The isolation and characterisation of equine bone marrow stem cell derived extracellular vesicles – evidence of an anti-inflammatory action on chondrocytes.

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Research article

Keywords: chondrocytes, anti-inflammatory, bone marrow, equine

Posted Date: February 10th, 2020

DOI: https://doi.org/10.21203/rs.2.23067/v1

The isolation and characterisation of equine bone marrow stem cell derived extracellular vesicles – evidence of an anti-inflammatory action on chondrocytes.

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Abstract

Background: Osteoarthritis (OA) in the horse is an economic and welfare issue and there are no current disease modifying drugs available. Stem cells have been suggested as potential therapeutics for OA, originally on the basis of their regenerative capacity. However, it is now hypothesised that MSCs exert their effects via paracrine factors including the production of extracellular vesicles, that can themselves recapitulate the MSC effects in the joint.

Results: In this study we have, for the first time, isolated and characterised extracellular vesicles from equine bone marrow stem cells. We have shown these vesicles can be taken up by autologous chondrocytes and have anti-inflammatory effects on gene expression following

chondrocyte exposure to tissue necrosis factor α and Interleukin 1β. No effects on chonodrocyte proliferation or migration was observed.

Conclusion: Extracellular vesicles can be isolated from equine bone marrow mesenchymal stem cells, they are taken up by chondrocytes and have an anti-inflammatory action.

Introduction

The use of mesenchyme stem/stromal cells (MSC) to treat orthopaedic disease in horses has become widespread in the last decade (1,2) with tendon/and ligament injuries, synovitis, osteoarthritis (OA) and cartilage lesions all being considered as candidates for MSC therapy. Whilst the majority of MSC therapies have been performed using autologous cells, the increasing commercialisation of stem cell therapies has also led to the launch of allogeneic MSC products (3).

OA in the horse is a major cause of lameness and poor performance. Characterised by changes in the joint including synovitis and subchondral bone abnormalities, it leads to degeneration of articular cartilage (4). Treatment of OA is challenging, with no disease modifying therapies available. The use of MSC to treat equine OA has increased in recent years and whilst intra-articular MSC are considered to be safe to administer, it is well recognised that, in the horse, these injections can cause transient inflammation and synovitis (5). Additionally there are other complications and disadvantages of using MSC including limited cell survival, immune-rejection, senescence-induced genetic instability or loss of function and the theoretical risk of malignant transformation (6,7).

The original theories to explain the beneficial effects of MSC proposed that their mechanism of action was primarily to engraft and regenerate damaged tissue. However, it has become clear that this 'engraftment and build' concept is not supported by evidence. It has now been demonstrated that MSC exert their effect via paracrine actions through the secretion of anti-inflammatory and regenerative factors (8,9), and that many of these paracrine actions have

been demonstrated to reside in small, membrane bound particles, extracellular vesicles (EVs), secreted by the MSC (10)

EVs are secreted by all cells and are involved in cell-cell communication (11). EVs are sub-classified depending on their size into exosomes (40-100nm), microvesicles (100-1000nm) and apoptotic bodies (100-5000nm) (12). EV act as a paracrine signalling system, stably transporting proteins, RNAs (rRNA, tRNA, miRNA, lnc(RNA)) and mitochondrial DNA through the extracellular environment (13,14). EVs attach to the recipient cell membrane and are internalised, exerting their effects by direct ligand-receptor interactions on the cell surface or by intracellular effects of their cargo including affecting gene expression through de novo translation and post-translation regulation of target mRNAs (15). Thus, EV can theoretically recapitulate the therapeutic effects of their parent cell (13,16).

In vivo, bone marrow derived mouse MSC EV have been reported to prevent cartilage breakdown (17) in the joint and, as cell-free, minimally immunogenic particles, EV may represent an opportunity for low cost off-the-shelf therapy for equine joint disease treatment and regeneration. The aim of this study was i) to isolate and characterise EV from horse bone marrow derived MSCs (BM-MSC) and ii) to investigate the effect of these EV on chondrocytes cultured in a pro-inflammatory environment *in vitro.*

Methods

Animals

Bone marrow and cartilage was collected post mortem from three adult horses that were euthanized for reasons other than orthopaedic disease at the Department of Veterinary Medicine, University of Cambridge, UK with full ethical consent from the owners. The femoro-patella joints were dissected and a visual inspection of the cartilage surface carried out to ensure no cartilage abnormalities were detected.

Cell Harvest and Culture

Bone marrow was aspirated from the medullary cavity of the distal femur and collected into 2,000U/ml sterile Heparin (Sigma, UK) at 4ºC. The marrow was passed through a 70µm nylon filter, before undergoing two rounds of centrifugation (300g, 5 minutes). The supernatant was discarded and the pellet re-suspended in phosphate buffered saline (PBS) (Sigma, UK).

Cartilage was removed from the articular surface of the distal femur using sharp dissection and collected into α-MEM at 4ºC. Cartilage fragments were washed in PBS, before being minced into <1mm^3 pieces. The minced cartilage was then incubated in culture media (below) with 0.2% collagenase II (Roche, Germany) overnight. The contents of the flask were then filtered (70µm nylon filter). Of the filtered substrate, any cells were pelleted out of solution via centrifugation (300g, 5 minutes). The pellet was re-suspended in 15ml of basal media and plated for cell culture.

All cells were cultured in a humidified incubator (Sanyo, UK) at 37˚C and 5% CO_2. Standard culture media consisted of αMEM (Invitrogen, UK), supplemented with 10% foetal calf serum

(FCS) (First Link, UK), 1% penicillin/streptomycin (Sigma, UK), 1% Glutamax (Thermofisher, USA), 5ug/ml ascorbic acid (Sigma, UK) and 50ng/ml beta Fibroblast growth factor (Peprotech, USA). Upon reaching 80% confluence, cells were passaged using trypLE. In brief, culture media was removed, and 100ul/cm^2 trypLE was added to the culture flask. The cells were incubated with trypLE for 10-15 minutes before mild agitation to detach the cells. The cell containing trypLE was then collected and centrifuged at 300g for 5 minutes. The supernatant was discarded, the cells re-suspended in PBS and counted with a haemocytometer.

Tri-lineage differentiation of MSCs

Upon reaching approximately 80% confluence at passage 2, the cells were passaged as above before being cultured in the appropriate differentiation medium.

Chondrogenic Differentiation

2X10^5 MSCs were centrifuged at 300g for 5 minutes to form a pellet and were cultured in Stempro Chondrogenic differentiation media (Life technologies, UK) for 21 days under standard cell culture conditions with media changes every 3-4 days. The pellet was then fixed in formalin for 1 hour before paraffin embedding, sectioning at 5μm, staining with Alcian Blue and imaged using light microscopy.

Osteogenic Differentiation

2X10^5 MSCs were plated into a 6 well plate with standard culture media for 24 hours. The media was then removed, the cells washed once with PBS before being cultured in Osteogenic differentiation media (Culture media, 3.5mM β glycerophosphate (Sigma, UK), 10nm Dexamethasone (Sigma, UK)) with media changes every 3-4 days. Cells were cultured in

differentiation media for 21 days before the media was removed, the cells washed once with PBS and fixed with formalin for 1 hour. After fixing, the cells were covered with 0.5% (W/V) alizarin red (pH 4.1). The cells were stained for 15 minutes before the alizarin red was removed and the cells washed in PBS 5 times before being left to air dry before analysis using light microscopy.

Adipogenic Differentiation

$1X10^5$ MSCs were cultured in 6 well plates for 24 hours in standard culture conditions before the media was removed, the cells washed and StemPro Adipogenesis media (Life technologies, UK) added to the cells. The media was changed every 3-4 days and the cells were cultured for 14 days before being fixed with formalin and stained with oil-red-o solution (0.1% V/V) for 1 hour. The stain was removed, the cells washed with PBS 5 times before imaging and analysis under light microscopy.

Differential Sequential Ultracentrifugation for EV Isolation

EVs were isolated from passage three BM-MSCs. At passage three, standard culture media was removed from the cells, cells were washed with PBS and then cultured in FCS free media for 48h under standard culture conditions. This media was then collected and centrifuged at 300g for 5 minutes, before the supernatant was collected and re-centrifuged at 2,000g for 20 minutes. The supernatant was then ultra-centrifuged at 10,000g for 45 minutes, the supernatant again collected and re-centrifuged at 100,000g for 90 minutes. The supernatant was then discarded, the pellet re-suspended in PBS and ultra-centrifuged again. The pellet was re-suspended in PBS and frozen at -80˚C. Figure 1 illustrates the process of EV isolation via differential sequential ultracentrifugation.

Nanoparticle Tracking Analysis (NTA)

NTA analysis was performed and analysed using a NanoSight LM10 Nanoparticle Analysis system (Malvern, UK) and NTA 1.4 analytical software as per the manufacturer's instructions. An aliquot of EV suspension was diluted to give an average particles/frame count of 50 before analysis.

Transmission Electron Microscopy

An aliquot of EV suspension was placed on a 'glow discharge disk' prepared by the Cambridge advanced imaging centre (CAIC). The EV loaded disk was then negatively stained with 2% uranyl acetate (Sigma, USA) for 2 minutes at room temperature, before washing with PBS ready for viewing.

BCA protein assay

The protein content of the EV suspension was measured using the Pierce BCA Protein Assay Kit as per the manufacturer's instructions (Thermoscientific, UK). An aliquot of EV suspension was used to quantify surface protein or added to 20% sodium dodecyl sulphate for 20 minutes (Sigma, UK) to lyse the EVs and quantify total protein. The percentage surface and internal protein was then calculated.

Internalisation

EVs from $4X10^4$ MSCs were labelled with the fluorescent dye PKH67 (Sigma, UK) via incubation at room temperature for 30 minutes. The reaction was then quenched via the addition of 10% Bovine Serum Albumin (BSA) (Sigma, UK). The EVs were pelleted via

centrifugation at 100,000g for 90 minutes and the supernatant removed. This removed any unbound dye. The pellet was then washed via the addition of 1ml of 10% BSA and re-centrifuged. The supernatant was removed and the pellet was then re-suspended in PBS and washed twice more via this centrifugation process before co-culture with equine chondrocytes for 1h under standard culture conditions. After 1 hour, cells were fixed with formalin for 1 hour before the cell membranes were stained with WGA-55 (Thermofisher, UK) as per manufacturer's instructions. Cells were then permeabilised by incubating in 10% Triton X and 10% BSA in PBS for 20 minutes before nucleus staining with DAPI. Cells were imaged via confocal microscopy (Leica SP5).

Effect of EV on chondrocytes grown under inflammatory conditions

$1.5x10^5$ equine chondrocytes were cultured in the presence of either 1ng/ml Interleukin-1β (IL-1β) or 1ng/ml Tumour Necrosis Factor-α (TNF-α). After 12 hours of culture, EVs isolated from $1.5x10^5$ MSCs were added to the culture medium, equivalent to a 1:1 ratio of chondrocytes:MSCs.

After 3 days of culture in the presence of EV, chondrocytes were lifted from their wells via the addition of trypLE and pelleted via centrifugation at 300g for 5 minutes. The supernatant was removed and the pellet re-suspended in 300µl of Qiazol (Qiagen, Germany). mRNA extraction was performed using the Direct-zol mRNA microprep kit as per manufacturer's instructions (Zymo, USA). cDNA synthesis was then performed using a QuantiTect Reverse Transcription Kit (Qiagen, Germany). MMP-13 and ADAMTS5 primers were obtained Primers sequences (5' to 3'); MMP13 Forward primer GGGAGCACTCATGTTTCCGA. Reverse primer GGGTTCGGGTCTTCATCTCC. ADAMTS4 Forward primer TGTGGTCACTATTCCTGCGG. Reverse primer TGAGGGCATAGGAGCCATCT (18).

qPCR analysis was performed using a QuantiFast SYBR Green PCR Kit (Qiagen, Germany) in a StepOnePlus Machine (ThermoFisher, UK). Target genes were normalised using the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT-1) and the relative gene expression was calculated by comparing $2^{-\Delta\Delta Ct}$ values.

Migration 'scratch' assay

Chondrocytes were cultured to confluence in standard or in the presence of either 1ng/ml IL-1β or 1ng/ml TNF-αinflammatory culture conditions in 6 well plates, as described above. EVs were added to give an equivalent of a 1:1 ratio of originally seeded chondrocyte:MSCs as previously described. A scratch was made in the centre of the well with a p1000 pipette tip. Scratch filling was assessed after 1h, 3h, 24h and 72h using light microscopy. Scratch filling was quantified via blinded quantification of the percentage healing.

Statistics

All statistical work was performed using a one way annova. If $P<0.05$ = *, $P<0.01$=** $P<0.005$=***.

Results

Bone-marrow derived MSC differentiation

Cells obtained from bone marrow were demonstrated to be adherent to tissue culture plastic and capable of tri-lineage differentiation. These results show that the cells isolated were an appropriate BM-MSC cell source for subsequent experiments (Figure 2).

EV production, isolation and characterisation

EV were obtained from BM-MSC from all three horses differential sequential ultracentrifugation. The mean yield of EVs was $4.61X10^{10}$/million MSCs (+/- $0.89X10^{10}$ =2XSD). EV were characterised in a number of ways. Firstly, TEM was used to directly visualise the EV. TEM confirmed that the particles isolated by differential sequential ultracentrifugation were circular structures with a heterogeneous size, characteristic of EVs (Figure 3A). In order to measure the size of the EV produced by differential ultracentrifugation, NTA was used. This showed that the mean size of the EV isolated was 182 nm(+/- 21.6nm =2XSD) (Figure 3B), with the size of the EV at the smallest 10^{th} percentile to be 124nm, at the 50^{th} percentile 164nm and at the 90^{th} percentile to be 253nm (Figure 3C). Total protein analysis using a BCA revealed that 73% +/- 18 =2XSD of the total EV protein content was on the surface of the EV (Figure 3D).

These data show that EV can be harvested from equine BM-MSC using ultracentrifugation and that their mean size is 182nm.

EV Internalisation

Confocal microscopy of chondrocytes co-cultured with pre-labelled EVs was used to confirm the uptake of MSC derived EV by autologous chondrocytes (Figure 4). These data show that BM-MSC secrete EV that can be internalised by chondrocytes in monolayer culture. EV could clearly be seen within the membrane (red) of the chondrocytes due to the Z stack images. The EV (green) could not be detected on the surface as per figure 4 top however, they could be seen in the middle and bottom stack images. This being said, it could not be confirmed if the EV were clustered around the nucleus or any other organelles.

The effect of bone marrow derived-MSC EVs on chondrocytes cultured under inflammatory conditions

Chondrocytes were cultured under standard or inflammatory conditions and RT-qPCR performed to assess the effects of culture on gene expression of MMP13 and ADAMTS4 in the presence of autologous EV derived from BM-MSC (Figure 5). Gene expression was calculated relatiove to chondrocytes cultured under standard conditions without EV being added. In chondrocytes treated with IL-1β, MMP-13 and ADAMTS4, gene expression was significantly increased compared to the control and chondrocytes cultured with EV alone. The increase in gene expression was significantly reduced when the cells were co-cultured with EV in the presence of IL-1β. In chondrocytes treated with TNF-α, MMP-13 gene expression was significantly increased compared to the control (chondrocytes cultured with EV alone) and this increase in MMP-13 gene expression was significantly reduced when the cells were co-cultured with EV in the presence of TNF-α. However, ADAMTS4 expression decreased in chondrocytes cultured in the presence of TNF-α compared to controls, and this down-regulation was significantly reduced in the presence of EV.

These data show that BM-MSC EV can influence gene expression in chondrocytes and that they can affect the inflammatory response to IL-1.

The effect of bone marrow derived-MSC EVs on chondrocytes migration when cultured under inflammatory conditions

A 'scratch' was made in a confluent monolayer of chondrocytes, EV added and the closure of the scratch gap measured. The addition of IL-1 β did not have any effect on scratch healing and was unaffected by the presence of EV however, the addition of TNF-α significantly increased the scratch healing compared to all other treatment groups. The addition of EV did not change the rate of scratch healing in the presence of TNF-α (Figure 6).

Discussion

This paper describes, for the first time, the isolation and characterisation of EV derived from equine BM-MSC and demonstrates their ability to alter gene expression in normal chondrocytes in the presence of IL-1β and TNF-α.

With regards to EVs in horses we found only six publications were extracellular vesicles had been isolated from any horse tissues(19–23). Of these six publications, four derive the EVs from adipose derived stem cells and the others are isolated from amniotic stem cells(19–21,23,24). With regards to the isolation of EVs, ultracentrifugation, size exclusion chromatography or precipitation however, we identified no publications regarding the isolation of equine bone marrow MSC EVs or their applications as a therapeutic(19–24).

Whilst MSC have been shown to be efficacious in the treatment of joint disease in the horse (25)(26), MSC therapy has inherent drawbacks including provoking an immune response. In addition the practical challenges of removing equine bone marrow and subsequent sterile cellular expansion prior to re-injection makes MSC therapy prohibitively costly in many cases. In contrast the use of BM-MSC EV as a potential off-the-shelf therapeutic (27) makes the development of EV therapy for the treatment of equine joint disease an attractive proposition.

In this study we have used differential ultracentrifugation technique to isolate a population of EV from BM-MSC. In a recent publication ultracentrifugation, ultrafiltration and charge based precipitation techniques were used to isolate a reported ‘exosome’ population from horse adipose derived MSC (22), with ultrafiltration giving the highest and ultracentrifugation the lowest yield. However, we consider that ultracentrifugation isolates EV in a reproducible

313 fashion and that the EV produced fall within a narrow size range comparable to EVs of other
314 species.
315
316 The nature of the EV isolated from bone marrow-MSC was shown using TEM and NTA
317 analysis. TEM provides important information in the characterisation of EV (12) and, in this
318 study, demonstrated the presence of the rounded vesicles of heterogenous size as reported by
319 other authors (4,22,28,29) NTA analysis was used to measure the size distribution and the
320 concentration of the isolated EV in the samples studied. Whilst not without some technical
321 limitations, NTA allows for the rapid measurement of these parameters and is widely used in
322 EV research (12). The mean size of the bone marrow derived MSC reported in this study
323 (182nm) is similar to that reported for EV isolated from adiopose derived MSC in the horse
324 which was variously reported as between 91-178 nm depending on the method of isolation (22)
325 and in other species (mean size 112nm (murine, (30), 231nm (human, (31)).
326
327 Having isolated EV from equine BM-MSC, we investigated the interactions between the EV
328 and a target cells in the joint, the chondrocyte. Autologous chondrocytes from normal joints
329 were grown in monolayer culture and incubated with fluorescent labelled EV for one hour. Z-
330 stack images, acquired with confocal microscopy, were used to identify EV in the cellular
331 environment, revealing that the EV were internalised and were located in the cell. In a number
332 of cells multiple EV were present. This uptake of BM-MSC EV has previously been shown in
333 osteoarthritic human chondrocytes (32) but not reported before in equine chondrocytes, normal
334 or osteoarthritic. These results confirmed that the isolated EV were internalised by the potential
335 target cell.
336

In order to demonstrate efficacy of EV isolated from bone marrow derived MSC we investigated the effects of EV on the gene expression of MMP-13 and ADAMTS4 produced by chondrocytes cultured in the presence of IL-1β or TNF-α. One of the main goals in the treatment of equine OA is to inhibit further inflammation in the joint. In this study we have used the inflammatory cytokines IL-1 and TNF-α to induce a catabolic response in chondrocytes. These cytokines increased MMP-13 and ADAMTS4 expression as has been reported previously (33). BM-MSC derived EV counteracted the inflammatory effects of IL-1β, significantly decreasing the upregulation of gene expression of both MMP-13 and ADAMTS4. This anti-catabolic effect is similar to that reported in equine chondrocytes for corticosteriods (33,34). The BM-MSC derived EV had a similar effect on MMP-13 gene expression induced by TNF-α i.e. they decreased the upregulation of gene expression, however, the EV were did not decrease the upregulation of ADAMTS4. This lack of effect on TNF-α induced ADAMTS4 was unexpected, although it has been previously reported that equine chondrocyte MMP-13 and ADAMTS4 can behave differently in the pro-inflammatory environment (35). Overall, the results of this study suggest that EV isolated from BM-MSC can counteract inflammatory effects of IL-1β but not the effect of TNF-α on ADAMTS4.

Finally we evaluated the ability of EV isolated from BM-MSC to influence the migration of chondrocytes in a simplistic 'scratch' assay (36). In this assay IL-1β did not have a discernible effect on chondrocyte migration, whereas TNF-α appeared to markedly enhance chondrocyte migration. Previous findings have shown that both IL-1β and TNF inhibit or have little effect on chondrocyte migration from non-fibrillated osteoarthritic cartilage (36), however no previous studies have been reported on normal equine chondrocytes and it is possible that TNF-α is acting to promote migration in these normal cells, as it does in other cell types (37). Whilst further work is required to investigate this response, no effect on cell migration was detected in the presence of BM-MSC EV.

This study has demonstrated that equine BM-MSC produce EV that can be isolated by ultracentrifugation, that the EV can be internalised into chondrocytes and have anti-catabolic properties. In this study, chondrocytes were co-cultured with EVs such that the number of EVs added to the cells was equivalent to those released from a 1:1 ratio of MSCs to chondrocytes and, as such, the 'dosage' or EV was speculative however it was the same ratio as that used by Vonk *et. al* (32). Further work is required to elucidate the optimum concentration of EV for maximum effect. However, the results of this study show that BM-MSC EV could be considered for the treatment of inflammatory joint disease in the horse.

Figure legends

Figure 1: Differential Sequential Ultracentrifugation for EV Isolation
Conditioned media is centrifuged at 300g for 5 minutes, 2,000g for 20 minutes at 4°C, 10,000g for 45 minutes, at 4°C under vacuum, 100,000g for 90 minutes at 4°C under vacuum x 2.

Figure 2 Tri-lineage differentiation of bone marrow derived MSC. A Staining with Oil-Red-O showing the presence of red fat vacuoles stained red confirming adipogenesis, B Staining with Alcian blue showing the presence of blue chondrocytes confirming chondrogenesis and C, staining with alizarin red showing the presence of red mineral staining confirming osteogenesis.

Figure 3: EV characterisation.
A) Transmission electron microscopy showing isolated EVS have a circular morphology and that different EVs have different sizes. Scale bar = 200nm **B**) Nanoparticle tracking analysis showing the size distribution of the EV population was 45-450nm characteristic of EVs. **C)** The mean particle size was 182.4.8nm with a heterogeneity of sizes across the sample, (**D)** Analysis of protein content of the EVs showed that the mean percentage of EV surface protein in comparison to the internal protein is 73% (+/-18% 2XSD) Error bars =2XSD

Figure 4: Confocal imaging of equine chondrocytes showing internalisation of bone marrow derived MSC EV.
Three images from the confocal z-stack (Top, middle and bottom respectively) showing EVs pre-stained with PKH67 (green) and internalised by an equine chondrocyte stained with the membrane stain WGA-555 (red) and nuclei stain (blue). This figure shows the EVs primarily located in the middle of the cell.

Figure 5: MMP-13 and ADAMTS4 gene expression
Catabolic gene expression in chondrocytes cultured in the presence of IL-1β and TNF-α in the presence and absence of autologous bone marrow MSC derived EV. MMP-13 gene expression is increased in the presence of IL-1β and TNF-αand significantly reduced when EV are added. Similarly, ADAMTS4 gene expression is increased in the presence of IL-1β and significantly reduced

when EV are added, however ADAMTS4 gene expression is reduced in the presence of TNF-α was significantly lessened when EV are added. Error bars =2XSD ***=p<0.005 *=P<0.05

Figure 6: Scratch healing

Percentage healing of a scratch made in a confluent chondrocyte monolayer cultured in the presence of IL-1β and TNF-α in the presence and absence of autologous bone marrow MSC derived EV for 30h. No significant difference between the treatment groups was observed in the presence of EV alone and IL-1β When TNF-α was added a significant increase in scratch healing was detected which was not affected by the presence of EV. C=Control, CE=Control + EVs, I = interleukin treated, IE = interleukin + EVs, T = TNFα treated, TE = TNFα + EVs. *** = P<0.005, error bars =2XSD

Declaration

Ethics approval and consent to participate

Not applicable but discussed in text

Consent for publication

Obtained

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

Funding

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement 761214. The material presented and views expressed here are the responsibility of the authors only. The EU commission takes no responsibility for any use made of the information set out. This study has also received funding from the BBSRC Bright Stars summer studentship award. The funding was split equally in the design of the study and the collection, analysis and interpretation of data and in writing the manuscript.

Authors' contributions

WEH & CHT did all the data acquisition and analysis jointly apart from where STL performed the osteoblast differentiation and took the image for Figure 2C. KJN assisted with

the harvest of the bone marrow from the horses. FMDH for her supervision throughout the project

Acknowledgements This research was supported by the Cambridge NIHR BRC Cell Phenotyping Hub for their advice and support in imaging.
Multi-specimen prep and imaging was performed at the Cambridge Advanced Imaging Centre. Thanks must go especially to Dr Karin Muller for her assistances especially. Thanks should also go to Mr Matthew Rhodes for his help with finding suitable horses for isolating the bone marrow from the horses.

Authors information

Not applicable

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Figures

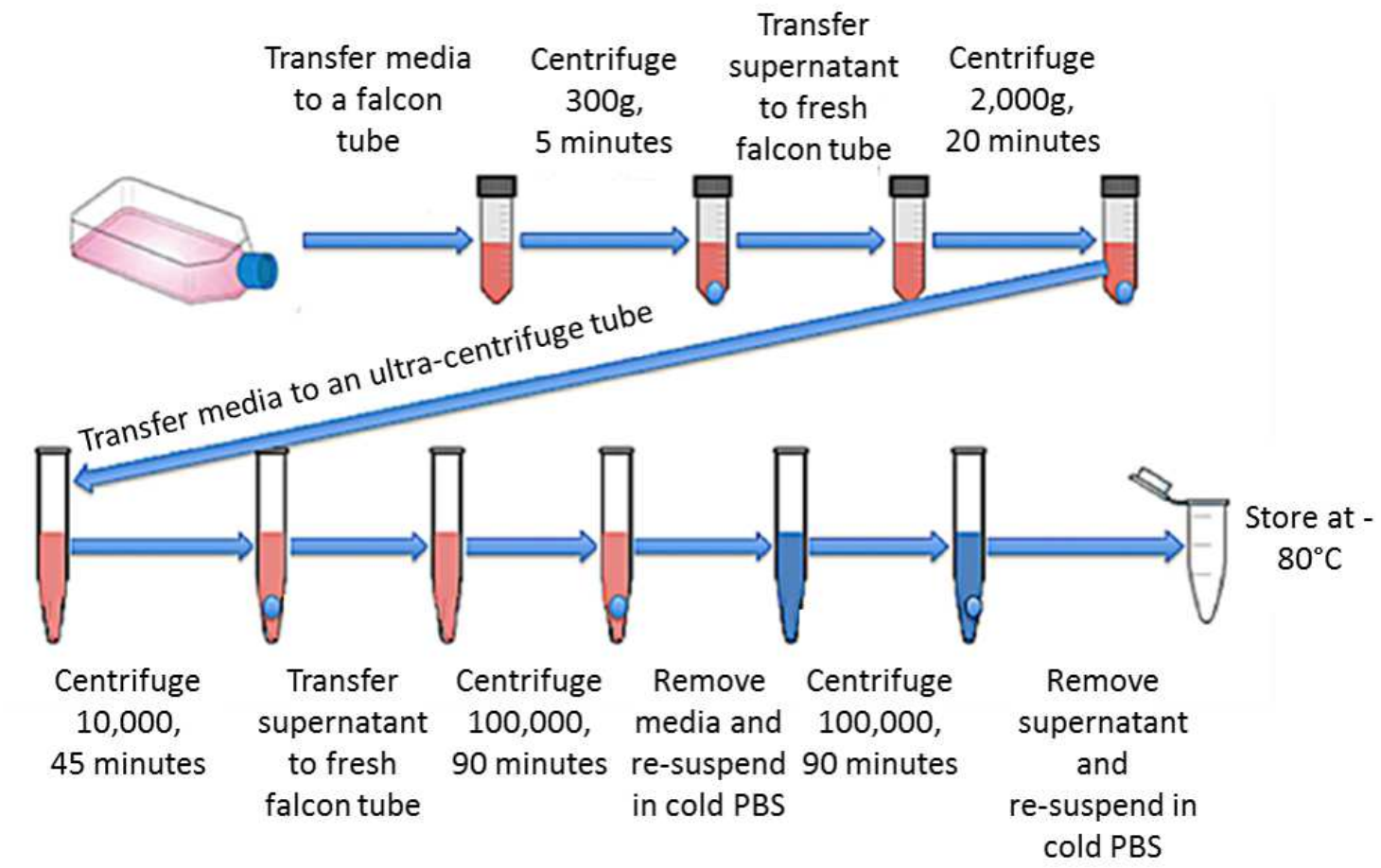

Figure 1

Differential Sequential Ultracentrifugation for EV Isolation. Conditioned media is centrifuged at 300g for 5 minutes, 2,000g for 20 minutes at 4°C, 10,000g for 45 minutes, at 4°C under vacuum, 100,000g for 90 minutes at 4°C under vacuum x 2.

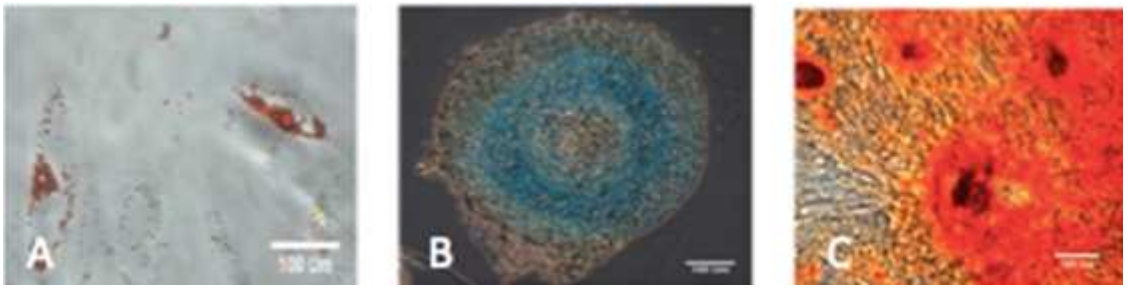

Figure 2

Tri-lineage differentiation of bone marrow derived MSC. A Staining with Oil- Red-O showing the presence of red fat vacuoles stained red confirming adipogenesis, B Staining with Alcian blue showing the presence of blue chondrocytes confirming chondrogenesis and C, staining with alizarin red showing the presence of red mineral staining confirming osteogenesis.

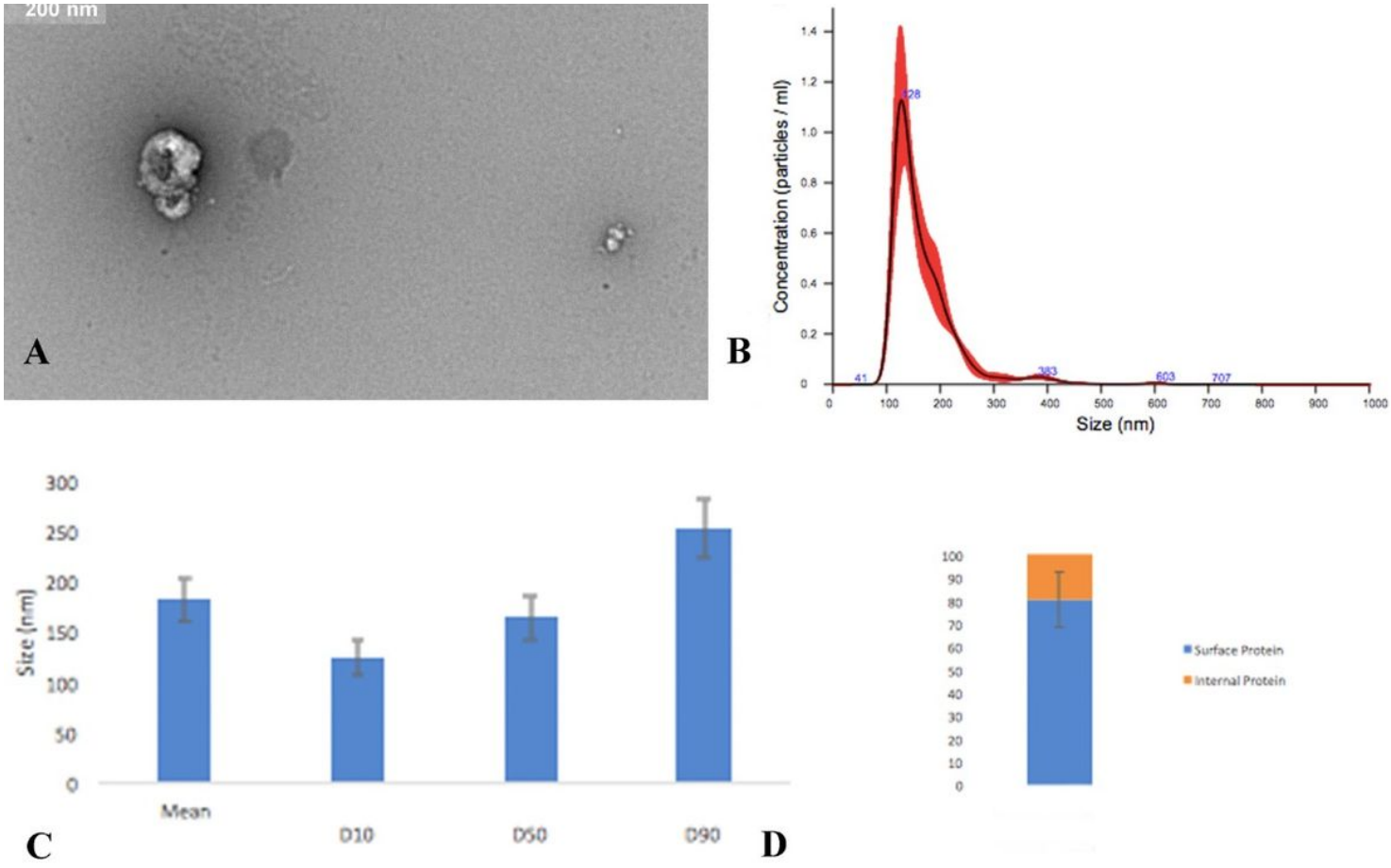

Figure 3

EV characterisation. A) Transmission electron microscopy showing isolated EVS have a circular morphology and that different EVs have different sizes. Scale bar = 200nm B) Nanoparticle tracking analysis showing the size distribution of the EV population was 45-450nm characteristic of EVs. C) The mean particle size was 182.4.8nm with a heterogeneity of sizes across the sample, (D) Analysis of protein content of the EVs showed that the mean percentage of EV surface protein in comparison to the internal protein is 73% (+/-18% 2XSD) Error bars =2XSD

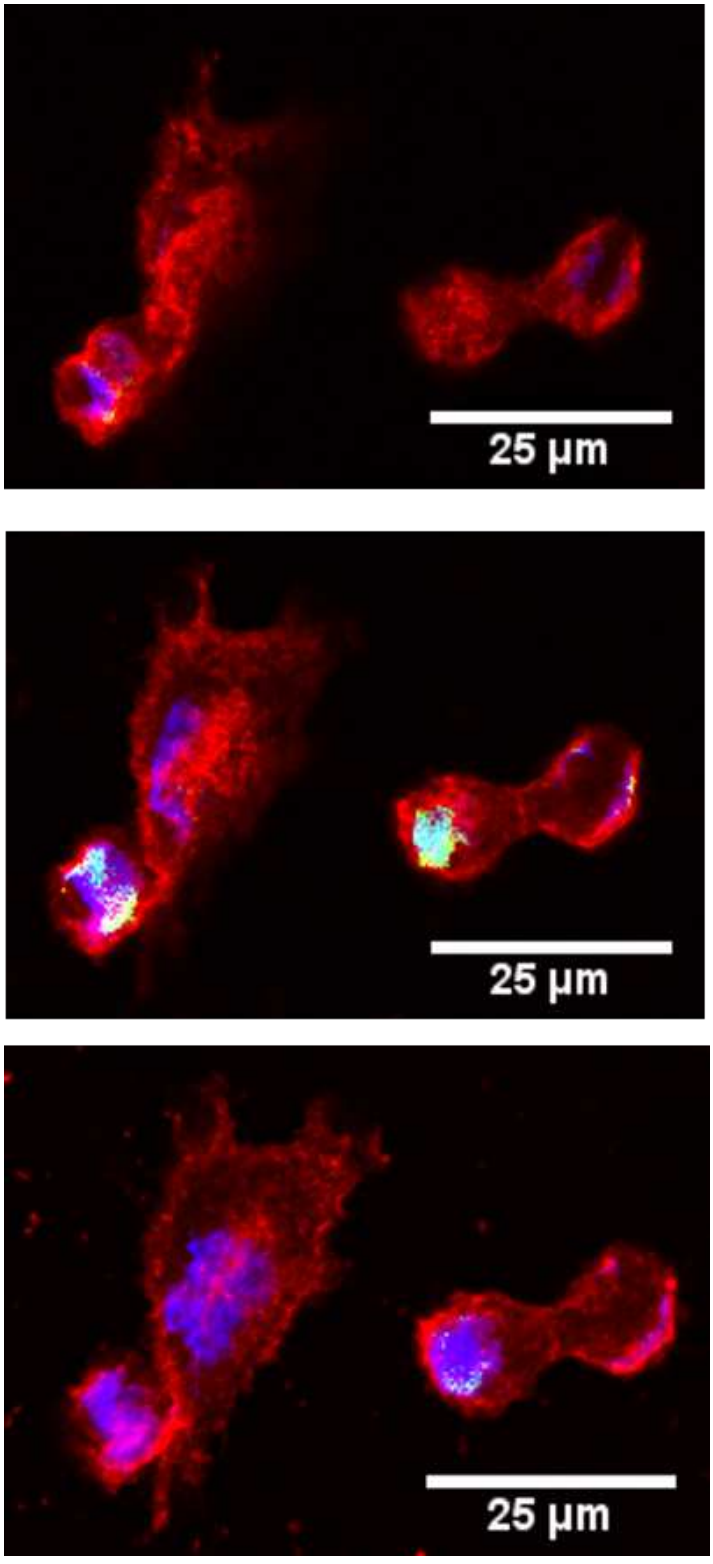

Figure 4

Confocal imaging of equine chondrocytes showing internalisation of bone marrow derived MSC EV. Three images from the confocal z-stack (Top, middle and bottom respectively) showing EVs pre stained with PKH67 (green) and internalised by an equine chondrocyte stained with the membrane stain WGA-555 (red) and nuclei stain (blue). This figure shows the EVs primarily located in the middle of the cell

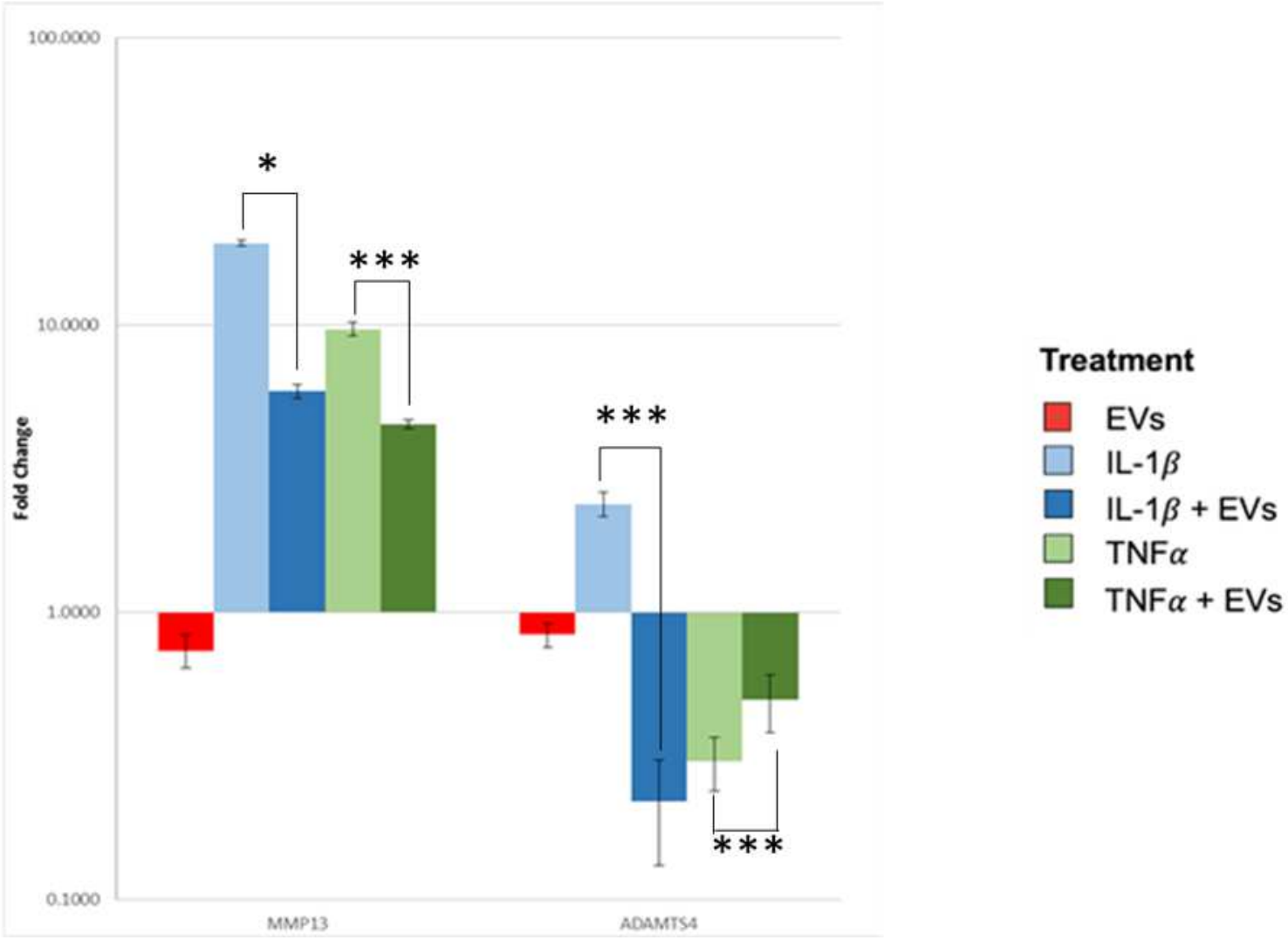

Figure 5

MMP-13 and ADAMTS4 gene expression Catabolic gene expression in chondrocytes cultured in the presence of IL-1β and TNF-α in the presence and absence of autologous bone marrow MSC derived EV. MMP-13 gene expression is increased in the presence of IL-1β and TNF-αand significantly reduced when EV are added. Similarly, ADAMTS4 gene expression is increased in the presence of IL-1β and significantly reduced when EV are added, however ADAMTS4 gene expression is reduced in the presence of TNF-α was significantly lessened when EV are added. Error bars =2XSD ***=p<0.005 *=P<0.05

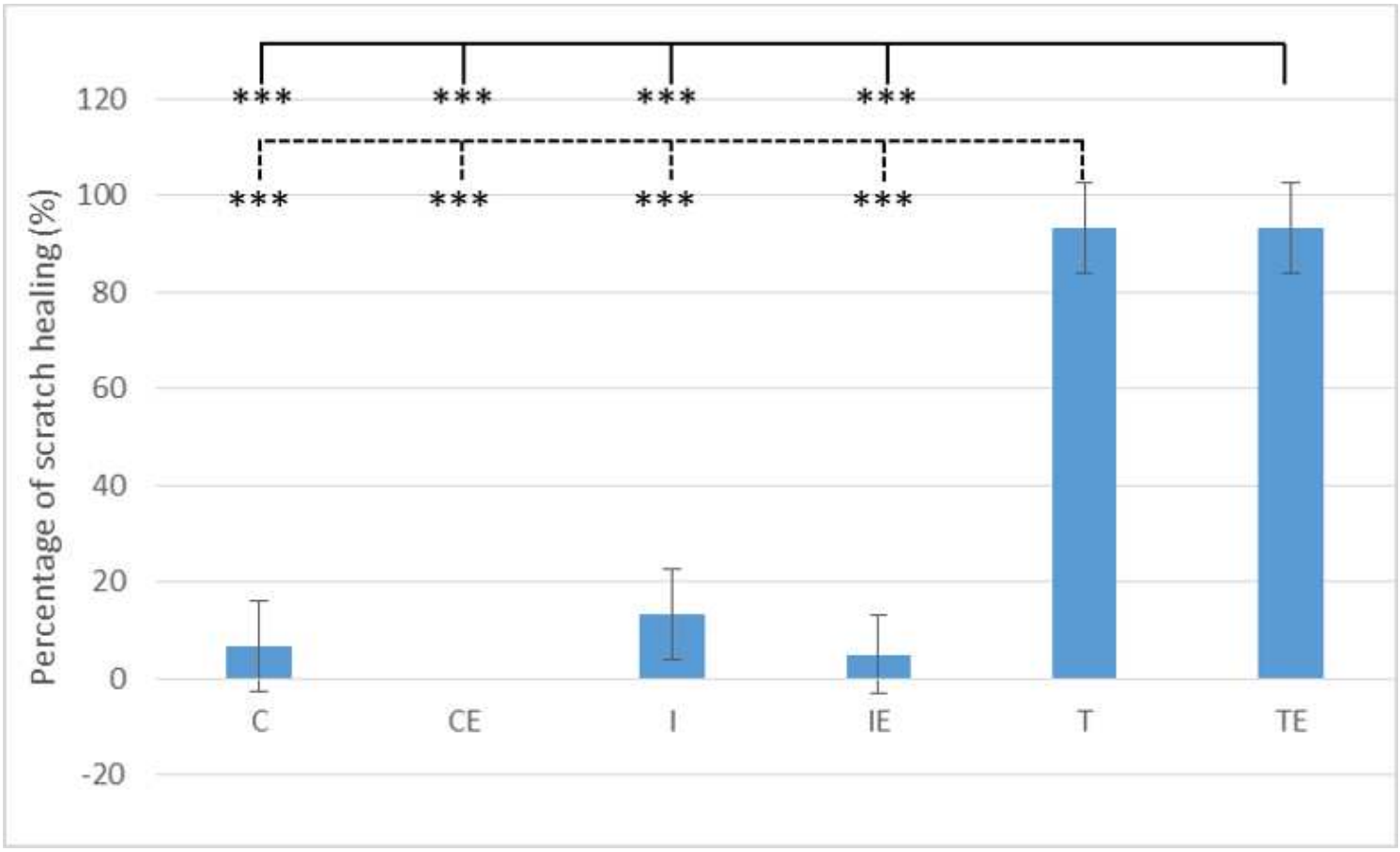

Figure 6

Scratch healing. Percentage healing of a scratch made in a confluent chondrocyte monolayer cultured in the presence of IL-1β and TNF-α in the presence and absence of autologous bone marrow MSC derived EV for 30h. No significant difference between the treatment groups was observed in the presence of EV alone and IL-1β When TNF-α was added a significant increase in scratch healing was detected which was not affected by the presence of EV. C=Control, CE=Control + EVs, I = interleukin treated, IE = interleukin + EVs, T = TNFα treated, TE = TNFα + EVs. *** = P<0.005, error bars =2XSD