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REVIEW

Immune response triggered by the ablation of hepatocellular carcinoma with nanosecond pulsed electric field

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Abstract **Nanosecond pulsed electric field (nsPEF) is a novel, nonthermal, and minimally invasive modality that can ablate solid tumors by inducing apoptosis. Recent animal experiments show that nsPEF can induce the immunogenic cell death of hepatocellular carcinoma (HCC) and stimulate the host's immune response to kill residual tumor cells and decrease distant metastatic tumors. nsPEF-induced immunity is of great clinical importance because the nonthermal ablation may enhance the immune memory, which can prevent HCC recurrence and metastasis. This review summarized the most advanced research on the effect of nsPEF. The possible mechanisms of how locoregional nsPEF ablation enhances the systemic anticancer immune responses were illustrated. nsPEF stimulates the host immune system to boost stimulation and prevail suppression. Also, nsPEF increases the dendritic cell loading and inhibits the regulatory responses, thereby improving immune stimulation and limiting immunosuppression in HCC-bearing hosts. Therefore, nsPEF has excellent potential for HCC treatment.**

Keywords nanosecond pulsed electric fields (nsPEF); hepatocellular carcinoma (HCC); immune response; recurrence; metastasis

Introduction

Hepatocellular carcinoma (HCC) is one of the most common and deadly malignant tumors in the world [1]. Resection and liver transplantation are still effective methods for early HCC treatment [2,3]. However, most liver cancers are diagnosed at an advanced stage, and only approximately 20% of these patients have the chance to undergo surgery. In addition, the postoperative recurrence rates of HCC are still high, requiring multiple treatments to prolong the life of patients. Therefore, finding novel therapies that can be applied repeatedly and are minimally invasive for HCC patients is important.

With the development of minimally invasive technologies, an increasing number of local ablation methodologies are being applied in the treatment of HCC. Most of them are thermal ablative techniques such as cryoablation, radiofrequency ablation (RFA), microwave ablation (MWA) [4], and focused ultrasound [5]. Notably, RFA has become the mostly widely used local ablation treatment in the clinic. Two randomized controlled studies between surgical resection and RFA have shown that the overall survival and disease-free survival rates are not statistically different between the two strategies. Furthermore, RFA leads to a shorter hospital stay and has lower incidence of posttreatment complications [6,7]. However, the 5-year recurrence rate of surgical resection and RFA is still high because of the highly malignant nature of HCC. Moreover, RFA has several problems. Small and distal metastases often escape destruction because of the locoregional nature of the very precise intervention of RFA. These micrometastases can be responsible for tumor recurrence after RFA treatment. RFA kills tumors by heat conduction, which can cause thermal damage to large vessels and heat sink effect, and the residual tumor may promote tumor metastasis [8]. Therefore, a new type of minimally invasive nonthermal technology should be developed for the treatment of HCC.

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Nanosecond pulsed electric field (nsPEF) is a novel modality for the treatment of tumor with the characteristic of being nonthermal and minimally invasive. Compared with RFA and other methods based on the extreme temperatures, nsPEF avoids the thermal damage of adjacent organs and structures caused by heat [9] and eliminates the residual cancer caused by the heat sink effect. The principle of nsPEF is to stimulate cell membrane and subcellular structures to produce membrane perforation, which induces apoptosis [10]. nsPEF has been demonstrated to successfully induce apoptosis in various tumor cells [11–13]. In addition, nsPEF effectively eliminated tumors in a series of animal experiments, such as murine liver cancer [14], rat liver cancer [15], mouse melanoma [16], mouse pancreatic cancer [17], and mouse breast cancer models [18]. Most importantly, a clinical trial demonstrated that nsPEF could successfully be used in the treatment of skin melanoma in human patients. The experiment showed significant efficacy and minimal invasion and did not induce pain [19].

nsPEF has promising therapeutic prospects for liver cancer treatment, which have been shown in both cells and animal experiments [13,20,21]. Animal experiments on tumor ablation by nsPEF have shown that it can lead to long-term nontumor survival without recurrence [22]. As the research progresses, researchers have found that nsPEF can stimulate the host to produce an immune response, thereby causing the immunogenicity cell death of tumor and killing the tumor of distant metastasis. nsPEF can produce a "vaccine-like" effect that can effectively prevent the recurrence and metastasis of tumors in an orthotopic HCC model [15]. Therefore, relevant studies are systematically reviewed to illustrate how nsPEF stimulates the host immune responses during HCC ablation. The possible mechanisms are also discussed, which could facilitate a better understating of the subject and provide guidance for future clinical treatments.

nsPEF causes apoptosis and immunogenic cell death

Recent research showed that nsPEF can induce apoptosis in tumor cells. For instance, Schoenbach *et al.* [23] claimed that nsPEF can induce the release of calcium ions in cells, which enhances gene expression and apoptosis of cells. Moreover, Nuccitelli *et al*. [24–26] found that the melanoma cells in SKH-1 mice post-nsPEF showed activation of caspase and other apoptotic markers such as DNA damage and the morphological changes of cell apoptosis, indicating that high-intensity pulsed electric field can induce the apoptosis of malignant tumors.

Apoptosis is the death pathway by which most cells undergo programmed cell death when they lose normal function and need to be replaced. In addition, apoptosis will not cause immune response due to its immune tolerance under normal physiologic conditions. nsPEF allows calcium ions to enter the cytoplasm from the endoplasmic reticulum (ER) and extracellular space, causing an increase in ER stress [27], reactive oxygen species (ROS) [17,28], and other factors that play a role in the cascade process of apoptosis. Currently, cell death is known to elicit an immune response through ER stress and ROS production, which is an immunogenic form of apoptosis. This phenomenon has been observed in many other different treatments, such as anthracycline, oxaliplatin, and ultraviolet radiation [29]. Under ER stress, cells undergo apoptosis and release signals, which are also known as danger-associated molecular patterns (DAMPs), that can be recognized by the immune system of the body. These DAMPs that play an important role in the immune response include three critical types: calcium reticulin translocation, ATP secretion, and high mobility group box 1. The emission of these signaling molecules causes the immune response of the body and thus kills tumor cells [30].

The host typically reacts with an immune response to eliminate tumors when it recognizes the antigens of the mutated genes that produce tumors. However, tumor cells deploy various mechanisms to evade the body's immune surveillance system. In the case where the tumor wins the battle, it ultimately develops quickly in local primary organ and remote metastasis. The emission of DAMPs caused by nsPEF can activate the immune system, thereby identifying tumor cells and triggering an immune response [11]. Therefore, nsPEF leads the host to be capable of evoking a robust antitumor response because nsPEF can boost the immune system's response to fight against tumors.

nsPEF alters the liver tumor microenvironment

The tumor microenvironment is a systematic concept that cancer behavior is determined not only by tumor genes but also by the environment around the tumor. In addition to tumor cells, the tumor microenvironment includes blood vessels around the tumor extracellular matrix, other benign cells, and some signaling molecules [31]. The microenvironment of HCC is thought to be composed of cellular and noncellular components. Cellular components include hepatic stellate cells, fibroblasts, and immune cells (i.e., regulatory T cells, cytotoxic T cells, tumor associated macrophage, and endothelial cells). The noncellular components are as follows: (1) growth factors including transforming growth factor β 1 and platelet-derived growth factor, (2) proteolytic enzymes such as matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases, (3) extracellular matrix proteins, (4) cytokines, and so on [32]. Majority of recent studies have emphasized that the

tumor microenvironment plays a significant role in the development and progression of HCC. In summary, the tumor microenvironment plays a vital role in the process of HCC formation, epithelial–mesenchymal transition, and tumor infiltration and metastasis [33–35].

The tumor microenvironment can make tumors evade immune surveillance, which can inhibit the body's antitumor immunity by activating the immunosuppressive pathways. These pathways include immune receptor-monitoring points of effector T cells and bone marrow cells and the emission of inhibitory cytokines and metabolites [36]. Recently, a number of different animal experiments found that local ablation tumor using nsPEF can change the tumor microenvironment, which may induce the variation of immune cells and cytokines and remove the immunosuppressive effect in the tumor microenvironment, and activate the reconstruction of local immune responses. Thus, nsPEF can induce immunogenic tumor cell death, effectively eliminate the tumor, and prevent the recurrence of tumor metastasis. Using an HCC nude mouse model, Yin *et al.* found that the number of resistance macrophage positive cells below the tumor subcapsular were significantly increased in the nsPEF treatment group compared with the control group. These findings indicate that nsPEF changed the composition of immune cells in the tumor microenvironment and triggered a local immune response to eliminate the tumor effectively, thereby extending the disease-free survival time of the mice [21]. Also, Chen *et al*. found that the infiltration of macrophages in the tumor microenvironment significantly increased after the nsPEF was applied to a xenogeneic nude mouse model of liver cancer [14]. The findings of these experiments indicate that the ablation of nsPEF on the tumor may cause the increase of macrophages in the tumor microenvironment.

In addition, nsPEF not only causes increased infiltration of macrophages in the tumor microenvironment but also changes the proportions of other immune cells. Chen *et al*. set up an HCC N1–S1 rat model and found that Granzyme-B increased obviously in the nsPEF-treated group after nsPEF treatment. The Granzyme-B continued to accumulate in the tumor microenvironment during the post-nsPEF follow-up [15]. Granzyme-B is considered as an indicator of infiltration by T and natural killer (NK) cells, which also indirectly reflect that nsPEF may cause the increase of T and NK cells in the tumor microenvironment, thus causing the innate and adaptive immune response of the body. Lassiter *et al*. performed an experiment in the same HCC animal model and found that the immune cells altered post-nsPEF in terms of the types and numbers in the tumor microenvironment, indicating that nsPEF can alter the tumor microenvironment of immunosuppression and induce an organism congenital and adaptive immune response to remove the tumor. nsPEF may stimulate the formation of memory T cells, so that the body can dramatically reduce the risk of tumor recurrence [12].

nsPEF stimulates a strong immune signal pathway activation

nsPEF induces tumor cells to secrete DAMPs, which are similar to "autophagy" signals that induce the combination of CD91 and CD69 on tumor and dendritic cells [37]. These changes facilitate the transfer of tumor-associated antigens and initiation of immunogenic tumor cell death. Simultaneously, these signals can alter the composition and number of immune cells in the tumor microenvironment that may stimulate the production of tumor specific cytotoxic T cells and enhance the antitumor immune responses of the body [38]. Chen *et al*. found that all 21 experimental rats had no recurrence after 20 weeks of observation after challenge experiments performed 7 weeks post-nsPEF in the rat model of N1–S1 HCC, reflecting that the nsPEF may have a protective effect by stimulating the immune response of the host [15]. They measured the blood and spleen lymphocytes post-nsPEF in the rats with the same HCC model experiments of Lassiter and found that the number of adaptive T cells in the blood and spleen in rat after the nanosecond pulse therapy a week later presented an increasing tendency, which reflects that the nsPEF induced the body's adaptive immune response. Later, they found that the strong toxicity of killing tumor cells of $CD8^+$ T cells increased significantly in the challenge experiments. In addition to postoperative immune response, the appearance of $CD4^+$ and $CD8^+$ memory precursor and short-lived effector cells also show that the nsPEF induces the body's immune memory mechanism, which can effectively avoid the recurrence of the tumor. The immune memory is specific to the N1–S1 HCC tumors. The experimental rats kept the immune memory without recurrence after 8 months of observation [39]. Nuccitelli *et al*. found that the immune response induced by nsPEF was $CD8^+$ dependent. Their experiments indicate that nsPEF can cause the body to produce specific $CD8^+$ toxic T cells, which can specifically kill tumor cells and prevent tumor recurrence [40].

The challenge experiments indicate that animals treated with nsPEF ablation mount an immune response against HCC, which is also known as vaccine-like effect, thereby preventing the recurrence and metastasis of HCC. Nuccitelli *et al*. injected tumor cells that have been treated by nsPEF *in vitro* in mice and found that these nsPEF-treated cells did not grow into a tumor lesion, unlike the nontreated tumor cells. In subsequent experiments, they waited three weeks for an immune response to develop, injected untreated and healthy tumor cells, and measured the subsequent tumor size over time. These secondary tumors failed to grow and instead shrunk until they were no longer visible, whereas the tumors treated with mitomycin

C in the control group were significantly larger [40]. The above experiments reflect that nsPEF treatment of tumors can stimulate the immune response to generate immune memory and vaccine-like effects, thereby preventing the recurrence and metastasis of tumors. Moreover, tumor cells treated by nsPEF *in vitro* can release antigens that can be specifically recognized by dendritic cells. nsPEF treatment strengthens the antitumor efficacy, which is similar to immunotherapy that uses antigen-loaded dendritic cells to treat liver cancer [41]. In general, nsPEF may serve as a tumor vaccine and provide a novel immunotherapy of HCC. nsPEF makes HCC tumor antigens exposed as an *in situ* cancer vaccine that can lead to the initiation of a systemic antitumor immune response by destroying readily accessible HCC. This response can affect and potentially eliminate occult and metastatic tumors in the lungs.

Researchers have not only used nsPEF in animal models of liver cancer to stimulate the immune response and prevent tumor recurrence and metastasis but also observed this phenomenon in animal models of other tumor types and conducted in-depth studies. Guo *et al*. conducted experiments on 4T1 breast cancer model. All 11 mice were resistant to the challenge experiments of 4T1 cells and did not relapse after nsPEF ablation. In addition, they also found that the number of $CD4^+$ effector memory T cells and $CD8^+$ effector and central memory T cells increased significantly. The number of antitumor specific IFN-γ also increased. Their results suggested that nsPEF can stimulate a robust immune response and reduce recurrence and metastasis [18]. The animal experiments are summarized in Table 1.

Conclusions and perspectives

nsPEF with high-intensity electric field has an impact on the organelles of HCC cells, which can induce ER stress in tumor cell and increase ROS, thereby inducing protease-dependent apoptosis of cells. Moreover, tumor cells treated by nsPEF can release DAMPs that can specifically combine with dendritic cells that can present the tumor antigen to killer T cells and form immune memory in the meantime. Simultaneously, nsPEF alters the tumor micro-environment and breaks the immunosuppression of the host. Consequently, local application of nsPEF can prevent the remote metastasis of the tumor. The possible mechanisms for inducing the immune response include the following: (1) nsPEF can induce apoptosis of tumor cells and release DAMPs and tumor-associated antigens. Simultaneously, nsPEF can alter the tumor

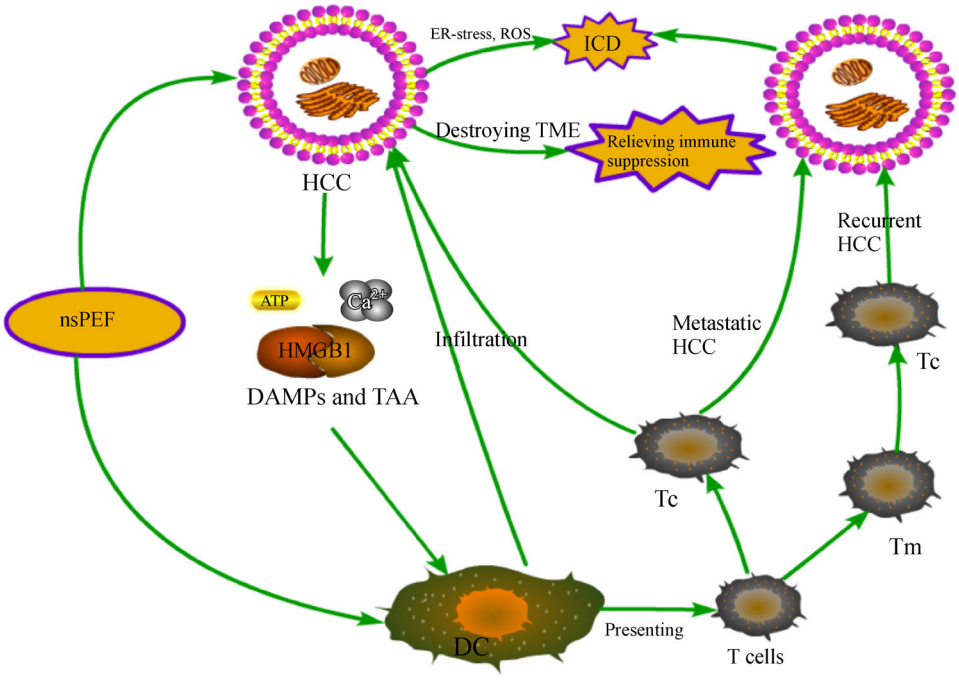

Fig. 1 Possible mechanism of the nsPEF-induced immune response. Abbreviations: nsPEF, nanosecond pulsed electric field; TAA, tumor-associated antigens; DC, dendritic cells; Tc, effector toxic T cells; Tm, memory T cells; TME, tumor microenvironment; ICD, immunogenic cell death; ER, endoplasmic reticulum; ROS, reactive oxygen species; DAMPs, danger-associated molecular patterns; HMGB1, high mobility group box 1.

Table 1 Significant findings from preclinical nsPEF studies

Researchers	Year	nsPEF parameters	Experimental animals and cell line	Immune response
Nuccitelli *et al*. [16]	2012	2000 pulses each, 100 ns long, and 30 kV/cm at a rate of 5–7 pulses	C57/BL6-HGF/SF transgenic mice with melanomas induced by UV radiation	Elevated $CD4^+$ T cells have been detected in tumor
Chen *et al*. [15]	2014	1000 pulses each, 100 ns long, and 50 kV/cm with repetition rates of 1 Hz	Orthotopic HCC model established in rats using N1–S1 HCC cells	Presence of Granzyme-B expressing cells in the tumor
Chen *et al*. [14]	2014	300 pulses each, 100 ns long in 0.5 Hz	Animal model of human subdermal xenograft HCCLM3 cells in BALB/c nude mouse	Macrophage infiltration in tumor
Nuccitelli *et al*. [40]	2015	(1) 400 pulses each, 100 ns long, and 15 kV/cm in delivering 50 A (2) 500 pulses each, 100 ns, and 50 kV/cm	(1) Orthotopic HCC model established in rats using McA-RH7777 cells (2) C57BL/6 female mice and B6 albino female mice along with the isogenic MCA205 fibrosarcoma cell line	Triggered a CD8-dependant inhibition of secondary tumor growth
Skeate *et al*. [38]	2018	Each 100 ns long and 30 kV/cm at a rate of 3 pulses	Human papillomavirus type 16 (HPV16)-transformed C3.43 mouse tumor cell model	Induced an antitumor response driven by $CD8^+$ T cells
Guo *et al*. [18]	2018	300–1000 pulses each, 100 ns long, and 50 kV/cm with the frequency of 1–2 Hz	Female Balb/c mice with 4T1 cell line	Destruction of suppressive tumor microenvironment (TME); activation of antigen-presenting cells and induction of a potent antitumor memory response
Lassiter *et al*. [39]	2018	1000 pulses (1–3 pulses/s) each, 100 ns long, and 48–52 kV/cm	Orthotopic HCC model established in rats using N1–S1 HCC cells	Activated innate and adaptive immune memory
Guo *et al*. [17]	2018	600–1200 pulses each, 200 ns long, and 30 kV/cm in 2 Hz	Female C57BL/6 mice with Pan02 cells	The number of immune cells in the TME was changed and multiple activation markers were upregulated

microenvironment and relieve immune suppression in tumor microenvironment. (2) Dendritic cells are activated by DAMPs in the treated tumor tissues. nsPEF can also directly stimulate dendritic cells around the tumor in addition to the indirect approach. (3) The activated cells migrate to lymph node tissues and present tumor antigens to T helper (TH) cells. (4) Many effector toxic and memory T cells are produced in large quantities to eliminate residual tumor cells and prevent tumor recurrence with minimal metastasis [18]. The possible mechanism of nsPEF-induced immune response is illustrated in Fig. 1.

Traditional ablation treatments such as RFA, cryoablation, and MWA have been used clinically. The differences of the immune responses induced by nsPEF and these ablations are summarized in Table 2 [42–46]. Compared with these ablations, nsPEF has the following advantages: (1) nsPEF does not damage the important organs and blood vessels, (2) nsPEF can avoid residual cancer due to heat sink effect, and (3) nsPEF can induce immune memory to prevent tumor recurrence and metastasis.

In summary, nsPEF is a nonthermal ablation therapeutic technique that is used to target local primary HCC malignancies for destruction. Due to its non-thermal nature, nsPEF can induce immunogenic tumor cell death and the emission of specific antigens from apoptosis of tumor cells that can combine with dendritic cells specifically, causing local and systemic immune responses. Furthermore, nsPEF can regulate innate immunity and adaptive immune cells and release the host's immune inhibition status that can affect the progress of the local and distant metastases and form long-term antitumor immune memory to prevent the residual carcinoma and tumor recurrence and metastasis. However, studies on immune response induced by nsPEF are now only based on *in vitro* or animal models. Therefore, much work on clinical experiments still needs to be done in the future. Nevertheless, nsPEF is potentially a new immunotherapeutic strategy for the effective treatment of HCC patients.

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Compliance with ethics guidelines

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Table 2 Comparison of the immune response induced by nsPEF and thermal ablation

	nsPEF	RFA	Cryoablation	MWA
Fundamental principles	Utilizing nsPEF to stimulate cell membrane and subcellular structures to produce membrane perforation	Utilizing high-frequency alternating current to generate high temperatures	Utilizing liquefied gases to induce the freezing–thawing cycle of targeted lesions	Utilizing electromagnetic waves to generate heat
Treatment temperature	Nonthermal	60–100 °C	<-40 °C	>100 °C
Mechanism of tumor cell injury	Apoptosis and immunogenic cell death	Central area necrosis, peripheral area necrosis, or apoptosis	Central area necrosis, peripheral area necrosis, or apoptosis	Mainly necrosis
Released signals	DAMPs: CRT, ATP secretion, and HMGB1	Intracellular antigens and DAMPs such as HSPs and HMGB1	Preserved intracellular organelles, antigens, and DAMPs such as DNA and HSPs	DAMPs: HSPs
Immune response	(1) The number of immune cells in the TME was changed, and multiple activation markers were upregulated (2) Elevated $CD4^+$ T and $CD8^+$ T cells were detected, and a $CD8^+$-dependent inhibition of secondary tumor growth was triggered (3) Activation of antigen-presenting cells and induction of a potent antitumor memory response	(1) Levels of interleukin-1β (IL-1β), IL-6, IL-8, and TNF were increased (2) The increasing levels of $CD4^+$ and $CD8^+$ T cells, and the decreasing levels of $CD25^+$, $FoxP3^+$, and regulatory T cells	(1) Activating the nuclear factor κ-light-chain-enhancer of activated B cells (NF-κβ) pathway (2) Stimulating T cells and promoting a systemic immune response (3) Levels of serum IL-1, IL-6, NF-κβ, and TNF-α were increased	The increasing levels of IL-1 and IL-6

Abbreviations: nsPEF, nanosecond pulsed electric field; RFA, radiofrequency ablation; MWA, microwave ablation; DAMPs, danger-associated molecular patterns; CRT, calcium reticulin translocation; HMGB1, high mobility group box 1; HSPs, heat shock proteins; IL, interleukin; TNF, tumor necrosis factor.

article and does not involve a research protocol requiring approval by the relevant institutional review board or ethics committee.

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