Capacitively Coupled Electrical Stimulation of Rat Chondroepiphysis Explants: A Histomorphometric Analysis

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Abstract

The growth plate is a cartilaginous layer present from the gestation period until the end of puberty where it ossifies joining diaphysis and epiphysis. During this period several endocrine, autocrine, and paracrine processes within the growth plate are carried out by chondrocytes; therefore, a disruption in cellular functions may lead to pathologies affecting bone development. It is known that electric fields impact the growth plate; however, parameters such as stimulation time and electric field intensity are not well documented. Accordingly, this study presents a histomorphometrical framework to assess the effect of electric fields on chondroepiphysis explants. Bones were stimulated with 3.5 and 7 mV/cm, and for each electric field two exposure times were tested for 30 days (30 minutes and 1 hour). Results evidenced that electric fields increased the hypertrophic zones compared with controls. In addition, a stimulation of 3.5 mV/cm applied for 1 hour preserved the columnar cell density and its orientation. Moreover, a pre-hypertrophy differentiation in the center of the chondroepiphysis was observed when explants were stimulated during 1 hour with both electric fields. These findings allow the understanding of the effect of electrical stimulation over growth plate organization and how the stimulation modifies chondrocytes morphophysiology.

Keywords: Growth plate; Electric fields; Columnar organization; Hypertrophy; Histomorphometric.

1. Introduction

The growth plate also known as epiphyseal plate is a cartilaginous tissue located between the epiphysis and the diaphysis, and it is responsible for the longitudinal growth and shape of long bones [1], [2]. Given its location, the epiphyseal plate is exposed to external stimuli that generate different signals involved in the physiological regulation of cell behavior and various pathological problems. The response of chondrocytes to biophysical stimuli results in the synthesis of the molecules that compose the extracellular matrix (ECM) of the growth plate, such as proteins (collagen type II and X, Ihh, BMP, WNT), proteoglycans (glycosaminoglycans (GAGs), aggrecan) and growth factors (VEGF, TGFβ, PTHrP) [3], [4]. Histologically, the growth plate is stratified in different zones according to the phenotype of the chondrocytes and the spatial arrangement of the ECM. Consequently, the growth plate is organized into four zones: reserve, proliferative (P-z), pre-hypertrophic (PH-z) and hypertrophic (Ht-z) [5]. Other morphological characteristics of the growth plate are the shape and width that change according to the species, the type of bone and age. For example, it has been shown that the morphology of the growth plate in the proximal femoral epiphysis of humans is concave at the age of four years, straight at the age of seven and convex at the beginning of the puberty [6]. Regarding the width of the growth plate, the physis is wider in early stages of development; while at the end of the adolescence the thickness of the physis decreases progressively, until it is completely reabsorbed by connecting the epiphysis and the metaphysis in a process known as epiphyseal fusion [3]. On the contrary, the growth plates in mammals vary according to the location within the bone. For example, the epiphyseal plate in the proximal femur is concave in the postnatal stages, while in the distal femur it has an irregular morphology. This architecture tends to be constant during bone growth; nevertheless, some changes can be observed 21 days after birth when the growth plate becomes thicker and the distal physis acquires a wavy morphology. Unlike the growth plate in humans, where complete ossification occurs once sexual maturity is reached, the epiphyseal plate is preserved throughout life in rodents [7].

The growth plate is an avascular, aneural and alinfatic tissue that has limited self-healing properties due to its poor vascularization, reduced migration and mitogenic characteristics of chondrocytes [8], [9]. Therefore, a damage in the ECM and/or chondrocyte death may result in dysfunction of the epiphyseal plate and subsequent defects in bone development [10], [11]. Considering that growth plate responds to external biophysical stimuli, studies have shown that compressive mechanical loading induces to histological changes at cellular level within the

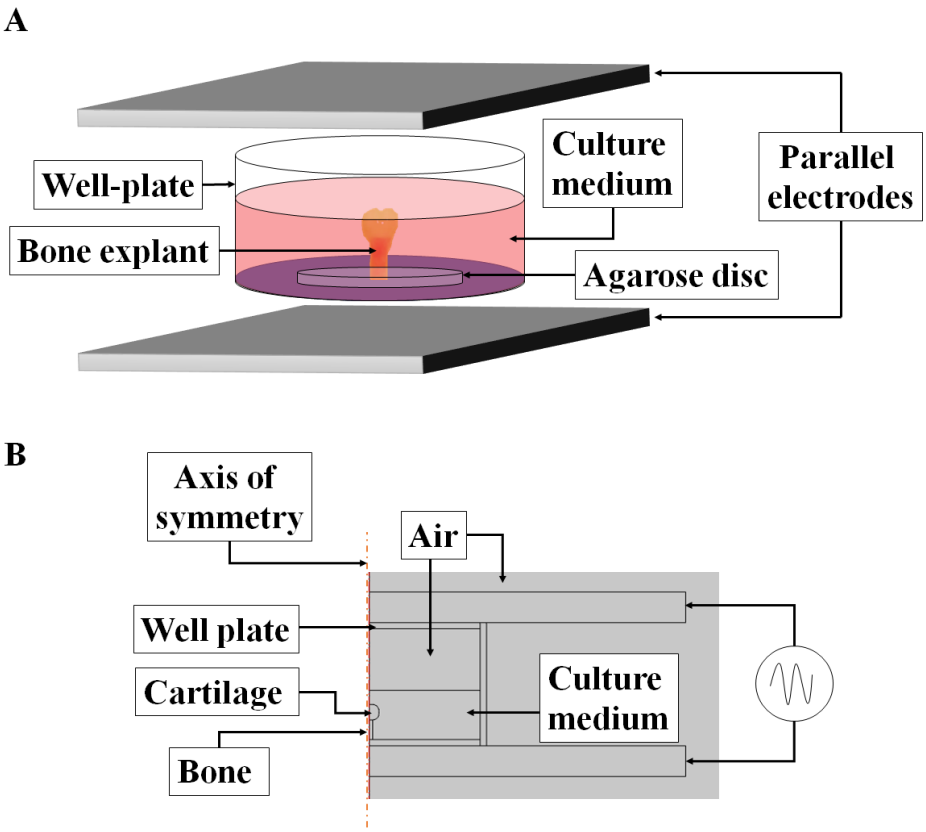
growth plate, specifically in the width of the P-z and Ht-z, as well as disruption of columnar arrangement [12]–[14]. The effects of compressive loading within the tissue include chondrocyte deformations, changes in the interactions among collagens and proteoglycans present in the ECM, and flux of water. The latter has been associated to the application of other types of biophysical stimuli such as tension, shear stress and hydrostatic pressure that alter the molecular activity of proteases and their inhibitors (e.g. TIMPs, MMPs), soluble factors, pro-inflammatory factors (e.g. Nitric Oxide, Prostaglandin E, Cyclooxygenase 2) and proteoglycans (e.g. GAGs) [15]–[17]. Although mechanical stimuli play a relevant role in growth plate development, it is difficult to extrapolate final conclusions regarding characteristics of the stimuli and cell response, considering that there is a high variability in terms of strain magnitude, loading duration and frequency. In fact, it has been described that cell deformations caused either by changes in cell volume or due to biophysical stimuli, lead to the activation of different types of ionic channels, such as intracellular calcium levels [18]–[22]. Similarly, other type of stimuli such as electric fields (EFs) may stimulate the ion channels of the chondrocyte cell membrane and control, extra and intracellularly, the pathway-signaling [21], [22]. It has been evidenced that EFs have an effect over growth plates zones altering mainly its morphology. For instance, an increase in length and width of distal femurs was observed when EFs stimulated the growth of the proliferative zone making it wider with a well-stratified columnar arrangement [23]. A similar study showed that EFs caused a mild proliferation of the hypertrophic zone towards the marrow cavity in the central area of the growth plate [24]. External devices have been also used to apply EFs and it has been evidenced that this electrical stimulus reduces bone growth, maintains cartilage in a quiescent state and promotes weight loss in animals without changes in bone growth [25], [26].

Even though *in vivo* electrical assays have evaluated the changes in the morphology of the growth plate and the development of long bones, there are many discrepancies in the results obtained; moreover, there are no studies that explore in detail the underlying effects that the EFs have over growth plate morphology and chondrocyte behavior. The main reason of this inconsistency is because factors such as EFs magnitudes, stimulation time per day, and the amount of days that the growth plate need to be under stimulation have not been well documented. Moreover, a morphological study has not been carried out to observe how the EFs alter the morphology of the growth plate zones. According to this, chondroepiphysis explants of femur and humerus were stimulated with 7 and 3.5 mV/cm at 60 kHz (sine wave-form) using two different stimulation schemes (30 minutes and 1 hour). These exposure times were applied four times per day during 30 days of culture. To accomplish this, a stimulation scheme, based on a capacitively coupling system, was designed and implemented to generate and homogeneously distribute the EFs over the explant cultures. Parameters such as frequency, duration and effective stimulation period to stimulate explants are based on previous findings, which have evidenced that EFs of lower intensity at 60 kHz stimulate chondrocyte dynamics, specially cell proliferation and molecular expression [27], [29], [31]. Moreover, it was evidenced that EFs of 16.5 and 33.5 mV/cm at 60 kHz have little effect on composition of the growth plate [23], but can alter the thickness of zones within the physis [23], [24], and the length of the bone [23], [26]. Results from this study indicated that depending on the EFs and the scheme used to stimulate the explants, the growth plate zones change in terms of width, cell arrangement and cell morphology. In addition, it was observed that EFs induce chondrocytes to enter in a pre-hypertrophy state in the center of the epiphysis. These findings are relevant since non-invasive therapies such as electrostimulation can be improved to restore pathological tissues. In fact, electrical stimulation of *in vitro* cultures may be used to understand how cell dynamics interact within the tissue and trigger certain molecular events that modulate tissue growth. This optimization brings a better understanding of long bone development and new therapeutic strategies for future cartilage tissue engineering and regenerative medicine.

2. Materials and Methods

2.1 Chondroepiphysis isolation and *in vitro* culture

2-day-old Wistar rats (n=20) obtained from the Veterinary Medicine Faculty of Universidad Nacional de Colombia were used in this study. The rats were sacrificed by decapitation at the Biomimetics Laboratory (Biotechnology Institute in Universidad Nacional of Colombia). Recommendations from the ethics committee were followed for this experimental procedure. First, femurs and humerus were isolated and washed with PBS containing 2% of antibiotics (streptomycin and penicillin, LONZA, Walkersville, MD USA). Next, the fibrous tissue and muscles were carefully removed from the epiphyses and the diaphysis. Just one epiphysis per bone was electrically stimulated in this study; therefore, the proximal epiphysis was removed from the femur, while the distal epiphysis was detached from the humerus. A large portion of the diaphysis of the bone was also extracted to preserve the ossification front of the growth plate. The extremities were fixed vertically into a 24 well-plate in order to apply the EFs perpendicularly to the chondroepiphysis (Schematic 1A). Bone fixation was performed with an agarose disc located at the bottom of the well-plate (agarose at 3% diluted in distilled water). Once the explants were located vertically, 2 mL of culture medium were added (DMEM-F12 with β-Glicerol phosphate 1 mM, ascorbic acid 50 µg/mL, Fetal Bovine Serum (FBS) 0.2%, and antibiotics 1 %). The explants were cultured at 37 °C and 5% CO_2 atmosphere. Culture medium was changed every other day. This procedure was implemented for controls and stimulated cultures.



Schematic 1. Capacitively coupling electrical system. **A)** Fixation of the explants using an agarose disc into the bottom of a single well. **B)** Axisymmetric system used for the computational simulation.

2.2 Electric field estimation

A computational simulation was implemented to estimate the EFs within the capacitively coupling system. The EFs were generated using voltages of 100 and 200 Vpp at 60 kHz (Schematic 1A). The capacitively coupling system was modelled as an axisymmetric configuration to calculate the EFs values in each part of the culture. The dielectric properties of the different materials that composed the capacitive system were considered (Table 1). The agarose disc was not taken into account in the computational simulation due to the fact that the

EF in this zone is negligible. A single well-plate from a 24 well-plate was simulated and the electrodes were big enough at the top and bottom of the well to ensure a homogeneous and isotropic distribution of the EF during the simulation (Schematic 1B). The computational simulation was implemented by a finite element analysis using an electromagnetic simulations software (COMSOL Multiphysics, Comsol Inc. Los Angeles, CA USA).

Table 1. Dielectric properties and measurements used in the simulation.

Component	Parameter	Value used in simulation	References
Stainless-steel	Electrode separation	20 [mm]	
	Electrode radius	50 [mm]	
	Thickness	5 [mm]	
	Relative permittivity	1	
	Electric conductivity	1.73913[MS/m]	
Bone marrow	Length and thickness	3.2 and 0.5 [mm]	
	Relative permittivity	102.8	[34]
	Electric conductivity	0.002901[S/m]	
Cartilage	Length and thickness	2.5 and 1.5 [mm]	
	Relative permittivity	2706.5	[34], [35]
	Electric conductivity	0.17732 [S/m]	
Culture medium	Height	8 [mm]	
	Relative permittivity	402.4 - j25000	[27]
	Electric conductivity	0.0834 [S/m]	
Well-plate	Length and height	17.5 and 20 [mm]	
	Thickness	1 [mm]	[27]
	Relative permittivity	3.5	
	Electric conductivity	6.9 [nS/m]	
Air	Relative permittivity	1	
	Electric conductivity	0[S/m]	

2.3 Electrical stimulation assay

Electrical stimulation was performed using a coupled system that delivered homogeneous EFs (Figure 1A) [27]. The electrical stimulation device was composed of two parallel stainless-steel electrodes located at the top and bottom of the culture well-plates (Figure 1A). The electrodes were placed in Teflon supports to eliminate any contact with the metallic surface of the incubator. The positive and negative terminals of the electrodes were connected to an electronic circuit (oscillator) that generated the voltages and frequencies required to create the EFs. The oscillator was energized with a dual source (Lendher – HY3003D-3, Shenzhen, China) and the signal verification before and after electrical stimulation was monitored using an oscilloscope (Keysight – DSO1052B, Santa Rosa, CA, USA). Control explant cultures were incubated in the same way as stimulated explant cultures, except that electrodes were not connected to the oscillator.

Stimulated cell cultures were exposed to EFs of 3.5 and 7 mV/cm at 60 kHz sine wave-form during 15 and 30 days of culture. The EFs were applied the first day of the isolation using two exposure times (30 minutes and 1 hour), according to a previous report [27]. Each stimulation time was applied four times per day according to the following scheme: 30 minutes of stimulation corresponds to 5.5 hours without stimulation, while 1 hour of stimulation corresponds to 5 hours without stimulation. A schematic description of the signal protocol is given in figure 1B. A temperature control assay was performed when explants were submitted to continuous EFs of 7 mV/cm during a period of 1 hour.

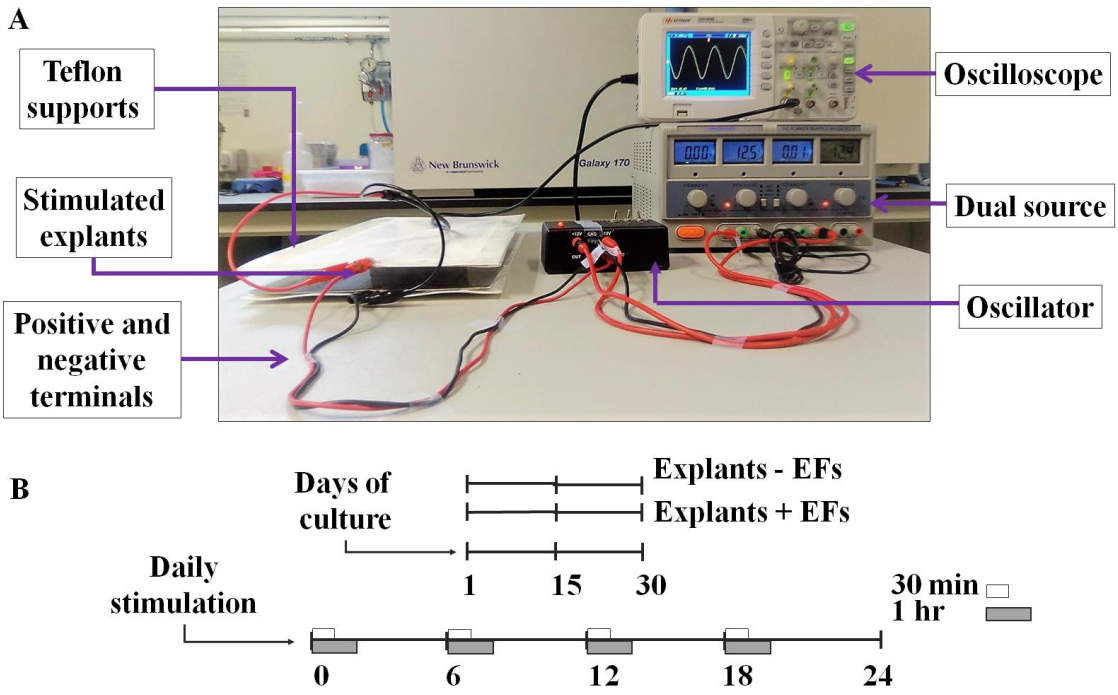


Figure 1. Schematic diagram of the *in vitro* stimulation. **A)** Capacitively coupling electrical stimulation *in vitro*. Connection of the dual source, oscillator and oscilloscope to the parallel electrodes. **B)** Diagram of the experimental design. Explants were randomly divided into 10 groups according to culture days, EFs and stimulation period: (1) 15 days – 3.5 mV/cm - 30 minutes, (2) 15 days - 7 mV/cm - 30 minutes, (3) 30 days – 3.5 mV/cm – 30 minutes, (4) 30 days - 7 mV/cm – 30 minutes, (5) 15 days – 3.5 mV/cm – 1 hour, (6) 15 days - 7 mV/cm - 1 hour, (7) 30 days - 3.5 mV/cm – 1 hour, (8) 30 days - 7 mV/cm – 1 hour, (9) 15 days – without stimulation and (10) 30 days - without stimulation.

2.4 Growth plate histological analysis

2.4.1 Masson's trichrome staining

Control and stimulated explants were stained with Masson's trichrome technique to observe chondrocyte nucleus, cytoplasm and collagen fibers in the chondroepiphysis. All samples were fixed in formaldehyde at 4% for 24 hours at room temperature. Then, explants were embedded in paraffin and sectioned at 5 µm for Masson's trichrome staining. An imaging analysis of growth plates were performed using a Nikon microscope with integrated camera (Nikon Eclipse E600W, Melville, NY USA) at two different magnifications (4X and 10X).

2.4.2 Quantitative analysis of growth plate zones

Considering the protocols described by [36] to analyze growth plates, some histomorphometric parameters were measured to quantify the size of P-z, PH-z and Ht-z, the total length of the epiphyseal plate, and the columnar organization in each growth plate zone (Figure 2A). For measuring zonal size (P-z, PH-z and Ht-z), the different zones were delimited manually in 10X-magnification images according to cell morphology. A minimum of 90 measures were obtained for each growth plate zone through evaluation of at least 3 images per explant (1 image x 3 independent sections). Then, ten parallel lines (green) were drawn in the direction of longitudinal bone growth to have different measures of each zone (Figure 2B). The size of the measured zone corresponded to the average length of each parallel line from the top to the bottom of each zone, which was separated by the hand-made lines. The total size of the growth plate was the result of the sum of the P-z, PH-z and Ht-z lengths. The quantification of chondrocytes and columns in each zone was performed by drawing three meshes (yellow grids) over 10X-magnification images

(Figure 2C). The square size varied for each zone corresponding to 36x36 µm squares for Ht-z and PH-z, and 100x100 µm squares for Ht-z. Using these grids, three parameters were measured: density of isolated cells (C_i), columnar cells (C_c) and column density (C_D). Moreover, a cartesian axis was drawn into the meshes to measure the inclination angle (α) of the columns in all zones (Figure 2D). Here, a cell column was defined as three or more stacked cells with a distance among them not greater than the average cell height within the field. All images were processed with Image Fiji Software and at least 3 images per individual (1 image x 3 independent sections) were analyzed. (NIH Image software, Bethesda, MD USA).

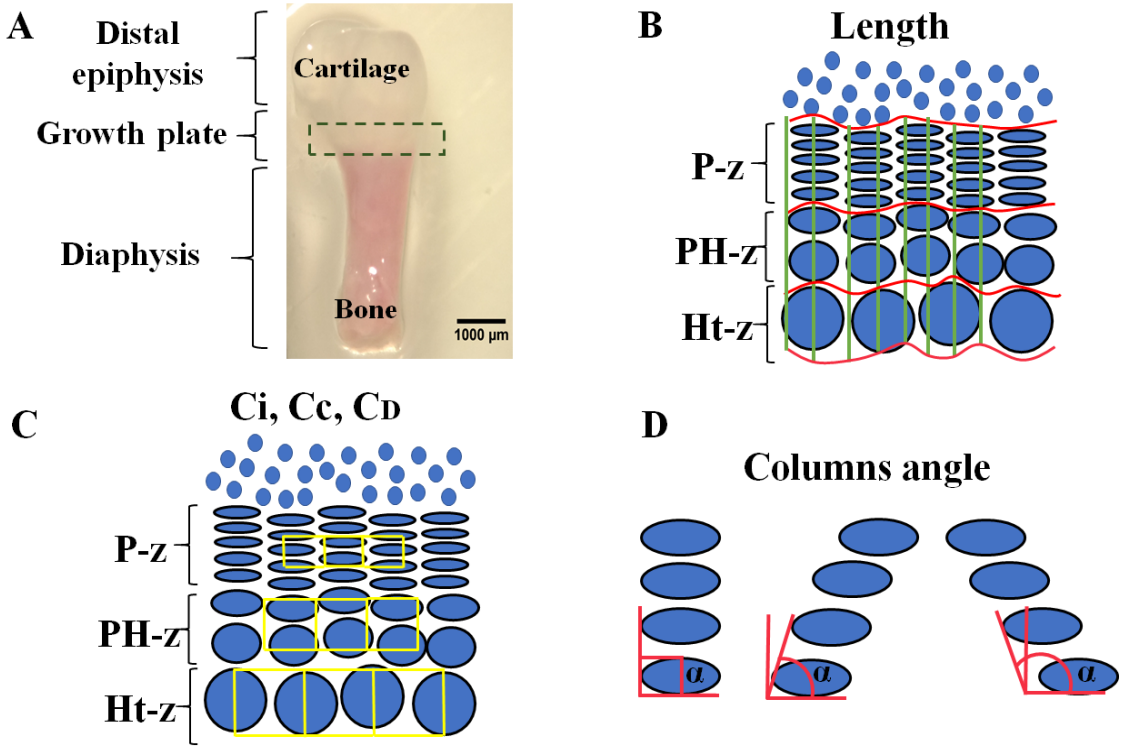


Figure 2. Representative diagram for the quantitative analysis of growth plate zones. **A)** Representation of explant bone used for cultures and its parts. **B)** Scheme that shows the treatment applied to the growth plate images to measure the height of the P-z, PH-z and Ht-z. **C)** Scheme that shows the treatment performed to the epiphyseal plate images to quantify the chondrocytes in each zone. **D)** Columnar arrangement and orientation.

2.5 Statistical Analysis

For the statistical studies, all analyses were performed with a mean ± SD (n= 3) according to the protocol described by [36]. Both a one-way ANOVA, and a Least Significant Difference (LSD) – Fisher post-test were carried out to analyze the histomorphometric parameters of the growth plate. Moreover, a Kruskal-Wallis nonparametric test or an unpaired two-tailed t-test were performed where applicable. Finally, a multifactorial ANOVA was done to observe which factors such as EFs, stimulation time or time culture have an effect over growth plate morphology and cell behavior. Statgraphics Centurion software was used for the statistical analysis (Statgraphics Technologies, Inc. The Plains, VA USA).

3. Results

3.1 Electric field estimation

The EF estimation in the capacitively coupling system for a single well-plate is shown in figure 3. This simulation was performed applying a voltage of 100 Vpp at 60 kHz sine wave-form

between the electrodes**.** The EF distribution in the capacitive system for the empty well-plate and with culture medium is shown in figure 3A. Measurements in five zones of the capacity system were performed to show the EFs in detail (Figure 3B). Results evidenced that in zone a (blue arrow in Figure 3B) exists a constant EF of 1.08 x10^5 mV/cm within the empty well-plate (Figure 3C left side), while in zone a' (blue arrow in Figure 3B) the EFs within the well-plate with bone explants and culture medium vary depending on where it is measured (Figure 3C right side).

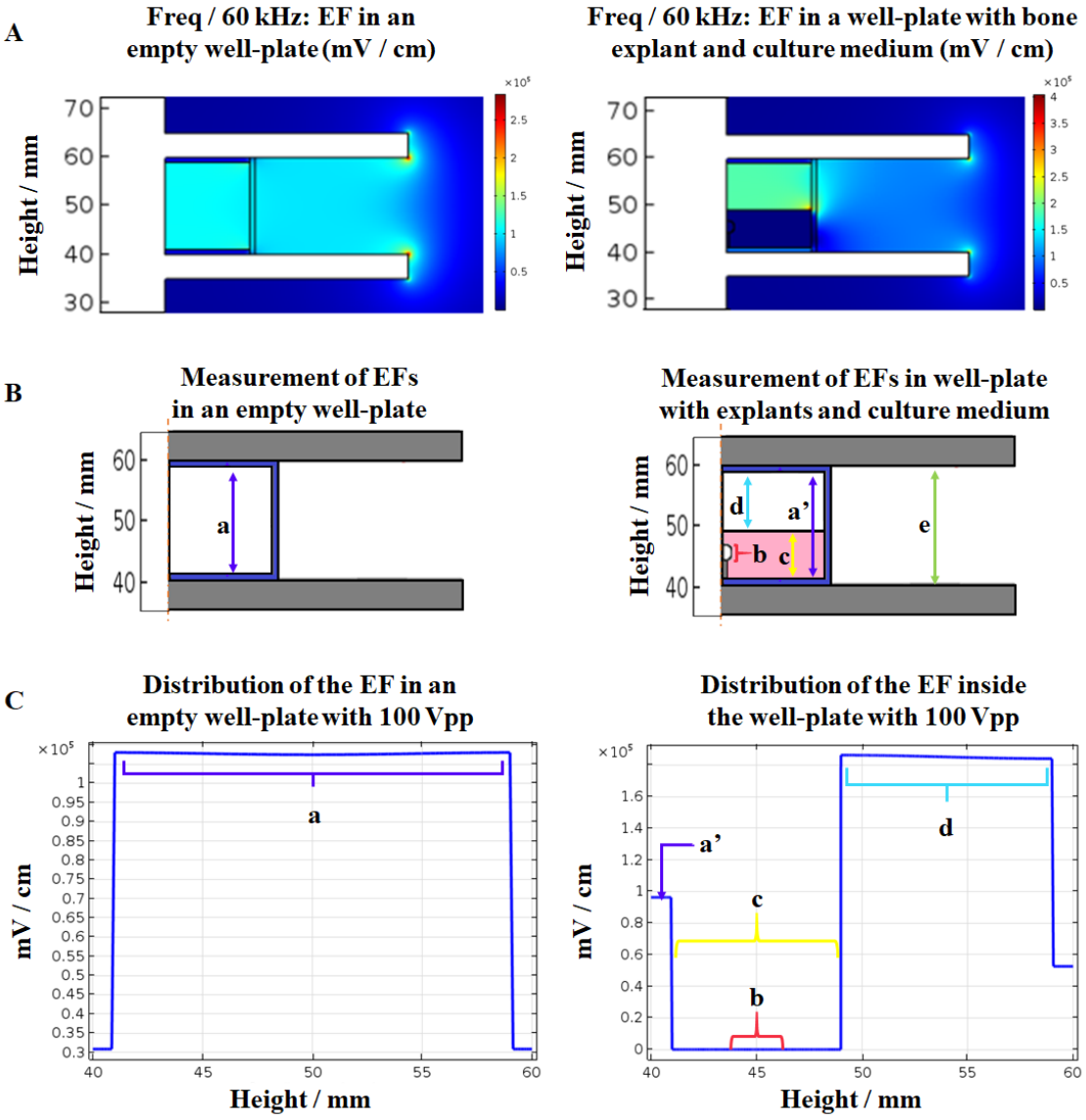


Figure 3. Representative scheme of the EFs in the capacitively coupling system. **A)** EFs in an empty capacitive system and with bone explants and culture medium. **B)** Measurement points of the EFs within and outside the well-plate. **C)** On the left, the EF in the empty capacitive system is illustrated; on the right, the EF distribution in the capacitor with the explant bone and culture medium is shown.

The EF values in the capacitive system are shown in figure 4. In zone b (red arrow in Figure 3B) there is an EF of 7 mV/cm in the center of the chondroepiphysis (Figure 4A), while in zone c (yellow arrow in Figure 3B) there are EFs from 13.5 mV/cm in the bottom of the well-plate to 7.5 mV/cm at the surface of the culture media (Figure 4B). In zone d (cyan arrow in Figure 3B) there is an EF of 1.8 x10^5 mV/cm in the air gap (Figure 4C). Finally, in zone e (green arrow in Figure 3B) there is an EF of 1.03 x10^5 mV/cm outside the well-plate between the electrodes (Figure 4D). As two EF intensities were tested in this study, the EFs generated by a voltage of 50 Vpp at 60

kHz sine wave-form are shown in the supplementary A and B. Due to the fact that EFs have a lineal increment, the intensities of EFs when applying 50 Vpp are half of those mentioned above.

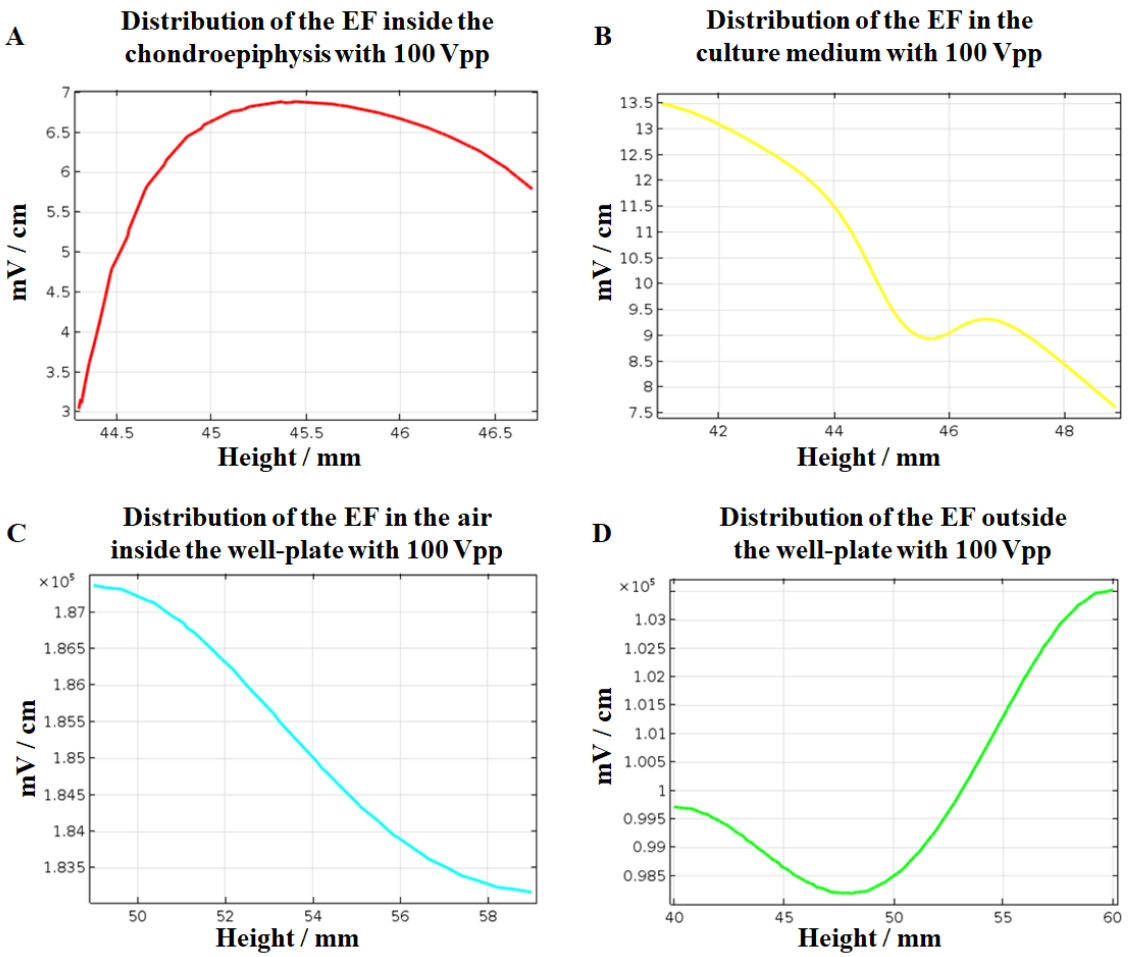


Figure 4. EF intensities within the capacitively coupled system. **A)** EF within the chondroepiphysis. **B)** The EF in the culture medium. **C)** The EF in the air gap between the culture medium and the top of the well-plate. **D)** The EF outside the well-plate between the electrodes.

3.2 Histomorphometric of the growth plate

3.2.1 Thickness and morphology of growth plate zones

The histomorphometric measurements of the growth plate width and the thickness of each zone in femur explants are shown in figure 5. The epiphyseal plate width of control bones decreased from D15 to D30, while the epiphyseal plate experienced a different behavior in stimulated explants depending on the EFs applied and culture time. For instance, the width of femur explants stimulated either with 3.5 or 7 mV/cm for 15 days did not change, while those stimulated for 30 days tended to be thinner, excepting the explants that were stimulated with 3.5 mV/cm for 30 minutes and 7 mV/cm for 1 hour where the width remained stable (Figures 5A).

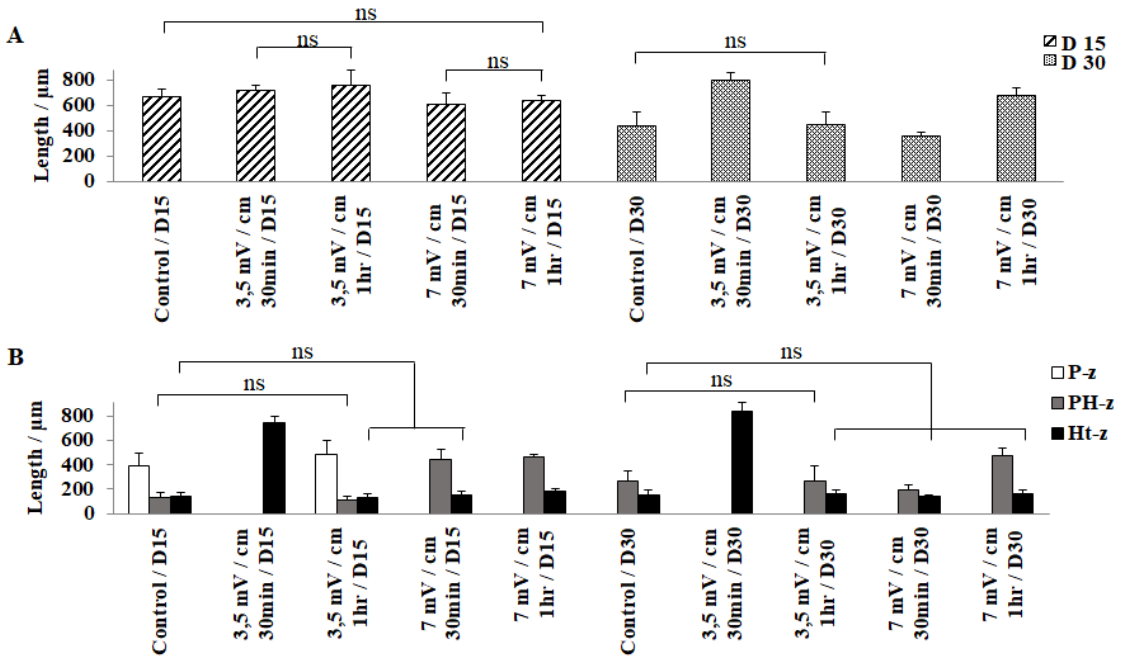


Figure 5. Measurement of growth plate lengths and thickness of P-z, PH-z and Ht-z. **A)** Length of distal femur growth plates. **B)** Thickness of distal femur epiphyseal plate zones. Statistically significant differences were found between controls and stimulated explants in terms of length and thickness after a stimulation with EFs of 3.5 and 7.5 mV/cm ($p < 0,05$), excepting those marked with "ns" (not significant).

Histologically, control bones showed highly proliferative flattened chondrocytes arranged into columns in the P-z, followed by pre-hypertrophic chondrocytes in the PH-z. Finally, chondrocytes that had stopped cell division and increased their size turned into hypertrophic in the Ht-z (Figure 6A). Thickness and cell morphology of growth plate zones in stimulated explants vary according to the EFs applied. For example, the P-z tends to disappear during culture in both controls and stimulated cultures (Figure 5B). This behavior was evidenced in figure 6A, where it is possible to observe that columnar chondrocytes are randomly distributed within the tissue, except in those epiphyseal plates stimulated with 3.5 mV/cm for 30 minutes where the P-z remained stable (See figure 6B). The PH-z and Ht-z were visible in the epiphyseal plate in both controls and stimulated cultures; however, each zone had a different behavior. The PH-z increased in thickness as the EFs were higher, while the Ht-z was higher with EFs of 3.5 mV/cm in comparison with those stimulated with EFs of 7 mV/cm (Figure 5B and 6B). The thickness of PH-z and Ht-z also changed according to the period of culture. For instance, the PH-z at D15 was higher in explants stimulated with 7 mV/cm in comparison with those stimulated with 3.5 mV/cm. However, a reverse behavior was observed at D30 in which the PH-z decreased in presence of EFs of 7 mV/cm, while this zone experienced an increase with EFs of 3.5 mV/cm. Regarding the Ht-z, the thickness of this zone in explants stimulated with 3.5 mV/cm was higher at D15, in comparison with those stimulated with 7 mv/cm. No significant changes were observed at D30 (Figure 5B). The growth plate width and the thickness of each zone in humerus explants are shown in supplementary C.

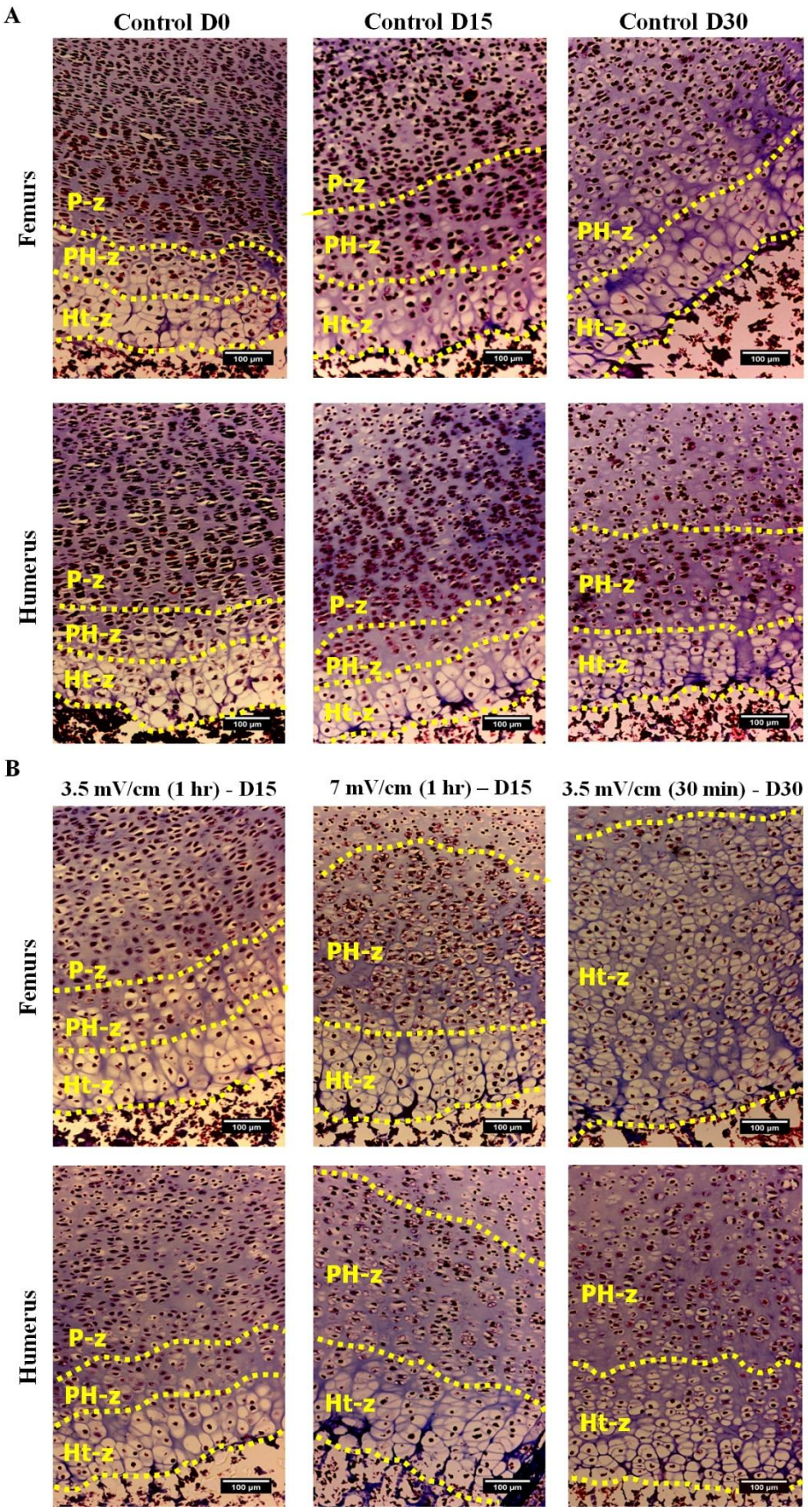


Figure 6. Histologies of growth plates at different times of culture. **A)** Growth plates of control cultures. **B)** Growth plates of femurs and humerus after stimulation with EFs. Yellow dotted lines separate the growth plate from calcified bone. Scale bar = 100 µm.

3.2.2 Columnar analysis of growth plate zones

Cells not forming columns (C_i) and the chondrocytes that stratified into columns (C_c) in femur explants are shown in figure 7. Results evidenced that the number of C_i vary depending on the zone of the growth plate, due to the fact that chondrocyte density tended to be higher at P-z and lesser in Ht-z at D15. A similar behavior was observed in explants stimulated with 3.5 mV/cm for 1 hour where there was the same proportion of C_i in the growth plate zones. It was possible to observe that chondrocytes which were not forming columns were higher in P-z and PH-z, while in Ht-z tended to be lesser (Figure 7A). Regarding the number of C_c, control cultures evidenced that there are more chondrocytes forming columns in P-z than in PH-z and Ht-z (Figures 7B). This behavior was similar in bones stimulated with 3.5 mV/cm during 1 hour. In bones exempt of P-z, the number of C_c varied according to the EF applied; for instance, chondrocytes forming columns tended to increase in PH-z and Ht-z with EFs of 7 mV/cm applied either for 30 minutes or 1 hour. The quantification of C_i and C_c of femurs explants are shown in supplementary D.

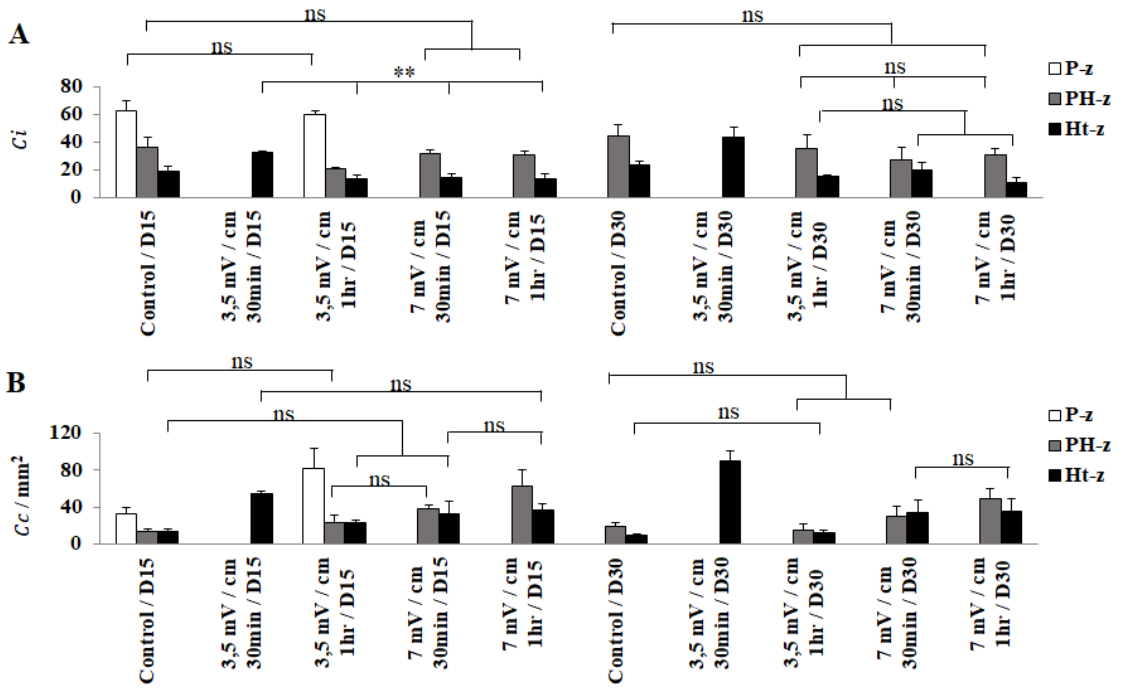


Figure 7. Measurement of C_i and C_c in growth plate zones of femur explants. **A)** Graphic bars of C_i within growth plate zones of femurs. **B)** Graphic bars of C_c within growth plate zones of femurs. Statistically significant differences were found between controls and stimulated explants after a stimulation with EFs of 3.5 and 7.5 mV/cm ($p < 0,05$**), except those marked with "ns" (not significant).

The parameters that indicate the number of columns (C_D) and their inclination angles α within the growth plate zones of femur explants are shown in figure 8. Some differences can be found between treatments in terms of the number of C_D per zones. For example, explants stimulated for 15 and 30 days had more C_D in the PH-z and Ht-z compared with the C_D in the P-z (Figures 8A). The parameter of C_D has a directly proportional behavior with C_c according to the observed between figure 7B and figure 8A. It means that columns in each zone depend directly on the number of stacked chondrocytes. Even though column of cells were observed in the different zones of the growth plate during culture (Figure 6), not all columns were perpendicularly aligned to the axis of bone growth. This behavior can be corroborated in figure 8B, where it was possible to observe that at D30 the variation coefficient of the angle decreased for stimulated cultures. It means that columns were formed but they experienced a random growth. In terms of the inclination angle of columns (α), it was observed that variation of the angle tended to be similar between controls and stimulated explants at D15. Nevertheless, variation decreased for stimulated

cultures at D30 α, which means that columns experienced a random growth (Figures 8B). This behavior was much more evident in humerus explants than in femurs (See supplementary E).

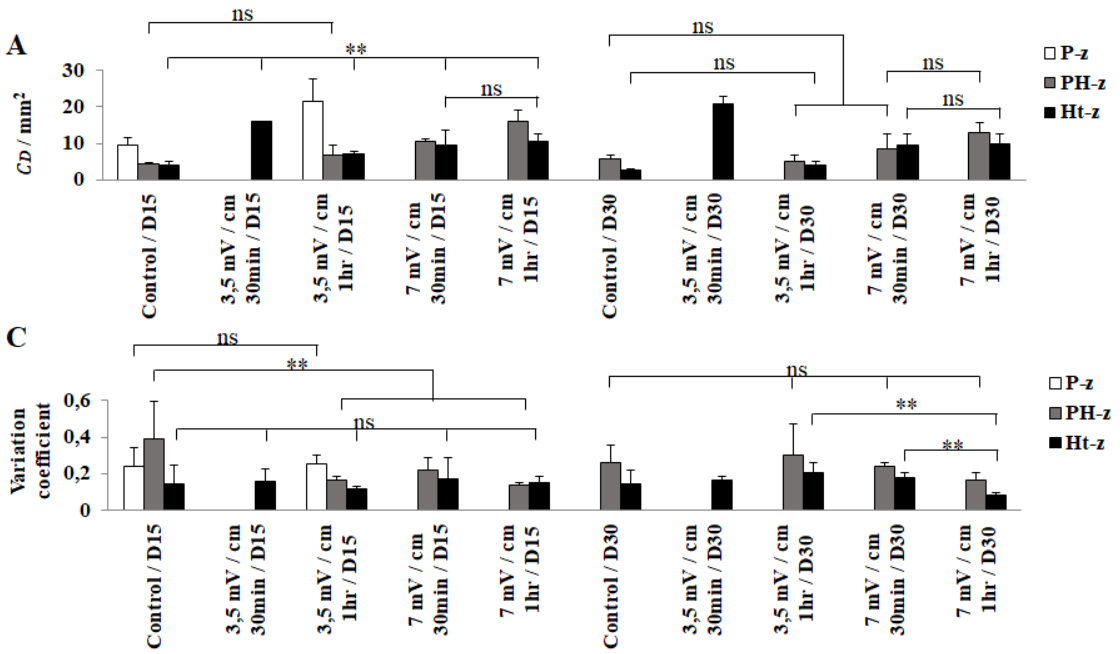


Figure 8. Measurement of C_D and inclination angle α of columns in growth plate zones of femur explants. **A)** Graphic bars of C_D within growth plate zones of femurs. **B)** Graphic bars of α within growth plate zones of femurs. Statistically significant differences were found between controls and stimulated explants after a stimulation with EFs of 3.5 and 7.5 mV/cm ($p <$ 0,05**), except those marked with "ns" (not significant).

The Masson's trichrome staining not only allowed to observe chondrocyte morphology in the growth plate zones, but also allowed to evidence a differentiation process in the chondroepiphysis of stimulated bones at D30 (Figure 9). A group of hypertrophic chondrocytes forming a circle was stained in the center of the epiphysis of bones stimulated with EFs of 3.5 and 7 mV/cm during 1 hour (Figure 9B and 9C). No evidence of this differentiation process was observed in control cultures (Figure 9A).

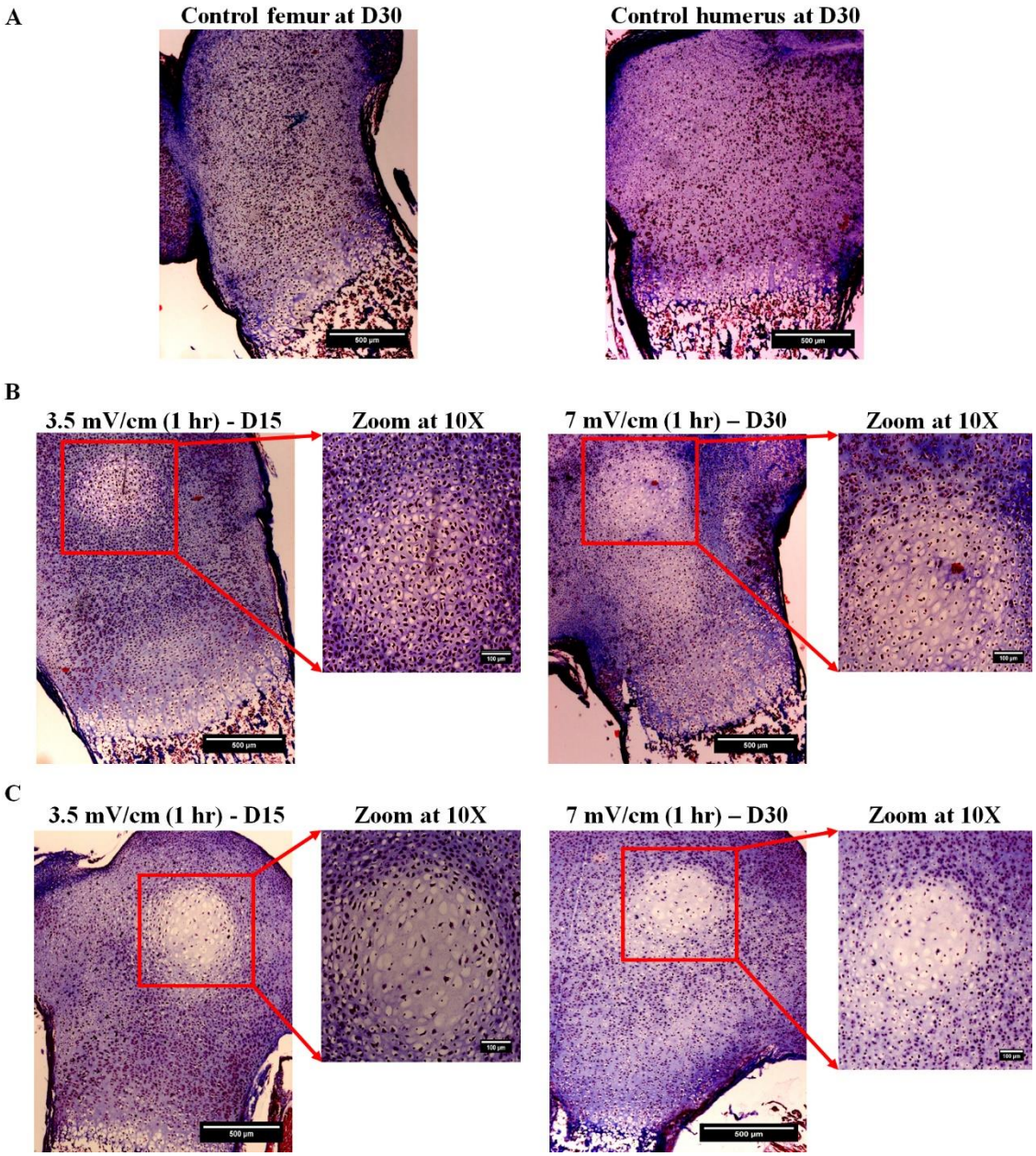


Figure 9. Representative scheme of histologies showing a hypertrophic cell condensation in the center of the chondroepiphysis of femurs and humerus. **A)** Histologies of femur and humerus controls at D30 of culture. **B)** Histologies of stimulated femurs at D15 and D30. **C)** Histologies of stimulated humerus at D15 and D30. Scale bar for bone histologies = 500 µm. Scale bar for zoom histologies at 10X = 100 µm.

1. Discussion

Biophysical stimuli belong to the wide range of environmental stimuli to which cartilage cells are exposed. In fact, it has been demonstrated that chondrocytes are able to sense and trigger specific responses to different types of stimulation such as compressive loads, tension, hydrostatic pressure, shear stress and electrostimulation. Regarding chondrocytes in the growth plate, the main biophysical stimulus sensed by these cells is mechanical loading. Experimental studies have shown that mechanical compression has effects over chondrocytes in terms of dynamics (proliferation, hypertrophy and apoptosis) [4], [37], [38], cell morphology [39], [40], protein synthesis of chondrocytes (Collagen type II, X and aggrecan) and enzyme expressions (MMP 13 and ADAMTS-4/5) [13], [41]. Accordingly, it is possible to argue that the absence of mechanical loading corresponds to alterations in growth plate function, leading to abnormal shape and length

of one or various skeletal elements. Studies *in vivo* and *in vitro* have also demonstrated that chondrocytes are also affected by electrical and electromagnetic stimuli, resulting in changes in cell dynamics, such as migration, differentiation, morphology, proliferation and gene expression [25], [27], [44], [45], [28]–[33], [42], [43]. To approach such issue, a histomorphometric approach was used to evaluate the effect of capacitively coupled EFs on bone explants cultured *in vitro*.

Results showed that the columnar zone on the stimulated bones were usually wider, and that cell columns were longer with many mature cells (hypertrophic). This result corroborates the findings of [23], in which an extra growth and longitudinal arrangement of immature cartilage cells in the Ht-z was observed. A similar work carried out by [24] showed that a direct stimulation on the bone increased the Ht-z of the growth plates; however, an abnormal growth of the epiphyseal plate was observed due to an incorrect application of the EFs according to the author. In this context, the correct intensity of the EFs applied are a relevant factor to consider, because high voltages can reduce bone growth and maintain cartilage in a quiescent state [26]. Taking this into account, the EFs, the stimulation time and the period of culture were well-stablished parameters in order to obtain a better understanding about the effect of electrical stimulation over the growth plate.

Considering parameters about the EFs and frequencies established in literature [25]–[27], [32], [33], it was possible to obtain relevant results about the morphophysiology of chondrocytes within the epiphyseal plates. Although these results are important to understand how cells react to an external stimulus, the mechanism by which cells sense an external signal and then, translate it into a molecular response remains unknown. It has been evidenced that ion channels might be associated to electrical signal transduction. Even though a plethora of ion channels has been identified in chondrocytes membranes, calcium channels are the main type of ion channels associated to transduction of biophysical stimulations. Electrical signals applied either in indirect or direct contact with the cells, exert their effect on the cell membrane by activating the VGCC leading to increase in the intracellular $Ca^{2}+$ levels [21], [46], [47]. The elevated influx of $Ca^{2}+$ activates the cytoskeletal calmodulin, an intracellular protein that regulates the signal transduction of calcium within the cell, allowing the production of certain transcription factors such as the SOX9 molecule. The activation of SOX9 triggers an intracellular production of the main molecules of the articular cartilage such as collagen type II and aggrecan [18], [20], [21]. Although this signaling pathway was not implicated in this study, this aspect should be the focus of future works to clarify how EFs act over the chondrocytes from an electro-biological point of view. Another signaling pathway activated by EFs is the Wnt/β-catenin. This protein, also recognized as a morphogen, is involved in several developmental processes including body axis patterning, as well as cell differentiation, proliferation and migration [48], [49]. In fact, a study demonstrated an increase of β-catenin in the chondrocytes nucleus after an stimulation with EFs [45]. These results allow to conclude that electrical stimulation is a promising tool, as this biophysical stimulus may potentially alter biochemical, biophysical and electrochemical properties of chondrocytes [18], [21], [29], [30], [32], [42].

The histomorphometric analysis was carried out in bone explants isolated after birth. At this stage the internal structures of bones, such as the primary ossification center and the epiphysis are already formed. Considering that bones have a different behavior *in vivo*, where the process of bone formation continues, the results obtained cannot be compared with growth plate of bones that have grown *in vivo* for 30 days. A bone presents a well-formed structure at D30, highlighting the primary and secondary ossification centers and growth plates. Although the aim of this study was not to reproduce the endochondral ossification process, the *in vitro* experiments allowed to observe the hypertrophic evolution of chondrocytes in response of a biophysical stimuli. Thus, it is noteworthy to mention that EFs have influence in the differentiation process not only over the growth plates, but also over the center of the epiphysis where chondrocytes increased their cellular volume (Figure 9). This finding is relevant due to the fact that EFs demonstrated to have influence over the pre-hypertrophy. This is an important event that plays a pivotal role, as it is responsible of the 70% of total longitudinal bone growth, in the initiation of the matrix mineralization, and in

the promotion of tissue vascularization [36]. Although the mechanisms by which capacitively coupled EFs stimulate chondrocytes differentiation are not yet known, the observed effects in the immunohistochemistry staining evidenced that chondrocytes experience a pre-hypertrophic process at the location of the secondary ossification center (Figures 9B and 9C).

Based on the above, understanding the influence of electrical stimulus in the biology of the growth plate not only provides useful information regarding their role in epiphyseal plate physiology *in vivo*, but also supplies new tools for tissue engineering and regenerative medicine focused on the development of new therapeutic approaches for the treatment of injuries in the growth plate.

2. Conclusion

The histomorphometric approach used to evaluate the effect of capacitive coupled EFs on bone explants revealed that growth plates morphology varies combining three main factors: 1) the EFs intensities, 2) stimulation time per day and 3) period of culture. Within this context, electrical stimulation has the ability to precisely control physical and biological properties of the growth plate by modifying either the thickness of the tissue or altering the cell response. Overall, the combination of biophysical techniques and *in vitro* culture models will not only allow the creation of novel tools to study the behavior of tissues, but also they can be used to create new therapies focused on the treatment of chronic pathologies of hyaline cartilage. The methodologies used to stimulate the regeneration of cartilage with EFs are increasingly approaching the development of technologies that meet the requirements of successful cartilage healing. In addition to that, electrical stimulation as a biophysical stimulus used in cartilage tissue engineering may improve clinical settings through the development of devices that stimulate *in vivo* tissues in regenerative therapies.

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Conflict of interest

The authors declare that they have no conflict of interest.

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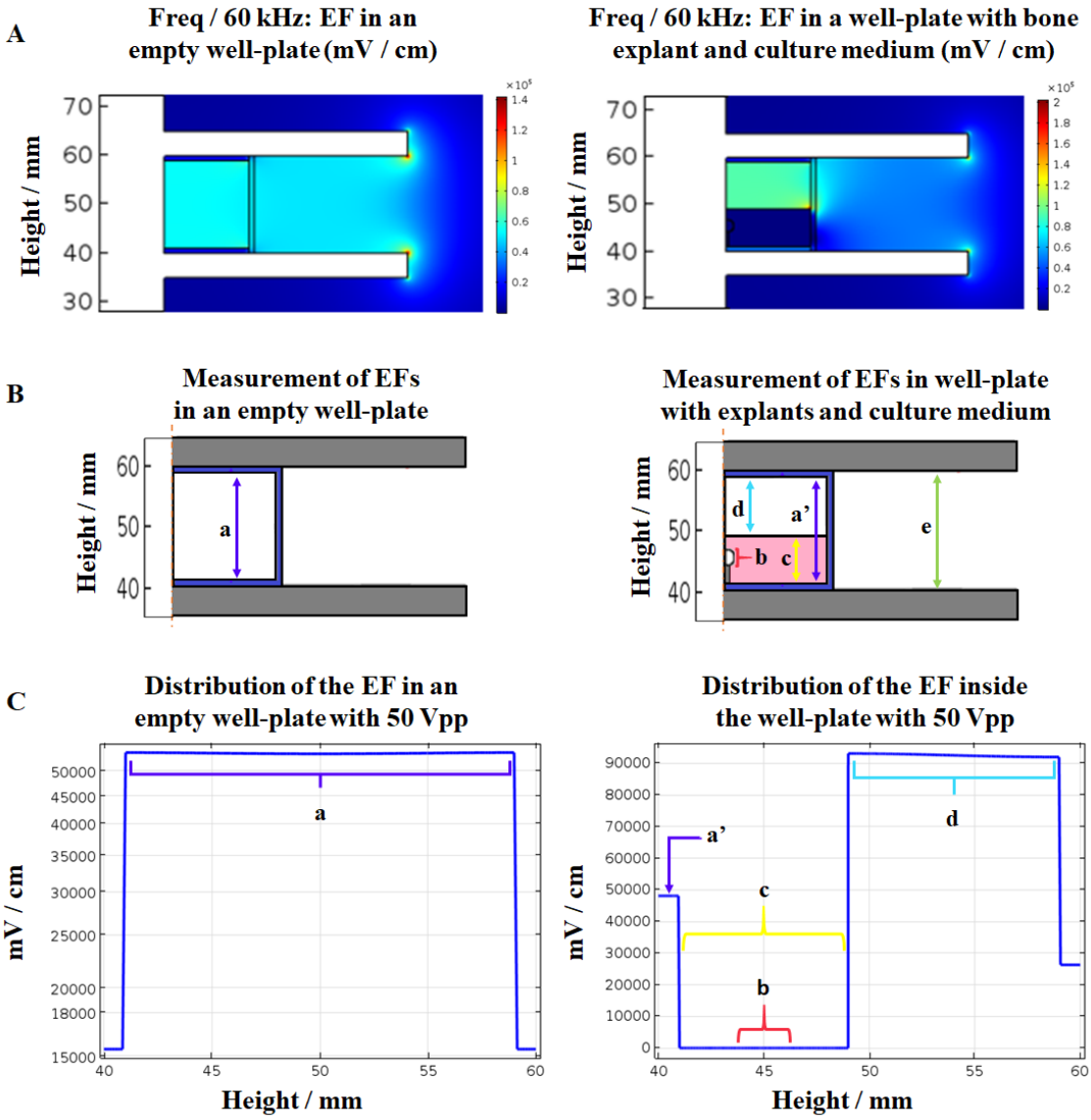
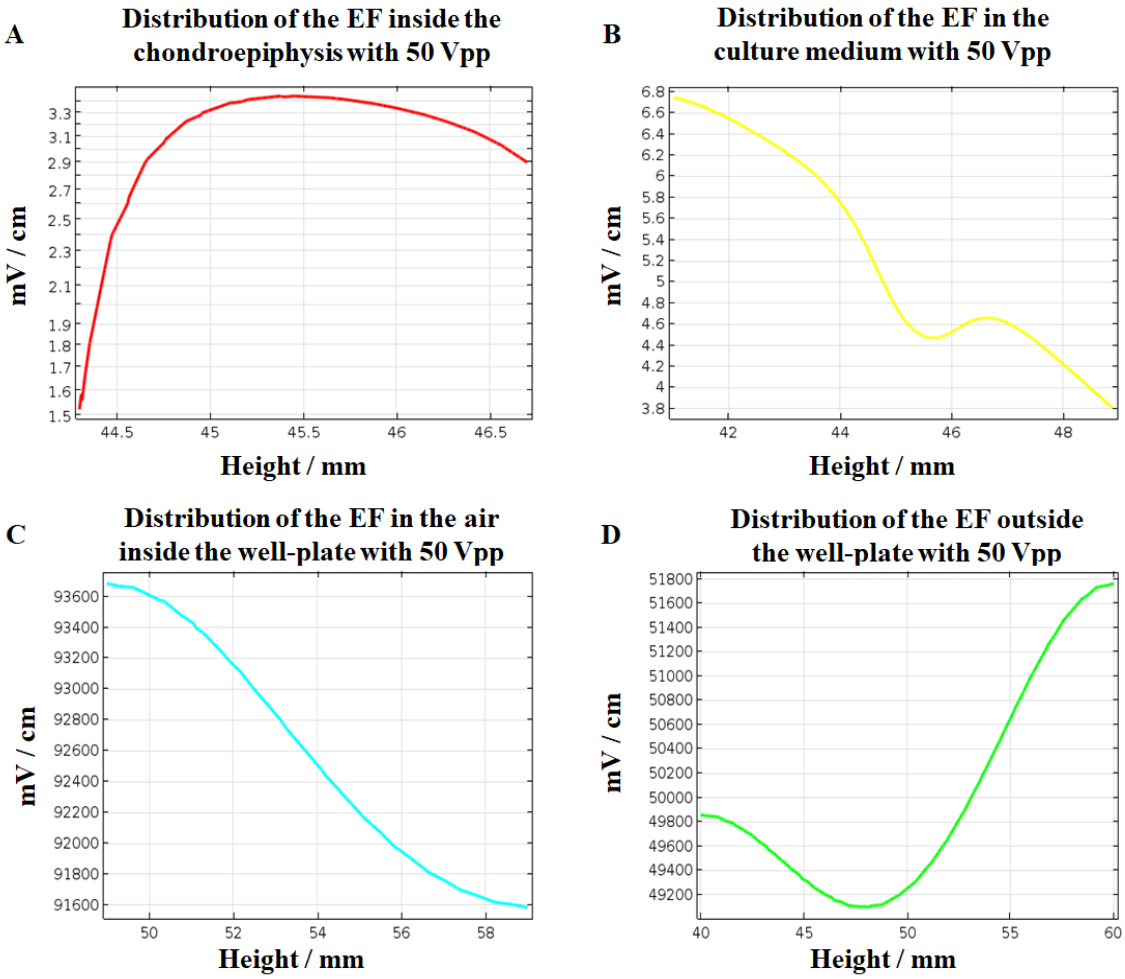
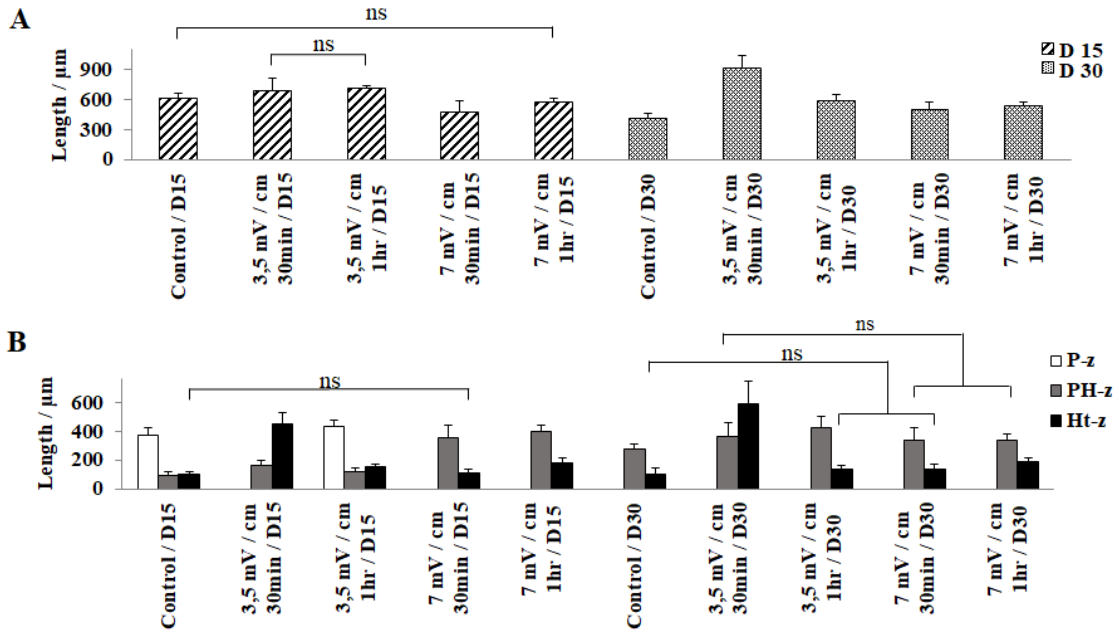
S- A. Representative scheme of the EFs in the capacitively coupling system generated by a voltage 50 Vpp at 60 kHz sine wave-form. **A)** EFs in an empty capacitive system and with bone explants and culture medium. **B)** Measurement points of the EFs within and outside the well-plate. **C)** On the left, the EF in the empty capacitive system is illustrated; on the right, the EF distribution in the capacitor with the explant bone and culture medium is shown.



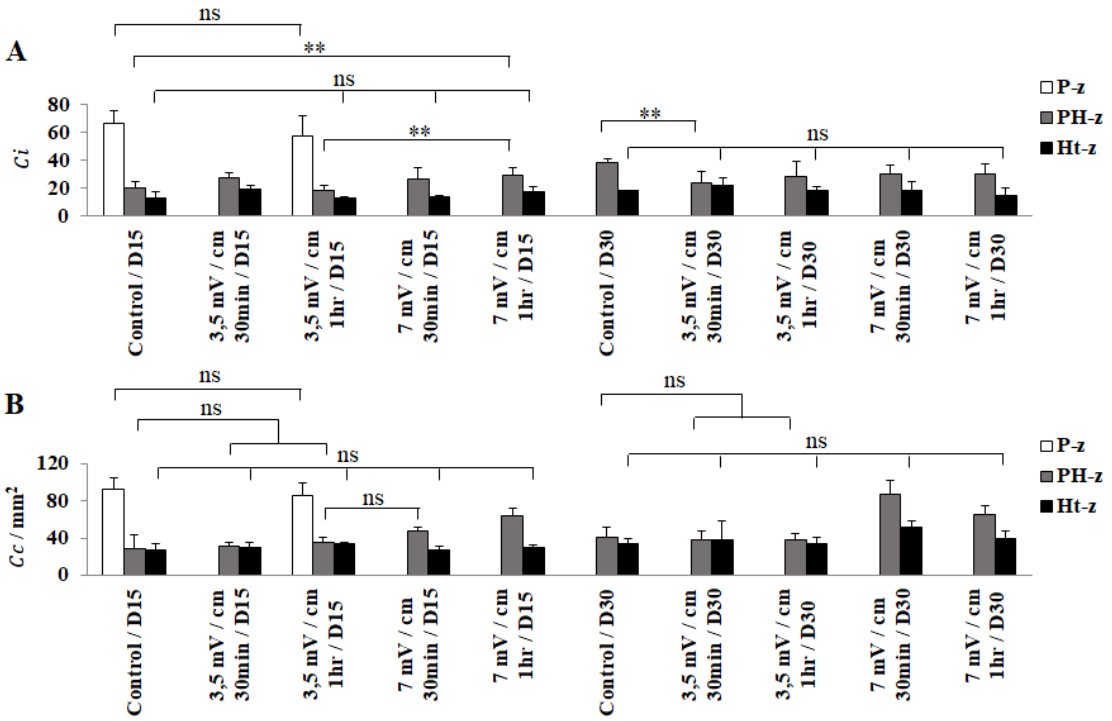
S- B. EF intensities within the capacitive coupled system applying a voltage 50 Vpp at 60 kHz sine wave-form. **A)** EF within the chondroepiphysis. **B)** The EF in the culture medium. **C)** The EF in the air gap between the culture medium and the top of the well-plate. **D)** The EF outside the well-plate between the electrodes.



S- C. Measurement of growth plate lengths and thickness of P-z, PH-z and Ht-z. **A)** Length of proximal humerus growth plates. **B)** Thickness of proximal humerus epiphyseal plate zones. Statistically significant differences were found between controls and stimulated explants in term of length and thickness after a stimulation with EFs of 3.5 and 7.5 mV/cm ($p < 0,05$), except those marked with "ns" (not significant).



S- D. Measurement of C_i and C_c in growth plate zones of humerus explants. **A)** Graphic bars of C_i within growth plate zones of humerus. **B)** Graphic bars of C_c within growth plate zones of humerus. Statistically significant differences were found between controls and stimulated explants after a stimulation with EFs of 3.5 and 7.5 mV/cm ($p < 0,05$**), except those marked with "ns" (not significant).



S- E. Measurement of C_D and inclination angle α of columns in growth plate zones of humerus explants. **A)** Graphic bars of C_D within growth plate zones of humerus. **B)** Graphic bars of α within growth plate zones of humerus. Statistically significant differences were found between controls and stimulated explants after a stimulation with EFs of 3.5 and 7.5 mV/cm ($p < 0,05$**), except those marked with "ns" (not significant).