Adopted Proposed Elaborations of CONSORT Items for Randomized, Controlled Trials of Herbal Medicine Interventions to a Rooster Comb Extract*

Section/Topic	Item No	Descriptor	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	4
Introduction			
Background	2a	Scientific background and explanation of the rationale	5
	2b	Including a brief statement of reasons for the trial with reference to the specific herbal medicinal product being tested and, if applicable, whether new or traditional indications are being investigated. Specific objectives or hypotheses	5-6
Methods			
Trial design		Description of trial design (such as parallel, factorial) including allocation ratio	6-7
		Important changes to methods after trial commencement (such as eligibility criteria), with reasons	-
Participants	3	Eligibility criteria for participants and the settings and locations where the data were collected	
		If a traditional indication is being tested, a description of how the traditional theories and concepts were maintained. For example, participant inclusion criteria should reflect the theories and concepts underlying the traditional indication	6-8
		Settings and locations where the data were collected	-
Interventions	4	Precise details of the interventions intended for each group and how and when they were actually administered. A detailed description of this item 4 for reporting	-

		Randomized Controlled Trials of Herbal Medicine Interventions on CONSORT item 4 appears in Supplementary Table S2	7
		The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	
Objectives	5	Specific objectives or hypotheses	6
Outcomes	6	Clearly defined primary and secondary outcome measures and, when applicable, any methods used to enhance the quality of measurements (eg, multiple observations, training of assessors)	
		Outcome measures should reflect the intervention and indications tested, considering, where applicable, underlying theories and concepts	8
		Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	
		Any changes to trial outcomes after the trial commenced, with reasons	
Sample size	7	How sample size was determined	11
		When applicable, explanation of any interim analyses and stopping guidelines	
Randomisation Sequence allocation	8	Method used to generate the random allocation sequence, including details of any restriction (eg, blocking, stratification)	7-8
Sequence generation	8b	Method used to generate the random allocation sequence, including details of any restriction (eg, blocking, stratification Type of randomisation; details of any restriction (such as blocking and block size)	-
Allocation concealment	9	Method used to implement the random allocation sequence(eg, numbered containers or central telephone), clarifying whether the sequence was concealed until interventions were assigned	7-8

Implementation	10	Who generated the allocation sequence, who enrolled participants, and who assigned participants to their groups	7-8
Blinding (Masking)	11a	Whether or not participants, those administering the interventions, and those assessing the outcomes were blinded to group assignment. When relevant, how the success of blinding was evaluated	7-8
		If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how If relevant, description of the similarity of interventions	
Statistical methods	12	Statistical methods used to compare groups for primary outcome(s); Methods for additional analyses, such as subgroup analyses and adjusted analyses	
		Statistical methods used to compare groups for primary and secondary outcomes	10-11
		Methods for additional analyses, such as subgroup analyses and adjusted analyses	
Results			
Participant flow (a diagram is strongly recommended)	13	Flow of participants through each stage (a diagram is strongly recommended) Specifically, for each group report the numbers of participants randomly assigned, receiving intended treatment, completing the study protocol, and analyzed for the primary outcome. Describe protocol deviations from study as planned, together with reasons	11
		For each group, losses and exclusions after randomisation, together with reasons	-
		Dates defining the periods of recruitment and follow-up	-

	14	Dates defining the periods of recruitment and follow-up. Why the trial ended or was stopped	
Recruitment			-
Baseline data	15	Baseline demographic and clinical characteristics of each group. Including concomitant medication, herbal and complementary medicine use. A table showing baseline demographic and clinical characteristics for each group	11 and Table 1
Numbers analysed	16	Number of participants (denominator) in each group included in each analysis and whether the analysis was by "intention-to-treat." State the results in absolute numbers when feasible (eg, 10/20, not 50%)	11
Outcomes and estimation	17	For each primary and secondary outcome, a summary of results for each group, and the estimated effect size and its precision (eg, 95% confidence interval)	11-13
		For binary outcomes, presentation of both absolute and relative effect sizes is recommended	-
Ancillary analyses	18	Address multiplicity by reporting any other analyses performed, including subgroup analyses and adjusted analyses, indicating those prespecified and those exploratory	-
Harms	19	All important adverse events or side effects in each intervention group	8

Discussion			
Limitations	20	Interpretation of results, taking into account study hypotheses, sources of potential bias or imprecision, and the dangers associated with multiplicity of analyses and outcomes *Interpretation of the results in light of the product and dosage regimen used* Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	14 and 16
Generalisability	21	Generalizability (external validity) of trial results (applicability) of the trial findings Where possible, discuss how the herbal product and dosage regimen used relate to what is used in self-care and/or practice	14
Overall evidence	22	General interpretation of the results in the context of current evidence. Discussion of the trial results in relation to trials of other available products Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	14-17
Other information			
Registration	23	Registration number and name of trial registry	3
Protocol	24	Where the full trial protocol can be accessed, if available	-
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	17-18

SUPPLEMENTARY MATERIAL AND METHODS

Cell culture and reagents

Murine C2C12 myoblasts were obtained from the European Collection of Authenticated Cell Cultures. Myoblasts were routinely cultured in a humidified incubator containing 5% CO_2 and 95% O_2 at 37 ºC in complete medium (CM). CM consists of high glucose-Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% of heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin (all reagents from Biowest, France).

High molecular weight-Hyaluronic Acid (HA) was obtained from Bioiberica S.A.U. with a mean molecular weight of 900.000 Da. Rooster Comb Extract (RCE) was obtained from Bioiberica S.A.U. containing 65% of HA (mean molecular weight of 900.000 Da). HA and RCE were dissolved in water to a final concentration of 5 mg/mL. Both solutions were shaken for 2 h at room temperature and for an additional hour at 50ºC to enhance the compounds' solubility, and stored at -20ºC.

The inhibitor of endogenous HA synthesis 4-Methylumbelliferone (4MU; Sigma-Aldrich, Spain) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 400 mM and stored at -20ºC.

The inhibitor of cell proliferation PD98059 (Calbiochem, Germany) was dissolved in DMSO at a concentration of 50 mM and stored at -80ºC.

Study of myoblasts proliferation

C2C12 myoblasts were cultured in CM until they reached 20% of confluency. At this time point, HA (0.5 mg/mL), RCE (0.83 mg/mL), and PD98059 (10 μM) were added to CM for 48 and 72 h. This medium was refreshed every 24 h until the end of the experiment.

PD98059 was used as a negative control of proliferation since it is a specific inhibitor of mitogen-activated protein kinases, a pathway that plays an important role in cell proliferation.[1]

An additional batch of experiments was performed in DM since C2C12 myoblasts incubated in this medium cease proliferation, exit cell cycle, and undergo differentiation to myotubes due to the reduced concentrations of growth factors.[2]

Cells proliferation was monitored by the CellTiter-Glo luminescent assay (Promega, Spain) based on quantification of ATP. The amount of ATP is directly proportional to the number of metabolically active cells present in culture and therefore, proportional to the proliferation process. At the end of the experiments, CellTiter reagent was added to cells (100 μL/well) and mixed on an orbital shaker for 2 min. The plate was then incubated at room temperature for 10 min and luminescence was monitored in a black plate in a Synergy HT reader (BioTek Instruments, Spain). Proliferation values are expressed as arbitrary units (AU).

Study of myoblasts differentiation

C2C12 myoblasts were cultured in CM until they reached a pre-confluent status (70-90% confluency). At this time point, 4MU (0.25, 0.5, and 1 mM) was added to CM for 24 h. Myogenic differentiation was then induced by replacing CM with differentiation medium (DM) consisting of DMEM supplemented with 2% of horse serum, 100 U/mL penicillin, and 100 mg/mL streptomycin (all reagents from Biowest, France). HA (0.1 mg/mL), RCE (0.164 mg/mL), and 4MU were added to DM for 24, 48, and 72 h. This DM was refreshed every 24 h until the end of the experiment.

4MU, a known inhibitor of endogenous hyaluronan synthesis, was used in this set of experiments to discard the endogenous HA effect on myoblasts differentiation. Consequently, it can be assured that the observed effects can be only attributed to the effect of exogenous HA and RCE.

Two additional batches of experiments were performed: a) in the absence of 4MU and b) using CM as a negative control of differentiation.

Cells differentiation was optically assessed using phase-contrast microscopy and by the determination of creatine kinase (CK) activity. For this purpose, cells were washed twice with cold phosphate buffered saline, lysed with 200 μL of lysis buffer (40 mM 2-N-morpholinoethanesulfonic acid, 50 mM Trizma, 1% v/v Triton X-100, and proteases

inhibitors), and then scrapped on ice. Cell lysates were centrifuged (16000 g, 10 min, 4 ºC) and pellets were discarded. CK activity was measured in the supernatants in the automated analyzer Cobas-Mira Plus (Roche Diagnostic System, Spain) using CK-NAC reagent (Spinreact, Spain), by the change in absorbance at 340 nm over 3 min (25-s intervals) at 37 ºC. CK activity was normalized to protein content measured by Bradford assay [3] using a 4-point standard curve with bovine serum albumin (Sigma-Aldrich, Spain). Therefore, CK activity is expressed as U/mg of protein (1 U = 1μmol of substrate hydrolyzed per minute).

Cell viability and cytotoxicity assessment

Cell viability was assessed by trypan blue exclusion (Merck, Spain) using phase-contrast microscopy.

Cytotoxicity was assessed by lactate dehydrogenase (LDH) Cytotoxicity Detection Kit (Roche Applied Science, Germany) as previously described.[4] LDH is an intracellular enzyme that is rapidly released into the cell culture supernatant when the plasma membrane is compromised. This results in a colorimetric reaction that can be measured at a wavelength of 492 nm. The activity of LDH released from cells was measured in cell-free supernatants collected at all time points tested (0, 24, 48, and 72 h after differentiation or proliferation induction) in a Synergy HT reader (BioTek Instruments, Spain).

SUPPLEMENTARY RESULTS

Myoblast differentiation and proliferation in CM and DM

C2C12 cells proliferation was studied after 48 and 72 h of incubation in CM and DM. Cells incubated in CM alone, but not in DM, proliferated in a time-dependent way ($P<0.05$). As expected, proliferation was lower when cells were incubated in DM alone (Supplementary Figure S1).

When it comes to myoblast differentiation, cells incubated in DM alone displayed a myotube morphology which became more evident as days advanced, especially after 48 h of incubation on ahead (Supplementary Figure S2). CK activity was higher when cells were incubated in DM than in CM. In both media, CK activity was time-dependent after 48h and 72h of incubation ($P<0.05$; Supplementary Figure S3). These results were optically verified by phase-contrast microscopy (data not shown).

Effect of 4MU on myoblast differentiation

Incubation of 4MU for 72h inhibited CK activity at 0.5 and 1mM by 42.90 and 52.23% respectively versus blank condition. No statistically significant changes in inhibition were found between both doses ($P<0.05$; Supplementary Figure S4). Consequently, 0.5 mM was chosen as the concentration used for the following experiments. These results were optically verified by phase-contrast microscopy (data not shown).

SUPPLEMENTARY REFERENCES

1 J. Girona, R. Rosales, N. Plana, P. Saavedra, L. Masana and J. C. Vallvé, *PLoS One*, 2013, **8**, 1–12.

2 N. A. Dumont, Y. X. Wang and M. A. Rudnicki, *Development*, 2015, **142**, 1572–1581.

3 M. Bradford, *Anal. Biochem.*, 1976, **7**, 248–54.

4 S. Fernández-Castillejo, P. Formentín, Ú. Catalán, J. Pallarès, L. F. Marsal and R. Solà, *Beilstein J. Nanotechnol.*, 2017, **8**, 675–681.

SUPPLEMENTARY TABLES

Supplementary Table S1. Total work of the affected joint, age subgroup analyses.

Characteristic	Position	Angle	Treatment	Baseline	Change at 12 weeks relative to baseline	Treatment difference	
				Mean±SD	Adjusted mean [95%CI] (% difference from baseline)	Adjusted mean [95%CI] (% difference from placebo)	*P*
Over 50	**Flexion**	**180**	**Placebo (n = 35)**	186.14±87.15	12.12 [-4.76; 29.01] (6.5%)	25.40 [2.66; 48.14] (13.6%)	0.0291
			RCE (n = 38)	167.81±94.45	37.52 [21.08; 53.96] (22.4%)		
		240	**Placebo (n = 35)**	147.00±81.11	25.42 [11.10; 39.75] (17.3%)	-6.08 [13.07; -25.22] (-4.1%)	0.5288
			RCE (n = 38)	142.89±81.59	19.35 [5.56; 33.14] (13.5%)		
	Extension	**180**	**Placebo (n = 35)**	349.23±153.13	23.75 [2.34; 45.16] (6.8%)	1.04 [-27.70; 29.79] (0.3%)	0.9425
			RCE (n = 38)	328.44±125.86	24.80 [4.09; 45.50] (7.6%)		
		240	**Placebo (n = 35)**	302.47±137.26	26.85 [7.73; 45.97] (8.9%)	-11.24 [-36.79; 14.32] (-3.7%)	0.3835
			RCE (n = 38)	295.89±120.86	15.62 [-2.78; 34.01] (5.3%)		
Under 50	**Flexion**	**180**	**Placebo (n = 35)**	200.75±132.91	28.77 [1.83; 55.71] (14.3%)	19.00 [-16.96; 54.97] (9.5%)	0.2953
			RCE (n = 35)	214.01±149.45	47.78 [19.80; 75.76] (22.3%)		
		240	**Placebo (n = 35)**	181.07±126.26	28.00 [1.50; 54.51] (15.5%)	12.60 [-23.01; 48.21] (7.0%)	0.4824
			RCE (n = 35)	203.56±142.96	40.60 [13.13; 68.08] (19.9%)		
	Extension	**180**	**Placebo (n = 35)**	400.93±207.39	17.34 [-27.12; 61.80] (4.3%)	61.62 [2.21; 121.00] (15.4%)	0.0423
			RCE (n = 35)	419.63±214.55	78.96 [32.90; 125.01] (18.8%)		
		240	**Placebo (n = 35)**	366.63±190.17	15.92 [-26.89; 58.73] (4.3%)	50.50 [-6.94; 107.90] (13.8%)	0.0839
			RCE (n = 35)	389.69±202.25	66.42 [22.08; 110.76] (17%)		

All results are expressed as means ± standard deviation and baseline adjusted least square means [95%CI].
RCE= Low-fat yoghurt supplemented with 80mg of natural rooster comb extract rich HA (65%)**.**

Supplementary Table S2. Consort Herbal Extension to guide them as to the level of detail and other information required about this extract.

Standard CONSORT Checklist	Standard CONSORT Checklist Item	Descriptor	Reported on Page Number	Row	Text
Methods	4.	Where applicable, the description of a herbal intervention should include			
Interventions	4.A.Product name	1. The Latin binomial name together with botanical authority and family name for each herbal ingredient; common name(s) should also be included	-	-	-
		2. The proprietary product name (ie, brand name) or the extract name (eg, EGb-761) and the name of the manufacturer of the product	7	133	Mobilee® ; Bioiberica S.A., Palafolls, Spain
		3.Whether the product used is authorized (licensed, registered) in the country in which the study was conducted	-	-	We approached the present manuscript when RCE had been approved by the European Commission as a Novel Food ingredient
	4.B. Characteristics of the product	1. The part(s) used to produce the product or extract.	5	90-91	Food grade rooster combs
		2. The type of product used (eg, raw [fresh or dry], extract)	5	90-91	Food grade rooster combs
		3. The type and concentration of extraction solvent used (eg, 80% ethanol, H_2O 100%, 90% glycerine, and others) and the herbal drug to extract ratio (drug: extract; eg, 2:1)	-	-	The details of the manufacturing process to obtain the rooster comb extract is considered know-how of the company
		4. The method of authentication of raw material (ie, how done and by whom) and the lot number of the raw material. State whether a voucher specimen (ie, retention sample) was retained and, if so, where it is kept or deposited and the reference number	-	-	The raw material was validated according to internal specification

4.C. Dosage regimen and quantitative description	1. The dosage of the product, the duration of administration, and how these were determined	7	132-135	80 mg/d of RCE, over a period of 12 weeks. The dose and treatment duration were determined according to previous studies (Martinez-Puig et al 2013; Sanchez et al 2014)
	2. The content (eg, as weight, concentration may be given as range where appropriate) of all quantified product constituents, both native and added, per dosage unit form. Added materials, such as binders, fillers, and other excipients; eg, 17% maltodextrin, 3% silicon dioxide per capsule, should also be listed	7	138-141	RCE contained HA (65%), hidrolized proteins (specially collagen) and other polysaccharides.
	3. For standardized products, the quantity of active/marker constituents per dosage unit form	7	138-139	a low-fat yoghurt (125 mL/d) supplemented with 80 mg/d of RCE
4.D.Qualitative testing	1. Product's chemical fingerprint and methods used (equipment and chemical reference standards) and who performed it (eg, the name of the laboratory used). Whether or not a sample of the product (ie, retention sample) was retained and, if so, where it is kept or deposited	-	-	The content of HA in the final yogurt was determined according to the method described by Coleman et al 1997
	2. Description of any special testing/purity testing (eg, heavy metal or other contaminant testing) undertaken. Which unwanted components were removed and how (ie, methods)	-	-	Heavy metals, dioxins, furans and PCB's were analyzed according to the official methods of European Pharmacopoeia
	3. Standardization: what to (eg, which chemical component(s) of the product) and how (eg, chemical processes or biological/functional measures of activity)	-	-	-
4.E. Placebo/control group	The rationale for the type of control/placebo used	7-8	142-143 and 153-154	Participant assignment to treatment or placebo arm was at a ratio of 1:1
4.F. Practitioner				

SUPPLEMENTARY FIGURE CAPTIONS

Supplementary Figure S1. Proliferation of C2C12 myoblasts incubated in Complete Medium (CM) and Differentiation Medium (DM). C2C12 myoblasts were cultured in CM or DM for 48 and 72h and media were refreshed every 24 h. At the end of experiments, proliferation was monitored by the CellTiter-Glo luminescent assay based on quantification of ATP. † $P<0.005$.

Supplementary Figure S2. Images of C2C12 myoblast differentiation process. C2C12 myoblasts were cultured in DM for a long period and images were captured at several time points using phase-contrast microscopy. Scale bars correspond to 1000 μm (x4 column) and 500 μm (x10 column).

Supplementary Figure S3. Differentiation of C2C12 myoblasts incubated in CM and DM. C2C12 myoblasts were cultured in CM until they reached a pre-confluent status (70-90% confluency). At this time point, myogenic differentiation was induced by replacing CM with DM for 24, 48, and 72 h; the DM was refreshed every 24 h. Cells' differentiation was assessed by the determination of creatine kinase (CK) activity. * $P<0.05$ versus 0h condition in CM; † $P<0.05$ versus 24h in the same culture media; ‡ $P<0.05$ versus 48h in the same culture media; § $P<0.05$ between conditions.

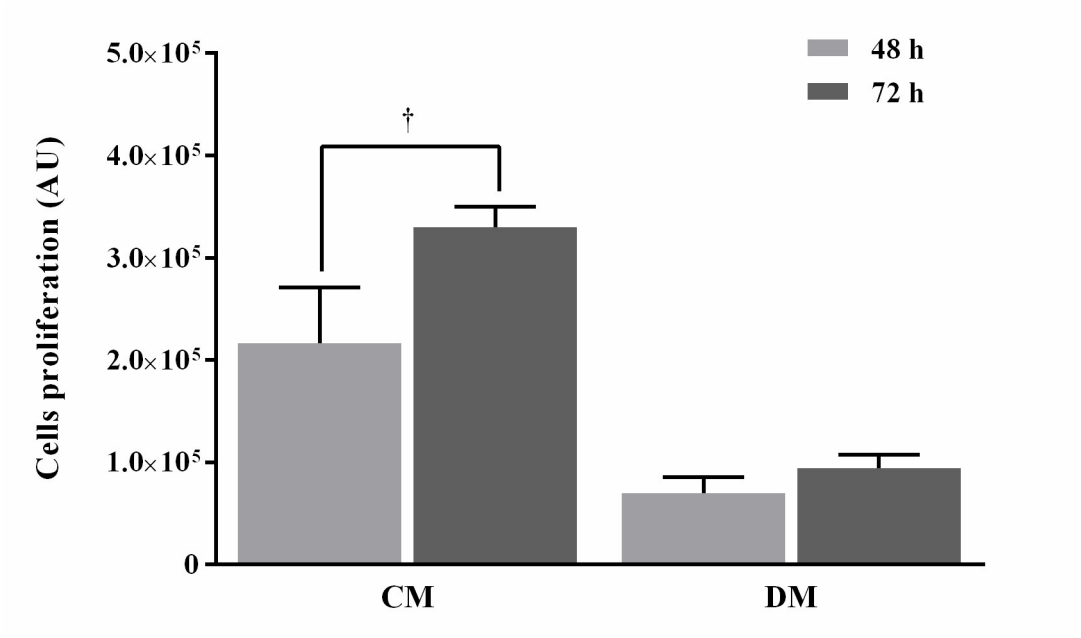
Supplementary Figure S4. Dose- and time-response to 4-Methylumbelliferone (4MU). C2C12 myoblasts were cultured in CM until they reached a pre-confluent status (70-90% confluency). At this time point, the inhibitor of endogenous hyaluronan synthesis 4MU (0.25, 0.5, and 1 mM) was added to CM for 24 h. Afterward, myogenic differentiation was induced by replacing CM with DM. 4MU was added to DM for additional 24, 48, and 72 h, and refreshed every 24 h. Cells differentiation was assessed by the determination of creatine kinase (CK) activity. *$P<0.05$ versus 0h; † $P<0.05$ versus 24h; ‡ $P<0.05$ versus 48h; § $P<0.05$ versus 0mM; £ $P<0.05$ versus 0.25mM; ¥ $P<0.05$ versus 0.5mM.

Supplementary Figure S5. Effects of HA and RCE on C2C12 myoblast differentiation. C2C12 myoblasts were cultured in CM until they reached a pre-confluent status (70-90% confluency). At this time point 4MU (0.5 mM) was added to CM for 24 h and then myogenic differentiation was induced by replacing CM with DM. HA (0.1 mg/mL), RCE (0.164 mg/mL), and 4MU were added to DM for 24, 48, and 72 h. The DM was refreshed every 24

h until the end of the experiment. Cells differentiation was optically assessed using phase-contrast microscopy and by the determination of creatine kinase (CK) activity. No statistical significant levels were observed.

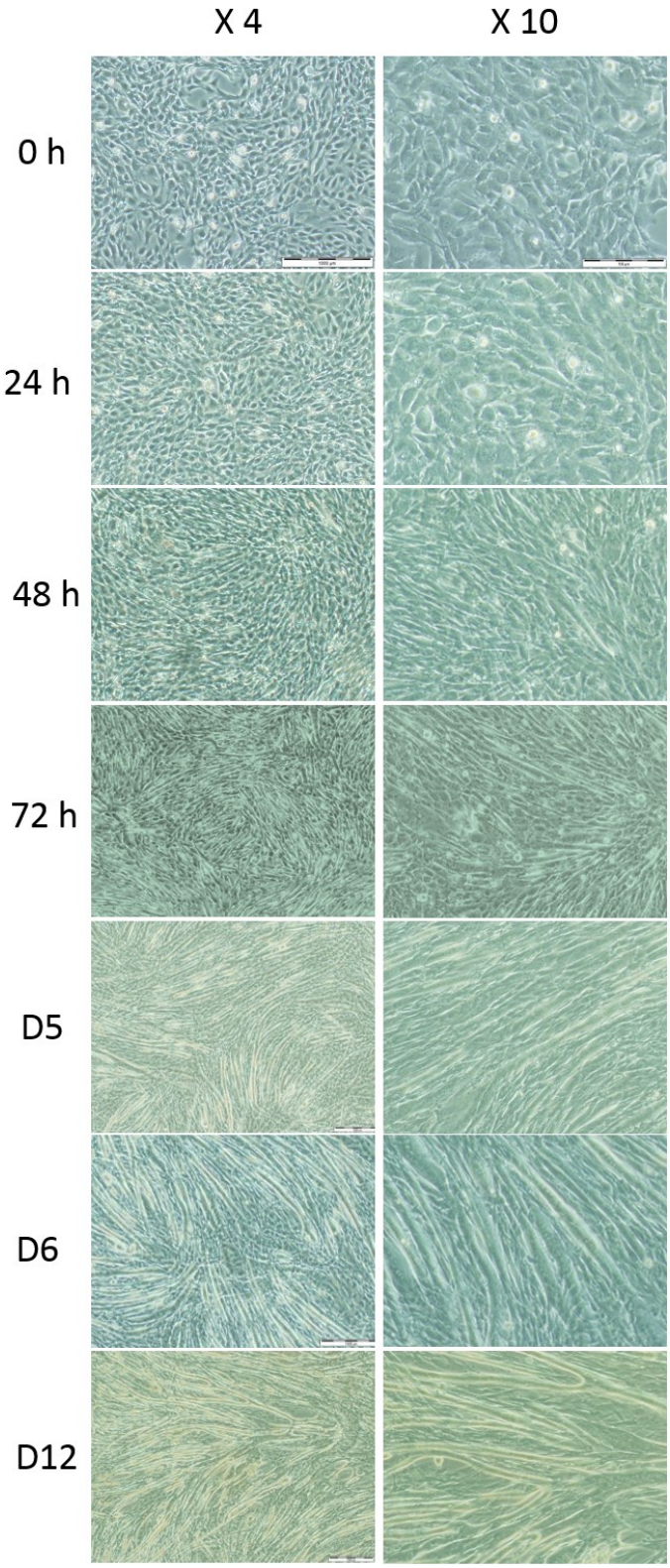
SUPPLEMENTARY FIGURES

Supplementary Figure S1. Proliferation of C2C12 myoblasts incubated in Complete Medium (CM) and Differentiation Medium (DM).

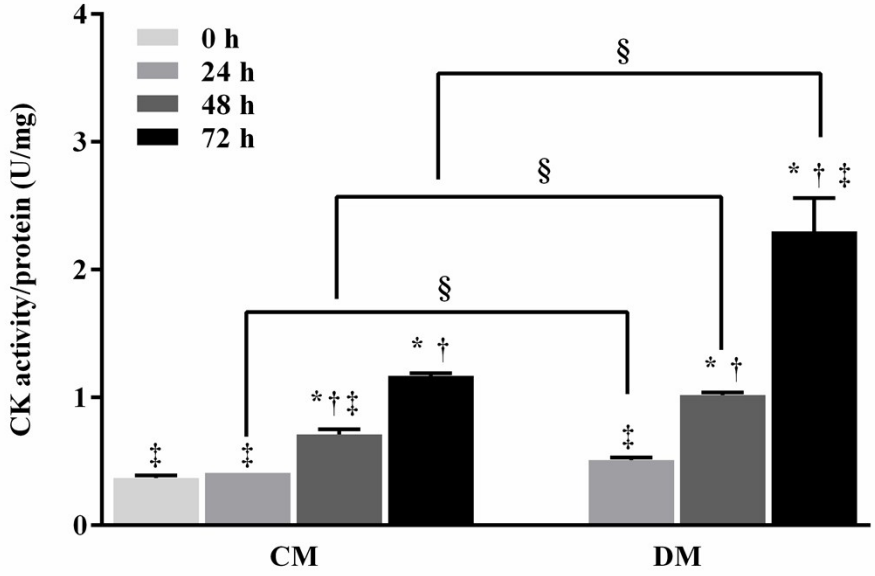


P<0.005

Supplementary Figure S2. Images of C2C12 myoblast differentiation process.



Supplementary Figure S3. Differentiation of C2C12 myoblasts incubated in CM and DM.



P<0.05 versus:

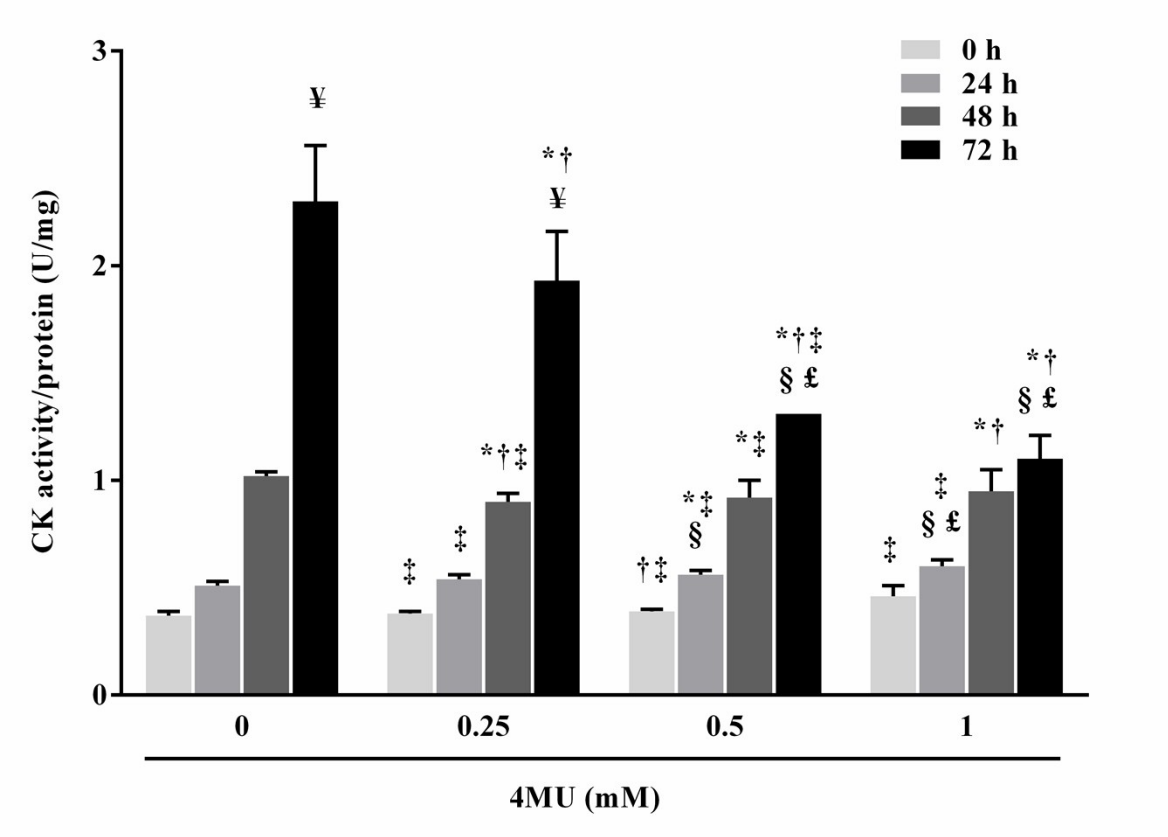
§ P<0.05 between conditions.

* 0h condition in CM;

† 24h in the same culture media;

‡ 48h in the same culture media;

Supplementary Figure S4. Dose- and time-response to 4-Methylumbelliferone (4MU).



Time-dependent differences:

*P<0.05 versus 0h;

† P<0.05 versus 24h;

‡ P<0.05 versus 48h;

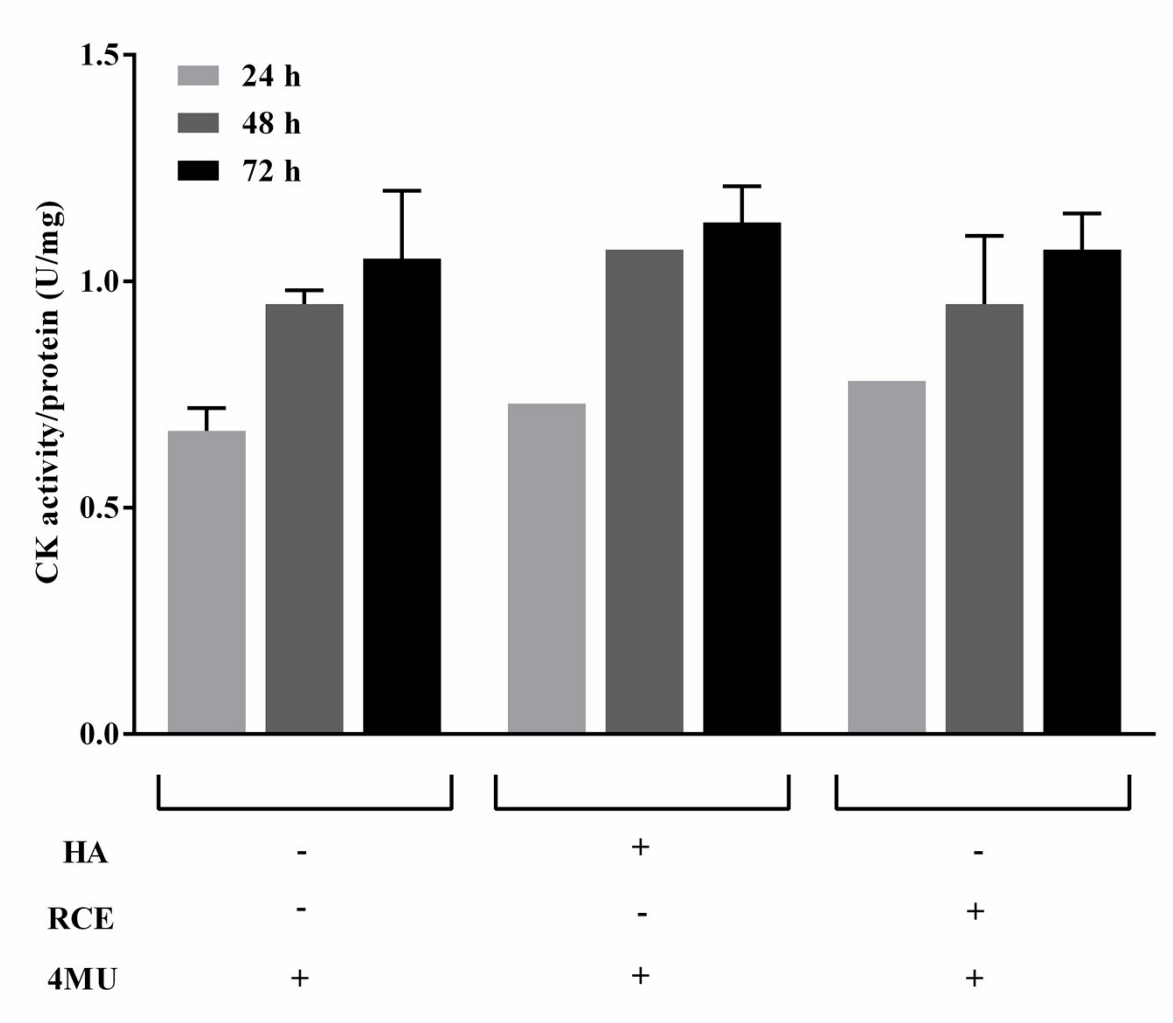
4MU dose-dependent differences:

§ P<0.05 versus 0mM;

£ P<0.05 versus 0.25mM;

¥ P<0.05 versus 0.5mM.

Supplementary Figure S5. Effects of HA and RCE on C2C12 myoblast differentiation.



No statistical differences were observed.