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Effectiveness of a low-fat yoghurt supplemented with rooster comb extract on muscle strength in adults with mild knee pain and mechanisms of action on muscle regeneration.

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Abbreviations:

4MU: 4-Methylumbelliferone

ADO: Available Data Only

BMI: Body Mass Index

CK: Creatine kinase

CM: Complete medium

DM: Differentiation medium

HA: Hyaluronic acid

ITT: Intention-To-Treat

LDH: Lactate dehydrogenase

MAPK: Mitogen-activated protein kinases

MAR: Missing at Random

OA: Osteoarthritis

RCE: Rooster Comb Extract

SD: Standard Deviation

VAS: Visual Analogue Scale

Electronic supplementary information is available.

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ABSTRACT

Purpose: To determine the effects of the intake of low-fat yoghurt supplemented with rooster comb extract (RCE) on muscle strength. **Methods and results:** 148 subjects, with mild knee pain, participated in a randomized, placebo-controlled, double-blind, and parallel study. Muscle strength, knee effusion, and pain perception were measured. C2C12 myoblasts were used to elucidate the mechanisms of action involved. RCE improved total work and mean power in men, and also peak torque in extension by 10%. RCE reduced synovial effusion by 11.8% and pain perception by 24.6%. Both RCE and HA increased myoblasts proliferation by a 29%, while RCE reduced myoblast differentiation, suggesting a beneficial role of RCE on muscle regeneration. **Conclusions:** Low-fat yoghurt supplemented with RCE improved muscle strength. This effect is partially explained by muscle regeneration enhancement, reduced synovial effusion, and reduced pain perception, which could exert a beneficial clinical impact on men affected by mild knee pain.

Keywords: Mechanisms of action; mild knee pain; muscle regeneration; myogenesis; osteoarthritis.

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1. INTRODUCTION

Knee osteoarthritis (OA) is a cause of chronic disability in older people, becoming a public health concern in western populations.[1] In knee OA, the loss of hyaline articular cartilage is the pathologic basis whereas muscular deficiencies are a frequent cause of instability and unbalance.[2] Muscular disability should be reduced to preserve joint stability and to prevent falls.[3] Muscular disability is related to a reduction of the quadriceps strength of the affected extremity,[4] therefore knee joint is more susceptible to further damage and pain.[5] Knee extensors (mainly the quadriceps muscles) are involved in physical activities such as running, jumping, or kicking the ball, whereas knee flexors (mainly the hamstrings muscles) are required in physical activities influencing the stride length and stabilizing the knee joint in changing direction, for acceleration and deceleration, and during landing.[6] In this context, the magnitude of muscle strength improvement is a cardinal element of the treatment of knee OA.[6,7] In this sense, identification of modifiable factors, such as quadriceps strength, that could mitigate the severity or pace of worsening of knee OA could potentially reduce the significant burden of subsequent disability.[8] Therefore, treatment of knee OA mild joint discomfort was aimed at alleviating pain, improving mobility, increasing the torque by enhancing muscle strength in the joints, and slowing the progression to OA.[7]

In a preliminary study, the effect of daily oral supplementation with natural rooster comb extract (RCE) naturally rich in Hyaluronic Acid (HA; 65%), collagen, and other polysaccharides included in a low-fat yoghurt matrix, was studied in 20 participants with mild knee joint discomfort (defined as Visual Analog Scale (VAS) between 10 and 40 mm), compared with 20 participants who consumed a non-supplemented yoghurt for a period of 12 weeks.[9] Compared with the placebo group, RCE consumption produced a significant increase in the maximum peak torque, total work, and mean power of the knee extensors at 240º/s, reflecting the effectiveness on quadriceps muscle strength evaluated objectively by an isokinetic dynamometer.[9] The improvement in knee muscle strength after the daily intake of a low-fat yoghurt supplemented of RCE (80 mg of RCE) in healthy volunteers with mild joint discomfort, was further verified in two subsequent studies.[10,11] Orally-

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administered HA has been described to be absorbed and ubiquitously distributed to organs and joints,[12] thus explaining why an HA-rich oral supplement can exert an effect at lower limb skeletal muscle, as observed after RCE intake. Consequently, RCE's mechanism of action, linked or not to its HA content, may offer new therapeutic tools for muscle regeneration or myogenesis in early stages of knee OA.[13]

Mammalian skeletal muscle has the ability to regenerate in response to muscular injury, such as in the case of knee OA. Regeneration of skeletal muscle is carried out by satellite cells, a population of stem cells that are mitotically quiescent that reside between the sarcolemma and the basal lamina of the muscle [14,15]. It has been shown that upon muscle injury present in several medical conditions such as knee OA, muscle satellite cells are activated. These activated cells, also known as myoblasts, enter the cell cycle and proliferate massively to generate the myogenic progenitors needed for muscle regeneration. Afterward, some myoblasts exit cell cycle and undergo differentiation to form multinucleated myotubes. However, after muscle injury proliferating myocytes not only differentiate to myotubes but they also keep proliferating to self-renew in order to maintain the satellite cell pool. The fate of the proliferating myocytes (to keep proliferating of to undergo differentiation) is a complex process highly regulated by myogenic regulatory factors. The activation of muscular satellite cells and the balance between myoblast proliferation and differentiation to myotubes are crucial to muscle regeneration [13]. To date, molecular mechanisms by which HA or HA-rich extracts influence muscle regeneration remain unclear.

With all the stated before, the hypothesis of the present study is that the daily consumption of low-fat yoghurt supplemented with 80 mg of RCE (containing 65% of HA) for 12 weeks improves knee muscular strength (in particular peak torque, total work, and power mean variables), joint synovial effusion, and subjective joint discomfort in adults with mild knee pain. To test this hypothesis the integration of the clinical findings on muscular strength using the individual data from each of the two aforementioned trials[10,11] was carried out. Moreover, we hypothesize that RCE and HA stimulate muscle regeneration by promoting some myogenesis stages such as myoblasts

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proliferation or differentiation to myotubes, as a possible mechanism for explaining the improvement of muscle strength observed in the clinical studies carried out in humans.

2. MATERIAL AND METHODS

2.1 Participants, design study, and intervention

After promising results obtained by Sanchez *et al.*,[11] an additional study was developed with the same protocol, previous standardization of researchers.[10] The individualized results from each participant of the two randomized, placebo-controlled, double-blind, and parallel nutritional intervention trials performed on patients with mild knee pain were analysed. The inclusion criterion was baseline pain evaluated by a VAS between 30 and 50 mm.[10,11] The exclusion criteria were: (1) the use of paracetamol or other drugs to control joint discomfort; (2) patients with active rheumatoid arthritis and any inflammatory arthritic conditions; (3) patients who have been treated with oral corticosteroids within 4 weeks prior to selection; (4) patients who have been treated with intraarticular corticosteroids within 12 weeks prior to selection; (5) patients with significant joint injury during the 3 weeks prior to screening (identified from medical history); (6) patients who were consuming drugs or dietary supplements for OA at the time of screening; (7) patients who depend on prescription drugs to control pain; (8) patients participating in a concurrent clinical trial, or having received a product being evaluated during the previous 30 days; (9) patients who are allergic to dairy products; (10) patients following an energy-restricted diet for weight loss; (11) pregnant or lactating individuals; (12) patients currently taking nutraceuticals with HA and/or other types of joint regenerators; and (13) patients suffering from axis alterations. Participants were randomized to receive either a low-fat yoghurt (125 mL/d) supplemented with 80 mg/d of RCE (Mobilee®; Bioiberica S.A., Palafolls, Spain) or the same low-fat yoghurt without RCE, over a period of 12 weeks.

RCE characteristics were described according to Consort Herbal Extension (see Supplementary Data file). To guarantee the appropriate dosage, the RCE was added before yoghurt fermentation in

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the manufacturing process. The concentration, structure, and stability of the RCE were confirmed before the yoghurt was made available to the participants. RCE (80mg/unit) contained 65% of HA, hydrolysed proteins (mainly collagen) and other polysaccharides. The content of HA in the final yoghurt product was determined according to the method previously described.[16] Each 100 g of the low-fat yoghurt contained: 3.25% protein; 0.2% fat; 4.45% carbohydrates; 30 kcal. The only difference between the product under investigation and control one was the RCE supplementation. The participants were asked to consume the yoghurt at the same time each day, preferably at lunchtime. The participants' diets were monitored using two 3-day dietary records, one prior to commencing the study, and the other one at the ending of the 12-week trial. Additionally, a list of foods and products rich in mucopolysaccharides and/or HA was provided to the participants with instructions to avoid these dietary items so as to pre-empt their influence on the test substance measurements.

2.2 Randomization

The randomization code was computer generated. The randomization list was based on a block randomization procedure (with block-size of 4) generated using PROC PLAN in the SAS program (version 9.2). To guarantee allocation concealment, the randomization list was guarded and was unavailable to the investigators involved in the study. Participant assignment to treatment or placebo arm was at a ratio of 1:1. The number sequence for the subject, centre, and treatment assignment were allocated via an interactive electronic response system hosted by the Nutrition and Health Technology Centre (CTNS). The unit responsible for the randomization took no further part in the study.

2.3 Adverse effects

The adverse events were coded according to the Medical Dictionary for Regulatory Activities (MedDra dictionary; version 9). RCE had been approved by the European Commission as a Novel Food ingredient in 2013.

2.4 Outcomes

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The main outcome was the evolution of muscle function over 12 weeks from baseline assessed by isokinetic evaluation of the affected knee joint. The secondary outcomes were the change from baseline in the echographic evaluation of the affected joint using an OA risk parameter scale and pain evaluation on the VAS scale. An additional outcome was to elucidate the mechanisms of action involved in such muscular changes through an *in vitro* cellular model with myoblasts. Moreover, the results obtained from this subject-level meta-analysis allowed the estimation of the optimum sample size required to design a new nutritional intervention trial to evaluate the efficacy of a RCE-supplemented low-fat yoghurt, on muscle strength of subjects with knee pre-OA.

2.5 Packaging characteristics

The investigational and control products were packed in 125 mL polyethylene terephthalate containers sealed with an aluminium foil cover. The test units were batched in cartons containing 6 units each. The labels on each included the following information: the EU code for products of animal origin, consume-by date, trial code/name of the promoter, the inscription "sample for nutritional investigation", storage conditions, blank space for noting information, consume-by date if necessary, and participant's code identification number in the study. The palatability and general acceptability of the low-fat yoghurt supplemented with RCE compared with the placebo were evaluated by means of a subjective acceptance questionnaire.

2.6 Ethics

The study was approved by the Clinical Research Ethical Committee of the *Hospital Universitari Sant Joan* and by Ethical Review Committee *Fundació Gol i Gurina*. The protocol complied with the Helsinki Declaration and good clinical practice guidelines of the International Conference of Harmonization (ICH GCP). All participants provided written informed consent prior to enrolment into the trial.

2.7 Knee muscular strength evaluation

As previously described in each published trial [10,11], evaluation of isokinetic tests followed the gold standard methodology comprising an isokinetic dynamometer (Biodex System 4; Biodex Medical

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Systems, New York, USA) and using five repetitions at two angular velocities (180°s−1, and 240°s−1). This allows a quantitative evaluation of muscle function through variables such as torque, work, and power. The isokinetic tests were determined at 240°s−1 as the preliminary study of Martinez-Puig *et al.* However, at low speeds (i.e., 0–180°s-1) peak force reflected pure muscle strength, while neuromuscular control comes into play at higher speeds (>180°s-1).[17] In consequence, to evaluate the muscle strength, the isokinetic tests were performed at angular speeds of 180ºs-1 and 240ºs-1. Participants adopted a seated position with the hips flexed at 90°. The degree of freedom of the knee was restricted to extension/flexion of 0 to−90º. A break of 2 min was allowed between sets of measurements. Based on the data retrieved from all the sets, the maximum total work (J), maximum peak torque (Nm) and the mean power (W) at 180 and 240°s−1 were determined. The maximum peak torque is defined as the maximum force produced by the tested musculature at the two different angular velocities. Total work is defined as the workload at a defined angular velocity, while the mean power is defined as total work over a specific period of time.[9]

2.8 Knee echography evaluation

A medical specialist performed ultrasonography to assess synovial effusion of the affected knee joint as previously described.[18]

2.9 Pain evaluation

Pain perception was assessed using the VAS.

2.10 RCE mechanism of action studies in an *in vitro* model of myoblast cells

2.10.1 Study of myoblasts proliferation

Murine C2C12 myoblasts were cultured for 48 and 72h in complete medium (CM) or differentiation medium (DM). C2C12 incubated in DM cease proliferation, exit cell cycle, and undergo differentiation to myotubes due to the reduced concentrations of mitogenic stimuli. All incubations performed in these both media were carried out in the presence of HA (0.5 mg/dL) or RCE (0.83 mg/dL) and in the presence (10 μM) or absence of PD98059. This molecule is a known inhibitor of

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mitogen-activated protein kinases (MAPK), a pathway involved in cell proliferation, that is activated by growth factors released by the damaged muscle[19]. Cells proliferation was monitored by the CellTiter-Glo luminescent assay (Promega, Spain), a test based on the quantification of ATP as a measure of metabolic activity of cells and therefore proliferation. An extended version of these methods is available in the Supplementary Data file.

2.10.2 Study of myoblasts differentiation

Murine C2C12 myoblasts were cultured in DM supplemented with HA (0.1 mg/dL) or RCE (0.164 mg/dL) for 24, 48, and 72h in the presence (0.5 mM) or absence of the inhibitor of endogenous HA synthesis 4-Methylumbelliferone (4MU). Cells differentiation was assessed optically using phase-contrast microscopy and by the determination of creatine kinase (CK) activity, an objective marker of myogenic differentiation.[20] An extended version of these methods is available in the Supplementary Data file.

2.10.3 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[21] and detailed in Supplementary Data file.

2.11 Statistical analysis

The subject-level meta-analysis included individualized data of all participants from the two studies.[10,11] Descriptive values are expressed as mean ± standard deviation (SD) or percentages, depending on the nature of the variable being measured. The continuous efficacy variables have been analysed by means of an analysis of covariance (ANCOVA) model with the baseline value as covariable and subject and trial as random effects. The main efficacy analysis was performed over the intention-to-treat (ITT) population using multiple imputation for missing values.[22] For each efficacy variable with missing values, 100 data sets were generated using the package mice[23] for software R version 3.1.1[24] with the predictive mean matching approach. A sensitivity analysis based

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on the available data only (ADO) approach was conducted for all variables in Table 2, providing similar results. The subgroup analyses were based on age (under or over 50 y), baseline BMI (under or over 30 kg/m^2), and gender. Main efficacy analyses were performed using SAS software version 9.2[25], and the level of significance was established at bilateral 5%. Sensitivity analysis based on multiple imputations was performed using R version 3.1.1.[24] The sample size analyses were performed using the software STATA/SE version 11.2 for Windows.[26] In the mechanistic study, ANOVA with Bonferroni and Dunnett correction was used to assess the effects of HA and RCE on differentiation, proliferation, and cytotoxicity. T-Tests were used to assess 4MU and PD98059 effects, and to assess differences between incubations performed in CM and DM. These analyses were performed using SPSS for Windows, version 23 (IBM corp., USA).

3. RESULTS

3.1 Participants

The baseline characteristics of 148 participants, 74 in the intervention group and 74 in the control group are described in Table 1.

3.2 Knee muscular strength evaluation

At baseline, the affected knee presented a lower total work (J) in flexion 180° s−1 by 12.79 (54.24) (mean difference (SD)), 6.67%, and in extension by 37.7 (78.31), 0.08% as compared to the unaffected counterpart. Similarly, the mean power (W) was also lower for the affected knee, by 4.31 (17.77), 7.37% in flexion; and by 15.37 (29.36), 13.04% in extension compared to the unaffected joint. Moreover, a lower peak torque (Nm), in flexion by 0.96 (7.99), 2.35%; and in extension by 4.76 (11.85), 6.36% was found in the affected knee versus the unaffected one. Also at baseline, participants presenting obesity had a lower total work in flexion at 180ºs-1 in mild pain knee compared to the normal knee (34.73 (61.12); 19.33%) and also in extension (49.38 (85.81); 13.34%).

Regarding the effects of RCE on muscle strength, after 12 weeks of treatment RCE group increased

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11.3% of total work in flexion (P=0.039), compared to control. These results are consistent with the trend observed in both trials, although this trend only reaches statistical significance in the present work when the sample size was increased, as depicted in Figure 1. However, the most relevant isokinetic data to evaluate the effect of RCE on muscle strength were those obtained from the segregation by gender as summarized in Table 2. In men, the intake of the RCE-supplemented yoghurt significantly improved all the studied parameters by a 13%, except the peak torque at flexion whose increase did not reach significance. In women, no statistically significant changes were found compared to placebo. When it comes to data segregated by age, in participants under 50 y the consumption of RCE increased total work by 15.4%, in extension (180ºs-1; P<0.041), whereas in those over 50 y a 13.6% increase was found in flexion (180ºs-1; P<0.026) as detailed in Supplementary Table S1.

3.3 Knee synovial effusion evaluation

At 12 weeks, the echographic evaluation of the affected knee revealed that consumption of low-fat yoghurt enriched with RCE reduced knee synovial effusion by 11.8% compared to control (P=0.038; Table 3). This reduction was particularly significant in women by 15.9% (P=0.035). Moreover, in those participants over of 50 y, the consumption of the RCE-enriched low-fat yoghurt reduced knee synovial effusion by 21.1% (P< 0.047) compared to controls.

3.4 Knee pain perception evaluation

At 12 weeks, the consumption of low-fat yoghurt supplemented with RCE reduced the pain perception by 24.6% (P=0.007) compared to control. The intensity of the reduction in pain perception was similar for men, women, younger and older than 50 y.

3.5 Dietary intake

The intake of energy, macronutrients, cholesterol, and alcohol did not change during the 12 weeks intervention period, and no significant differences were observed between groups.

3.6 Effect of HA and RCE on myoblast proliferation

Differences on myoblast proliferation and differentiation cultured in CM and DM, along with the

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effects of 4MU on myoblast differentiation to myotubes are comprehensively detailed in Supplementary Data file (Supplementary Figures S1, S2, S3, and S4).

Myoblast proliferation increased by a ~ 29% after incubations with HA (0.5 mg/dL; both at 48 and at 72 h) and RCE (0.83 mg/dL; 72 h) ($P<0.05$) in CM, as depicted in Figures 2A and 2B, respectively. In these experiments, the MAPK inhibitor PD98059 decreased myoblasts proliferation between 23.60% and 43.13%. Regarding incubations performed in DM (Figure 2C), HA had no effect while RCE increased proliferation by a 90.03% and 82.80% at 48 and 72 h respectively *versus* blank condition, and these increases were statistically significant also versus HA ($P<0.05$). Higher doses of HA (0.5 mg/dL) and RCE (1.7 mg/dL) were tested in these experiments but no dose-dependent effect was observed. No cytotoxicity was observed at any of the concentrations tested (data not shown).

3.7 Effect of HA and RCE on myoblast differentiation

C2C12 myoblasts incubated in DM alone differentiate in a time-dependent way and this differentiation was inhibited by 4MU (see Supplementary Data file). HA had no effects on CK activity at any time point, while RCE decreased this activity (36.19%) after 72h of incubation ($P<0.05$; Figure 3). Moreover, neither HA nor RCE could revert the differentiation inhibition produced by 4MU at any time tested (Supplementary Figure S5). These results were optically verified by phase-contrast microscopy (data not shown). Higher doses of HA (0.25 mg/dL) and RCE (0.41 mg/dL) were tested in these experiments but no dose-dependent effect was observed. No cytotoxicity was observed (data not shown).

3.8 Impact of muscle strength, effusion, and pain on sample size calculations

The optimal sample sizes had been calculated, according to the results obtained in this subject-level analysis in reference to each analysed parameter. In all cases, the level of significance was set at 5% bilateral and the statistical power at 80%. The sample size estimates performed on the basis of an ANCOVA model adjusted by the baseline value and starting from the isokinetic parameter outcomes obtained on the knee with mild pain, showed that from muscle strength by isokinetic

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326 measurements, observed in present study: a) the most optimistic scenario was obtained for total
327 work at 180º/s in flexion position, as consequence of the maximum improvement observed by
328 11.3% for which a sample size of 68 participants per group in a parallel design was needed; b) the
329 most pessimistic scenarios were obtained for peak torque parameter, at any measurement
330 conditions, leaded to the maximum size sample of 263 participants. The sample size estimated from
331 observed knee joint effusion reduction, by echographic evaluation and from pain perception
332 diminution, measured by VAS scale, was about 65-70 participants per group, in a parallel design,
333 according to the results obtained in this subject-level meta-analysis.
334
335 **4. DISCUSSION AND CONCLUSION**
336 The present study verifies the hypothesis that the daily consumption of low-fat yoghurt
337 supplemented with 80 mg of RCE for 12 weeks not only improves significantly knee muscular
338 strength but also reduces synovial liquid effusion and pain perception compared to placebo. In
339 particular, the consumption of a low-fat yoghurt supplemented with RCE increases total work in
340 flexion (19.9 %) and extension (16.2 %) at 180º/s-1 in men. It also increases the flexion at 180º/s-1
341 in participants over 50 y (13.6 %) and increases the extension at 180º/s in participants under 50 y
342 (15.4 %). In addition, in men increases the mean power in flexion and in extension (21.1% and
343 18.1% respectively), and peak torque in extension (13%). There is scarce information available
344 about the clinical significance of knee isokinetic measures. However, it is well known that when the
345 comparison between two isokinetic variables is greater than 10% is functionally significant.[27,28]
346 Thus, an improvement of 10% in the affected knee muscle strength observed after the RCE
347 intervention can be translated into beneficial effects with clinical impact.
348 So far, it has been described that the role of the quadriceps in the extension of the knee is the main
349 variable affected in knee OA.[2,6,29] To the best of our knowledge, our results indicate for the first
350 time that not only quadriceps but also the hamstring muscles are equally affected in the knee OA. In
351 particular, isokinetic variables assessed at baseline revealed differences in the affected versus the

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unaffected knee not only in extension (being the quadriceps the main extensor muscles) but also in flexion (being the hamstring the main flexor muscles). Affected joint showed less muscle strength as indicates a lower knee peak torque, lower total work, and lower mean power versus the unaffected knee. Our data could be translated into significant clinical impact since hamstring muscles training may also ameliorate the muscle strength in patients affected by knee OA and the symptoms associated to it. To our knowledge, in this study is reported for the first time that obesity is a key factor influencing muscle strength in knee OA. From our results, those participants presenting obesity had a lower total work in flexion and extension compared to non-obese participants at baseline.

Regarding the effects of RCE intake, this is the first time that an increase in knee total work in flexion (180° s−1) is observed after RCE intake regardless the gender. In our previous studies, Sánchez et al.[11] found no statistical differences, while Solà et al.[10] showed this improvement in muscle strength but only in men. Our new results obtained when sample size was increased bear relevance because total work is the measurement of muscle strength with more clinical impact since it is involved in the daily activities. In the daily life, knee flexions and extensions are performed in the angles assessed in the present study (180° and 240 s−1), which are determined by the knee total work. Moreover, knee total work at low speeds such as 180°s-1 reflects pure muscle strength, while neuromuscular control comes into play at higher speeds (>180°s-1)[17]. This evidence supports the assertion that total work is a good indicator of functional joint capacity. Despite the relevance of this isokinetic measurement, total work is not usually evaluated in population studies assessing knee OA, being therefore difficult to compare our results with those from other authors[30]. Since the increase in the affected knee muscle strength observed after the RCE intervention is greater than 10% (*i.e.*, 11.3%), this improvement can be translated into beneficial effects with clinical significance[27,28].

When muscle strength data were segregated by gender, an improvement in not only total work but also in mean power and in peak torque (both the flexion and extension) was observed in men

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affected of mild joint discomfort after RCE intake. This suggests an additional clinical impact by enhancing both the hamstring (flexion) and the quadriceps (extension) muscle strength since muscle-strength loss seems to be responsible for the weakness involved in knee OA and for the joint instability. The gender differences observed could be explained by the fact that male muscle strength declines progressively and linearly with age, while female muscle strength decreases from around the age of 41 years, due to the loss of the ovarian function associated to the menopause[31,32]. Moreover, our study reveals for the first time differences in muscle strength according to age. In this regard, RCE intake increased total work in flexion in participants over 50 y and in extension in participants under 50 y. That is, RCE seems to provide dissimilar beneficial effects on hamstring and quadriceps muscles according to the age of the subjects affected by mild knee pain. These age-related findings have significant clinical impact since training of different muscle groups should be recommended according to the age of the patient affected by knee OA.

Another beneficial effect observed after the RCE consumption is the reduction of the pain perception in the whole sample, confirming the results observed by Sánchez *et al.*[11]. A possible explanation could be the decrease of synovial effusion observed in the present study after the intake of RCE since the presence of synovitis assessed by magnetic resonance imaging has been correlated with knee pain and its severity. Moreover, the reduction of synovial effusion in the knee joint indicates a decrease in inflammation and therefore in pain presence.[2] To the best of our knowledge, this is the first time that RCE is shown to exert a different effect on synovial effusion according to age and gender, since RCE enhanced greater decreases of synovial effusion in females and in participants over 50 y, in comparison with their counterparts.

A key point in the clinical parallel studies is the calculation of the number of participants needed to identify a significant clinical impact. Regarding the assessment of muscular strength in knee OA, thus far there is scarce information regarding sample size needed to detect significant clinical findings. The present work provides data for the first time that allows proper sample size calculations for isokinetic parameters. In particular, the total work in flexion (180ºs-1) was found to

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be the variable which needs the smallest sample size (that is 68 participants per group) while other variables, need a higher size sample, being the peak torque the one which needs maximum sample size (that is 263 participants per group).

A possible mechanism of action for the beneficial effects observed after the consumption of RCE involves a nutrigenomic approach. About 157 coding genes were found to be differentially expressed in blood cells from participants that ingested a low-fat RCE-supplemented yoghurt compared to those from the placebo group. These differences were observed after the intervention (fold change ≥1.2) but not before.[11] In particular, reduced gene expression of glucuronidase-beta (GUSB), matrix metallopeptidase 23B (MMP23B), xylosyltransferase II (XYLT2), and heparan sulfate 6-O-sulfotransferase 1 (HS6ST1) was found in the RCE-supplemented *versus* the placebo group. Correlation analysis indicated a direct relationship between blood cell gene expression of MMP23B and pain intensity, along with an inverse relationship between blood cell gene expression of HS6ST1 and indicators of knee muscular strength. Therefore, the expression of specific genes in blood cells, in particular those related to glycosaminoglycan metabolism (such as HS6ST1) and extracellular matrix dynamics (such as MMP23B), are potential biomarkers of beneficial effects on articular health [11].

Additional mechanisms of action of the beneficial effects observed after the consumption of RCE are proposed in this study for the first time. From our results, both HA and RCE promoted myogenesis by increasing myoblasts proliferation to the same extent (~ 29%). Since equivalent doses of HA and RCE were tested in our experiments, the effect observed after RCE can be entirely attributed to its content in HA rather than to additional components present in RCE. Moreover, HA had no effect on myoblasts differentiation while RCE decreased such differentiation by a 36.19%. Under certain conditions, such as muscle injury present in chronic diseases as knee OA, skeletal muscle regenerates by activating satellite cells from the muscle. These activated cells, called myoblasts, proliferate massively to generate the myogenic cells needed for complete muscle regeneration, exit cell cycle, and undergo differentiation to form multinucleated myotubes.[13,33,34]

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However, following muscle injury, not all the proliferating myocytes are further differentiated into myogenic progenitors needed to regenerate the muscle. Some activated myocytes are kept in cell cycle proliferating to maintain and restore the satellite cell pool. The fate of the proliferating myocytes (to keep proliferating or to undergo differentiation) is a complex process highly regulated by myogenic regulatory factors and it is crucial to accomplishing a proper muscle regeneration. From our results RCE components seem to modulate muscle regeneration at two different stages of myogenesis: HA present in RCE seems to activate myoblasts entrance into cell cycle promoting initial proliferation, while components present in RCE other than HA may be involved in the further decision of cells to proliferate or to differentiate. In this sense, these components present in RCE keep cells proliferating in the cell cycle rather than promoting cell cycle exit to differentiate. The magnitude of the effect of RCE on proliferation is such that even in DM myoblasts proliferate (by a 90%) instead of differentiating as it may be expected due to the reduced concentrations of mitogenic stimuli or growth factors in the DM. However, these mechanistic experiments are preliminary and further experiments are warranted to confirm the mechanism of action proposed in the present study.

One limitation of this analysis is that it has been based on only two trials, although both studies were identical in their execution. However, further studies are needed to elucidate reasons for the observed age-, BMI- and sex-related differences in the responses observed and to provide further insight into the muscle function.

In conclusion, our results verify the hypothesis that low-fat yoghurt supplemented with RCE consumption improves muscle strength and reduces synovial effusion and pain perception providing clinical benefits, especially in men affected by mild knee pain. Moreover, it is safe to assume that the improvement in muscle strength observed in the human studies is due to an increase in muscle regeneration, as indicates the increase in myoblasts proliferation caused by the HA content in RCE observed in the *in vitro* model of this work. These findings could provide the basis of new dietary therapeutic objectives in the treatment of early osteoarthritis.

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6. AUTHORSHIP

RS, as Principal Investigator, had full access to all of the data in the study and takes responsibility for the integrity of the data and the validity of the analyses. RS, R-MV, AP, IP, AR, LA, FP, IM, and DM-P were responsible for the overall study design including project concept, development of overall research plan, and study monitoring. DM, SF-C, AP, NT, MR, MG, MM, IP, GB, JF, LP-M, RG, M-CC, AR, and IM provided hands conducting the experiments and data collection.AR and DM-P provided essential materials and logistic support necessary for conducting the study. DM and SF-C analysed data and performed statistical analyses of the data. DM, SF-C, CC, DM-P, and RS drafted the article and revised it critically for important intellectual content. All co-authors critically

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revised the manuscript for important intellectual content and approved the manuscript to be submitted for publication.

7. CONFLICTS OF INTEREST

DM-P is employed by Bioiberica S.A., Palafolls, Spain. The other authors have declared that no conflict of interest exists. The authors also declare there have not been any other involvements such as employment, consultancy and patent products that can be construed as a conflict of interest.

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9. FIGURE CAPTIONS

Figure 1: Efficacy of the RCE-supplemented yoghurt on total work at 180°/s in flexion of both trials independently and combined. Difference between groups on the temporal evolution from baseline.

Figure 2. Effects of HA and RCE on C2C12 myoblasts proliferation. C2C12 myoblasts were cultured in CM until they reached 20% of confluency. At this time point HA (0.5 mg/mL; Figure 2A), RCE (0.83 mg/mL; Figure 2B), 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. An additional batch of experiments was performed in DM (Figure 2C). At the end of experiments, proliferation was monitored by the CellTiter-Glo luminescent assay based on quantification of ATP. * $P<0.05$ versus blank condition (condition without the presence of HA, RCE, nor PD98059); † $P<0.05$ between conditions.

Figure 3: Effects of HA and RCE on C2C12 myoblasts differentiation. 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. HA (0.1 mg/mL) or RCE (0.164 mg/mL) were added to DM for 24, 48, and 72 h. This DM was refreshed every 24 h until the end of the experiment. Cells differentiation was optically assessed using a phase-contrast microscopy and by the determination of creatine kinase (CK) activity. * $P<0.05$ versus blank condition (condition without the presence of HA nor RCE).

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573 **Table 1. Baseline characteristics of participants.**

Variable	Placebo (n=74)	RCE (n=74)
Age; years	47.91±12.49	48.70±11.51
Weight; Kg	69.16±14.44	71.14±13.38
Height; cm	162.41±8.38	161.82±8.63
Body Mass Index; Kg/m^2	26.01±3.74	27.02±3.72
Gender; male, n (%)	24 (32.40%)	27 (36.50%)
Race; Caucasian, n (%)	73 (98.60%)	74 (100.00%)
> 50 years, n (%)	37 (49.33%)	38 (50.67%)
BMI > 30 Kg/m2, n (%)	5 (14.71%)	9 (26.47%)

574

575 The results are described as mean ± standard deviation or n (%). RCE= Low-fat yoghurt
576 supplemented with 80 mg of a natural rooster comb extract containing 65% of HA (1 /d).

577 **Table 2. Changes in isokinetic variables of knee with mild pain segregated by gender.**

Gender	Parameter	Position	Treatment	Baseline Mean±SD	Change at 12 weeks relative to baseline Adjusted mean [95%CI]	Treatment difference Adjusted mean [95%CI]	*P*
Males	Peak torque (N m)	Extension	Placebo (n=24)	101.19±39.87	-0.06 [-7.91; 7.79] (-0.1 %)	13.19 [2.54; 23.85] (13.0 %)	0.016
			RCE (n=27)	109.95±34.03	13.13 [13.13; 20.34] (11.9 %)		
		Flexion	Placebo (n=24)	50.73±20.81	4.35 [-0.21; 8.91] (8.6 %)	4.06 [-2.20; 10.32] (8.0 %)	0.198
			RCE (n=27)	60.41±18.52	8.41 [4.22; 12.60] (13.9 %)		
	Total work (J)	Extension	Placebo (n=24)	505.19±231.63	14.23 [-40.36; 68.820] (2.8 %)	81.83 [7.87; 155.80] (16.2 %)	0.031
			RCE (n=27)	532.75±180.25	96.06 [45.90; 146.22] (18.0 %)		
		Flexion	Placebo (n=24)	255.89±135.53	21.37 [-7.62; 50.36] (8.4 %)	50.83 [11.36; 90.29] (19.9 %)	0.013
			RCE (n=27)	297.06±130.20	72.20 [45.53; 98.87] (24.3 %)		
	Mean power (W)	Extension	Placebo (n=24)	155.63±77.07	11.86 [7.58; 31.30] (7.6 %)	28.20 [1.79; 54.61] (18.1 %)	0.037
			RCE (n=27)	173.83±66.03	40.06 [22.16; 57.97] (23.0 %)		
		Flexion	Placebo (n=24)	78.47±46.38	8.98 [-1.74; 19.69] (11.4 %)	16.52 [1.91; 31.13] (21.1 %)	0.028
			RCE (n=27)	93.97±44.09	25.50 [15.63; 35.36] (27.1 %)		
Females	Peak torque	Extension	Placebo (n=50)	59.99±18.26	6.78 [3.83; 9.72] (11.3 %)	-1.87 [-6.11; 2.38] (-3.1 %)	0.385
			RCE (n=47)	57.05±18.49	4.91 [1.87; 7.95] (8.6 %)		
		Flexion	Placebo (n=50)	35.40±13.83	2.60 [0.26; 4.94] (7.3 %)	0.57 [-2.83; 3.97] (1.6 %)	0.740
			RCE (n=47)	30.20±10.77	3.17 [0.76; 5.58] (10.5 %)		
	Total work	Extension	Placebo (n=50)	312.62±111.26	26.66 [7.32; 45.99] (8.5 %)	10.19 [-17.76; 38.15] (3.3 %)	0.471
			RCE (n=47)	280.91±95.40	36.85 [16.88; 56.82] (13.1 %)		
		Flexion	Placebo (n=50)	163.47±84.79	16.98 [2.46; 31.50] (10.4 %)	11.95 [-9.17; 33.06] (7.3 %)	0.264
			RCE (n=47)	128.95±70.07	28.93 [13.95; 43.91] (22.4 %)		
	Mean power	Extension	Placebo (n=50)	97.93±38.06	14.21 [7.64; 20.79] (14.5 %)	-0.76 [-10.25; 8.73] (-0.8 %)	0.873
			RCE (n=47)	87.66±36.95	13.45 [6.66; 20.24] (15.3 %)		
		Flexion	Placebo (n=50)	48.58±25.90	8.18 [3.37; 12.99] (16.8 %)	1.68 [-5.30; 8.66] (3.5 %)	0.634
			RCE (n=47)	38.47±23.09	9.86 [4.90; 14.82] (25.6 %)		

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578 All results are expressed as means ± standard deviation and baseline adjusted least square means
579 [95%CI]. RCE= Low-fat yoghurt supplemented with natural rooster comb extract (80mg/day) rich
580 in HA (65%).
581

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582 **Table 3. Changes in effusion of knee with mild pain determined by echographic evaluation**

Treatment	Baseline (mm)	Relative change at 12 weeks relative to baseline (%)	Treatment difference	*P*
	Mean±SD		**Adjusted mean [95%CI]**	
Placebo	7.78±4.61	-0.68 [-1.30; -0.06] (-8.7%)	-0.92 [-1.79; -0.05] (-11.8%)	0.038
RCE	8.02±4.39	-1.60 [-2.21; -0.99] (-20.0%)		

583

584 All results are expressed as % of change relative to baseline and baseline adjusted least squares
585 means [95%CI]. RCE= Low-fat yogurt supplemented with natural rooster comb extract (80 mg/d)
586 rich in HA (65%).

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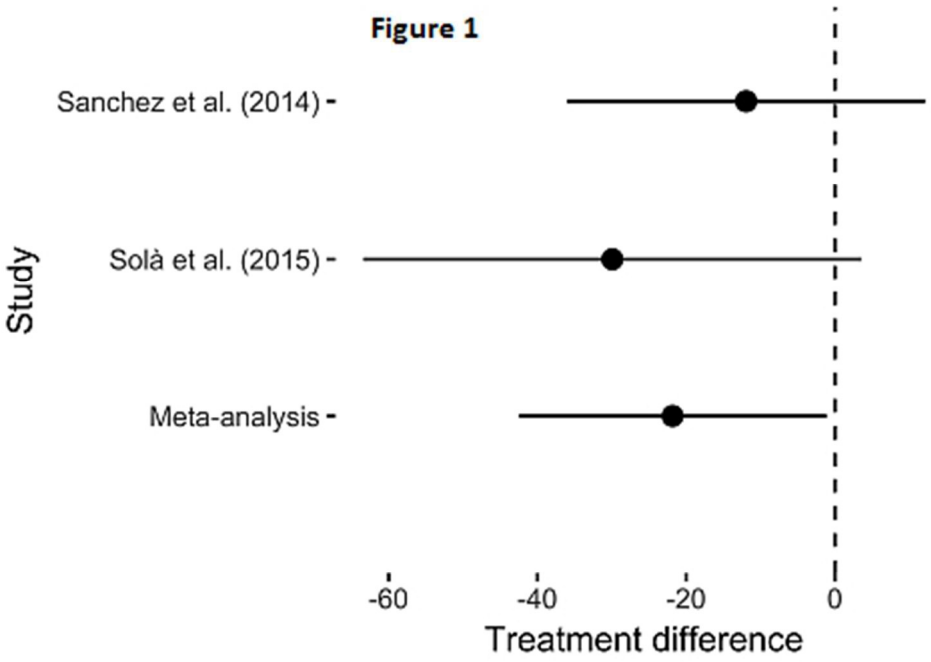

Figure 1

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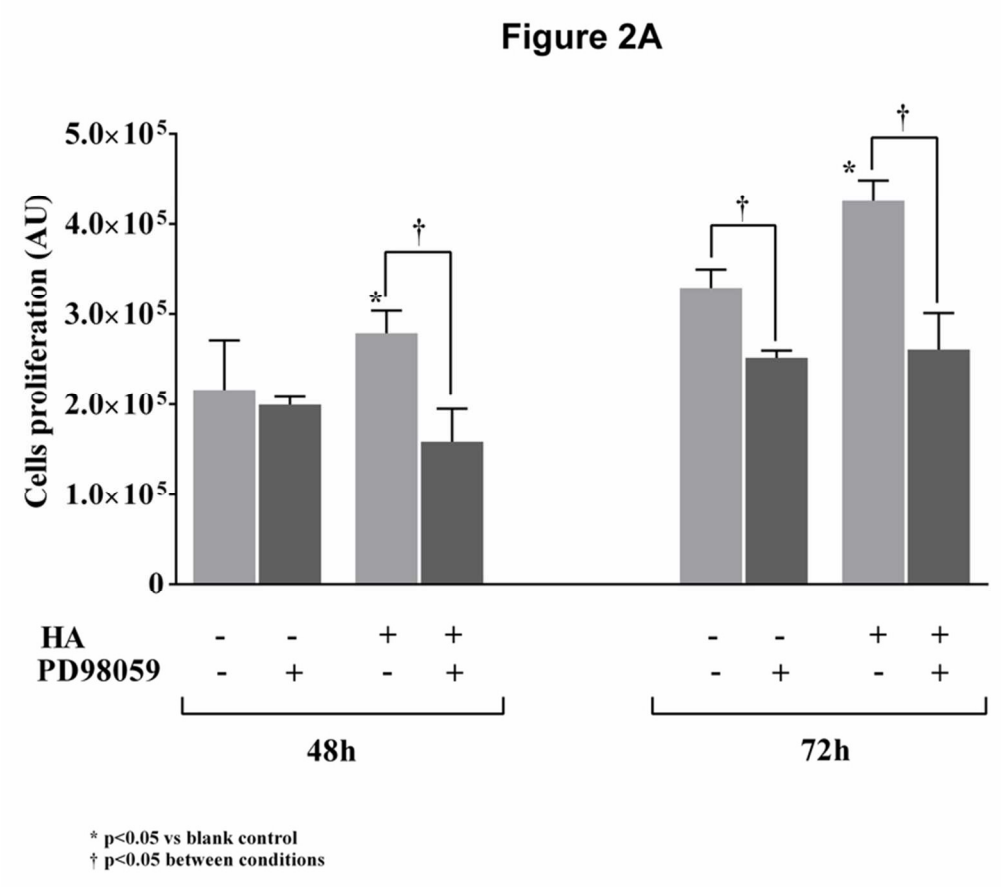

Figure 2A

73x65mm (300 x 300 DPI)

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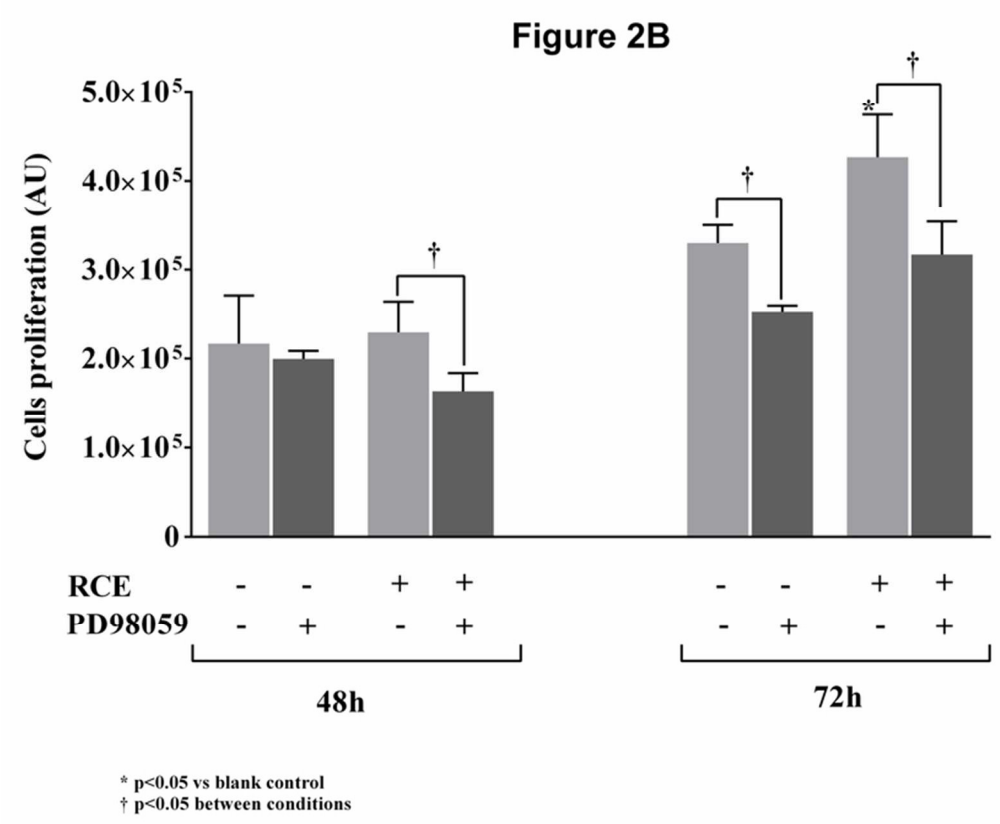

Figure 2B

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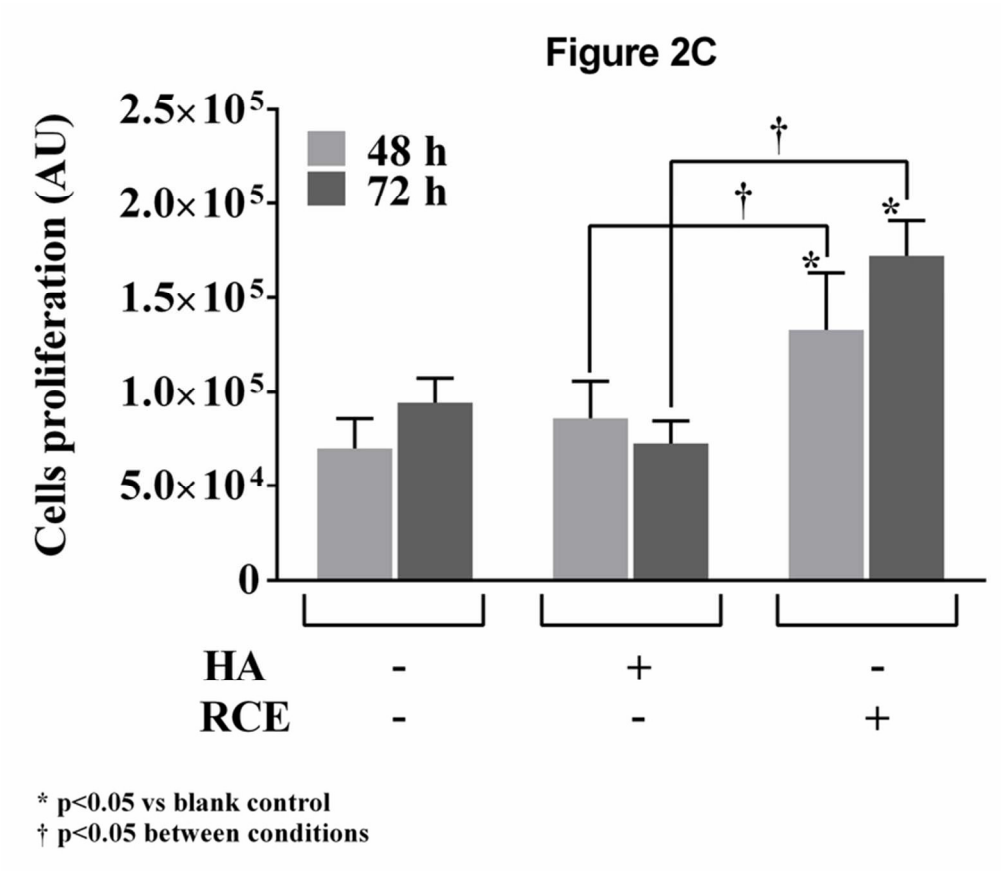

Figure 2C

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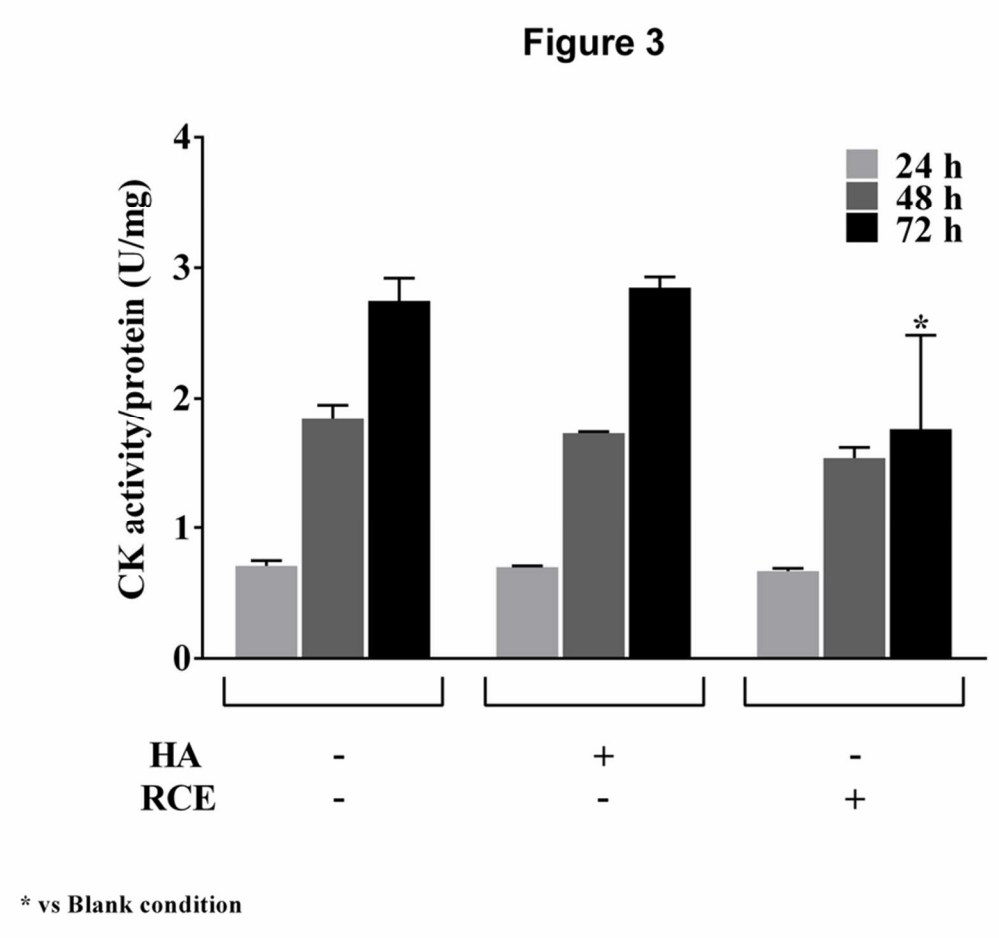

Figure 3

77x73mm (300 x 300 DPI)

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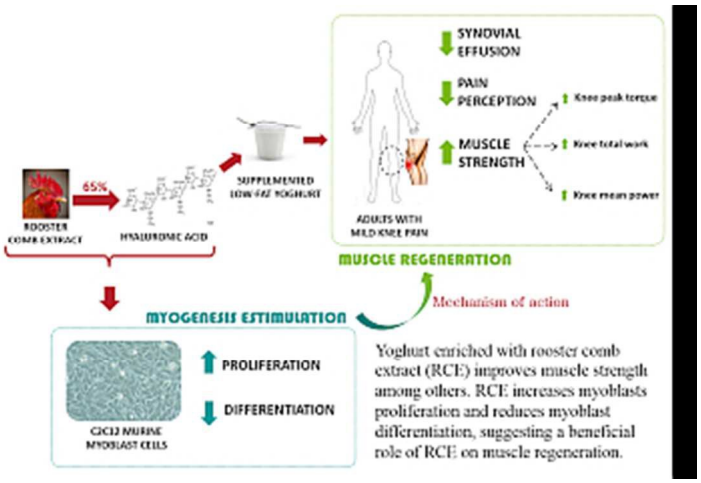

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