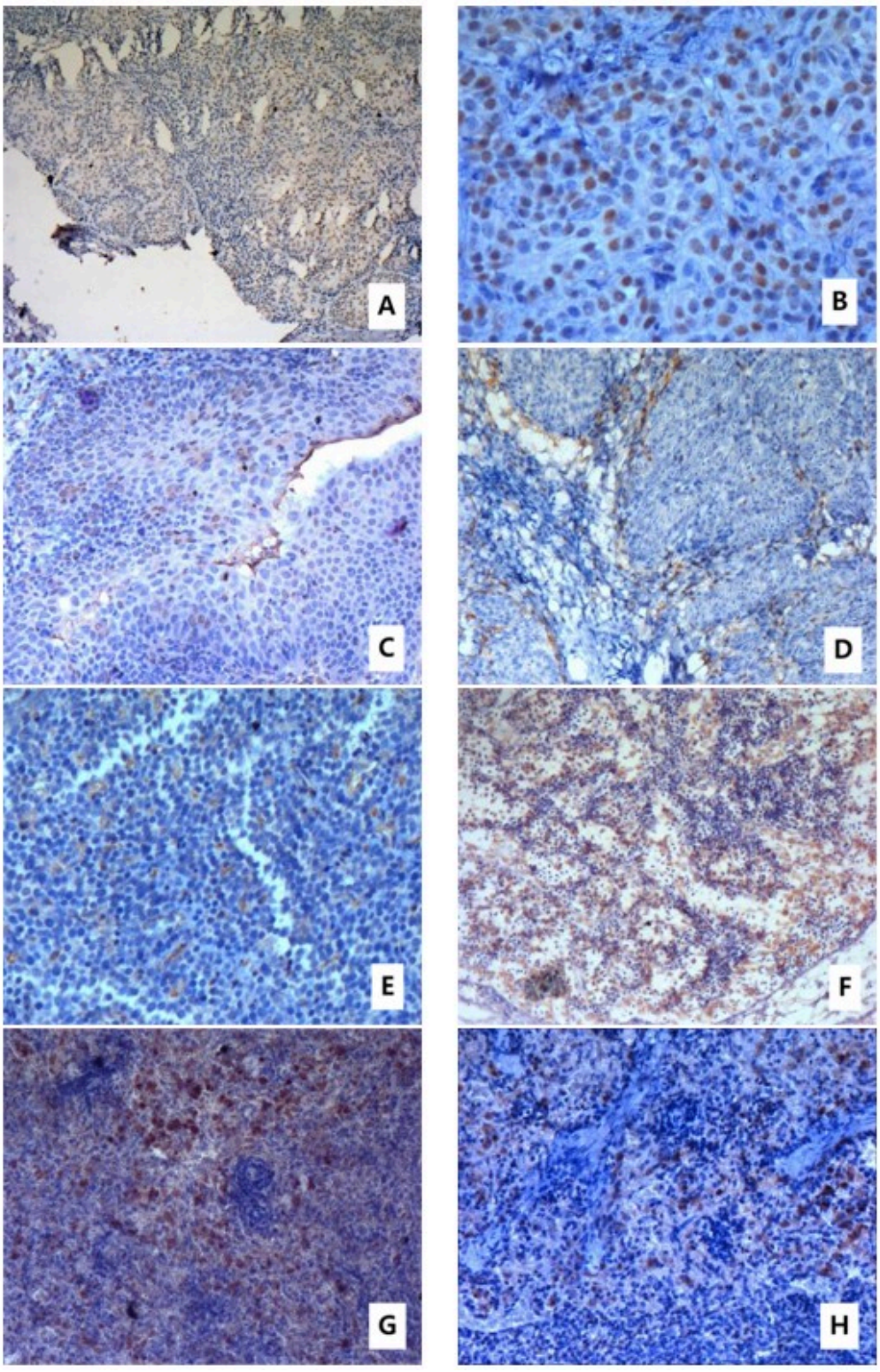


Figure 5

Pathological section of the combined treatment group: A is bladder tumor apoptotic cells (TUNEL), light microscope (×200). B is PCNA immunohistochemical staining of bladder tumor cells, light microscopy (×400). C is tumor cell HSP70 immunohistochemical staining, light microscopy (×200). D is the immunohistochemical staining of S-100 protein in tumor tissue and muscle layer, light microscope (×200). E is the immunohistochemical staining of CD_8 in tumor tissue and muscle layer, light microscope (×200). F is the immunohistochemical staining of S-100 protein in the iliac artery and paraaortic lymph nodes in the bladder drainage area, light microscope (×200). G is spleen S-100 protein immunohistochemical staining, light microscopy (×200). H is spleen CD_8 immunohistochemical staining, light microscopy (×200).