Distinct immunoreactions after microwave ablation of primary tumor with different heating parameters in VX2 tumor model

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Research

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Abstract

Background: Thermal ablations of solid tumors in situ can activate the immune system and produce a specific immune response against tumor. Microwave ablation (MWA) with different parameters can ablate tumors with similar sizes and cause different local inflammatory effects. Our aim was to determine whether MWA of primary tumor in different energy modes can induce different immunological effects.

Methods: Seventy rabbits with VX2 tumors implanted subcutaneously underneath the right second nipple were treated with high-power MWA (40W×1 minute), low-power MWA (20W×2 minutes), surgical resection, or left without treatment as control. Survival time was evaluated by log-rank test. On day 14 after ablation, immunohistochemistry and flow cytometry were used to evaluate the T-cell immune responses. Besides, the cytokine patterns were also identified from enzyme-linked immunosorbent assay.

Results: Tumor eradication was achieved completely in MWA groups as proven by nicotinamide adenine dinucleotide diapforase (NADH-diaphorase) staining. The rabbits in the control group showed significantly more pulmonary metastases and poorer survival than the three treatment groups; however, no significant difference was observed among three treatment groups. MWA groups induced more intratumoral and systemic CD4+ and CD8+ T cells than the control group. MWA caused more systemic CD4+ T cells than operation. High-power MWA induced more intratumoral CD4+ and CD8+ T cells and systemic CD4+ T cells than low-power MWA. MWA induced increased levels of IL-2, IL-12 and decreased levels of IL-4, IL-6, IL-10 compared with control group. Importantly, a significant higher level of serum IL-12 was found in high-power MWA than that in low-power MWA group.

Conclusions: High-power MWA induced enhanced Th1-type immune response in comparison to low-power MWA. High-power MWA may be selected for the treatment of solid tumors, although future studies are still needed to confirm our results.

Background

Minimally invasive therapies, including radiofrequency ablation (RFA), cryotherapy, high-intensity focused ultrasound, and laser therapy, have been widely used in the treatment of solitary tumors[1–3]. Compared to other minimally invasive therapies, MWA is simple to operate and has a high potential for complete tumor ablation with many advantages, such as improved convection profile, consistent high intratumoral temperature, large ablation volume, and short ablation time, and it is an effective local therapy for several solid tumors including small breast cancer[4, 5].

Importantly, thermal ablations of solid tumors in situ can activate the immune system and produce a specific immune response against tumor[6]. Denaturation and necrosis of intracellular proteins occur after ablation, and a large amount of tumor-specific antigens are released to induce adaptive immune responses[7, 8]. However, ablation-induced immune response is weak, and the antitumor immunity

induced by MWA has not been clearly documented[9]. Interestingly, MWA with different parameters can completely ablate tumors with similar sizes, and how to select the parameter for MWA remains unknown.

After ablation with different ablation methods and heat doses, tissue responses and cytokines released have been proved to be different[10–12]. Interestingly, MWA of normal rat livers with different ablation parameters can cause different local inflammatory effects[13]. It has not been reported that whether changes in the ablation parameters of MWA can affect the immune response. The purpose of this study was to investigate whether MWA of different output powers and durations can induce different T-cell immune responses, distant metastasis and survival time in subcutaneous VX2 tumor model.

Methods

Cell lines and culture

The VX2 cell line, used for tumor implantation, was obtained from the Surgery Department of the First Affiliated Hospital of Nanjing Medical University. The cells were cultured in RPMI 1640 (Hyclone, USA) supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin, and 100 units/mL penicillin (Shanghai Sangon, China) at 37 °C with 5% CO2.

Animal and tumor model

Treatment procedures of animals

The larger and shorter diameters of the tumor were measured by using a caliper. After the larger diameter of the tumor reached about 15 mm (12 to 20 days), altogether the 65 rabbits involved in this study was given the following treatments: (1) control group (n=16), tumor-bearing without any treatments (one for NADH-diaphorase staining), (2) low-power MWA group (n=17), an output power of 20W was used along the tumor's long axis for 2 mins (two for NADH-diaphorase staining), (3) high-power MWA group (n=17), an output power of 40W was used along the tumor's long axis for 1 min (two for NADH-diaphorase staining), (4) operation group (n=15), in which tumors were removed. For low-power MWA, the output power of 20 W was applied for 2 mins by the use of a microwave generator (ECO-100E, Yigao Microwave Electric Institute, Nanjing, China) with the microwave irradiation frequency of 2,450 MHz. For high-power MWA, the output power of 40 W was applied for 1 mins with the same microwave irradiation frequency.

Pathologic Evaluation

After MWA, the tumor specimen was sliced sequentially into 5-mm sections. The sections were snap-frozen, and cryosections of 8-μm thick were made for cell viability staining with slices, and stained with NADH-diaphorase (Sigma, USA). A section of tumor from control group was used as a negative control. The frozen unfixed sections were mounted on glass slides and covered with 150μl of incubation media for 20 minutes at room temperature. This incubation medium was prepared as described previously [14]. After incubation, viable cells showed an intense blue cytoplasmic pigment, which were absent in nonviable cells.

Lung metastasis analysis

The animals were autopsied within six hours after death. The lung specimens were fixed in 10% formalin solution, embedded in paraffin, sectioned into 4 μm slices, and stained with hematoxylin and eosin (H&E). The histological slides were evaluated by one experienced pathologist.

Immunohistochemical analysis

The tumor tissues were fixed in 4% formalin solution and paraffin embedded. Paraffin sections were stained with Rabbit-anti-CD8 (RM-9116-SO, Thermo Fisher, USA) and

Mouse-anti-CD4 (ab194998,ABCAM,British) followed by horseradish peroxidase (HRP)-conjugated goat anti-rat IgG (Santa Cruz Biotechnology, Santa Cruz, USA) and 3,3'-diaminobenzidine (DAB kit, Beyotime, Nanjing, China). The numbers of positive cells were counted in five randomly selected fields at 400-fold magnification. Results from the five areas were averaged and used in the statistical analysis.

Flow cytometric analysis

The rabbits were anesthetized and blood was drawn from the ear vein in EDTA tubes 14 days after operation. The blood were lysed with RBC lysis buffer (BD Biosciences, San Jose, USA) to remove red cells and obtained single cell suspension. Subsequently, all single cell suspensions were resuspended in Flow cytometry staining buffer. Then single cell suspensions were stained with surface markers at 4 °C for 30 min, after Fc blocking, to characterize the immune cell subsets: CD4(clone KEN-4, BIO-RAD), PE(clone A85-1, BD), FITC-anti-CD5 (clone KEN-5, BIO-RAD) and APC-anti-CD8A (clone 12.C7, Novusbio). Flow cytometric analysis was performed by using a FACS flow cytometer (Beckman Coulter, Miami, USA), and data analyzed by FlowJo software.

ELISA assay

Blood was collected from ear vein into EDTA tubes. Plasma was obtained by centrifugation at 1000 ×*g* for 10 mins and stored at -80°C until analysis. Concentrations of INF-γ, IL-4, IL-10, IL-6 and IL-12 in plasma were measured by enzyme-linked immune sorbent assay (ELISA) using High Sensitivity ELISA Kit (m1027824, mm030302, mm030702, mm030202, m1027340, MLBIO, CHINA) according to manufacturer's instruction respectively. TNF-α and IL-2 were quantifified using High Sensitivity ELISA Kit (DY5670, DY6994, R&D, USA).

Statistical analysis

Software (SPSS 16.0, Chicago, IL) was used for statistical analysis in the current study. The differences between the four groups were compared by repeated measurement ANOVA. Pairwise comparisons were made using the bonferroni corrected t-test. The Kaplan-Meier method and the log-rank test were used for the end-point survival analysis. Results with *P* values less than 0.05 were considered significant. **P* < 0.05; ** *P* < 0.01; *** *P* < 0.001; **** *P* < 0.0001.

Results

General aspects

All the animals well tolerated during the experimental procedures. Epidermal burns were found in six rabbits in the high-power MWA group and two in the low-power MWA group, while no necrosis of the skin was observed. No other immediate adverse effects occurred in all experimental subjects.

MWA eradicates the local tumor

NADH-diaphorase staining revealed a negative reaction in the MWA cases, confirming complete thermal damage to the lesions (Fig.1). However, staining revealed positive reactions in the control cases (Fig.1). During the follow-up, no local recurrence was observed in the two MWA groups.

MWA prolongs the survival and reduces lung metastasis

The survival of rabbits in different treatment groups were compared (Fig.2). Both MWA and surgery resulted in prolonged survival compared to the control group. But there were no significant differences among the two MWA groups and the operation group (Fig.2A). The rabbits in the control group showed significantly more pulmonary metastases than that in the three treatment groups, while there was no significant difference among the three treatment groups (Fig.2B,2C).

High-power MWA promotes intratumoral $CD4^+$ and $CD8^+$ T-cell infiltrations

The intratumoral infiltrations of $CD4^+$ and $CD8^+$ T cells were evaluated by immunohistochemistry on day 14 after ablation (Fig.3A). The numbers of intratumoral $CD4^+$ and $CD8^+$ T cells were significantly higher in the two MWA groups than in the control group (All $P < 0.001$, Fig.3B,3C). Moreover, high-power MWA enhanced $CD4^+$ and $CD8^+$ T cells infiltrating into the tumors compared with low-power MWA (both $P < 0.01$).

High-power MWA augments systemic $CD4^+$ T-cell immune responses

To determine the impact of MWA on systemic immune response, the levels of $CD4^+$ and $CD8^+$ T cells in the blood were evaluated. On day 14 after ablation, the percentages of peripheral $CD4^+$ T cells of rabbits treated with MWA were significantly increased compared with operation group and control group ($P < 0.001$, respectively, Fig. 4A, B). High-power MWA also dramatically raised $CD4^+$ T cells compared with low-power MWA ($P < 0.001$). The levels of peripheral $CD8^+$ T cells of rabbits treated with MWA and operation were significantly increased compared with that in control group ($P < 0.001$, respectively). However, the proportions of $CD8^+$ T cells in the two MWA groups were at similar levels to that in the operation group.

High-power MWA induces Th1-type cytokines

Elevations of Th1-type cytokines IL-2 were observed in all treatment animals compared with the control animals ($P < 0.001$) (Fig5), while there was no significant difference among the treatment groups. Another Th1 related cytokine IL-12 level in MWA groups were significantly higher than that in the operation group ($P < 0.001$). Moreover, the serum IL-12 level in high-power MWA group was significant higher than that in low-power MWA group ($P< 0.05$). No differences of serum IFN-γ and TNF-α levels were found among the treatment and control groups. Significantly lower levels of serum IL-4, IL-6, and IL-10 were observed in the two MWA groups compared with the other two groups, while there were no differences between the two MWA groups.

Discussion

Thermal ablation has become a reliable method for the local treatment of solid tumors by virtue of its minimally invasive, fast recovery and satisfied local effect[15]. After ablation of the lesion, the immune system of the body is also activated weakly, removing the immune suppression of the tumor on the body, and producing specific anti-tumor immune response[6, 16, 17]. Whether MWA of different output powers and durations can induce different T-cell immune response is unknown. To the best of our knowledge, our study firstly reported that MWA with different parameters can induce different T-cell immune responses for the same-sized ablation zone.

In our study, we found that surgery induced enhanced CD8 + T cells, but not enhanced CD4 + T cells or Th1-type cytokines. These results suggested that tumor burden reduction may reverse CD8 + cytotoxic T cell response, and MWA can induce the Th1-type T cell response but not surgery. Prior studies shows that thermal ablation could induce Th1 immune response[18, 19]. The balance of Thl/Th2 is of great significance for tumor immunity[20, 21]. In the process of tumor formation and development, the secretion of IL-4 and IL-6 is increased, and Th2 cytokines are dominant, which inhibits the production of Th1 cytokines such as IFN-γ, reduces the ratio of Thl/Th2, and the balance is gradually shifted to Th2[22, 23]. The results of this study indicated that MWA of VX2 tumor reduced the expression of Th2 cytokines, and the high-power MWA furtherly promoted the expression of Th1 cytokine IL-12. MWA, especially in the high-power group, is more likely to shift the balance of Th1/Th2 toward the direction dominated by Th1 cells to enhance the body's anti-tumor immune function. The difference may be related with the different heat generated by microwave energy. High-power MWA may generate a higher central temperature than low-power MWA. The thermal tissue gradient possibly generate a wider rim of periablational tissue that is exposed to lower nonlethal levels of heating, similar to what has been presented in early ablation literature[24]. All these results suggested that high-power MWA can generate enhanced Th1-type immune response in comparison to low-power MWA, and high-power MWA may be selected for tumor ablation in the future.

Importantly, both MWA and operation promoted the survivals of experimental animals compared with the control, while there was no significant difference among the treatment groups. Compared with the operation group, MWA of the local tumor can activate the immune system, but it couldn't bring survival benefits, indicating that the anti-tumor immune effect generated by thermal ablation was weak, which

was consistent with the phenomenon observed by Machlenkin et al[9]. MWA of the tumor may be a trigger of immune response, and MWA combined with immune checkpoint inhibitors may be promising strategies for solid tumors.

In addition, it was found that each group had an extremely high distant metastasis rate, which was similar to the high metastasis rate of other related studies, which may be related to the extremely aggressive VX2 tumor cell line itself[25]. Importantly, surgical trauma and non-specific inflammatory response can indirectly affect cytokine levels[26], and similar levels of cytokines were observed in surgery group and control group in this study, suggesting that this temporary inflammatory response have little impact in subcutaneous VX2 tumor model.

There were several limitations in our study. Other ablation technologies including radiofrequency ablation and cryoablation have also been reported to induce potentially beneficial immunologic effects. Therefore, further studies of the differences between ablation modalities in a wide range of tumor types are needed. Moreover, each ablation energy source can be applied using numerous applicator designs and with a multitude of energy parameters, including durations and intensities. All of these may potentially affect the outcomes of the treatment.

In conclusion, high-power MWA induced enhanced Th1-type immune response in comparison to low-power MWA in subcutaneous VX2 tumor model, although there was no difference in distant metastasis and survival. Considering the weak immunostimulating effect of MWA, the combination therapy with immune enhancers may have good prospects.

Conclusions

In this study, we showed that MWA with different parameters can induce different T-cell immune responses for the same-sized ablation zone. High-power MWA can generate enhanced Th1-type immune response in comparison to low-power MWA, and high-power MWA may be selected for tumor ablation in the future. MWA of the tumor may be a trigger of immune response, and MWA combined with immune checkpoint inhibitors may be promising strategies for solid tumors.

Abbreviations

MWA:microwave ablation; RFA:radiofrequency ablation; NADH-diaphorase:nicotinamide adenine dinucleotide diapforase; H&E:hematoxylin and eosin; ELISA:enzyme-linked immune sorbent assay.

Declarations

Acknowledgement

Not applicable.

Authors’contributions

JX, HW, SW and WZ conceptualized and designed the research. JX, HW, MQ, HC, G M, and MY performed the experiments. JX, HW, HP and HX analysed the data. JX, HW, YZ and LL interpreted results of the experiments. JX, HW, SW and WZ edited and revised the manuscript. All authors read and approved the fifinal manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All protocols and studies involving animals were approved by Nanjing Medical University Institutional Animal Care and Use Committee.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

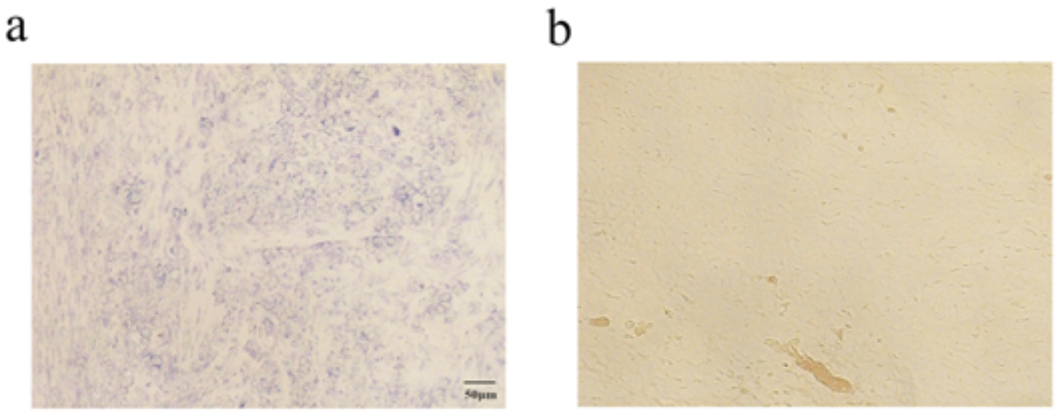

Figure 1

a) Photomicrographs from the control cases shows the α-NADH-diaphorase- positive tissue. b) Section of ablated tissue shows a negative reaction to α-NADH-diaphorase stain or no viable tumor cells present after MWA therapy.

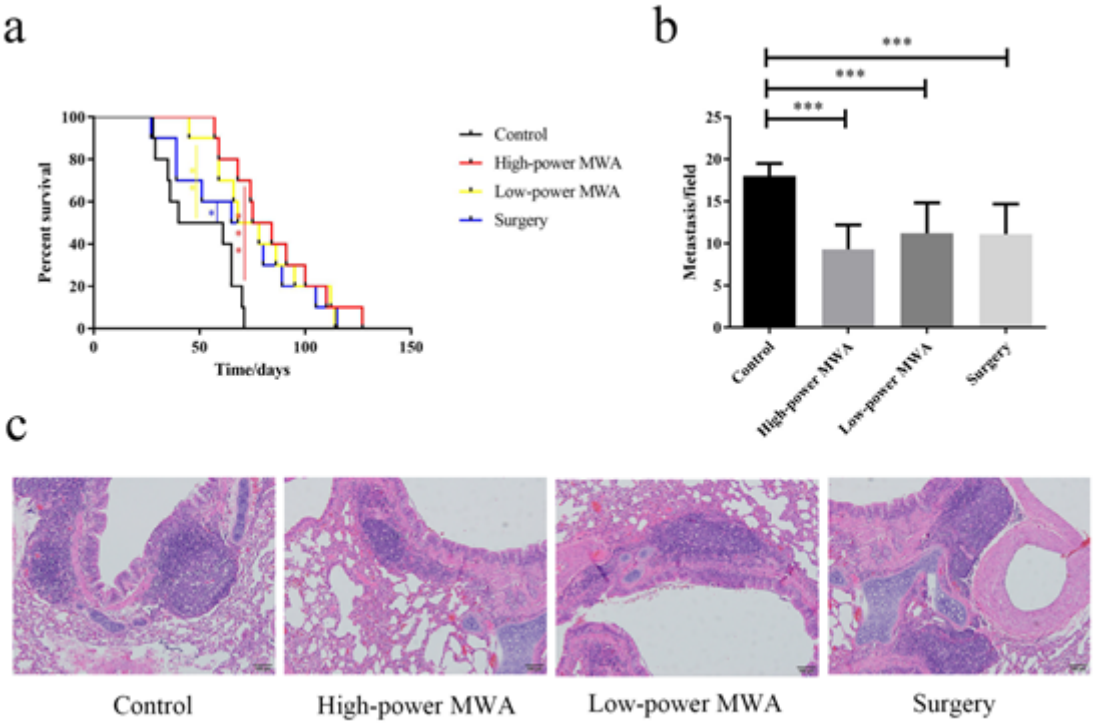

Figure 2

a) Kaplan-Meier curves showing survival of untreated rabbits and rabbits treated with MWA and operation. Points, mean; error bars, SEM. *P < 0.05; ***P < 0.001. One representative experiment out of three is shown. b) Numbers of lung metastases in different groups. c) Metastatic nodules demonstrated by hematoxylin and eosin (H&E) stain (40×) .

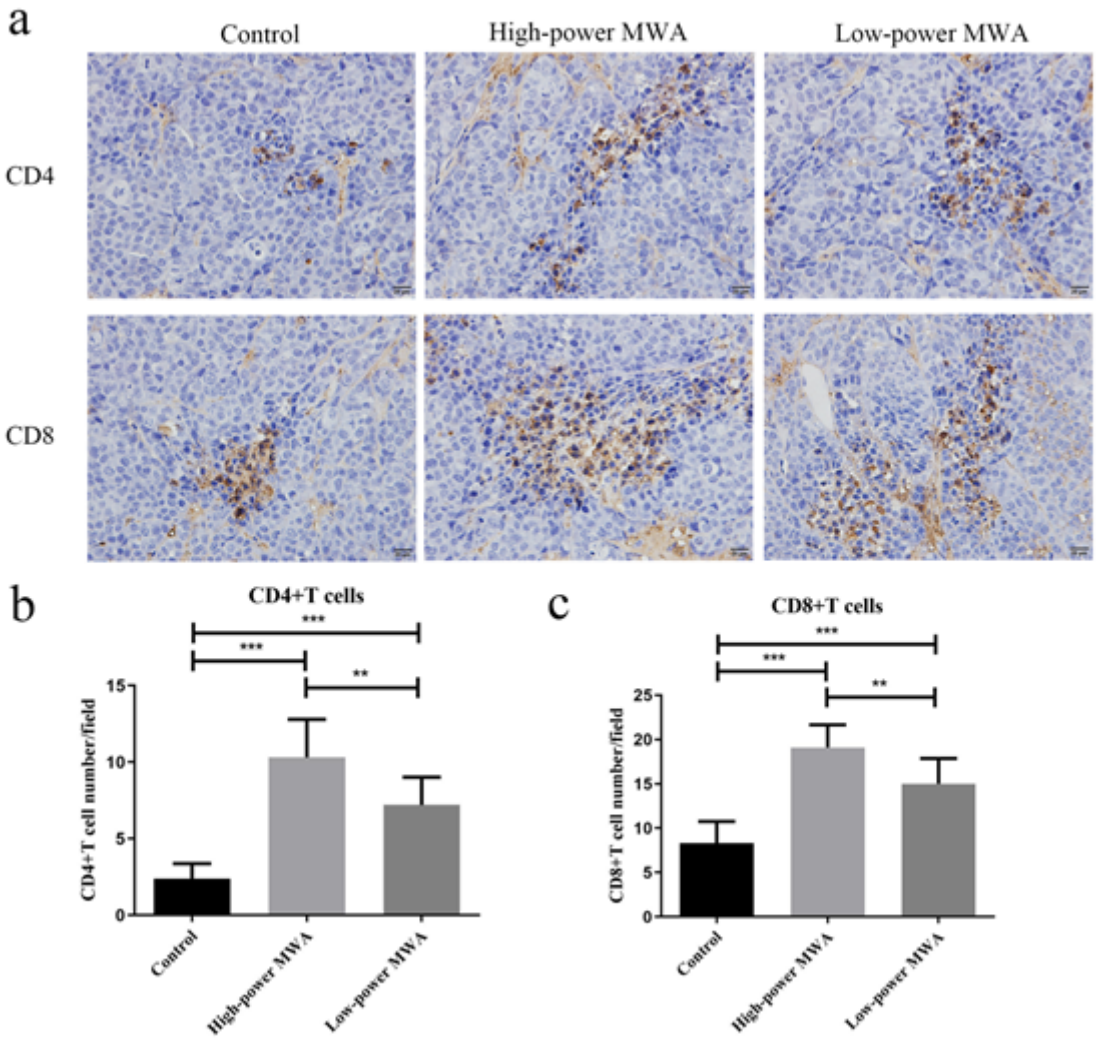

Figure 3

a) Representative microphotographs of CD4 and CD8 staining in each group. Immunohistochemical staining was performed on tumor specimens that were harvested 14 days after treatment. Original magnification, ×100. b,c) Density of CD4+ and CD8+ cell was determined. Columns, mean; error bar, SEM. ***P < 0.001.

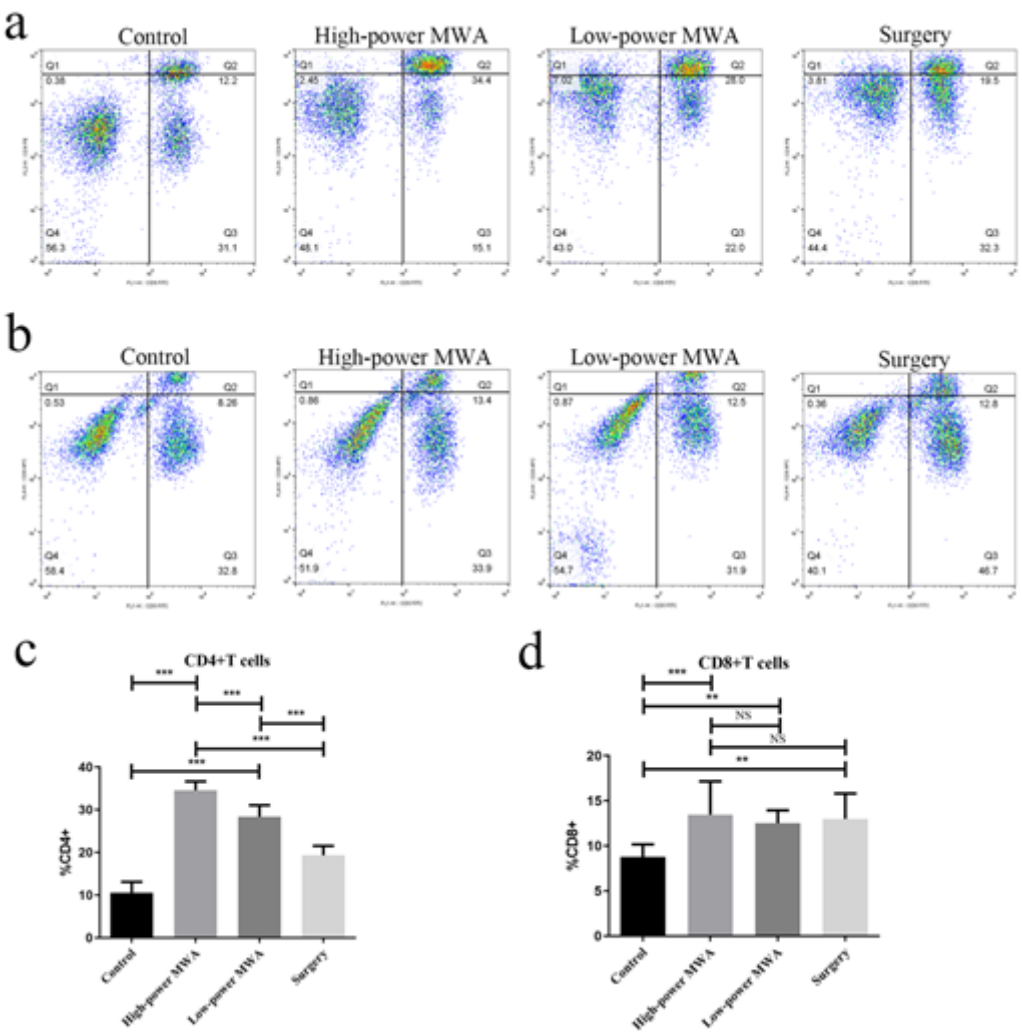

Figure 4

a) Representative flow cytometric plots showing CD4+T-cell on days 14 after MWA. b) Percentage of CD4+ cells on days 14 after MWA. c) Representative flow cytometric plots showing CD8+T-cell on days 14 after MWA. d) Percentage of CD4+ cells on days 14 after MWA. Column, mean; error bars, SEM. *P < 0.05; **P < 0.01; ***P < 0.001; NS not significant. Data were pooled from two independent experiments with 10 rabbits per group.

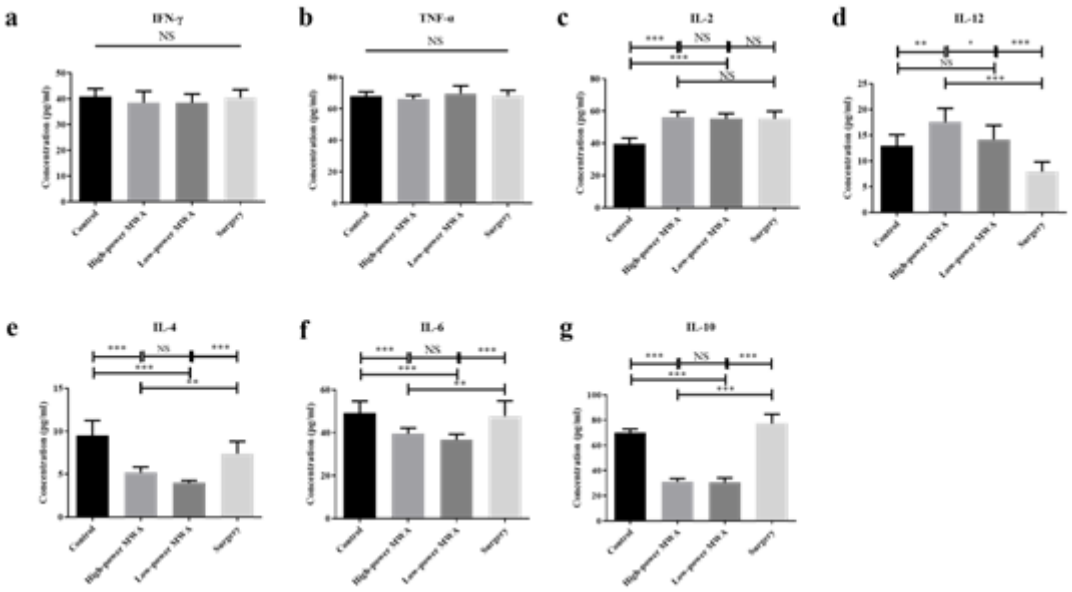

Figure 5

Bar graphs show serum levels of seven cytokines after ablation. Cytokine levels were determined by using an enzyme-linked immune sorbent assay, or ELISA, protocol in untreated rabbits and rabbits treated with MWA and operation.