Abstract

Biological effects of pulsed electrical stimulation (PES) on cells and tissues have been intensively studied with the aim of advancing their biomedical applications. These effects vary significantly depending on PES parameters, cell and tissue types, which can be attributed to the diverse variety of signaling pathways, ion channels, and epigenetic mechanisms involved. The development of new technology platforms, such as nanosecond pulsed electric fields (nsPEFs) with finely tuned parameters, have added further complexity. The present review systematically examines current research progress in various aspects of PES, from physical models to biological effects on cells and tissues, including voltage-sensing domains of voltage-gated channels, pore formation, intracellular components/organelles, and signaling pathways. Emphasis is placed on the complexity of PES parameters and inconsistency of induced biological effects, with the aim of exploring the underlying physical and cellular mechanisms of the physiological effects of electrical stimulation on cells. With chondrogenic differentiation of stem cells and cartilage regeneration as examples, the underlying mechanisms involved were reviewed and analyzed, hoping to move forward towards potential biomedical applications. Hopefully, the present review will inspire more interest in the wider clinical applications of PES and lay the basis for further comprehensive studies in this field.

Keywords: Electrical stimulation, electric fields, chondrocytes, chondrogenic differentiation, cartilage, stem cells.

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MAPK    | mitogen-activated kinase
MSCs    | mesenchymal stem cells
NEUROG2 | neurogenin 2
NMES    | neuromuscular electrical stimulation
NIN     | non-invasive interactive neurostimulation
NO      | nitric oxide
nsPEFs  | nanosecond-pulsed electric fields
OA      | osteoarthritis
PD-ECGF | platelet-derived endothelial cell growth factor
PEG     | polyethylene glycol
PES     | pulsed electrical stimulation
PIP2    | phosphatidylinositol 4,5-bisphosphate
ROS     | reactive oxygen species
snRNPs  | small nuclear ribonucleoprotein particles
TGF-β   | transforming growth factor beta 1
VEGF    | vascular endothelial growth factor
VOCCs   | voltage-operated calcium channels
VSDs    | voltage-sensing domains

Introduction

PES has long been studied in the context of clinical therapy for various diseases, including wound healing (Ud-Din and Bayat, 2014), bone and cartilage regeneration (Chao and Inoue, 2003; Ciombor and Aaron, 2005), recovery of motor neurons (Rossini et al., 2015), contractile properties of injured heart tissues (Hirt et al., 2014), and rehabilitation of muscle contractile function (Kern et al., 2016), as well as tumor therapy through triggering permanent membrane lysis or loss of homeostasis (Cemazar et al., 2013). Several PES devices have been approved by the US FDA for use in the treatment of various conditions, such as tumors, arthritis, and pain, with durations varying from μs to ms (Table 1). Accumulation of Joule heating energy is still a major hurdle for clinical application of electrical stimulation (Voldman, 2006; Weaver et al., 1999), which in turn demands critical restrictions on PES parameters, even though mammalian cells can survive up to a 1-3 °C increase in temperature. Relatively broad or unfocused biological targets limit the exploration of the mechanistic effects of electrical stimulation, as well as their widespread applications in clinical therapy (Okino and Mohri, 1987; Yao et al., 2009). Some reviews have also focused on treatment of conditions such as OA, fracture healing, and muscle pain by PES (Ebrahim et al., 2014; Negm et al., 2013). PES has also been used for plant and animal tissue drying processes and food preservation (Toepfl et al., 2006). Furthermore, high intensity PES can be used as a highly effective process for decontamination of liquid food (Sobrino-López and Martín-Belloso, 2010).

The application of PES, with varying parameters, may lead to significantly varied outcomes under different physiological conditions (depending on cell phenotypes, attachment, and extracellular environment), which limits wider biomedical usage. For example, PES with voltages ≥3 V and frequencies ≥130 Hz has been reported to ameliorate Parkinson’s disease, while PES with a frequency of 5 Hz significantly worsens akinesia (Moro et al., 2002). PES has been reported to prevent atrophy of long-term denervated muscles, resulting in increased muscle cross-sectional area (Ashley et al., 2007). On the other hand, PES (frequency of 20 Hz and durations longer than 2 ms) can also lead to atrophy of muscle fibers in rats after immediate sciatic nerve injury, with a decrease in muscle excitability (Gigo-Benato et al., 2010).

Various methods have been used to apply PES both in vitro and in clinical practice (Fig. 1) (Balint et al., 2013; Griffin and Bayat, 2011). The simplest way to apply PES is to arrange that electrodes are in direct contact with cells cultured in vitro (Fig. 1a). However, some by-products such as ROS could be detrimental. Capacitive coupling for in vitro experiments consists in subjecting cells in a Petri dish to two parallel layers of electrode plates (Fig. 1b). Such an approach can generate a homogenous electrical field, when compared with direct coupling. To achieve a high field strength, a gap cuvette (Fig. 1c) was developed for in vitro experiments due to its short gap width of 2 or 4 mm. Such a cuvette can be used in electroporation for cell suspension and field strengths can be in kV/cm range. However, it cannot be used for repeated electrical stimulation because cells need to be suspended. In clinical practice, electrodes could be placed at the defect sites invasively, with a power source nearby (Fig. 1d), or could be placed outside the skin (Fig. 1e).

Cartilage regeneration and OA pose a formidable healthcare challenge. Interestingly, the fact that the application of PES, with appropriate parameters, could enhance regeneration of cartilage and ameliorate OA may open the door to potential clinical applications (Haddad et al., 2007). However, knowledge on the physical and cellular mechanisms of the biological effects of PES is still limited (Schoenbach et al., 2007). To make sense of the multitude and complexity of data within the scientific literature relating to PES and the observed beneficial therapeutic effects, the underlying cellular and molecular mechanisms associated with the application of PES were rigorously delineated, with a focus on chondrocytes, chondrogenic differentiation, and cartilage as models.

Physical models of the effects of electrical stimulation on cells

Conformational changes to biomolecules induced by electric fields elicit further biological responses. Several physical models for electrical stimulation of living cells, using varying cell parameters and electric...
Table 1. Devices for PES approved by the FDA. HDE: humanitarian device exemption.

<table>
<thead>
<tr>
<th>Names of devices</th>
<th>Anatomical locations</th>
<th>Conditions</th>
<th>Parameters</th>
<th>Device class</th>
<th>Implanted device</th>
<th>Product code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bladder system</td>
<td>Urinary bladder</td>
<td>Urination</td>
<td>Frequency 7 or 9 MHz, Pulse duration 20-400 µs, Intensity 4.6 V</td>
<td>HDE</td>
<td>Yes</td>
<td>PAT</td>
</tr>
<tr>
<td>Cochlear implant with combined electrical stimulation and acoustic amplification</td>
<td>Ear</td>
<td>Hearing</td>
<td>Frequency 4-80 Hz, Pulse duration 40-1000 µs, Intensity 3 V</td>
<td>3</td>
<td>Yes</td>
<td>PGQ</td>
</tr>
<tr>
<td>Dorsal root ganglion stimulator for pain relief</td>
<td>Dorsal root ganglion</td>
<td>Pain</td>
<td>Frequency 150 Hz, Pulse duration 60/150/300 µs, Intensity 4.6 V</td>
<td>2</td>
<td>No</td>
<td>NTU</td>
</tr>
<tr>
<td>Intended to evaluate the functionality of human pain reception and transmission of sensory pathways</td>
<td>Wrist</td>
<td>Tremor symptom</td>
<td>Frequency 20 Hz, Pulse duration 60/150/300 µs, Intensity 3 V</td>
<td>3</td>
<td>Yes</td>
<td>PSR</td>
</tr>
<tr>
<td>Implanted phrenic nerve stimulator for central sleep apnea</td>
<td>Phrenic nerve</td>
<td>Central sleep apnea</td>
<td>Frequency 20 Hz, Pulse duration 60/150/300 µs, Intensity 3 V</td>
<td>3</td>
<td>Yes</td>
<td>PSR</td>
</tr>
<tr>
<td>External vagal nerve stimulator for headache</td>
<td>Neck</td>
<td>Headache</td>
<td>Frequency 5000 Hz, Pulse duration 30 ms, Intensity 24 V, 60 mA</td>
<td>2</td>
<td>No</td>
<td>PKR</td>
</tr>
<tr>
<td>Limited output transcutaneous piezoelectric stimulator for skin reactions associated with insect bites</td>
<td>Skin</td>
<td>Pain</td>
<td>Frequency 1-120 Hz, Pulse duration 340 µs, Intensity 0.7 V/cm</td>
<td>2</td>
<td>No</td>
<td>OSG</td>
</tr>
<tr>
<td>External pacemaker pulse generator</td>
<td>Right atrium and right ventricle</td>
<td>Heart disease</td>
<td>Frequency 130 Hz, Pulse duration 60 ms, Intensity 3 V</td>
<td>3</td>
<td>Yes</td>
<td>MHY</td>
</tr>
<tr>
<td>Stimulator, electrical, implanted, for essential tremor</td>
<td>Deep brain structures</td>
<td>Tremor</td>
<td>Frequency 100 Hz, Pulse duration 100 µs, Intensity 0.2-0.5 mA/cm²</td>
<td>2</td>
<td>No</td>
<td>OCF</td>
</tr>
<tr>
<td>Stimulator, electrical, implanted, for parkinsonian symptoms</td>
<td>Internal globus pallidus (gpi) or the subthalamic nucleus (STN)</td>
<td>Parkinson</td>
<td>Frequency 1-120 Hz, Pulse duration 340 µs, Intensity 0.7 V/cm</td>
<td>2</td>
<td>No</td>
<td>NGX</td>
</tr>
<tr>
<td>Transcutaneous electrical nerve stimulator for pain relief</td>
<td>Joint</td>
<td>Arthritis</td>
<td>Frequency 100-300 kHz, Pulse duration 0.7 V/cm</td>
<td>3</td>
<td>No</td>
<td>NZK</td>
</tr>
<tr>
<td>Transcutaneous electrical nerve stimulator for pain relief</td>
<td>Skin</td>
<td>Pain</td>
<td>Frequency 1-120 Hz, Pulse duration 340 µs, Intensity 0.7 V/cm</td>
<td>2</td>
<td>No</td>
<td>NGX</td>
</tr>
<tr>
<td>Stimulator, low electric field, tumor treatment</td>
<td>Head</td>
<td>Tumor</td>
<td>Frequency 1-100 Hz, Pulse duration 0.7 V/cm</td>
<td>3</td>
<td>No</td>
<td>PCC</td>
</tr>
<tr>
<td>Powered muscle stimulator</td>
<td>Skin</td>
<td>Muscle conditioning</td>
<td>Frequency 1-120 Hz, Pulse duration 340 µs, Intensity 0.7 V/cm</td>
<td>2</td>
<td>No</td>
<td>NGX</td>
</tr>
<tr>
<td>Transcutaneous electrical nerve stimulator to treat headache</td>
<td>Skin</td>
<td>Headache</td>
<td>Frequency 1-100 Hz, Pulse duration 0.7 V/cm</td>
<td>2</td>
<td>No</td>
<td>PCC</td>
</tr>
<tr>
<td>Transcutaneous electrical nerve stimulator for pain relief</td>
<td>Skin</td>
<td>Pain</td>
<td>Frequency 100 Hz, Pulse duration 100 µs, Intensity 0.7 V/cm</td>
<td>2</td>
<td>No</td>
<td>GZJ</td>
</tr>
</tbody>
</table>
fields, have been established. Physical models of membrane potential were established as early as 1953 when elliptical cells were exposed to direct electrical currents (Fricke, 1953), while the Schwan equation, based on models of spherical cells, represents one step forward by including extracellular fluid, plasma membrane, and cytoplasmic parameters (Schwan, 1956). The Schwan equation is as follows:

\[ \Delta \psi = 1.5 E_0 R \cos \phi \]

where \( R \) is the cell radius, \( \phi \) is the angle between the position vector and the applied electric field, \( E_0 \) is the field strength of the applied electric field, and \( \Delta \psi \) is the change in membrane potential.

The multilayer dielectric model of spherical cells lays the foundation for a deeper understanding of the biological effects of PES, which include physical parameters of cell electrical conductivity, relative permittivity, cellular radius, and membrane thickness, as well as cell various organelles and their individual membrane thickness. Based on the multilayer dielectric model of the spherical cell, several conclusions can be drawn (Yao et al., 2009):
1. membrane potentials are positively correlated with the field density of PES;
2. pores in the plasma membrane are formed within a transmembrane voltage range of about 0.5-1 V;
3. the effects of PES with a duration change of s to ps shift from plasma membranes to intracellular structures.

This, in turn, induces complex biological effects, such as activation or inhibition of signaling pathways, phosphorylation of proteins, manipulation of cell phenotypes, while longer durations or higher field strengths will induce apoptosis and necrosis (Weaver et al., 2012) (Fig. 2). The upper boundary of physical parameters is mainly limited by thermal effects and cell death, while the lower boundary is limited by biological effects. Although duration and field density are two key parameters, based on these models and corresponding equations, frequency and timing of electrical stimulation are also critical due to their effect on the action area of PES (Weinberg, 2013). PES with a high frequency (10^4-10^9 Hz) would affect the inner membranes, while PES with a low frequency (< 10^8 Hz) may affect the plasma membrane (Yao et al., 2009).

Field strength, duration, frequency, and time of stimulation are fundamental physical characteristics of PES. Field strength is the intensity of PES and it may range from mV/cm, V/cm, kV/cm to MV/cm for different types of application and studies. Duration of PES ranges from ps, ns, μs, ms to s. Long duration plus high voltage would cause thermal effects. Frequency is an important parameter for PES, as PES with a high frequency (above 10 kHz) could affect intracellular organelles. The time span of stimulation is also important, which may be minutes to hours daily. Repeated stimulation would cause significantly different effects due to cumulative effects. For example, a pulse-number-dependent downregulation of mitochondrial function and cell numbers was observed by Hall et al. (2007) for nsPEFs. 3 d of PES would not cause significant cell death, while 7 d of PES would cause significant cell death (Kwon et al., 2016). The formation of pores caused by PES increases due to the increase in pulse number (Yogesh, 2016). On the other hand, PES with a low field strength may require several hours daily to produce significant biological effects (Table 2).

The aforementioned physical model only works well when all individual cells have exactly the same physical properties, as well as disperse uniformly in the cell suspension (Yao et al., 2009). However, distribution of the electric field on individual cells is not even, as cell suspension may not have optimal homogeneity while tissues are absolutely non-homogenous. The effects of PES are also dependent on cell attachment, as well as changes in cell morphology (Casciola et al., 2017). The distribution of PES is difficult to measure, but could be simulated with commercially available software, such as HFSS.
and reduces conductance of $K^+$ channels, which may be due to conformational changes causing supra-physiological voltage in the membrane proteins of channel-gating systems (Chen, 2004). Furthermore, PES could denature voltage-sensitive membrane proteins with charged groups in some of their component amino acids which are sensitive to the plasma membrane potential (Chen, 2005). This may be due to breakage of chemical bonds between amino acids in the voltage-dependent membrane proteins, thus reducing their ability to open potassium ion channels simultaneously. From another point of view, nsPEFs inhibit voltage-gated $Ca^{2+}$ and $Na^+$ channels with reduction of the transmembrane ion gradient due to an increase in non-inactivating “leak” current (Nesin et al., 2012). Channel subtypes should also be considered, as voltage-gated potassium channels respond to electrical stimulation variably, depending on channel kinetics (Cameron et al., 2017). Reduction in the opening process of ion channels is less reversible in comparison to the process of inducing reversible pores by electroporation, which reclose within $\mu$s or ms. This mechanism is clinically significant as it helps illustrate how injuries caused by electric shock often show no signs of tissue damage but still result in malfunction (Chen, 2005). The presence of naturally occurring proteins with magnetic response hint at a possibility that there may be more proteins that have intrinsic capacity to respond to electrical stimulation (Qin et al., 2016).

Membrane

PES with varying durations has selective effects on either the plasma membrane or intracellular membranes. In terms of duration of pulses, PES is classified into several groups, including $\mu$s, sub-$\mu$s, ns, and ps (Yao et al., 2009). Living cells respond to PES variably since they are conceptualized as a combination of capacitors and resistors with

ANSYS, release 15.0 (ANSYS, Canonsburg, PA, USA) and COMSOL Multi-physics (COMSOL, Burlington, VT, USA) (Buyong et al., 2015; Casciola et al., 2017).

**Effects of electrical stimulation on cells and their microenvironment**

The effects of PES on different cellular components have been explored extensively, including VSDs, plasma membrane, intracellular components, and signaling pathways, which are often interwoven. PES induces conformational changes of voltage-sensitive proteins, pore formation in the membrane, and calcium influx within seconds of its application, which, in turn, leads to activation of signaling pathways, cell migration, and cell death within minutes or hours.

**VSDs**

Conformational changes of proteins are early stage key biological effects of PES which are still unexplored. VSDs, key components of voltage-gated channels, usually are comprised of four transmembrane helices (S1-S4) with a pore domain (helices S5 and S6). The S4 segment of voltage-gated channels with the motif [RK]-X(2)-R-X(2)-R-X(2)-[RK] is sensitive to electric fields (Murata et al., 2005). The basic residues of S4 segment are rapidly changed from hyperpolarized potentials, with an internally connected aqueous crevice, to depolarized potentials, with an externally connected aqueous crevice (Zhang et al., 2012). Electrical stimulation induces high levels of intracellular $Na^+$ ions by the activation of Na/K ATPase, which is responsible for at least 35 % of the voltage-induced pores in the plasma membrane (Teissie and Tsong, 1980). However, electrical stimulation with a high intensity induces a negative-shift of channel open-threshold and reduces conductance of $K^+$ channels, which may be due to conformational changes causing supra-physiological voltage in the membrane proteins of channel-gating systems (Chen, 2004). Furthermore, PES could denature voltage-sensitive membrane proteins with charged groups in some of their component amino acids which are sensitive to the plasma membrane potential (Chen, 2005). This may be due to breakage of chemical bonds between amino acids in the voltage-dependent membrane proteins, thus reducing their ability to open potassium ion channels simultaneously. From another point of view, nsPEFs inhibit voltage-gated $Ca^{2+}$ and $Na^+$ channels with reduction of the transmembrane ion gradient due to an increase in non-inactivating “leak” current (Nesin et al., 2012). Channel subtypes should also be considered, as voltage-gated potassium channels respond to electrical stimulation variably, depending on channel kinetics (Cameron et al., 2017). Reduction in the opening process of ion channels is less reversible in comparison to the process of inducing reversible pores by electroporation, which reclose within $\mu$s or ms. This mechanism is clinically significant as it helps illustrate how injuries caused by electric shock often show no signs of tissue damage but still result in malfunction (Chen, 2005). The presence of naturally occurring proteins with magnetic response hint at a possibility that there may be more proteins that have intrinsic capacity to respond to electrical stimulation (Qin et al., 2016).
multilayer dielectric models (Schoenbach et al., 2007; Yao et al., 2009). Charging time constant is the time required to charge a plasma membrane (Deng et al., 2003). The equations for charging time (τ) of the outer membrane and the inner membrane are as follows (Yao et al., 2009). According to the multilayer dielectric model of a spherical cell, the constant τ_{cell} of the outer membrane (plasma membrane) can be expressed as

\[
\tau_{\text{cell}} = \frac{1}{2\gamma_c} + \frac{1}{\gamma_{\text{nc}}} \frac{e_i e_m}{d_m} R_c
\]

The constant τ_{nuc} of the inner membrane (organelle membranes) can be expressed as

\[
\tau_{\text{nuc}} = \frac{1}{2\gamma_c} + \frac{1}{\gamma_{\text{nuc}}} \frac{e_i e_m}{d_n} R_n
\]

\(\gamma_c, \gamma_{\text{nc}}\) and \(\gamma_{\text{nuc}}\) are the conductivities of extracellular medium, cytoplasm and organelle cytoplasm, respectively. \(e_i, e_m\) and \(e_{\text{nm}}\) are the permittivities of vacuum, plasma membrane and organelle membrane, respectively. \(d_m\) and \(d_n\) denotes the thicknesses of the cell and organelle membranes, respectively. \(R_c\) and \(R_n\) are the radii of the cell and organelle, respectively. According to the above equations, the transmembrane potential of the membrane is as follows. At the end of the pulse, the outer membrane transmembrane potential is

\[
V_m = 1.5R_c E \left( 1 - e^{-\frac{\tau}{\tau_{\text{cell}}}} \right) \cos \theta
\]

and the inner membrane transmembrane potential is

\[
V_n = \frac{1.5 \tau_{\text{cell}} R_n E}{\tau_{\text{cell}} - \tau_{\text{nuc}}^2} \left( e^{-\frac{\tau}{\tau_{\text{cell}}} - e^{-\frac{\tau}{\tau_{\text{nuc}}}}} \right) \cos \theta
\]

E is the field intensity of the external electric field. θ is the polar angle measured with respect to the direction of the field. τ is the duration of the electric fields. Voltage of the plasma and organelle membranes are affected by the relationship of the size of \(\tau, \tau_{\text{cell}}\) and \(\tau_{\text{nuc}}, \tau_{\text{cell}}\) is in the range of hundreds of ns, while \(\tau_{\text{nuc}}\) is in the range of about tens of ns. τ may be different for different cells, because different cells in different environment have different physiological parameters. If τ is much larger than \(\tau_{\text{cell}}\), then the outer membrane can be fully charged by electric fields. If τ is much smaller than \(\tau_{\text{cell}}\), then the outer membrane is poorly charged by electric fields. That is the same for the inner membrane. \(\tau_{\text{cell}}\) is in the range of hundreds of ns for most cells and \(\tau_{\text{nuc}}\) is in the range of tens of ns for most cells.

Based on the charge time of the plasma membrane, PES with durations of μs or longer, mainly exert effects on the cell outer membrane, while PES with a sub-μs duration exert effects on both the inner and outer membrane with a sufficient PES intensity (at the kV/cm level) (Yao et al., 2009). nsPEFs with intensities up to 300 kV/cm have better penetrative capacity and mainly affect the inner cell membrane (Chopinet and Rols, 2015; Schoenbach, 2018). Theoretically, electrical stimulation with even shorter durations of ps could be endowed with even higher voltages. Ps-pulsed electrical stimulation with an intensity lower than 1 kV/cm hardly affects the plasma membrane (Yao et al., 2009). The intensity of electric fields is limited to less than 1 kV/cm, while the duration ranges from μs to ms (Zimmermann, 1986). Relatively short durations and high field strengths (hundreds kV/cm) can potentially affect the intracellular membrane while restraining total energy and narrowing down the previously broad biological effects. In any case, precise control of PES is essential for various potential clinical applications and more attention should be paid to nsPEFs.

PES has the unique ability to induce reversible pore formation in plasma membranes within seconds.

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**Table 2. Effects of PES on chondrocytes.**

<table>
<thead>
<tr>
<th>Model</th>
<th>Electric parameter</th>
<th>Results</th>
<th>References</th>
</tr>
</thead>
</table>
| Fetal bovine articular chondrocytes | Frequency: 60 Hz
Time: 0.5 h
Intensity: 20 mV/cm | COLII and AGG ↑
PG and collagen content ↑ | (Wang et al., 2004) |
| Adult bovine articular chondrocytes | Frequency: 60 kHz
Intensity: 20 mV/cm
Time: 4, 6 or 22 h | COLII and AGG ↑ | (Xu et al., 2009) |
| Pig chondrocytes               | Frequency: 1 Hz
Pulse: 5; pulse duration: 100 ns
Intensity: 10 kV/cm or 20 kV/cm | Dedifferentiation
COLI ↑, AGG ↓, COLII ↓, and SOX9 ↓ | (Zhang et al., 2014) |
| Human chondrocytes             | Frequency: 4150 Hz
Time: 30 min
Intensity: 0.1 to 10 mV/cm
Pulse duration: 10 ms | Proliferation ↑ | (Fitzsimmons et al., 2008) |
| Human chondrocytes             | Frequency: 10 Hz
Current: 10 µA
Time: 3 or 6 h | Chondrocyte adhesion ↑
Long-term cell densities ↑ | (Khang et al., 2008) |
| Bovine articular chondrocytes  | Frequency: 60 kHz
Intensity: 0-45 mV/cm
Time: 24 h | 15-30 mV/cm: proliferation ↑
45 mV/cm: proliferation ↓ and proteoglycan ↑ | (Armstrong et al., 1988) |
including the outer plasma membrane, as well as intracellular organelles, such as mitochondria, endoplasmic reticulum, and nuclear membranes. PES induces changes in membranes immediately. Changes are reversible under PES with a low voltage and short duration, while irreversible under PES with a high voltage and long duration (Pakhomov et al., 2015). Pores that form on plasma membranes are temporary and reversible when an electric field strength of about 0.1-20 kV/cm and pulse durations of 10 ns-10 ms are applied (Kotnik et al., 2015). These temporary pores have been widely used in biomedical applications, including delivery of biomolecules, such as DNA, RNA, and proteins – commonly referred to as electroporation or electrofusion (Cemazar et al., 2013; Schuehhammer et al., 2014) – as well as drugs (Okino and Mohri, 1987). The voltage applied in electroporation can range between 200 V and 350 V, with 260 V being the most widely used (Nature Methods, 2006). The electroporation technique is based on electroporation at 300 V pulse of the plasma membrane and is mediated by PEG (Hui and Stenger, 1993; Yu et al., 2008). The effects of electroporation on different cell types, cell sizes, and intensity of electric field have been studied. Traditionally, the duration of electric fields used for electroporation range between 10 and 100 μs (Jordan et al., 2013; Rems et al., 2013), while nsPEFs increase efficiency of cell electroporation through electroporation of the contact areas between cells (Rems et al., 2014). Size of pores created by nsPEFs on the membrane are smaller than that caused by PES with longer durations (Vasilkoski et al., 2006).

Intracellular components and organelles
PES affects intracellular components and organelles, such as the cytoskeleton. PES increases speed of cell migration of both inner and outer meniscus cells (Yuan et al., 2014) and ROS levels in both extracellular (electrochemical) and intracellular compartments (Pakhomova et al., 2012). PES [10 ns, high voltages (> 150 kV/cm)] disrupts pre-messenger RNAs by inducing nuclear speckles of snRNPs (Chen et al., 2007). PES with a low field strength (2.5 V/cm) accelerates cell migration within hours (Hayashi et al., 2016). PES with a duration of ns can induce disassembly of actin structures and cell swelling resulting from cell permeabilization (Pakhomov et al., 2014). Whereas, PES effects on ribosomes remain unexplored.

PES can induce apoptosis through dysfunctioning mitochondria. PES with a high field strength (kV/cm) and a short duration of ns range decreases mitochondria membrane potential (Beebe et al., 2012). The loss of mitochondria membrane potential may be a consequence of increased inner mitochondrial membrane permeability (Batista Napotnik et al., 2012). PES with a duration of ps can induce the release of cytochrome C from mitochondria, with activation of caspase 3 and 9, resulting in apoptosis (Hua et al., 2012).

PES also affects epigenetics through acetylation, histone modifications, alteration of the structural organization of chromatin, demethylation, as well as affecting RNA. PES downregulates histone deacetylase activities in HL-1 cells, with a significant reduction in Cx43 expression and cell-cell communication (Meraviglia et al., 2015). PES can also reversibly downregulate the activity of histone 1 kinase in pig oocytes (Leal and Liu, 1998). PES can prevent the tumorigenic transition of embryonic stem cells through downregulation of phospho-H3 and Ki67 immuno-reactivity (Yamada et al., 2007). PES promotes global DNA demethylation and downregulates DNMT1 expression (unpublished data).

Signaling pathways
PES exerts effects on cells through activation or inhibition of various signaling pathways (Liu and Song, 2014). PES activates protein kinase C signaling pathway by inducing PI2 hydrolysis (Tolstykh et al., 2013). PES inhibits VEGF signaling pathway via downregulation of VEGF and PD-ECGF, thereby disrupting pro-angiogenic and anti-angiogenic balance, which may consequently inhibit cancer development and suppress tumor blood vessel growth (Chen et al., 2007). PES activates MAPK signaling pathway. PES not only upregulates phosphorylation of ERK and p38 (Zhao et al., 2006), but can also activate JNK, another member of the MAPK family, with fast increased phosphorylation level within minutes, which then decreases within 1 h (Morotomi-Yano et al., 2011a; 2011b). As the signaling pathways activated by PES may become inactivated within minutes or hours, the effect caused by PES may last for minutes, hours, or days. PES-preconditioning promotes cartilage regeneration within weeks through enhancing differentiation of MSCs (Ning et al., 2019). This is the consequence of the biological effects initiated by PES during the early stage after stimulation. Meanwhile, phosphorylation of MAPK caused by PES is reversible (Morotomi-Yano et al., 2011a). PES can also induce dedifferentiation of chondrocytes through activation of Wnt/β-catenin signaling pathway (Zhang et al., 2014). Activation of β-catenin signaling pathway enhances NEUROG2 expression in brain injury after PES treatment (Matsumoto et al., 2013) (Fig. 3).

PES exerts effects on calcium-related signaling pathways through the release of Ca2+ ions from endoplasmic reticulum compartments (Semenov et al., 2013; Vernier et al., 2003). PES with high field strength can induce calcium release within seconds (Zhang et al., 2014). PES has been reported to induce release of Ca2+ leading to an increase in cytoskeletal CaM levels (Brighton et al., 2001). PES can also induce calcium influx from the external medium (Bourguignon et al., 1989). PES enhances proliferation of cardiac fibroblast and myocardial fibrosis through activation of the CaN-NFAT pathway, regulated by Ca2+/CaM (Chen et al., 2012). PES increases synthesis
of NO and production of cGMP through calcium/CaM and inhibits Ca^2+/CaM, thereby blocking these effects (Fitzsimmons et al., 2008).

PES can also regulate the process of apoptosis and its associated biochemical markers. PES activates caspase-dependent signaling pathways in apoptosis, with the activation of caspase-12, -9, and -3, leading to release of cytochrome c, upregulation of Bax and downregulation of Bcl-2 in HeLa cells (Chen et al., 2013).

Physiological microenvironment

PES can alter blood flow, temperature, and pH of tissues. Blood flow is significantly increased following application of electrical stimulation in wound healing (Ud-Din et al., 2015). Increased blood flow following increasing capillary density is observed when the transcutaneous oxygen partial pressure is increased by 82.4% during treatment of CVI (Jünger et al., 1997). Thermal effects are integral in traditional PES, but on the other hand, thermal effects are dependent on tissue properties as well as stimulation parameters (i.e., frequency, pulse duration, field strength) (Schoenbach et al., 2004). Electrical stimulation increases temperature around the electrodes in the visual prosthesis system (Celik and Karagöz, 2014). Temperature is increased around the electrodes (both anode and cathode) and the pH value varied from 3 (near the anode) to 12 (near the cathode) in the electrode array for local control of solid tumors (Soba et al., 2018). Low-voltage electrical stimulation can promote muscle tenderness and accelerate glycolysis, with a significant fall in pH during the first 6 h post mortem (Polidori et al., 1999).

PES has been reported to cause deleterious effects on cells, including cell death and cell fragmentation (Yang et al., 2018). PES with high field strength and long duration can result in thermal effects due to Joule heating (Yarmush et al., 2014), with consequent cell death, as proteins denature when the temperature reaches about 43-45 °C. Also, nsPESs with high field strength lead to cell death within minutes, which is mainly due to cell fragmentation (Ning et al., 2019). PES with high field strength and long duration can also induce cell death, following both necrotic and apoptotic ways (Pakhomova et al., 2013).

Effects of PES on chondrocytes, mesenchymal stem cells, cartilage explants, and OA

PES promotes cartilage regeneration and ameliorate OA through modulation of extracellular matrix and various biological factors, such as BMPs, TGF-β, and IGF2, which in turn induce anti-inflammatory and anabolic effects to improve articular cartilage regeneration together with amelioration of inflammation (Haddad et al., 2007; Massari et al., 2007). Cell-based repair strategies induce cartilage regeneration (Johnstone et al., 2013). Chondrocytes and MSCs (or their chondrogenic progenies) are important cell sources (de Vries-van Melle et al., 2014; Estes et al., 2010; Guilik et al., 2010). Both ADSCs and BMSCs have demonstrated their unique responses to growth-factor-induced chondrogenic differentiation, respectively to TGF-β3 and BMP6 (Diekman et al., 2009). However, there are obstacles to using primary chondrocytes for cell culture applications, including limited cell numbers and dedifferentiated phenotypes during in vitro expansion (Chung and Burdick, 2008). Challenges faced in culturing bone-marrow-derived MSCs or ADSCs in vitro include low efficiency of chondrogenic differentiation and heterogeneous phenotypes (Perdisa et al., 2015; Somoza et al., 2014), while proper application of PES could potentially overcome these obstacles.

Chondrocytes

PES can induce varied or even opposite effects on chondrocytes, including increased proliferation and increased or decreased differentiation (represented as expression of CollI or ratio of CollII/Coll and GAG production). PES for 24 h at 15-30 mV/cm increases proliferation of primary chondrocytes harvested...
from growth plate cartilage, with upregulated \(^{3}H\) thymidine uptake, and enhances proteoglycan deposition, with upregulated \(^{35}S\) sulfate uptake, while PES for 24 h at 45 mV/cm inhibits proliferation and enhances proteoglycan deposition (Armstrong et al., 1988). PES (0.5 h, 20 mV/cm, 60 Hz) can increase COLII and AGG expression in fetal bovine chondrocytes in a micromass culture system (Wang et al., 2004). PES (4, 6 or 22 h, 60 kHz, 20 mV/cm) induces up-regulation of both COLII and AGG expression levels through extracellular Ca\(^{2+}\) influx through voltage-gated calcium channels rather than intracellular Ca\(^{2+}\) repositories (Xu et al., 2009). PES (3 or 6 h, 10 Hz, 10 µA) enhances chondrocyte adhesion and long-term cell densities for up to 2 d (Khang et al., 2008). On the other hand, nsPEFs (5 pulses, 1 Hz, 100 ns at 10 or 20 kV/cm) decreases gene expression levels of COLII, AGG and SOX9 through activation of Wnt/β-catenin signaling pathway and intracellular calcium ion efflux (Zhang et al., 2014) (Table 2).

This obvious inconsistency could be attributed to either varying parameters of PES or cell types with ever-changing intra- and inter-cellular structures. Generally, PES with low electric field strength and long duration promotes proliferation with effects mainly on calcium homeostasis, cell skeleton, or membrane, while PES with high electric field strength affects intracellular structures or more specific targets.

### Chondrogenic differentiation of MSCs

PES has been optimized to promote chondrogenic differentiation of MSCs (Table 4). Piezoelectric scaffolds, exhibiting low voltage output, enhance chondrogenic differentiation of MSCs with upregulation of intense proteoglycan staining and COLII, while piezoelectric scaffolds with high voltage output enhance osteogenic differentiation (Damaraju et al., 2017). This emphasizes the importance of PES parameters in chondrogenic differentiation. PES (20 min daily for 7 d, 20 mV/cm, 60 kHz), with or without TGF-β3, promotes chondrogenic differentiation, with enhanced COLII and SOX9 expression levels (Esfandiari et al., 2014). PES (20 min, 1 kHz, 20 mV/cm) leads to chondrogenic differentiation of ADSCs (Mardani et al., 2016). TGF-β, BMPs and other signaling pathways have been implicated in PES treatment. PES (1, 5 or 25 V/cm, 5 Hz) promotes chondrogenic differentiation via upregulated SOX9, COLII, and AGG, with TGF-β signaling, BMP signaling and extracellular ATP signaling via P2X\(_{7}\) receptors (Kwon et al., 2016). Electrical stimulation induces calcium oscillations through modulation of VOCCs, which are implicated in chondrogenesis (Uzieliene et al., 2018). PES (n-scale with high intensity) enhances chondrogenic differentiation of MSCs with significantly upregulated expression levels of SOX9, COLII, and AGG, together with down-regulated expression of COLX through activation of c-Jun and Stat3 signaling pathways (Ning et al., 2019). ns-PEFs promote chondrogenic differentiation of MSCs through activation of MAPK signaling pathways (Morotomi-Yano et al., 2011a).

PES can promote chondrogenic differentiation through upregulation of related growth factors. PES (50 and 100 mV/mm) also increases FGF2 secretion and upregulates phosphorylation levels of Smad2 and Smad3 in fibroblasts (Wang et al., 2017). PES (50 mV/mm) upregulates the secretion of FGF1 and FGF2 in fibroblasts (Rouabhia et al., 2013). PES (5 V, 0.1 ms, 10 min) together with a heat shock can activate insulin signaling through phosphorylation of IRS (Morino et al., 2008). However, disruption of the secondary structure of insulin is observed at higher electric field strengths (above 0.25 V/mm) within 1 µs (Wang et al., 2014) and the binding capacity of insulin to receptors is significantly reduced by long PES duration (0.7 V/m, 50 Hz, 20 min) (Li et al., 2005). So, whether insulin is involved in chondrogenic differentiation of MSCs by PES remains to be explored. Perhaps low intensity PES can activate insulin signaling pathway to promote chondrogenic differentiation while high intensity PES can not.

### Cartilage explants and OA

PES can increase matrix production and reduce matrix destruction in cartilage explants. PES (60 kHz, 20 mV/cm) increases production of proteoglycan and collagen in the absence or presence of IL-1β, while dramatically inhibiting expression of matrix

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### Table 3. Effects of PES on cartilage explants.

<table>
<thead>
<tr>
<th>Model</th>
<th>Other factors</th>
<th>Electrical parameters</th>
<th>Results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf cartilage disks</td>
<td></td>
<td>Frequency: 1, 10, 100, 100(^{3}), and 10(^4) Hz, Intensity: 10-30 mA/cm(^2)</td>
<td>Protein synthesis ↑</td>
<td>(Macginitie et al., 1994)</td>
</tr>
<tr>
<td>Bovine articular cartilage explants</td>
<td></td>
<td>Frequency: 60 Hz, Intensity: 20 mV/cm, Time: 1 h on and 5 h off, 4 times/d for 3 d</td>
<td>Production of proteoglycan and collagen ↑, AGG and COLII ↑</td>
<td>(Brighton et al., 2006)</td>
</tr>
<tr>
<td>Human osteoarthritic cartilage explants</td>
<td>With or without IL-1β</td>
<td>Frequency: 60 kHz, Intensity: 20 mV/cm, Time: 1 h on and 5 h off, 4 times/d for 3 d</td>
<td>With IL-1β: production of proteoglycan and collagen ↑, AGG and COLII ↑</td>
<td>(Brighton et al., 2008)</td>
</tr>
</tbody>
</table>
mechanisms in the observed effects of electrical stimulation on OA need to be further explored to optimize the treatment, such as thermal effects, cell migration, blood flow etc. Perhaps PES with a low frequency (≤ 100 Hz) is able to improve physical function but not pain intensity (Negm et al., 2013), which calls for mechanistic exploration and studies using PES with high frequency and short duration or other parameters to avoid harmful changes.

### Perspectives

Most research studies on PES were stalled after identification of specific signaling pathways involved, without elucidating the initiator molecule or initial conformational changes of biomolecules. Some studies have moved forward to identifying a complex array of signaling pathways involved, with the biological effects observed being just the outcome of multiple well-orchestrated processes. A comprehensive overview of biological mechanisms, as well as the corresponding physical stimuli involved, are needed to enable more extensive clinical applications by using novel toolkits. The target may be a protein subunit or some other molecular subunit that requires identification by high-throughput assays.

Integration with novel technology platforms is necessary to facilitate clinical applications of PES, i.e. high-throughput screening, microfluidics platforms, single-cell analysis, and bioinformatics. Novel technologies such as nsPEFs, which could precisely trigger specific activities within the cell and organelle internally, should be further investigated. PES with different parameters induces variable effects on different organelles, which in turn elicit variable biological effects on cells, tissues, and organs. The specific parameters of PES will depend on the clinical conditions of individual patients, different tissue states, and cell-types. Therefore, utilizing PES with appropriate parameters, such as specific stimulation frequencies and specific durations, must be optimized and standardized by high-throughput assays and large-scale screening platforms.

### Table 4. Effects of PES on the chondrogenic differentiation of MSCs.

<table>
<thead>
<tr>
<th>Model</th>
<th>Electrical parameter</th>
<th>Results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Piezoelectric scaffolds Human MSCs</td>
<td>Frequency: 60 KHz Intensity: 20 mV/cm Time: 20 min daily for 7 d</td>
<td>COLII and SOX9 ↑</td>
<td>(Esfandiari et al., 2014)</td>
</tr>
<tr>
<td>Human ADSCs</td>
<td>Frequency: 1 kHz Intensity: 20 mV/cm Time: 20 min</td>
<td>COLII and SOX9 ↑</td>
<td>(Mardani et al., 2016)</td>
</tr>
<tr>
<td>ADSCs</td>
<td>Frequency: 5 Hz Intensity: 1, 5 or 25 V/cm</td>
<td>COLII and SOX9 ↑</td>
<td>(Kwon et al., 2016)</td>
</tr>
<tr>
<td>Mouse MSCs</td>
<td>Frequency: 1 Hz Intensity: 10 kV/cm, 20 kV/cm Duration: 10 ns, 100 ns</td>
<td>COLII and SOX9 ↑</td>
<td>(Ning et al., 2019)</td>
</tr>
<tr>
<td>Pig MSCs</td>
<td>Frequency: 1 Hz Intensity: 20 mV/mm</td>
<td>COLII and SOX9 ↑</td>
<td>(Damaraju et al., 2017)</td>
</tr>
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</table>

metalloproteinase in full-thickness osteoarthritic adult human articular cartilage explants (Brighton et al., 2008). Brighton et al. (2006) observed that PES (60 KHz, 20 mV/cm) increases collagen production and expression levels of COLII and AGG within adult bovine articular cartilage explants (Table 3).

PES used for neuromuscular stimulation increased muscle thickness and pennation angle in 45 women (age 66-75 years) with knee OA and it resulted in a significant increase in functional capacity, as measured by the 6 min walk test and Timed Up and Go Test (Melo Mde et al., 2015). PES resulted in high global effectiveness of OA treatment in two patients (Fary et al., 2009). However, the third patient exhibited no change, emphasizing the importance of heterogeneity. PES resulted in a significant downregulation of various OA symptoms in hands, with respect to pain, swelling, grip strength, and pinch force (Holt et al., 2018).

The effects of PES on OA pain relief remains to be explored. Neuromuscular electrical stimulation reduces pain (de Oliveira Melo et al., 2016). PES can alleviate pain in OA. PES (at least 6 h/d for 3 months) significantly reduced pain in patients with knee OA in clinical trials (Garland et al., 2007). Although PES could improve physical functions in subjects with knee OA, it may not reduce pain. PES attenuated knee OA symptoms in a dose-response manner after 750 h in a clinical trial with 288 patients who had failed nonsurgical therapy (Farr et al., 2006). However, contrary results were also reported in which PES could not provide symptomatic relief for OA (Fary et al., 2008; 2011). This difference may be due to limitations of the sample size and heterogeneity, such as age or BMI. Maybe the type of electrical stimulation could change the effects of PES on pain relief. Among electrical stimulation therapy, from h-TENS, l-TENS, NMES, IFC, PES to NIN, IFC seems to be the best treatment modality in terms of effectiveness, as compared with PES and other electrical stimulation modalities (Zeng et al., 2015). Amplitude-modulated frequency generated by IFC can permeate more deeply, which could improve pain relief, as the main analgesic component of IFC (Johnson et al., 2003).
comprehensive biological mechanistic studies, to enable PES technology to be applied clinically for enhancing cartilage repair and regeneration. The diameter of chondrocytes is about 20 µm, while that of MSCs is 10-15 µm. This means that higher field strengths are required for chondrocyte than MSCs. Lower field strength (mv/cm), moderate duration (μs-ms), and hours-long stimulation would be preferred for cartilage repair and chondrogenic differentiation of MSCs, as ns-pulsed electric fields with high field strength may cause dedifferentiation of chondrocytes (Table 2) (Zhang et al., 2014).

Wnt/β-catenin, TGF-β, MAPK, and FGF signaling pathways are critical for cartilage repair and chondrogenic differentiation of MSCs (Tang et al., 2015) (Fig. 3). TGF-β/Smad signaling pathway is activated by PES during chondrogenesis of MSCs (Kwon et al., 2016). Wnt/β-catenin is activated in primary chondrocytes by nsPESs (Zhang et al., 2014). MAPK signaling pathway is activated in enhanced chondrogenic differentiation of MSCs induced by nsPEFs (Ning et al., 2019). PES (50 mV/mm) promotes secretion of FGF-1 and FGF-2 in fibroblasts (Rouabhia et al., 2013). TGF-β and FGF signaling pathways need to be further explored during chondrogenic differentiation of MSCs induced by PES, particularly for upstream and downstream signaling pathways. Possible signaling pathways need to be explored (Fig. 4).

Acknowledgement

Authors would like to thank Rossana Rico for revision of the manuscript and Prof Jue Zhang and Jinsong Guo for constructive suggestions. This work was supported by the China National Natural Science Foundation (81772334).

No competing financial interests exist.

There are no ethical issues pertaining to human and animal rights.

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**Editor’s note:** All comments/questions by the reviewers were answered by making changes in the text. Hence, there is no Discussion with Reviewers section.

The Scientific Editor responsible for this paper was Christine Hartmann.