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Multiple regression analysis predicts the dynamic of chondrocytes stimulated by magnetic and electric fields

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Purpose: The aim of this study was to implement a multiple regression analysis to find mathematical models that estimate the proliferative rate and the molecular synthesis of chondrocytes when these cells are stimulated either by magnetic or electric fields. *Methods*: Data derived from previous studies performed in our laboratory were used for statistical analyses, which consisted of applying magnetic fields (1 and 2 mT) and electric fields (4 and 8 mV/cm) to chondrocytes. Data from cell proliferation and glycosaminoglycan expression were used to adjust and to validate each mathematical model. *Results*: The root square model efficiently predicted the chondrocyte dynamics, evidencing determination coefficients of R^2 = 92.04 for proliferation and R^2 = 70.95 for glycosaminoglycans when magnetic fields were applied, and R^2 = 88.19 for proliferation and R^2 = 74.79 for glycosaminoglycans when electric fields were applied. *Conclusions*: The reduced, interactive, quadratic and combined models exhibited lower R^2 values, nevertheless, they were useful to predict proliferation and glycosaminoglycan synthesis, as the right-skewed distribution, determined by the F parameter, evidenced a $F_{\text{rejected}} < F_{\text{computed}}$. The models are efficient since the prediction of chondrocyte dynamics is comparable to the cell growth and to the molecular synthesis observed experimentally. This novel formulation may be dynamic because the variables that fit the models may be modified to improve *in vitro* procedures focused on cartilage recovery.

Key words: *multiple regression analysis, electric fields, magnetic fields, chondrocyte dynamics*

1. Introduction

Biophysical stimuli such as magnetic fields (MFs) and electric fields (EFs) have been implemented to stimulate chondrocyte dynamics. On one hand, MFs have been applied to *in vitro* cultures to preserve chondrocytes morphology [22], increase DNA synthesis [29], and enhance proliferation and glycosaminoglycan (GAGs) synthesis [34]. MFs have been applied to cartilage explants either to improve synthesis of proteoglycans and stimulate anabolic activities [30], [31] or to counteract catabolic activities and enhance osteoarthritis [37]. Application of MFs has been investigated in chondrocytes cultured in scaffolds. For example, osteoarthritic chondrocytes encapsulated within alginate structures expressed more proteoglycans when MFs were applied [15]. In studies by Schmidt-Rohlfing et al. [42], and Nicolin et al. [35], it was possible to observe that MFs applied to articular chondrocytes cultured in collagen membranes increased cell proliferation, synthesis of collagen type II and proteoglycans in the pericellular region. MFs have been applied *in vivo* in the recovery of knee osteoarthritis. For instance, MFs preserved the articular cartilage morphology [10], [13], [14], retarded the development of lesions [10],

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and improved joint motion, pain and tenderness [34], [39], [46], [47]. On the other hand, EFs have been applied *in vitro* to increase chondrocyte proliferation and GAGs synthesis [2], [6], [7], [40]. Wang et al. [52], elucidated that EFs increased aggrecan and collagen type II synthesis. EFs have been applied to cartilage explants and scaffolds. For instance, cartilage explants stimulated with EFs experienced an increase in proteoglycans (34%) and collagen type II (71%) after 14 days of stimulation [8]. Osteoarthritic human cartilage explants were stimulated with EFs. Results showed 1.4 and 1.5-fold increase in proteoglycans and collagen type II, respectively [9]. EFs have been applied to scaffolds used for cartilage repair, however, the reports in literature are limited and the results have been contradictory. For instance, chondrocytes seeded into agarose gels and electrically stimulated increased in cell density and GAGs synthesis [45]. In contrast, Akanji et al. [1], investigated the effects of direct current on cell proliferation and matrix synthesis, using a 3D chondrocyte-agarose scaffold. Their results demonstrated that EFs have no influence over protein synthesis, cell proliferation and mRNA expression.

Based on the above, MFs and EFs modulate chondrocyte dynamics favoring cell proliferation, chondrocyte morphology and molecular synthesis. However, there are relevant stimulation parameters to find the best combination to stimulate chondrocytes [48]. These parameters include intensities of MFs and EFs to stimulate chondrocytes, stimulation time per day, and the days that the cells need to be under stimulation. By controlling and varying these parameters, it is going to be possible to estimate either the best proliferation rate or the desired increase in molecular synthesis. For this reason, it is necessary to use alternative methods that make it possible not only to mimic cell biology experiments, but also to predict cell dynamics when the variables previously mentioned vary. Accordingly, several mathematical models were implemented to simulate cell dynamics, such as exponential logistic growth equations [20], [24], [25], [53], growth [3], [43], linear regression [38], monod [5], [11], [44] and agent-based models [18], [19], [28]; and a combination of models [36], [41], [54]. Nevertheless, there is no model that simulates cell response when external biophysical stimuli are applied to cell cultures. Therefore, in this study, a multiple regression analysis was implemented to find the equations that estimate the proliferative rate and the molecular synthesis of GAGs when chondrocytes are stimulated by MFs and EFs. The implementation of a combined experimental and mathematical approach allows to predict the MFs and EFs that stimulate *in vitro* alternative chondrocytes, reducing the number of experimental assays and providing a cost-effective in cartilage tissue engineering. Additionally, the standardization of a well-stablished protocol allows for the analysis of different MF and EF intensities and stimulation times to better understand the impact of biophysical stimulation on cell dynamics during the process of articular cartilage maintenance.

2. Materials and methods

2.1. Data acquisition for statistical analysis

Data derived from previous studies carried out in our laboratory were used in this study. Regarding the magnetic stimulation, data of cell proliferation and GAGs synthesis were acquired from the study [12]. Here, chondrocytes were stimulated with MFs of 1 and 2 mT at 60 Hz. These magnitudes were applied to chondrocytes for 1, 3 and 5 h every 6 h for 8 days. Concerning the electrical stimulation, data of cell population and GAGs expression were obtained from the paper [49]. Here, chondrocytes were exposed to EFs of 4 and 8 mV/cm at 60 kHz sine waveform for 8 days. The EFs were delivered daily, starting from the first day of culture, using exposure times of 30 min, 1 and 5 h.

2.2. Regression model

Using data derived from the proliferation and GAGs quantification of stimulated and non-stimulated cell cultures, a multiple regression analysis was performed to observe the effect of independent variables (MFs/EFs, time of stimulation and days of culture) over the dependent variables (proliferation and GAGs synthesis). The data analyzed in the model were the number of cells/dish and the expression of GAGs/cell in each time measured.

Data were analyzed using different models. The first analysis consisted in applying a reduced model (Eq. (1)), which was compared with the following models. Independent variables were modelled using an interactive model (Eq. (2)). Thereafter, data were analyzed through a quadratic model (Eq. (3)). Finally, data were analyzed by combining equations (2) and (3) to observe how the interaction between the predictors of each model influence the response of the dependent variables (Eq. (4)).

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3, \qquad (1)$$

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_1 x_2 + \beta_5 x_1 x_3 + \beta_6 x_2 x_3, \tag{2}$$

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_1^2 + \beta_5 x_2^2 + \beta_6 x_3^2, \tag{3}$$

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_1 x_2 + \beta_5 x_1 x_3 + \beta_6 x_2 x_3 + \beta_7 x_1^2 + \beta_8 x_2^2, \tag{4}$$

where y represents either number of cells/dish or amount of GAGs/cell, x_1 describes either MF or EF strengths, x_2 indicates days of culture (D_c), x_3 is stimulation time (T_s), and $\beta_{0–9}$ are coefficients obtained from the regression analysis. A test comparing nested models was developed to observe whether the models are useful to predict the number of cells or the expression of GAGs of stimulated and non-stimulated cultures. The test consisted in corroborating the alternative and null hypothesis, which are described by Eq. (5).

$$H_0 : \beta_0 = \beta_1 = \beta_2 = \beta_3 = \beta_4 = \beta_5 = \beta_6 = \beta_7 = \beta_8 = \beta_9 = 0, \qquad H_1 : \text{at least one of those } \beta_i = 0. \tag{5}$$

Then, the right-skewed distribution determined by the F parameter was calculated to do a comparison between F_{rejected} and F_{computed}. The F_{computed} was calculated using Eq. (6), in which SSER is the sum of squared errors for the reduced model, SSEC is the sum of squared errors for the complete model, $k - g$ is the number of β parameters specified in H_0, $k + 1$ is the number of β parameters in the complete model and n is the sample size.

$$F_{\text{computed}} = \frac{(\text{SSER} - \text{SSEC})/(k-g)}{\text{SSEC}/(n-(k+1))}. \tag{6}$$

After analyzing the models previously described, data were transformed to stabilize residuals and avoid heteroscedasticity property. Data transformation was performed by applying the root square model to the number of cells/dish and the total of GAGs/cell (Eq. (7)). Then, transformed data were analyzed using Eq. (7), where a sensibility analysis was performed to eliminate all outliers and predictors that do not accomplish the level of significance $p \leq 0.05$.

$$\sqrt{y} = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_1 x_2 + \beta_5 x_1 x_3 + \beta_6 x_2 x_3 + \beta_7 x_1^2 + \beta_8 x_2^2 + \beta_9 x_3^2. \tag{7}$$

Experimental data was analyzed using Minitab 18.1 (Minitab, Inc., PA, USA), while the equations obtained to model proliferation rate and GAG's synthesis were implemented in MATLAB R2019b (MathWorks, Natick, MA, USA).

2.3. Statistical analysis

All experimental analyzes were performed using an average of 3. Cell proliferation was analyzed through ANOVA. In the cases in which the data showed significance, statistical verification tests were carried out to make use of the model. The statistical tests were: analysis of variances, F test, Shapiro–Wilk test and Durbin–Watson test. Data that did not meet the aforementioned assumptions were analyzed by non-parametric statistic through a F test that bought the slopes of the proliferation curves. A one-way ANOVA was implemented to analyze the synthesis of GAGs, identifying the difference between groups; similarly, assumptions of normality and homogeneity of variances were verified. In addition, paired comparisons were made using the DMS test. The significant differences were considered significant with a $p \leq 0.05$. Regarding the computational data, a prediction of the fitted models was implemented, considering a 95% of confidence interval.

3. Results

3.1. Magnetic stimulation

Data obtained from proliferation and GAGs quantification assays to perform the regression analysis are shown in Table 1. A comparison was performed between experimental data and prediction models regarding cell number and GAGs production. On the one hand, differences in cell proliferation were found at days 5 and 8 between non-stimulated and stimulated cultures applying 2 mT for 1 h. On the other hand, differences in GAGs synthesis were found at day 5 between non-stimulated and stimulated cultures applying 2 mT for 5 h, while at day 8 there were differences between non-stimulated and stimulated cultures applying 2 mT during 1, 3 and 5 h.

Coefficients obtained from the reduced model for both number of cells/dish and content of GAGs/cell are in Eqs. (8) and (9). Results demonstrated that the reduced model can explain both number of cells/dish and concentration of GAGs/cell (Fig. 1).

Table 1. Proliferation rate and GAGs quantification of chondrocytes stimulated with MFs

Field [mT]	Day of culture	Time of exposure [h]	Nº Cells	GAGS/cell [pg/cell]
1	**2**	**3**	**4**	**5**
0	2	0	41500	43
0	2	0	43000	39
0	2	0	37500	40
0	5	0	58500	32
0	5	0	59500	26
0	5	0	65000	31
0	8	0	154500	20
0	8	0	123000	24
0	8	0	114500	26
1	2	1	36000	61
1	2	1	31000	52
1	2	1	35500	46
1	5	1	62500	34
1	5	1	46500	48
1	5	1	43000	57
1	8	1	102000	26
1	8	1	102000	23
1	8	1	110500	26
1	2	3	32500	47
1	2	3	47500	36
1	2	3	29500	61
1	5	3	58500	28
1	5	3	48500	46
1	5	3	39500	47
1	8	3	98000	27
1	8	3	113000	23
1	8	3	109000	25
1	2	5	32000	44
1	2	5	14500	87
1	2	5	33000	36
1	5	5	60000	33
1	5	5	58500	37
1	5	5	61500	34
1	8	5	85000	24
1	8	5	109000	28
1	8	5	103500	25
2	2	1	30500	26
2	2	1	27500	78
2	2	1	29500	83
2	5	1	37500	55
2	5	1	46500	38
2	5	1	37500	54
2	8	1	95500	28
2	8	1	62000	43
2	8	1	84500	33
2	2	3	37000	58
2	2	3	37000	43
2	2	3	43500	40
2	5	3	90000	19
2	5	3	61000	28
2	5	3	67500	33
2	8	3	189500	11

Table 1 continued

1	**2**	**3**	**4**	**5**
2	8	3	180500	11
2	8	3	134500	14
2	2	5	27000	71
2	2	5	33000	57
2	2	5	27000	77
2	5	5	74000	50
2	5	5	72000	56
2	5	5	64500	62
2	8	5	126500	39
2	8	5	145500	33
2	8	5	111000	41

$$\text{Nº Cells/dish} = -2286 - 365\,MF + 13\,873\,D_c + 1077T_s, \quad (8)$$

$$\text{GAGs/cell} = 54.87 + 5.27\,MF - 4.56\,D_c + 0.47T_s. \quad (9)$$

Coefficients obtained from the interactive model for both number of cells/dish and content of GAGs/cell are in Eqs. (10) and (11). Results demonstrated that the interactive model could explain the number of cells/dish, but it cannot explain the concentration of GAGs/cell ($F_{\text{rejection}} = 2.76 > F_{\text{computed}} = 0.48$) (Fig. 2).

$$\text{Nº Cells/dish} = 17\,316 - 14\,831\,MF + 11\,947D_c - 10\,043T_s + 595\,MF * D_c + 7154\,MF * T_s + 452D_c * T_s, \quad (10)$$

$$\text{GAGs/cell} = 48.04 + 8.96\,MF - 3.08D_c + 0.64T_s - 0.88\,MF * D_c + 0.43\,MF * T_s - 0.14D_c * T_s \quad (11)$$

Regarding the quadratic model, the coefficients obtained for number of cells/dish and content of GAGs/cell are in Eqs. (12) and (13). Results demonstrated that the quadratic model could explain both number of cells/dish and concentration of GAGs/cell (Fig. 3).

$$\text{Nº Cells/dish} = 45\,024 - 78\,172\,MF - 5571D_c + 27\,000T_s + 29\,391\,MF^2 + 1944D_c^2 - 3997T_s^2, \quad (12)$$

$$\text{GAGs/cell} = 52.98 + 38.2MF - 4.01D_c - 18.47T_s - 11.25MF^2 - 0.056D_c^2 + 3.13T_s^2. \quad (13)$$

Applying the combined model to the data, coefficients obtained for number of cells/dish and content of GAGs/cell are in Eqs. (14) and (15). Results demonstrated that the combined model could explain both number of cells/dish and concentration of GAGs/cell (Fig. 4).

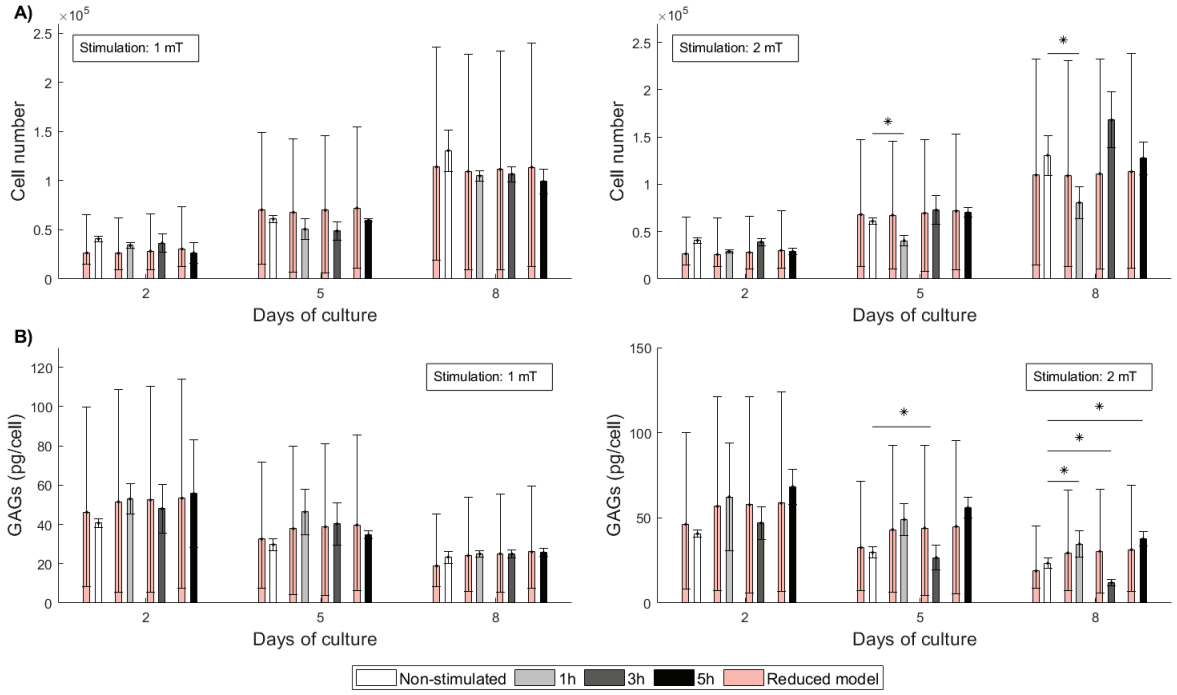

Fig. 1. Representative scheme of cell proliferation and GAGs synthesis of non-stimulated and stimulated cultures with MFs compared with data obtained by reduced model. Predictions (red bars) for each experimental data (grey scale bars) are located on the left side, A) Cell population of chondrocytes, B) GAGs synthesis of chondrocytes. Each scale bar corresponds to the average of three different samples. Experimental data were statistically analyzed, evidencing significant differences between stimulation schemes and controls ($p < 0.05$*). The prediction probability of the model was evidenced with error bars, considering a confidence interval of 95%

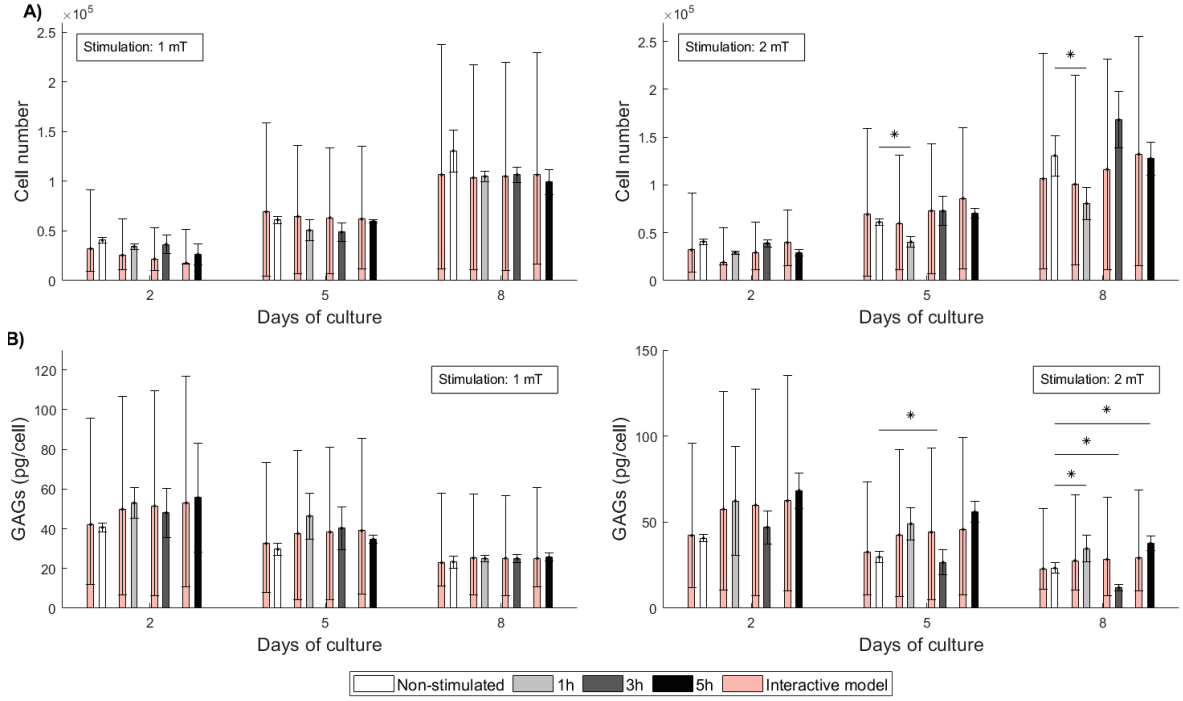

Fig. 2. Representative scheme of cell proliferation and GAGs synthesis of non-stimulated and stimulated cultures with MFs compared with data obtained by interactive model. Predictions (red bars) for each experimental data (grey scale bars) are located on the left side, A) Cell population of chondrocytes, B) GAGs synthesis of chondrocytes. Each scale bar corresponds to the average of three different samples. Experimental data were statistically analyzed, evidencing significant differences between stimulation schemes and controls ($p < 0.05$*). The prediction probability of the model was evidenced with error bars, considering a confidence interval of 95%

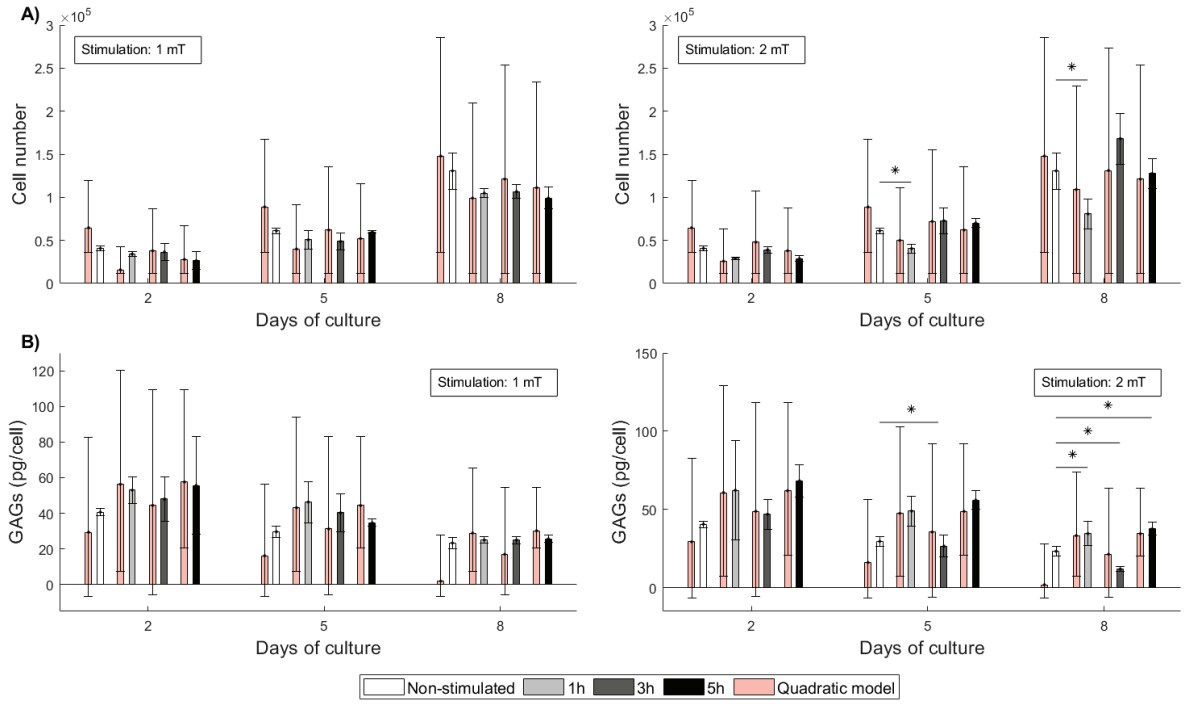

Fig. 3. Representative scheme of cell proliferation and GAGs synthesis of non-stimulated and stimulated cultures with MFs compared to data obtained by quadratic model. Predictions (red bars) for each experimental data (grey scale bars) are located on the left side, A) Cell population of chondrocytes, B) GAGs synthesis of chondrocytes. Each scale bar corresponds to the average of three different samples. Experimental data were statistically analyzed, evidencing significant differences between stimulation schemes and controls ($p < 0.05$*). The prediction probability of the model was evidenced with error bars, considering a confidence interval of 95%

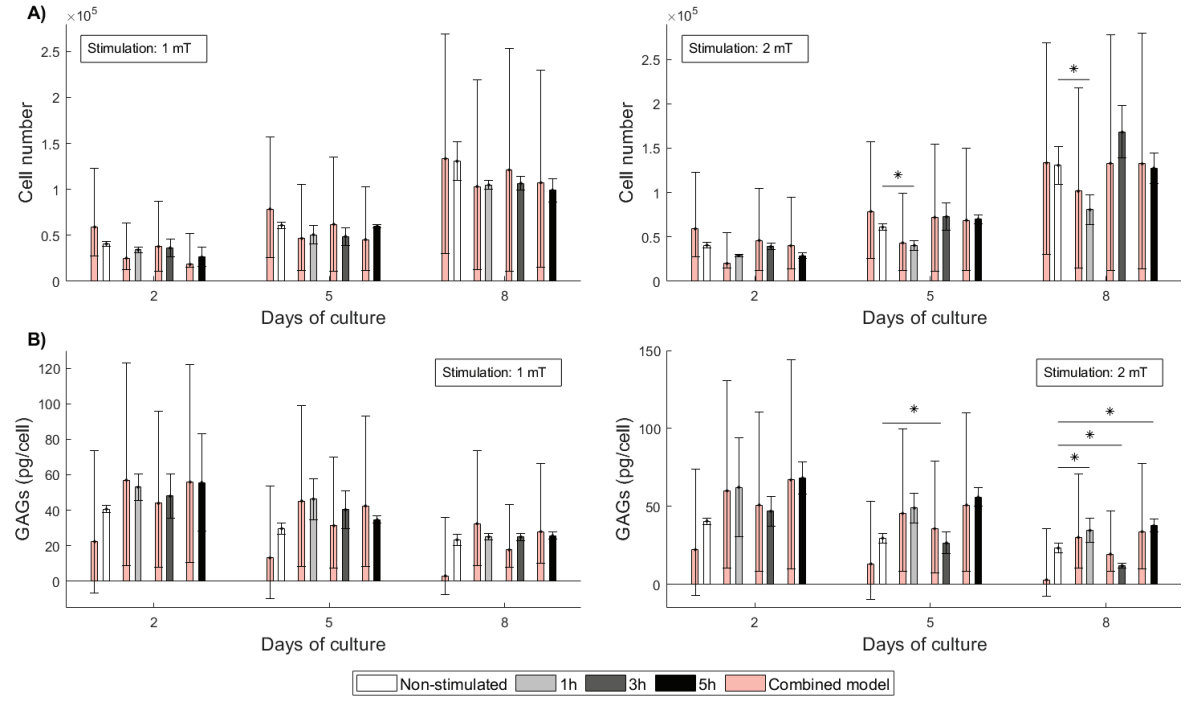

Fig. 4. Representative scheme of cell proliferation and GAGs synthesis of non-stimulated and stimulated cultures with MFs compared to data obtained by combined model. Predictions (red bars) for each experimental data (grey scale bars) are located on the left side, A) Cell population of chondrocytes, B) GAGs synthesis of chondrocytes. Each scale bar corresponds to the average of three different samples. Experimental data were statistically analyzed, evidencing significant differences between stimulation schemes and controls ($p < 0.05$*). The prediction probability of the model was evidenced with error bars, considering a confidence interval of 95%

$$\text{Nº Cells/dish} = 54656 - 55989\,MF - 7493D_c + 14679T_s + 595MF * D_c + 6708MF * T_s + 452D_c * T_s + 14297\,MF^2 + 1944D_c^2 - 3997T_s^2, \quad (14)$$

$$\text{GAGs/cell} = 45.56 + 50.02MF - 2.52D_c - 20.81\,T_s - 0.875MF * D_c + 2.03MF * T_s - 0.14D_c * T_s - 15.82MF^2 - 0.056D_c^2 + 3.13\,T_s^2. \quad (15)$$

The simulation of the reduced, interactive, quadratic and combined models evidenced the determination coefficients R-squared (R^2) and adjusted *R*-squared

Table 2. Constants and coefficients obtained from the regression output of stimulated cultures with MFs

		Reduced model	Interactive model	Quadratic model	Combined model	Root square model
R^2	No cell/dish	72.78	77.94	83.23	85.28	92.04
	GAGs /cell	49.12	50.41	59.58	61.59	70.95
R^2_{ADJ}	No cell/dish	71.40	75.70	81.43	82.78	91.29
	GAGs /cell	46.53	45.10	55.25	55.07	67.72

Table 3. Constants and coefficients obtained from the regression output in cell cultures stimulated with MFs. F_{rejected} was calculated with an $\alpha = 0.05$

		Interactive model	Quadratic model	Combined model
SSER	No cell/dish	2.73E + 10		
	GAGs/cell	9E + 3		
SSEC	No cell/dish	2.21E + 10	1.68E + 10	1.48E + 10
	GAGs /cell	9E + 3	7E + 3	6E + 3
F_{rejected}	No cell/dish	2.76	2.76	2.25
	GAGs /cell			
F_{computed}	No cell/dish	4.36	11.36	7.50
	GAGs /cell	0.48	4.83	3.04

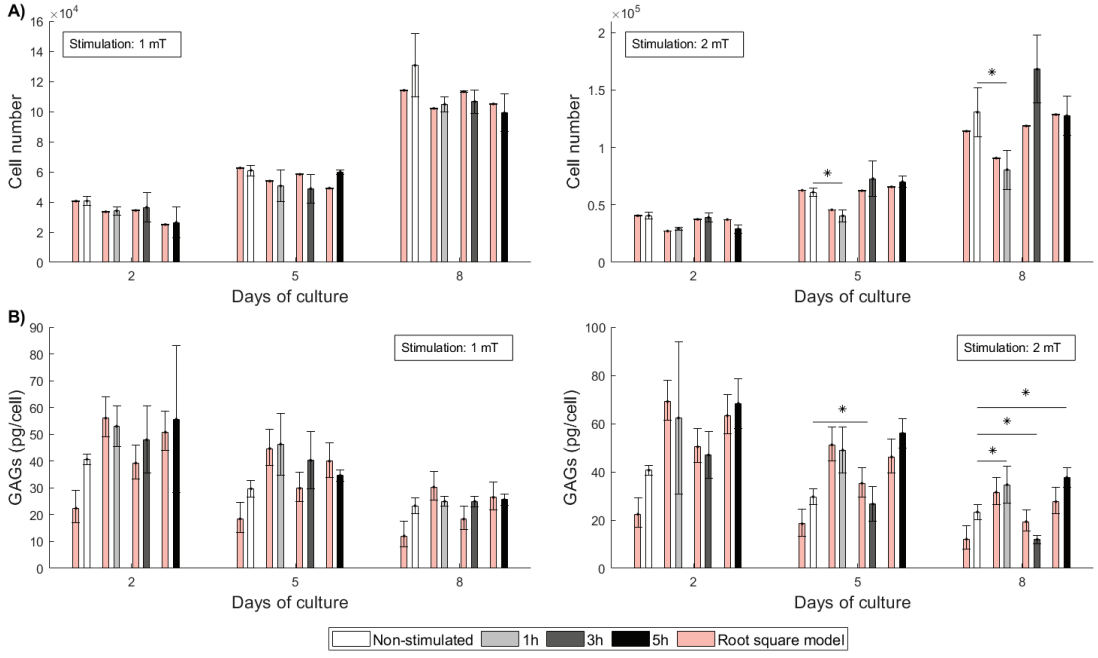

Fig. 5. Representative scheme of cell proliferation and GAGs synthesis of non-stimulated and stimulated cultures with MFs compared to data obtained by the root square model. Predictions (red bars) for each experimental data (grey scale bars) are located on the left side, A) Cell population of chondrocytes, B) GAGs synthesis of chondrocytes. Each scale bar corresponds to the average of three different samples. Experimental data were statistically analyzed, evidencing significant differences between stimulation schemes and controls ($p < 0.05$*). The prediction probability of the model was evidenced with error bars, considering a confidence interval of 95%

(R^2_{ADJ}) for number of cells/dish and content of GAGs/cell (Table 2). Here, R_2 provides information about how much the variation in the dependent variable could be explained by the model. In addition, the difference between R^2 and R^2_{ADJ} considers the sample size and the number of parameters used in the model.

Constants and coefficients to calculate right-skewed distribution (F) of interactive, quadratic and combined models are in Table 3. Models that evidenced an $F_{\text{computed}} > F_{\text{rejected}}$ were intended to reject H_0, concluding that interactive, quadratic and combined terms contribute to the prediction of number of cells/dish and GAGs/cell.

Finally, data were transformed using the root square model and analyzed by using the combined model. Coefficients obtained from the model for both number of cells/dish and content of GAGs/cell were used in Eqs. (16) and (17) (Fig. 5). These equations were obtained by eliminating outliers and predictors that do not accomplish levels of significance $p \leq 0.05$. Coefficients R^2 and R^2_{ADJ} for number of cells/dish and content of GAGs/cell are in Table 2.

$$\sqrt{\text{Nº}\,\frac{\text{Cells}}{\text{dish}}} = 194.02 - 31.50MF + 13.21MF * T_s + 1.23D_c * T_s + 2.15D_c^2 - 3.65T_s^2, \quad (16)$$

$$\sqrt{\frac{\text{GAGs}}{\text{cell}}} = 6.21 + 3.95MF - 1.65T_s - 0.12MF * D_c - 0.96\,MF^2 - 0.021D_c^2 + 0.26\,T_s^2 \quad (17)$$

3.2. Electrical stimulation

Data collected from proliferation and GAGs quantification assays to perform the regression analysis are listed in Table 4. On one hand, differences in proliferation rate were found at days 4 and 6 between non-stimulated and stimulated cultures applying 4 mV/cm for 30 min. Finally, a decrease in cell population was observed in cell cultures stimulated with 4 and 8 mV/cm during 5h at day 6. On the other hand, a decrease in GAGs synthesis were found at days 2, 4 and 6 in both non-stimulated and stimulated cultures during 30 min and 1 h, while a constant synthesis of GAGs was observed in stimulated cultures during 5 h compared to non-stimulated cultures; specially, at day 6 were GAGs were higher in stimulated cultures compared to controls.

Table 4. Proliferation rate and GAGs quantification of chondrocytes stimulated with Efs

Field [mV/cm]	Day of culture	Time of exposure [h]	Nº Cells	GAGS/cell [pg/cell]
1	**2**	**3**	**4**	**5**
0	2	0.5	55000	246.665
0	2	0.5	70000	315.700
0	2	0.5	70000	195.027
0	4	0.5	85000	262.999
0	4	0.5	110000	147.378
0	4	0.5	100000	182.594
0	6	0.5	205000	42.652
0	6	0.5	180000	60.684
0	6	0.5	150000	68.547
0	8	0.5	705000	26.223
0	8	0.5	420000	43.101
0	8	0.5	475000	36.761
4	2	0.5	50000	92.821
4	2	0.5	40000	138.462
4	2	0.5	65000	95.069
4	4	0.5	165000	53.768
4	4	0.5	195000	33.005
4	4	0.5	225000	54.815
4	6	0.5	250000	34.462
4	6	0.5	285000	35.178
4	6	0.5	290000	33.687
4	8	0.5	540000	29.250
4	8	0.5	435000	28.647
4	8	0.5	825000	16.814
8	2	0.5	55000	280.794
8	2	0.5	75000	212.742
8	2	0.5	65000	246.784
8	4	0.5	90000	199.090
8	4	0.5	125000	136.519
8	4	0.5	115000	166.197
8	6	0.5	165000	48.329
8	6	0.5	180000	73.504
8	6	0.5	205000	45.779
8	8	0.5	530000	19.642
8	8	0.5	375000	46.564
8	8	0.5	790000	22.265
0	2	1	65000	394.625
0	2	1	80000	366.894
0	2	1	70000	293.895
0	4	1	150000	288.551
0	4	1	105000	235.495
0	4	1	145000	290.102
0	6	1	215000	72.137
0	6	1	185000	108.034
0	6	1	190000	73.333
0	8	1	455000	15.561
0	8	1	495000	34.302
0	8	1	520000	19.512
4	2	1	75000	511.945
4	2	1	70000	421.745
4	2	1	80000	492.036
4	4	1	110000	343.003
4	4	1	100000	477.816
4	4	1	100000	276.976

Table 2 continued

1	2	3	4	5
4	6	1	160000	63.590
4	6	1	175000	63.590
4	6	1	175000	57.949
4	8	1	490000	16.647
4	8	1	440000	37.538
4	8	1	505000	18.703
8	2	1	70000	328.498
8	2	1	65000	351.048
8	2	1	60000	627.986
8	4	1	125000	390.358
8	4	1	105000	189.846
8	4	1	160000	29.670
8	6	1	195000	112.821
8	6	1	190000	216.850
8	6	1	215000	184.615
8	8	1	445000	32.017
8	8	1	480000	64.263
8	8	1	535000	55.092
0	2	5	55000	295.353
0	2	5	85000	146.473
0	2	5	67500	253.347
0	4	5	162500	89.888
0	4	5	147500	122.920
0	4	5	217500	67.484
0	6	5	317500	26.122
0	6	5	372500	31.766
0	6	5	295000	31.863
0	8	5	735000	26.335
0	8	5	430000	50.475
0	8	5	425000	43.446

Table 2 continued

1	2	3	4	5
4	2	5	60000	77.350
4	2	5	35000	106.960
4	2	5	30000	180.342
4	4	5	40000	83.974
4	4	5	40000	100.000
4	4	5	95000	50.202
4	6	5	40000	116.026
4	6	5	45000	105.983
4	6	5	90000	45.869
4	8	5	145000	45.270
4	8	5	155000	36.559
4	8	5	225000	16.638
8	2	5	45000	108.832
8	2	5	30000	184.615
8	2	5	25000	206.154
8	4	5	15000	437.607
8	4	5	60000	100.855
8	4	5	35000	169.231
8	6	5	55000	98.368
8	6	5	90000	80.057
8	6	5	40000	225.000
8	8	5	55000	300.962
8	8	5	55000	300.962
8	8	5	55000	300.962

Coefficients obtained from the reduced model for both number of cells/dish and content of GAGs/cell were used in Eqs. (18) and (19). Results demonstrated that the reduced model could explain both

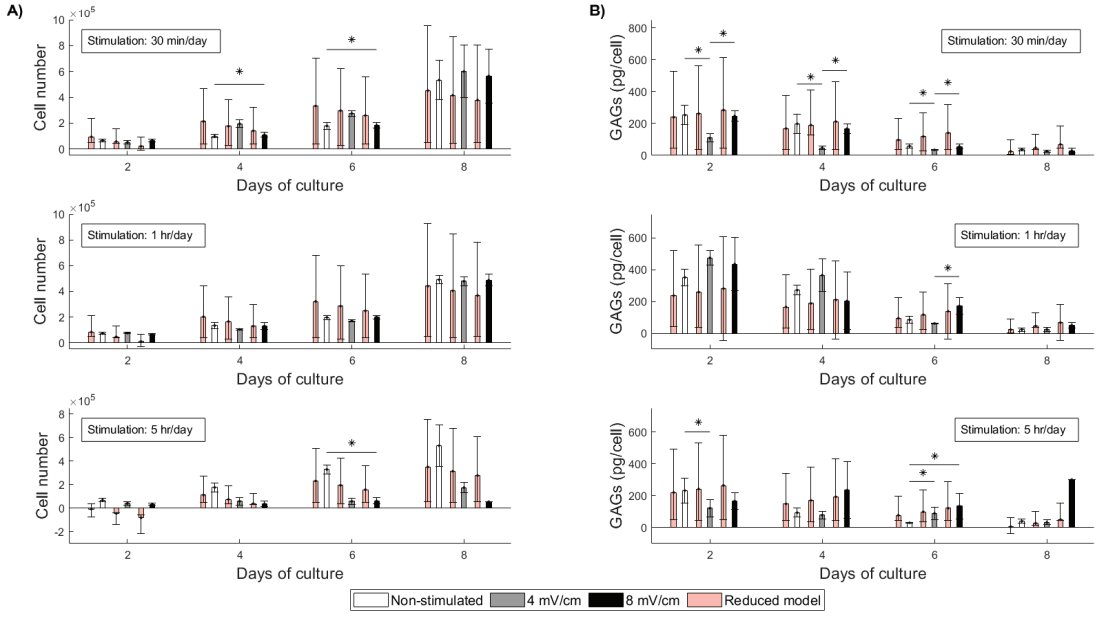

Fig. 6. Representative scheme of cell proliferation and GAGs synthesis of non-stimulated and stimulated cultures with EFs compared to data obtained by the reduced model. Predictions (red bars) for each experimental data (grey scale bars) are located on the left side, A) Cell population of chondrocytes, B) GAGs synthesis of chondrocytes. Each scale bar corresponds to the average of three different samples. Experimental data were statistically analyzed, evidencing significant differences between stimulation schemes and controls ($p < 0.05$*). The prediction probability of the model was evidenced with error bars, considering a confidence interval of 95%

number of cells/dish and concentration of GAGs/cell (Fig. 6).

$$\text{Nº Cells/dish} = -14\,283 - 9149EF + 59\,657D_c - 22\,607T_s, \quad (18)$$

$$\text{GAGs/cell} = 313.9 + 5.52\,EF - 35.72\,D_c - 4.31T_s. \quad (19)$$

Coefficients obtained from the interactive model for both number of cells/dish and content of GAGs/cell are in Eqs. (20) and (21). Results demonstrated that the reduced model could explain both number of cells/dish and concentration of GAGs/cell (Fig. 7).

$$\text{Nº Cells/dish} = -23\,0671 + 19646EF + 91\,065D_c + 51\,495T_s - 2792EF * D_c - 6848EF * T_s - 9342D_c * T_s, \quad (20)$$

$$\text{GAGs/cell} = 469.5 - 10.95EF - 61.60D_c - 57.8T_s + 1.99\,EF * D_c + 3.02\,EF * T_s + 8.28D_c * T_s. \quad (21)$$

Regarding the quadratic model, coefficients acquired for number of cells/dish and content of GAGs/cell are in Eqs. (22) and (23). Results demonstrated that the quadratic model could explain both number of cells/dish and concentration of GAGs/cell (Fig. 8).

$$\text{Nº Cells/dish} = 260332 - 15365\,EF - 62449D_c + 64329T_s + 777\,EF^2 + 12211D_c^2 + 7330T_s^2, \quad (22)$$

$$\text{GAGs/cell} = 231.6 - 16.92\,EF - 74.7D_c + 261T_s + 2.66\,EF^2 + 4.11D_c^2 - 46.76T_s^2. \quad (23)$$

Applying the combined model to the data, coefficients obtained for number of cells/dish and content of GAGs/cell are in Eqs. (24) and (25). Results demonstrated that the combined model can explain both number of cells/dish and concentration of GAGs/cell (Fig. 9).

$$\text{Nº}\,\frac{\text{Cells}}{\text{dish}} = 43\,944 + 13\,431EF - 31\,041D_c + 9773T_s - 2792EF * D_c - 6848EF * T_s - 9342D_c * T_s + 777EF^2 + 12\,211D_c^2 + 7330T_s^2, \quad (24)$$

$$\frac{\text{GAGs}}{\text{cell}} = 395 - 35.2\,EF - 102.6\,D_c + 209.1\,T_s + 2.65\,EF * D_c$$

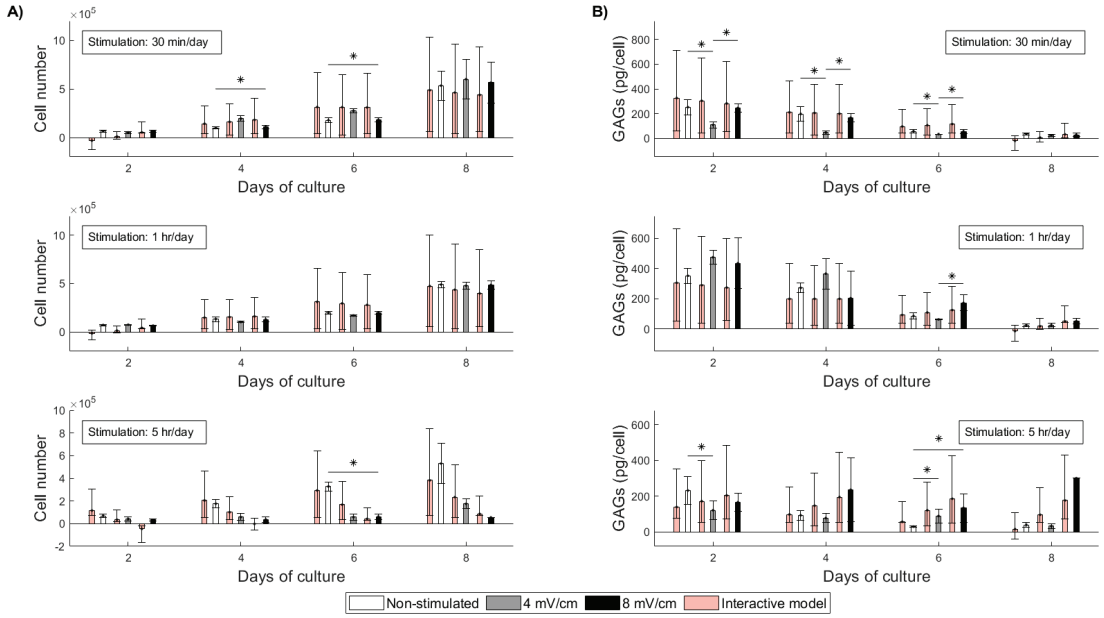

Fig. 7. Representative scheme of cell proliferation and GAGs synthesis of non-stimulated and stimulated cultures with EFs compared to data obtained by the interactive model. Predictions (red bars) for each experimental data (grey scale bars) are located on the left side, A) Cell population of chondrocytes, B) GAGs synthesis of chondrocytes. Each scale bar corresponds to the average of three different samples. Experimental data were statistically analyzed, evidencing significant differences between stimulation schemes and controls ($p < 0.05$*). The prediction probability of the model was evidenced with error bars, considering a confidence interval of 95%

$$+ 2.44\, EF * T_s + 8.12\, D_c * T_s$$
$$+ 2.61\, EF^2 + 4.1\, D_c^2 - 46.48\, T_s^2. \quad (25)$$

Simulation of the reduced, interactive, quadratic and combined models evidenced the determination coefficients R^2 and R^2_{ADJ} for number of cells/dish and content of GAGs/cell (Table 5).

Constants and coefficients to calculate right--skewed distribution (F) of the reduced, interactive, quadratic and combined models are in Table 6. Mod-

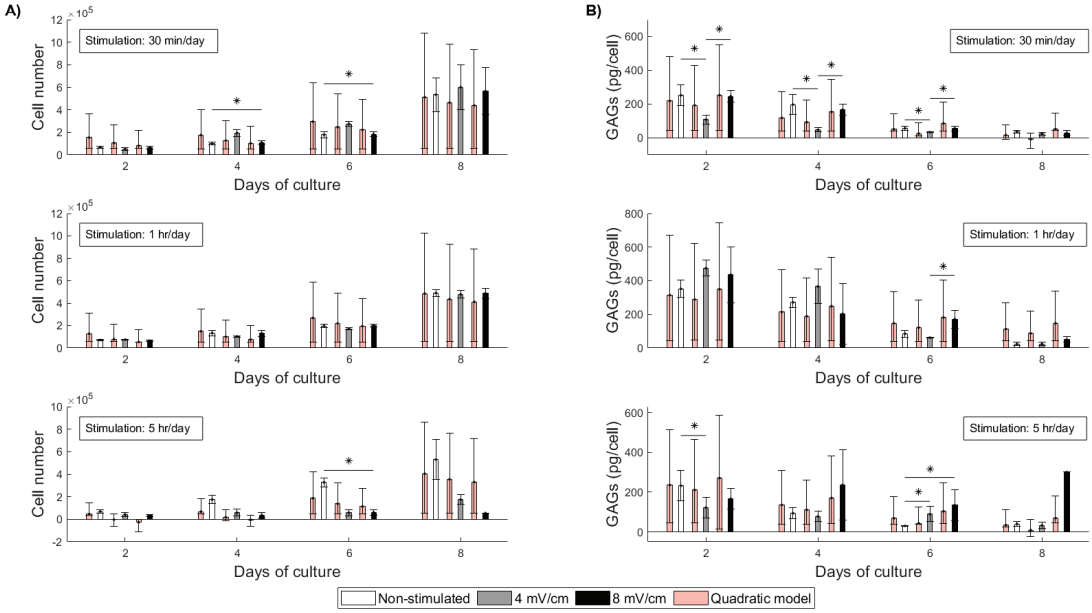

Fig. 8. Representative scheme of cell proliferation and GAGs synthesis of non-stimulated and stimulated cultures with EFs compared to data obtained by the quadratic model. Predictions (red bars) for each experimental data (grey scale bars) are located on the left side, A) Cell population of chondrocytes, B) GAGs synthesis of chondrocytes. Each scale bar corresponds to the average of three different samples. Experimental data were statistically analyzed, evidencing significant differences between stimulation schemes and controls ($p < 0.05$*). The prediction probability of the model was evidenced with error bars, considering a confidence interval of 95%

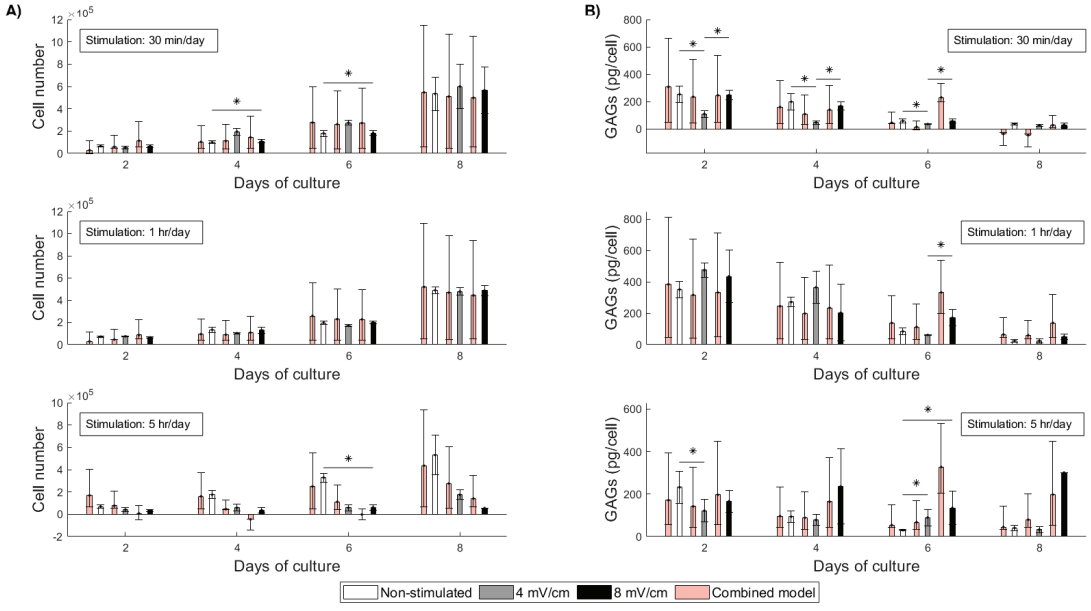

Fig. 9. Representative scheme of cell proliferation and GAGs synthesis of non-stimulated and stimulated cultures with EFs compared to data obtained by the combined model. Predictions (red bars) for each experimental data (grey scale bars) are located on the left side, A) Cell population of chondrocytes, B) GAGs synthesis of chondrocytes. Each scale bar corresponds to the average of three different samples. Experimental data were statistically analyzed, evidencing significant differences between stimulation schemes and controls ($p < 0.05$*). The prediction probability of the model was evidenced with error bars, considering a confidence interval of 95%

Table 5. Constants and coefficients obtained from the regression output of stimulated cultures with EFs

		Reduced model	Interactive model	Quadratic model	Combined model	Root square model
R^2	No cell/dish	61.79	74.34	69.11	81.66	88.19
	GAGs /cell	38.01	49.20	56.10	70.45	74.79
R^2_{ADJ}	No cell/dish	60.69	72.81	67.28	79.97	87.47
	GAGs /cell	36.22	46.18	53.39	67.62	72.66

Table 6. Constants and coefficients obtained from the regression output in cell cultures stimulated with EFs. F_{rejected} was calculated with an $\alpha = 0.05$

		Interactive model	Quadratic model	Combined model
SSER	No cell/dish	1.38E + 12		
	GAGs /cell	1.1E + 6		
SSEC	No cell/dish	9.31E + 11	1.12 + 12	6.65E + 11
	GAGs /cell	97E + 4	92E + 4	70E + 4
F_{rejected}	No cell/dish	2.68	2.68	2.17
	GAGs /cell			
F_{computed}	No cell/dish	16.45	7.98	17.68
	GAGs /cell	7.41	10	11.32

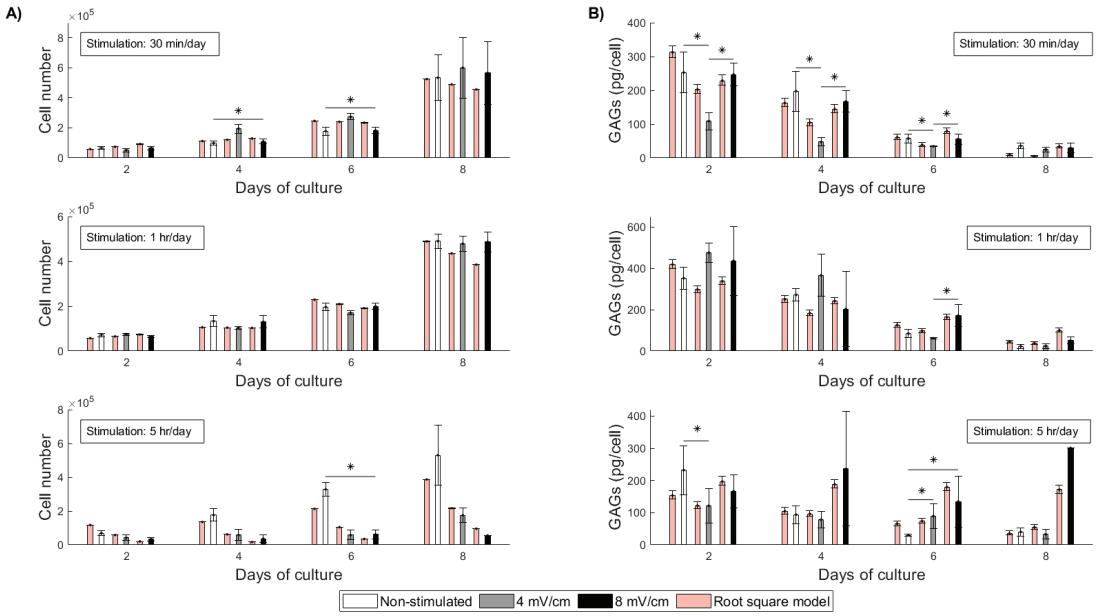

Fig. 10. Representative scheme of cell proliferation and GAGs synthesis of non-stimulated and stimulated cultures with EFs compared to data obtained by the root square model. Predictions (red bars) for each experimental data (grey scale bars) are located on the left side, A) Cell population of chondrocytes, B) GAGs synthesis of chondrocytes. Each scale bar corresponds to the average of three different samples. Experimental data were statistically analyzed, evidencing significant differences between stimulation schemes and controls ($p < 0.05$*). The prediction probability of the model was evidenced with error bars, considering a confidence interval of 95%

els that evidenced an $F_{\text{computed}} > F_{\text{rejected}}$ were intended to reject H_0, concluding that interactive, quadratic and combined terms contribute to the prediction of number of cells/dish and GAGs/cell.

Finally, data were transformed using the root square model and analyzed by using the combined model. Coefficients obtained from the model for both number of cells/dish and content of GAGs/cell are in Eqs. (26) and (27) (Fig. 10). This equation was obtained by eliminating outliers and predictors that do not accomplish levels of significance $p \leq 0.05$. Coefficients R^2 and R^2_{ADJ} for number of cells/dish and content of GAGs/cell are in Table 5.

$$\sqrt{\text{Nº}\,\frac{\text{Cells}}{\text{dish}}} = 213.60 - 16.02EF - 2.31\,EF * D_c - 7.33EF * T_s - 7.52D_c * T_s + 8.42D_c^2 + 6.81T_s^2, \quad (26)$$

$$\sqrt{\frac{\text{GAGs}}{\text{cell}}} = 19.03 - 1.68EF - 2.62D_c + 7.46T_s + 0.114EF * D_c - 0.116EF * T_s + 0.31D_c * T_s + 0.135EF^2 - 1.683T_s^2. \quad (27)$$

4. Discussion

Several mathematical and computational methods have been implemented for investigating chondrocyte dynamics. On the one hand, the logistic growth model was used by Grote et al. [21] to simulate the dynamic formation of oriented patches in chondrocytes. The Monod model, a mathematical approach that relates population growth rate to the concentration of a limiting resource, was implemented to simulate chondrocytes mitosis cultured in aterocollagen gels [27]. Similarly, the Contois model simulates the inhibition growth caused by cell density, implying saturation of growth due to spatial competition for resources [4]. This model has been implemented to simulate growth kinetics of chondrocytes seeded into polymer matrix [17]. On the other hand, cellular automata have been implemented to simulate chondrocyte dynamics in monolayer [50] and collagen constructs [27], and cells responsible for bone remodelling [51]. Markov chain models have been implemented to simulate, by a perturbational analysis, the role of molecules in the hypertrophic process of chondrocytes [26]. Different models have been implemented to simulate cell behaviour; however, there are not studies that simulate chondrocyte dynamics after stimulation by MFs and EFs. Accordingly, this study is the first attempt that elucidates different equations to predict chondrocyte proliferation and GAGs synthesis when stimulated by MFs and EFs. Each model was analysed to find the best equation that predicts cell population and GAGs synthesis. Therefore, the comparison between F_{rejected} and F_{computed} was performed to validate the contribution of β terms in the prediction of proliferation and GAGs synthesis. Regarding the equation that models the effect of MFs over expression of GAGs/cell, it was found that the interactive model had an F_{computed} = 0.48, smaller than F_{rejected} = 2.76. It means that β terms do not contribute to prediction of GAGs/cell. Comparing these results with the combined model, the F_{computed} = 3.04 was higher than F_{rejected} = 2.76, evidencing that β terms of combined model contribute to prediction of GAGs/cell. This behavior was not observed for those equations that simulated either proliferation or GAGs synthesis stimulated by MFs and EFs; however, the potential prediction of each equation varied according to the model used. In this case, R^2 were used to provide information about how the dependent variables variation could be explained by the models. The root square model was the equation that better predicted number of cells and GAGs. Regarding the root square model for MFs, it was evidenced that about 92.04 % and 71.06 % of the sample variation in cell population and GAGs synthesis, respectively, can be attributed to using *MF*, T_s, T_s^2 and D_c^2 variables to predict proliferation and GAGs synthesis. Concerning the root square model for EFs, it was evidenced that about 88.19 % and 74.79 % of the sample variation in cell population and GAGs synthesis, respectively, can be attributed to using *EF*, EF^2, T_s, T_s^2, D_c, and D_c^2 variables to predict proliferation and GAGs synthesis. A study by Freed et al. [16] found an equation to simulate chondrocyte proliferation in biodegradable polyglycolic acid scaffolds. Results evidenced that about 99.9 % of the sample variation in cell population can be attributed to using culture time as variable to predict cell density.

Models presented in this study are a combination of interactive and quadratic terms or a combination of both. In equations where interactive terms were considered, R^2 increased significantly, indicating that the models better predict either number of cells or GAGs synthesis when MFs or EFs are applied. However, not all interactive terms are relevant within the equations to enhance the prediction. For example, in Eq. (10), interaction terms such as $MF * D_c$ (p = 0.714) and $D_c * T_s$ (p = 0.465) are not statistically significant (data not shown), and can be removed from the equation [32]. Although the terms were not removed, it was possible to observe that all terms contribute to the prediction of proliferation, given that F_{computed} = 4.36 was higher than F_{rejected} = 2.76. SSER and SSEC parameters may be considered to assess whether the interactive and quadrative terms contribute to the model. In this context, SSEC should be smaller than SSER, and the difference SSER − SSEC – large [32]. For instance, in Table 3, the quadratic model evidenced SSEC = 1.68E + 10 and SSEC = 7E + 10 for number of cells and GAGs/cell, respectively. These values were smaller compared to SSER = 2.73E + 10 for cell population and SSER = 9E + 10 for GAG concentration. Similar values for SSER and SSEC were found in cultures stimulated with EFs. Considering that models fit these assumptions, it is possible to conclude that equations efficiently predict cell growth and GAGs synthesis when chondrocytes are

stimulated with MFs and EFs. Nevertheless, a greater range of experimental assays are needed to increase R^2 and avoid negative values.

Interpretation of β coefficients is crucial to understand how these values influence over equations and allow the prediction. For instance, in Eq. (9) the picograms [pg] amount of GAGs expressed by one chondrocyte is given by GAGs/cell = 54.87 + 5.27MF – 4.56D_c + 0.47T_s. Here, β_1 = 5.27, indicating that of GAGs/cell increases, the amount of 5.27 times for 1 unit of MF when D_c and T_s are fixed. A similar interpretation of β_2 = 4.56 can be expressed, where the amount of GAGs/cell decreases by 4.56 for 1 unit of D_c when MF and T_s are fixed. Regarding β_3 = 0.47, pg of GAGs/cell will increase 0.47 for 1 unit of T_s when the MF and D_c are fixed. Finally, β_0 = 54.87 has into account the pg of GAGs/cell when MF, D_c and T_s are zero. This interpretation can be performed for all equations in this study. Even though the main application of this equations lies on the prediction of number of cells and amount of GAGs/cell when chondrocytes are stimulated with MFs and EFs, it is possible to use these models to test the following hypotheses: 1) the mean of the number of pg of GAGs/cell increases as the filed strength increases when D_c and T_s are constant and 2) the number of cells per dish decreases as stimulation time T_s increases when D_c and MF are constant. These assumptions need to be confirmed by performing computational simulations to accept or reject the null hypothesis.

Limitations of using a regression analysis could be observed in the linear relationships of dependent and independent variables, as this kind of models assume that there is a straight-line relationship between the variables [32]. This assumption in cell proliferation can be incorrect given that cells in monolayer have two different phases. The Lag phase (from day zero to day fourth) where cells become accustomed to the ambient conditions, and the Log phase (from day fourth to one week) where cells experience an exponential growth [50]. In this study, the reduced and interactive models could not predict efficiently cell proliferation; however, the quadratic, combined and root square models can better predict number of cells due to the exponential variables. Mathematical models can only be used to qualitatively predict cell proliferation, and computational results have limited application for predicting outcomes of alternative therapy regimens with any precision [23]. We observed this behaviour in models where the prediction calculated a negative value. Another limitation using models to predict cell dynamics regards to observational data derived from *in vitro* experiments, as data is subjected to randomness due to experimental noise and imprecision from measuring devices [23]. Although experimental analyses were performed using the same measuring devices and same reagents, it is relevant to consider these variables to find models that better predict cell behaviour. In fact, equation coefficients vary for cell type, as each cell has a different dynamic depending on the host tissue.

Given that models were fitted using data derived from experimental assays in laboratory, these equations are useful to predict chondrocyte behavior in therapies carried out in the same conditions such as chondrocyte autologous implantation, where cells are grown *in vitro* to be implanted in the injured cartilage [33]. For equations with a clinical relevance, it is necessary to feedback the models with data obtained clinically [23]. Future perspectives may be focused on developing mathematical models that simulate chondrocyte behaviour in three-dimensional environments, where chondrocytes not only maintain its ideal morphology, but also efficiently express the main molecules such as collagen type II, SOX 9 and aggrecan, since these molecules are crucial for cartilage maintenance.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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