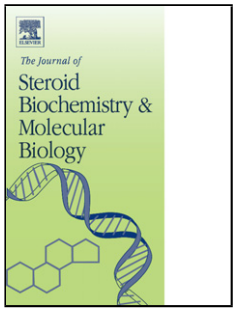

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Pharmacokinetics of intra-articular vitamin D analogue calcipotriol in sheep and metabolism in human synovial and mesenchymal stromal cells

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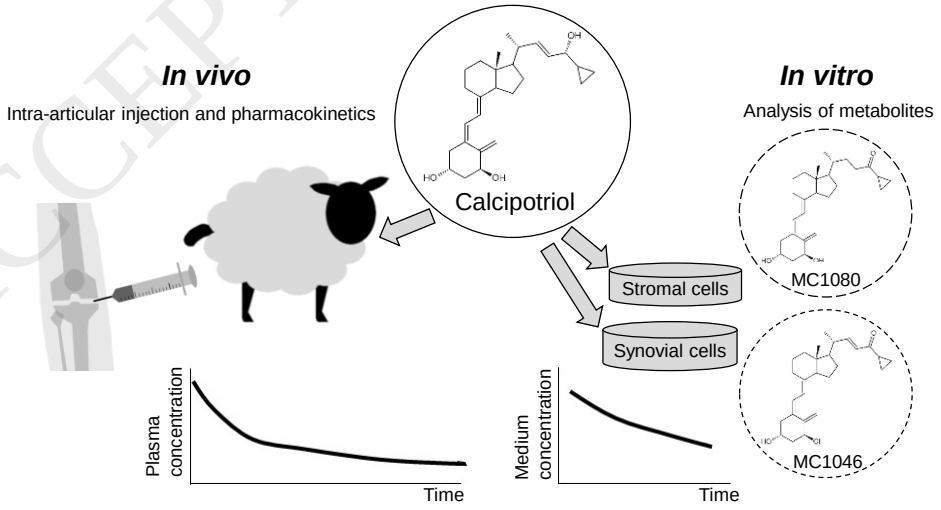
Graphical abstract



Highlights

- Calcipotriol produced no adverse effects in sheep after an intra-articular dose.
- Both synovial and mesenchymal stromal cells metabolize calcipotriol in vitro.
- Calcipotriol stays in the joint for long with small systemic exposure (1-13%).

ABSTRACT

Calcipotriol (MC903) is a side chain analogue of the biologically active 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$]. Due to its anti-inflammatory and anti-proliferative effects on stromal cells, calcipotriol is a promising candidate for the local treatment of arthritis. In this preliminary work, we studied the pharmacokinetics and safety of calcipotriol after an IV (0.1 mg/kg given to one sheep) and intra-articular dose (0.054 mg/kg, 0.216 mg/kg and 0.560 mg/kg given to three sheep). The terminal half-life of calcipotriol was approximately 1 hour after an IV dose. After intra-articular dosing, the systemic absorption was between 1 and 13% during the observed 24 hours. Hypercalcemia or other clinical adverse effects did not occur in any animal during the study, and no macroscopic or microscopic alterations were seen in the synovium of the calcipotriol-injected knees compared to the vehicle knees. The *in vitro* metabolism of calcipotriol was analyzed with LC-MS from human synovial and mesenchymal stromal cell cultures. Both cell types were able to metabolize calcipotriol with MC1080 and MC1046 as the main metabolites. CYP24A1 transcripts were strongly induced by a 48-hour calcipotriol exposure in mesenchymal stromal cells, but not consistently in synovial stromal cells, as determined by RT-qPCR. Calcipotriol proved to be safe after a single intra-articular dose with applied concentrations, and it is metabolized by the cells of the joint. Slow dissolution of calcipotriol crystals in the joint can extend the pharmaceutical impact on the synovium, cartilage and subcortical bone.

Keywords: Calcipotriol, Mesenchymal stromal cell, Synovial stromal cell, Intra-articular dosing, Pharmacokinetics, Metabolism

1 INTRODUCTION

Calcipotriol is a side-chain analogue of 1,25-dihydroxyvitamin-D_3 [$1,25(OH)_2D_3$], which differs from its physiological counterpart by the presence of the 22-23 double bond, a 24-hydroxyl group and a cyclopropane ring at the end of the side-chain [1]. It has similar differentiation-inducing and antiproliferative properties as $1,25(OH)_2D_3$ in vitro [2, 3, 4], but its calcemic effect is much less than $1,25(OH)_2D_3$. [3, 4, 5]

One of the main reasons that has stimulated research into vitamin D analogues was to find compounds capable of inducing the effects of $1,25(OH)_2D_3$ excluding its hypercalcemic effect. Calcipotriol is one of these synthetic analogues that displays the physiological properties of $1,25(OH)_2D_3$ while being 100-200 times less hypercalcemic [3, 4, 6]. Currently, vitamin D analogues are used in treatment of psoriasis, secondary hyperparathyroidism and osteoporosis [7]. Calcipotriol is used in the topical treatment of psoriasis where it has been found to be both safe and effective [8]. However, new applications are being evaluated. For instance, calcipotriol has been shown to alleviate induced liver fibrosis in mice [9] and multiple skin disorders in human subjects [10].

Calcipotriol is known to have two less active principal metabolites *in vitro*, MC1046 and MC1080, both of which are 24-ketones, and in the latter, the 22-23 bond is also saturated. This was observed in experiments with transformed keratinocytes, osteosarcoma, kidney and hepatoma cell lines [1]. Both of these metabolites have a low affinity for the vitamin D receptor (VDR), whereas calcipotriol and $1,25(OH)_2D_3$ bind to VDR with almost equal affinities [1, 4]. The complete metabolic pathway of calcipotriol is not entirely clear, but it is known that calcitroic acid is one of the end-products [1]. This suggests that there might be some overlap in the metabolism of calcipotriol and $1,25(OH)_2D_3$.

In rats, the half-life of calcipotriol is 4 min, whereas the half-life of $1,25(OH)_2D_3$ is 13 min after an IV administration [4]. After an oral dose, the half-life of calcipotriol is 0.6 h, much shorter than the half-lives of $1,24(OH)_2D_2$, $1,25(OH)_2D_2$, and $1,25(OH)_2D_3$, which are 4.9, 5.1 and 5.8 h, respectively [11]. This highlights the rapid elimination of calcipotriol *in vivo*, contributing to its low calcemic effect.

It has been observed that both calcipotriol and $1,25(OH)_2D_3$ have a long-lasting antiproliferative effect on synovial stromal cells and they inhibit the proinflammatory cytokine secretion *in vitro*

despite the presence of TNF-α. Also, the synovia of both RA and OA patients have a high expression of VDR. [12] Since calcipotriol exerts anti-inflammatory and antiproliferative effects on stromal cells [12] while its calcemic effects are almost insignificant, we started to explore its possible application in the local treatment of arthritis.

Pharmacokinetics and tolerability of calcipotriol after intra-articular injections have not been studied earlier. For that purpose, we carried out this preliminary study in sheep. Sheep were chosen on the grounds of mammalian metabolism and similar weight and joint structure to humans [13].

It was also not previously known whether calcipotriol could be locally metabolized in the joint. Therefore, another goal was to measure the rate of calcipotriol metabolism in an *in vitro* model and to identify the main metabolites in synovial and mesenchymal stromal cells (SSCs and MSCs, respectively).

2 MATERIALS AND METHODS

2.1 Animals

The study protocol was reviewed and approved by the National Animal Experiment Board of Finland (license number ESAVI/2526/04.10.07/2016). The animal care and experimental procedures were in line with Finnish [14, 15] and European legislation [16] and recommendations. The reporting of animal experiments in this study is in compliance with the ARRIVE guidelines [17].

Four 3-year-old Åland landrace ewes (Lammastila Sikka Talu, University of Turku, Rymättylä, Finland) were used. None of the sheep had been out in pasture due to the early spring season, in its stead dry hay was available *ad libitum*. On the farm, the sheep's diets were supplemented with mineral feed containing approximately 1600 IU vitamin D_3 per day (Vilomix Lammas Mira, Suomen Rehu, Finland).

After transport, the sheep were acclimated to the Laboratory Animal Centre of the University of Oulu for a week before the start of the experiment. The sheep were always housed, whenever possible, in a group pen of 7.2 m^2. On the day when the catheters were inserted, the sheep were kept in individual pens of 3.6 m^2. The other three sheep were, however, in direct contact with the fourth animal through a 20 cm horizontal gap in the partition wall and no individual sheep was left alone in the animal room during the experiment. The room temperature was 18 ± 2°C, the ventilation rate 15 times/hour and humidity was 50±10 %. The light-dark cycle was 12:12 with the lights off at 6 pm.

The sheep were given tap water and hay *ad libitum* and the diet was supplemented with a daily dose of mineral feed containing 1800-3000 IU of vitamin D_3 (Lammas Hertta, Suomen Rehu, Finland).

At the beginning of the experiments, the sheep weighed 43.9 kg, 50.0 kg, 49.9 kg and 48.2 kg (Table 1).

2.2 Administration of calcipotriol and collection of blood samples

For the administration of calcipotriol and collection of blood samples, both jugular veins were catheterized with 14 gauge IV cannulae, after numbing the site with s.c. lidocaine. Calcipotriol was administered IV to one sheep and intra-articularly to three sheep. The IV calcipotriol was given as a 30-second bolus into the left jugular vein to determine the half-life of elimination. All samples for pharmacokinetic analysis and other blood samples were collected from the right jugular vein. Venous blood (4 ml aliquots) was collected at 15 time points: predose (0 min), 1, 2, 5, 10, 15, 30, 60 min and then at 2, 3, 5, 8, 24, 48, and 72 h after dose.

Intra-articular injections were given into both knee joints while the animal was sedated with medetomidine (calcipotriol in the right knee and the vehicle in the left knee), but the calcipotriol dose was different for each sheep (0.054 mg/kg, 0.22 mg/kg and 0.54 mg/kg). The doses were selected to be high enough to be clearly observable while being lower than doses known to cause hypercalcemia. The sheep moved and behaved normally after the procedure and therefore no post-treatment analgesic was needed. After the 30 min blood sample, the sedation was reversed with atipamezol hydrochloride in all four sheep.

After the intra-articular administration of calcipotriol, the time points of blood collection were otherwise the same as after IV administration but the 1-10 min time points were omitted. All pharmacokinetic samples were collected into 10mL EDTA tubes and were kept on ice until centrifuged within 30 minutes to obtain plasma and stored at -80 ºC until analyses. Whole blood (hemoglobin and blood count), serum samples ($25(OH)D_3$, $1,25(OH)_2D_3$ and Ca-ion) and plasma samples (phosphate, creatinine, transaminases) for pharmacodynamics analyses were collected at

baseline (before injections), 24-, 48- and 72-hour time points after injections and stored at -80 ºC. Three of the sheep were euthanized by pre-anesthesia with i.m. medetomidine and ketamine, followed by an overdose of pentobarbital IV 13-14 days after the administration of calcipotriol. The sheep that was treated with the IV dose of calcipotriol was rehomed after veterinary inspection, as there was no need for joint tissue collection.

2.2.1 Analyses of pharmacokinetic samples

Quantification of calcipotriol concentrations in plasma samples at each time point (0-24h) was conducted using an LC-MS/MS analytical system (Instrument: Waters Acquity UPLC + Waters TQ-S Triple Quadrupole mass spectrometer, Column: Waters Acquity BEH Shield RP18 with guard filter) at Admescope, Oulu, Finland. The mass spectrometer was operated in the positive electrospray ionization (ESI) mode at a capillary voltage of 1000 V, source temperature of 150 °C and desolvation temperature of 550 °C. Desolvation gas flow was set to 900 L/h, cone gas flow to 150 L/h and collision gas flow to 0.18 mL/min. The used elution solvents were 2 mM ammonium formate (A) and 15% isopropanol in acetonitrile (B). Elution was started by keeping B at 20% for the first 0.5 min and directing flow to waste via the direct valve. B% was increased to 80% at 2.5 min and directly raised to 95% min with an isocratic hold for 3.5 min. Column oven temperature was 45 °C and injection volume 4 µl. Calcipotriol was detected with the following MRM-reactions: m/z 395>105, m/z 395>133 and m/z 395>151. The used collision energies for the MRM reactions were 42, 30 and 20 eV, respectively. Calcipotriol-d4 was used as internal standard and was detected with the following MRM-reactions: m/z 399>155, m/z 399>133 and m/z 399>151 with used collision energies of 42, 32 and 24 eV, respectively. Data was processed with Masslynx 4.1 software. The analytical method demonstrated good selectivity and a negligible matrix effect as

well as a low limit of quantification at 0.12 ng/ml. The plasma concentration versus time data was calculated using WinNonlin software.

The samples were prepared by liquid–liquid extraction. An aliquot of 200 µl of plasma sample was mixed with 8µl of an internal standard solution (DMSO containing 500 ng/ml calcipotriol D_4) followed by an addition of 1250 µl diethyl ether:cyclohexane (7:3) and a 60-minute incubation at room temperature. The sample vials were introduced into a mixture of dry ice and ethanol, resulting in a frozen water layer, which allowed transfer of 1000 µl of ether-cyclohexane-phase into another vial. The solvent was dried under nitrogen and the residue diluted in 100 µl of acetonitrile. Aliquots of 90 µl were transferred into a 96-well plate prior to the analysis.

Stock solutions of standard samples and the quality controls were prepared in DMSO to obtain a concentration of 1 mg/ml. Working solutions were made in DMSO by serial dilution of stock solutions and finally adding 8 µl of spiking solution into 192 µl blank sheep plasma to obtain concentrations 0.004, 0.012, 0.04, 0.12, 0.4, 1.2, 4, 12, 40, 120, 400, 1200 and 4000 ng/ml. These samples were further mixed with 8µl of the internal standard solution (DMSO containing 500 ng/ml calcipotriol D_4) and prepared similarly as samples.

2.2.2 Analyses of pharmacodynamic parameters

Measurements of 25-hydroxyvitamin D_3 [$25(OH)D_3$] and $1,25(OH)_2D_3$ were done from serum samples using a chemiluminescent immunoassay (Diasorin, Stillwater, USA). The samples were analyzed in Nordlab, Oulu, Finland. Both methods detect the D_2 and D_3 forms of these metabolites. Ionized calcium levels were measured from serum samples using an ion-specific electrode and phosphate levels by a photometric method (Nordlab, Oulu, Finland).

2.3 Histology and synovial fluid analysis

Immediately after euthanasia, tissue samples were collected from the heart, lung, kidney, liver and synovia from all three sheep (10, 11 or 12 days after intra-articular injections of sheep ID 7154, 7135 and 7111, respectively). Synovial tissue and synovial fluid samples were collected from the calcipotriol-injected and vehicle knees. Formalin fixed paraffin embedded tissues were cut into 3.5 µm sections and stained with hematoxylin-eosin.

A sample of the medial cartilage from both calcipotriol and vehicle knees of each animal was collected. One histological section from each individual cartilage was used in the analysis. Imaging of the samples was done using Leica Application Suite (version 4.3.0) software and Leica DFC320 digital camera connected to Leica DM LB2 microscope equipped with 10x objective. The images were saved in TIFF format with 2088x1550 resolution and analyzed using QuPath (version 0.1.2) software.

The cartilage surface was split to 200µm x 200µm square-shaped areas placed tangentially to the surface of the cartilage, and cell densities within these areas were analyzed as individual samples. The cell densities between the calcipotriol and vehicle knees were compared with Mann-Whitney test due to varying, partly small sample size and the non-normality of the data in some of the knees. Cartilage areas of poor quality (<200µm thick, detached surface layer or otherwise unfeasible quality) were not subjected to analysis. In total, 45 + 47 (calcipotriol + control) areas were analyzed from sheep 7111, 15 + 12 from sheep 7135, and 33 + 44 from sheep 7154. To avoid selection bias in photographing and choosing the square areas from the photographs, it was decided that a single section of each knee was photographed entirely, and all feasible square-shaped areas were included in the analysis. This explains the variation in the sample sizes.

Synovial fluid samples were first analyzed by polarized microscopy (Nicon Diapflot DMT Abrio Cri PLM-System) for crystals. For identification of calcipotriol in crystals, 30 µL of the synovial fluid was mixed with 100 µL of isopropanol and centrifuged at 14 000 rpm and the supernatant was

analyzed by LC-MS at Oulu University. An aliquot of 10µl synovial fluid was extracted with 100µl 2-propanol, phases were separated by centrifugation and 5µl of the 2-propanol phase was subjected to LC-MS in an Aquity UPLC system coupled to a SynaptG1 Q-Tof type mass spectrometer. The chromatography column was a BEH C18, 2.1x100, eluted with an acetonitrile gradient (A: 0.1% Formic Acid, B: 0.1% Formic Acid in Acetonitrile; 60%A to 100%A in 4 min, flow 0.4ml/min). The mass spectrometer was operated in the positive mode collecting 0.2s scans in the centroid mode from m/z 50 to 1000. Calcipotriol was analyzed by the extracted ion chromatogram of its accurate mass with confirmation by calcipotriol standards. Detection limit for calcipotriol was determined by measuring serial dilutions of calcipotriol.

2.4 Primary cell cultures

Primary cultures of synovial stromal cells and mesenchymal stromal cells were established from the tissue samples collected from patients with rheumatoid arthritis (RA) or osteoarthritis (OA) that had undergone a total knee replacement surgery in Oulu University Hospital during the years 2012-2016. All RA patients fulfilled the American College of Rheumatology criteria for RA (1987). OA patients suffered from pain and functional disability of the joint and the diagnosis was verified by x-ray. The Ethics Committee of Oulu University approved the study protocol. All patients gave their informed consent for the use of sample material. The cells were collected from two male and five female patients. Sex of the patients was not considered as a relevant factor in the analysis of data.

For synovial stromal cell cultures, twelve 2-3 mm pieces of synovial tissue were digested with 4 ml of collagenase (100 U/ml) and 20 µl DNase (the final concentration of 50 U/ml) at 37°C for 2-3 hours and centrifuged. The pellet was suspended in complete media (α-MEM 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin) and cells were allowed to adhere overnight. For mesenchymal stromal cell cultures bone marrow aspirates from the femoral shaft and

epicondylar region were suspended in complete medium, seeded in a cell culture flask and cells were allowed to adhere overnight. The non-adherent cells were rinsed away the next day and adherent cells were further cultured in the complete media. A half of the media was changed two times every week. All cell lines were stored in liquid nitrogen before use. All experiments were conducted between the third to fifth passages.

Altogether four SSC and four MSC cell lines (2 patients with OA and 2 with RA) were used in the analysis of reverse transcriptase quantitative PCR (RT-qPCR). For the metabolite study, MSC cell lines of RA (two cell lines) and OA (one cell line) patients were cultured together in the same flask to achieve enough MSC cells for the assay, but cocultures of SSCs (three cell lines) were from only RA patients. These were different cell lines from those used in RT-qPCR.

2.4.1 Calcipotriol metabolism *in vitro* in synovial and mesenchymal stromal cells

The metabolism of calcipotriol was analyzed by LC-MS in Admescope, Oulu, Finland. (Instrument: Waters Acquity UPLC + Thermo Q-Exactive Orbitrap mass spectrometer, Column: Waters Acquity BEH Shield RP18 with guard filter). The mass spectrometer was operated in positive electrospray ionization (ESI) mode at a capillary voltage of 4000 V, capillary temperature of 320 °C and an auxiliary gas heater temperature of 500 °C. The sheath gas, auxiliary gas and sweep gas flows were set to 50, 10 and 5 arbitrary units, respectively. The mass spectrometer was operated in the data-dependent MS^2 mode, which acquires full-scan MS and MS/MS fragment ion data in the same run. Resolution of 35 000 (full width at half maximum at m/z 200) and mass range of m/z 70-1000 and were used for the full scan. Resolution of 17 500 and collision energies of 10 and 30 were used in the $ddMS^2$ mode. The used elution solvents were 2 mM ammonium formate (A) and 15% isopropanol in acetonitrile (B). Elution was started by keeping B at 10% for the first 0.5 min and directing flow to waste by direct valve. B% was increased to 40% at 3.0 min and to 95% in 3.5 min

with isocratic hold to 4.5 min. The column oven temperature was 45 °C with an injection volume of 4 µl.

Ion chromatograms were extracted from the QE-orbitrap-MS total ion chromatograms using calculated monoisotopic accurate masses for protonated molecular ions with 5 ppm window. The metabolites (expected + unexpected) were mined from the data, using software-aided data processing (Thermo Compound Discoverer 2.0, including structure-intelligent dealkylation tool & mass defect filter) with manual confirmation. The structures of the observed metabolites were tentatively identified using obtained accurate mass and fragment ion data.

The area of the spike of calcipotriol was calculated and taken as a measure of relative amount. The amount of calcipotriol was measured at the time-points of 0h, 3h, 24h, 48h and 72h after changing the calcipotriol-containing medium to the culture flask.

Calcipotriol was administered in three different concentrations: 0.1 µM, 0.3 µM and 1.0µM to both SSCs and MSCs separately. Each sample was duplicated, leading to six flasks with 10 ml basic medium supplemented with different concentrations of calcipotriol. In addition, a control measurement without cells was conducted at each concentration to ensure that calcipotriol did not break down spontaneously.

The first-order rate constants k (min-1) of the metabolism were obtained from the slope of time vs. ln (% remaining) plot using Excel software. All time points (log-scale) were used in fitting the rate constant k (based on visual inspection of the curves). The in vitro half-life (t1/2) of study compound is defined as: t1/2 =ln2/k. Intrinsic *in vitro* clearance was calculated as follows: Clintinc =k*V/(M), where V is the volume of the incubation and M is the number of cells in the incubation.

2.4.2 Measurement of CYP24A1 expression by quantitative reverse transcription PCR

Both the SSCs and MSCs had four separate exposure groups. The cells were cultured in basic medium (control), basic medium with 10 nM calcipotriol, medium with 10ng/ml of TNF-α or medium with both supplementations. In total, eight cell lines (4 SSCs and 4 MSCs) were studied as triplicates (planned n=4 for each exposure group) to acquire reliable single values. Due to low expression of mitochondrial 1,25 dihydroxyvitamin D_3 24-hydroxylase (CYP24A1) in some groups, no data was acquired (Cq>40 in all replicates) from some of the cell lines with specific exposure. For this reason there was n=2 in MSCs (control and TNF-α) and n=3 in SSCs (in the calcipotriol group and TNF-α group). In the other four exposure groups, n=4.

After 48-hour exposure with TNF-α (10 ng/ml) and/or calcipotriol (10 nM) in complete media, the RNAs were extracted from the cell pellets with Nucleo Spin RNA kit (Macherey-Nagel) and cDNA was synthetized with the RevertAid First Strand cDNA synthesis kit (Thermo Scientific). The target transcripts were amplified using CFX96 Touch real-time Detection System (Bio-Rad) as triplicates using SYBR-Green detection (Bio-Rad). The following primers were used for the CYP24A1 gene: Forward primer CCTATCGCGACTACCGCAAA and reverse primer ATCGGCCAAGACCTCATTGAT and the RPS20 (ribosomal protein S20) gene: Forward primer GGTGGAAGTCACCATTGCAG and reverse primer TCCACCTGAAAAGTGGAAGTCT. Reverse transcriptase negative and non-template controls were used to exclude interfering non-specific amplification. RPS20 was selected as a reference gene for normalization since its expression has proved to be stable in both in SSCs and MSCs.

The size of the PCR product was checked in a 1.0% agarose gel and corresponded to 149 bp. The R^2 values of CYP24A1 and RPS20 were 0.986 and 0.988, respectively, indicating a good linearity in RT-qPCR. Two individual Cq values for the reference gene were excluded from analysis due to differing approximately 10 Cq units from their replicates, and were thus considered pipetting errors. Fold changes were calculated using the ΔΔCT method [18].

2.5 Data and statistical analysis

SPSS statistics software (version 23) (IBM corp. Armonk, NY, USA) was used for data analysis. P-values <0.05 were considered statistically significant. Technical replicates (duplicates in *in vitro* metabolism and triplicates in RT-qPCR) were used to ensure the reliability of single values.

Randomization or blinding was not done for the animal data. For the pharmacokinetic part of the work, it was considered unnecessary, because the study concentrated on obtaining preliminary safety data rather than measuring the efficacy of calcipotriol as a potential drug.

In vitro metabolism and RT-qPCR were not subjected to statistical analysis due to low sample size. Instead of studying the exact speed of calcipotriol metabolism or the exact fold change in CYP24A1 expression, we were interested whether the SSCs and MSCs are able to metabolize calcipotriol at all, and whether CYP24A1 is expressed on a measurable level after calcipotriol supplementation, indicating its role in the metabolism of calcipotriol.

2.6 Materials

Acetonitrile and isopropanol (Merck, Darmstadt, Germany), calcipotriol monohydrate (Sigma-Aldrich, Germany), Calcipotriol D_4 (Clearsynth, Mumbai, India), MC1046 (MedChem Express, Monmouth Junction, NJ, USA) and MC1080 (Clearsynth, Mumbai, India) were the highest purity available. The local anesthetic was 1% lidocaine hydrochloride (Lidocain 10mg/ml, Orion, Espoo, Finland). Medetomidine hydrochloride (Domitor vet 1mg/ml, Orion, Espoo, Finland) and ketamine (Ketaminol vet 50mg/ml, MSD Animal Health, Espoo, Finland) were used as sedatives, and pentobarbital sodium (Mebunat vet 60mg/ml, Orion, Espoo, Finland) was used for euthanasia. Atipamezol hydrochloride (Antisedan vet 5mg/ml, Orion, Espoo, Finland) was used for sedation reversal. The Pharmacy of Oulu University Hospital prepared all of the IV and intra-articular doses of calcipotriol. The IV preparation was made by dissolving 4.4 mg of calcipotriol monohydrate (0.1 mg/kg) in a mixture of 1 ml of ethanol (96%) and 0.2 ml of polysorbate 80% (Sigma-Aldrich). The

solution was made ready by adding 0.9% NaCl up to final volume of 10 ml. The doses of 2.7 mg (0.054 mg/kg), 10.8 mg (0.22 mg/kg) and 27.0 mg (0.56 mg/kg) of calcipotriol monohydrate were weighed into three separate 10 ml vials and kept at 6 ºC until use. Just prior to the experiment, 0.2 ml 99.6% ethanol was injected into each vial and after 1 min of stirring, 1% lidocaine solution was added up to total volume of 5 ml. The vehicle dose was prepared in the same way without calcipotriol monohydrate.

TNF-α (Sigma-Aldrich) was dissolved in sterile PBS containing 0.1% BSA and stored at -80 °C until use. Calcipotriol monohydrate (Santa Cruz Biochemistry, USA), collagenase (100 U/ml) (Worthington Biochemical Corporation, Lakewood, NJ, USA) and DNase (final concentration of 50 U/ml) (Sigma-Aldrich) were stored at -20 °C.

3 RESULTS

3.1 Pharmacokinetics of calcipotriol in sheep after IV and intra-articular doses

When calcipotriol (0.1 mg/kg) was administered IV to one sheep, its apparent terminal half-life was approximately one hour. (Figure 1a; Table 1). Thus, elimination was rather rapid as compared with the intra-articular doses, which provided lower and steadily declining plasma concentrations (Figure 1b, 1c). A very low concentration of compound (<1 ng/mL) was detected in some of the samples taken pre-dose. However, this was considered as analytical carry-over, and these concentrations have therefore been excluded from data analysis and graphs.

The intra-articular injections were administered to three sheep (doses of 0.054 mg/kg, 0.216 mg/kg and 0.54 mg/kg in one of the three animals) (Figure 1b, 1c). Neither the maximum plasma concentration nor the truncated AUC (area under curve) of the concentration curve over time (from time zero to the time where the last quantifiable plasma concentration was observed) acted in a dose-dependent manner. The truncated bioavailability (systemic absorption) was rather low, varying from approximately 1% to 13 % with the lowest value determined in the sheep treated with the highest dose (Figure 1a, Table 1). The differences in bioavailability were explained by the consistency of the formulation, i.e. the largest concentration had the most viscous consistency. The *in vivo* metabolites of calcipotriol could not be analyzed by LC-MS due to low concentrations.

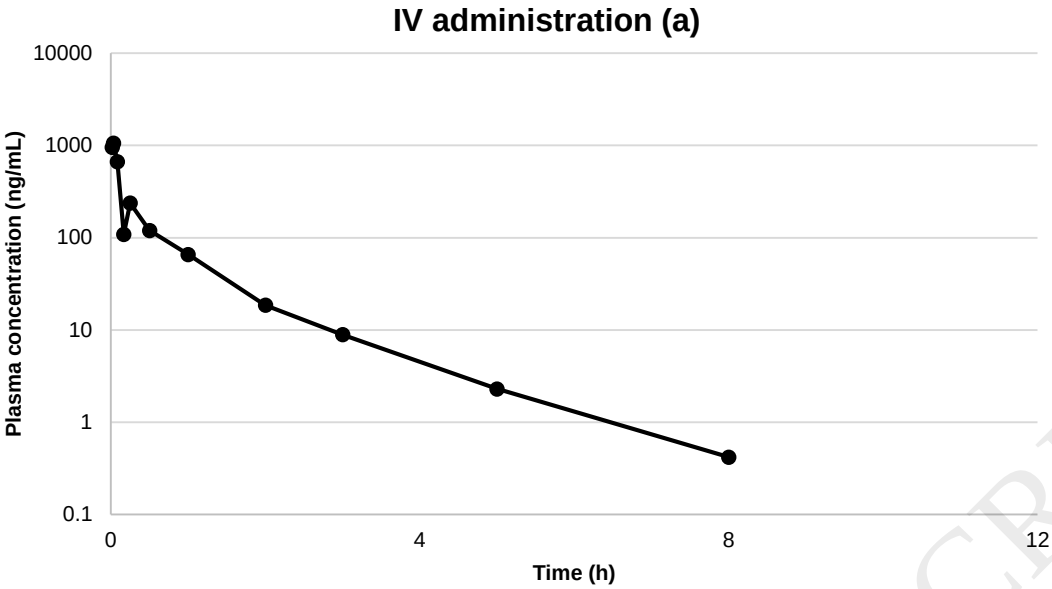
Table 1. Pharmacokinetic properties of calcipotriol after IV and intra-articular (IA) administration.

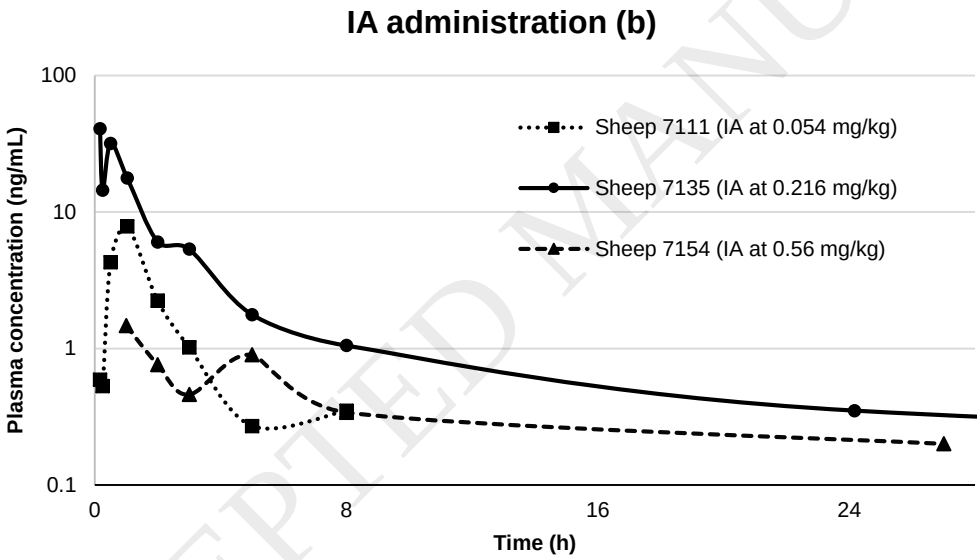
Dose_route		**IV**	**IA**	**IA**	**IA**
Sheep ID		7139	7111	7135	7154
Weight	kg	43.9	50.0	49.9	48.2
Dose (mg/kg)		0.1	0.054	0.216	0.56
Dose (mg)		4.39	2.7	10.8	27.0
$AUC_{0\text{-}last}$ †	h·ng/ml	267	11.9	72.8	10.5
$AUC_{0\text{-}8h}$ ‡	h·ng/ml	267	11.9	50.9	5.49
$AUC_{0\text{-}inf}$	h·ng/ml	268	c.n.c. §	c.n.c.	c.n.c.
CL	ml/min/kg	274	---	---	---
C_{max}	ng/ml	---	7.86	40.7	1.47
T_{max}	h	---	1.03	0.17	1.00
C_{last}	ng/ml	0.42	0.35	0.15	0.20
T_{last}	h	8.00	8.00	73.3	27.0
$t_{1/2}$ (no of data points)	h	1.10	c.n.c.	c.n.c.	c.n.c.
V_{ss}	l/kg	12.6	---	---	---
BA#	%	---	8.21	12.6	0.70

† Truncated AUC from time zero to the time where the last quantifiable plasma concentration was observed.

‡ Truncated AUC from time zero to the 8 h time point.

§ c.n.c. – could not be calculated due to lack of data





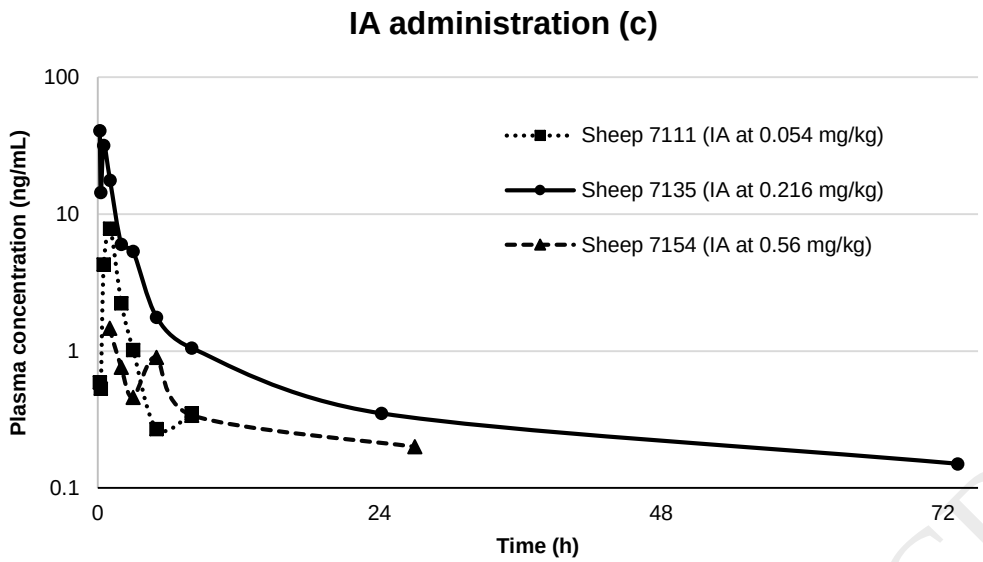


Figure 1. Intravenously dosed calcipotriol and the systemically absorbed portion of intra-articularly dosed calcipotriol are rapidly metabolized.

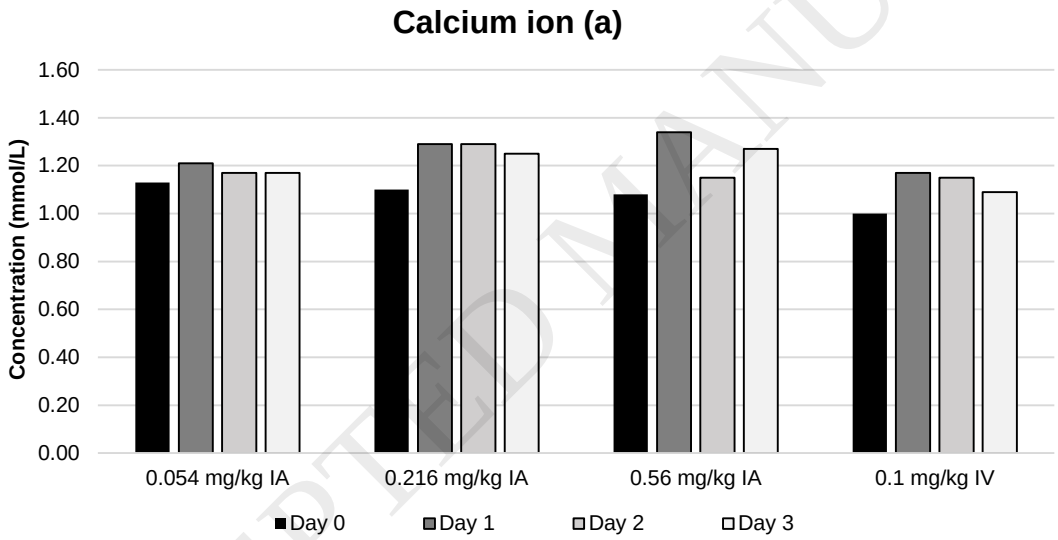
Plasma concentrations of calcipotriol after an IV dose of 0.1 mg/kg (a) and after intra-articular (IA) administration at three dose levels from time 0 to 28 h (b) and from 0 to 75 h (c).

3.2 Pharmacodynamics of calcipotriol in sheep after IV and intra-articular doses

Baseline 25(OH)D_3 (includes also 25(OH)D_2) levels were unmeasurably high, > 373 nmol/L, in all four sheep, and 1,25$(OH)_2D_3$ levels ranged 22-34 pmol/L. To our knowledge, reference ranges for sheep using these assays have not been published.

None of the sheep developed hypercalcemia, regardless of dose and the route of delivery (Figure 2a). However, a slight elevation in calcium-ion levels was measured from the 24h time-point onwards. The maximum elevation in the calcium-ion level was observed in the sheep receiving the

highest intra-articular dose (ID 7154) (from baseline 1.08 mM to 1.34 mM at 24h). Phosphate-ion levels were increased more clearly (Figure 2b). It should be noted that the baseline levels of phosphate-ion varied from 1.80 mM to 3.29 mM. Similarly to the calcium-ion concentrations, the maximum increase in the concentration of phosphate-ions was measured in the sheep receiving the highest intra-articular dose (from baseline 2.59 mM to 4.49 mM at 24h). No alterations were found in complete blood count, alkaline phosphatase, alanine aminotransferase or creatinine from baseline to follow-up samples (data not shown). The negative effect of calcipotriol on endogenous $1,25(OH)_2D_3$ level could not be analyzed by the immunoassay used in this study since the method also detects exogenously given calcipotriol.



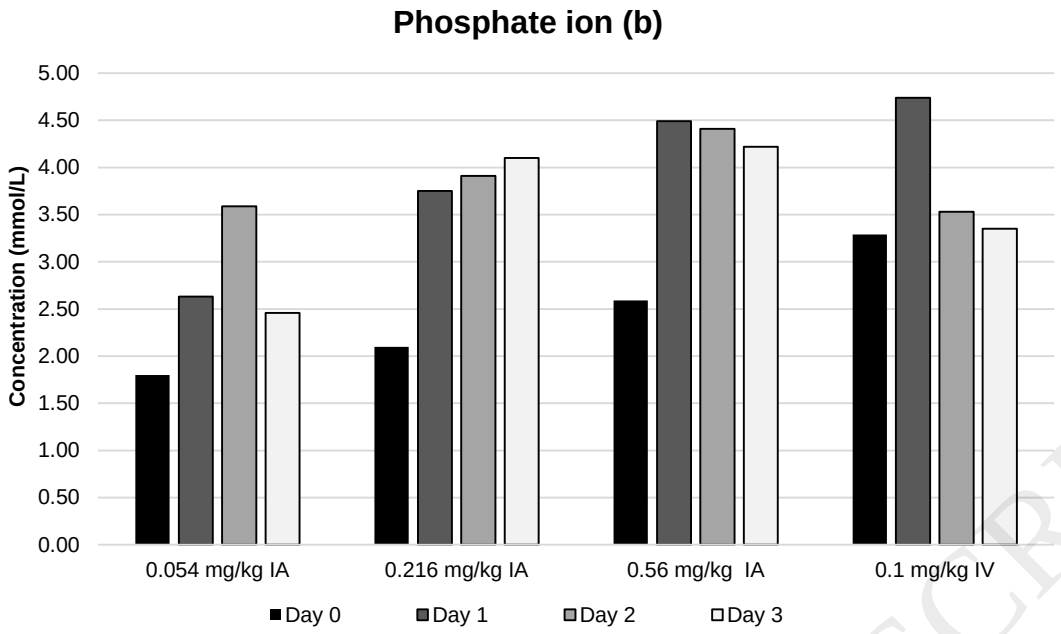


Figure 2. Calcipotriol increases serum phosphate ion levels after intra-articular and intravenous doses, while having a minor effect on calcium ion level.

(a) The plasma concentrations of calcium ion in all of the four sheep before (Day 0) and after intra-articular (IA) or IV dosing of calcipotriol. There is a slight increase in all concentrations after the dose, and the increase seems to be dose-dependent to some extent.

(b) The plasma concentrations of phosphate level in all of the four sheep before (Day 0) and after intra-articular (IA) or IV dosing of calcipotriol. There is a clear increase after each dose, with the largest increase being observed with the largest IA dose. Note that after IA dosing, the plasma levels remain high for a longer time due to the slow dissolution of calcipotriol crystals, whereas after the IV dose, the phosphate levels return to baseline after 48 hours

3.3 Tissue dissection and histological examination

As verification of successful intra-articular injections, crystals were detected by polarized light microscopy from all post-mortem samples of synovial fluid taken from the calcipotriol-injected joints but not from the placebo joints, with the greatest amount of crystals found in the synovial fluid of the sheep given the highest intra-articular dose of calcipotriol, shown in Figure 3d (ID 7154). There were only a few visible crystals in the joints of the two sheep receiving the lower doses (ID 7111 and 7135), indicating that almost all crystals were dissolved at the termination of the experiment. This is consistent with the data in Figure 1 showing the better systemic absorption of calcipotriol with lower intra-articular doses (ID 7111 and 7135) compared with sheep 7154 with a higher dose. Unfortunately, the molecules in the crystals could not be identified by LC-MS. The detection limit for pure calcipotriol monohydrate was at 200 nM. Calcipotriol and vehicle injected joints showed no differences in visual inspection or in the histological examination (Figure 4a-d).

However, the cell density was significantly higher in the cartilage that was exposed to calcipotriol than placebo in the sheep with the highest (ID 7154) intra-articular dose, but not in the other two sheep. The unit of measurement for the cell density was the amount of cells in an area of 200µm x 200µm. The cell densities of the cartilages were 27.4 ± 6.58 and 25.6 ± 3.78 cells per area ($p=0.401$) in sheep 7111, 24.9 ± 2.94 and 44.4 ± 28.39 ($p=0.755$) in sheep 7135, and 36.8 ± 7.77 and 24.3 ± 6.34 ($p<0.001$) in sheep 7154 for calcipotriol and control, respectively. The density is expressed as mean ± SD.

There was a slight variation in the shade of colors in hematoxylin-eosin staining of cartilage, but the variation showed no systematic difference between calcipotriol and placebo injected joints. The heart, kidney, liver and lung sections of the sheep showed no signs of tissue alterations (data not shown).

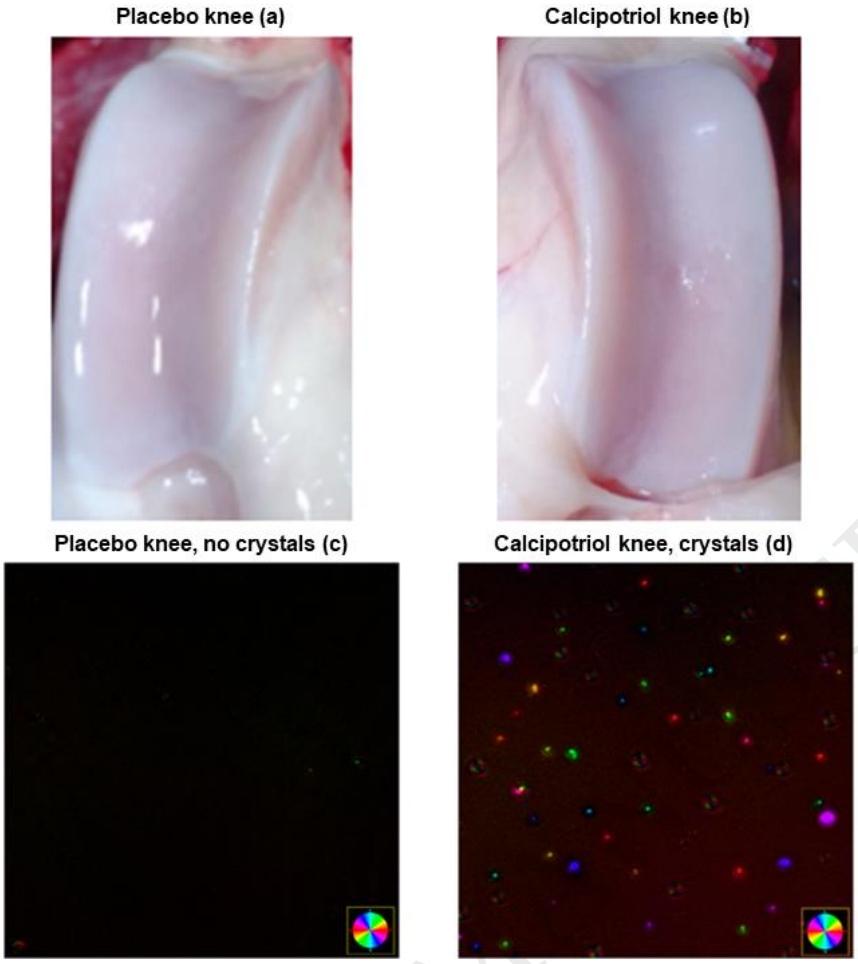


Figure 3. Calcipotriol crystals remain in the calcipotriol-injected joint without causing visible differences in the surface of the joint.

The dissected knee joints of the sheep with highest intra-articular dose (ID 7154) (a, b) and the view in polarized microscopy (c, d) after euthanasia on day 13. There is no apparent macroscopic difference between the joint injected with placebo solution (a) and the joint injected with calcipotriol (b). However, in polarized microscopy, hardly any signal is observed in the synovial fluid taken from the placebo joint (c), whereas there are multiple polarizing crystals in the calcipotriol joint (d) verifying the presence of calcipotriol crystals in that location.

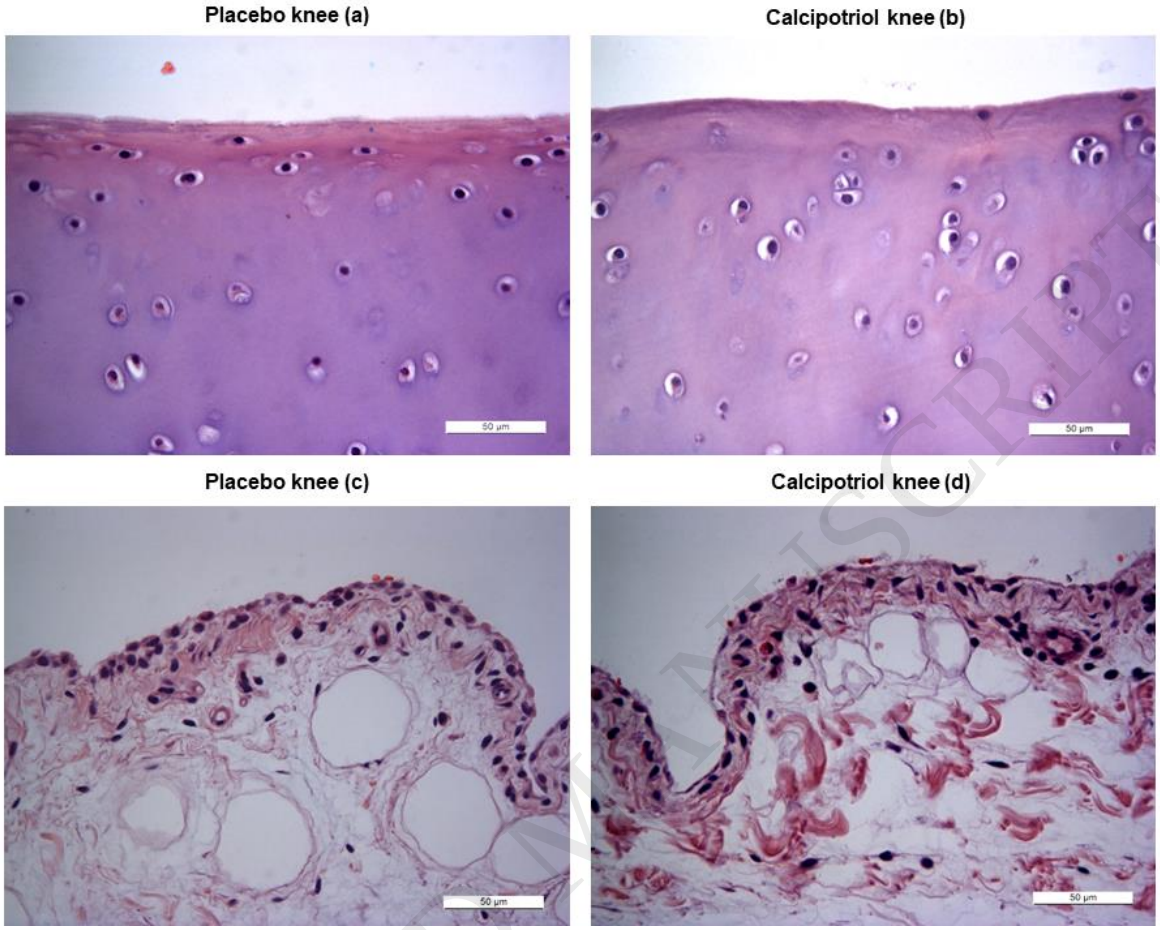


Figure 4. Calcipotriol did not cause apparent microscopic changes in the cartilage and synovial tissues.

Histological samples from the knees of the sheep with the highest intra-articular dose (ID 7154) after euthanasia on day 13. In the cartilage samples (a, b), there were no obvious superficial differences, but the cell density was higher in the calcipotriol-injected knee than in placebo ($p<0.001$) with the highest calcipotriol concentration, but not with the two lower concentrations (pictures not shown here). Furthermore, in the samples from synovium (c, d), no differences were observed by visual inspection. The samples were stained with hematoxylin-eosin.

3.4 Metabolism of calcipotriol in synovial and mesenchymal stromal cells

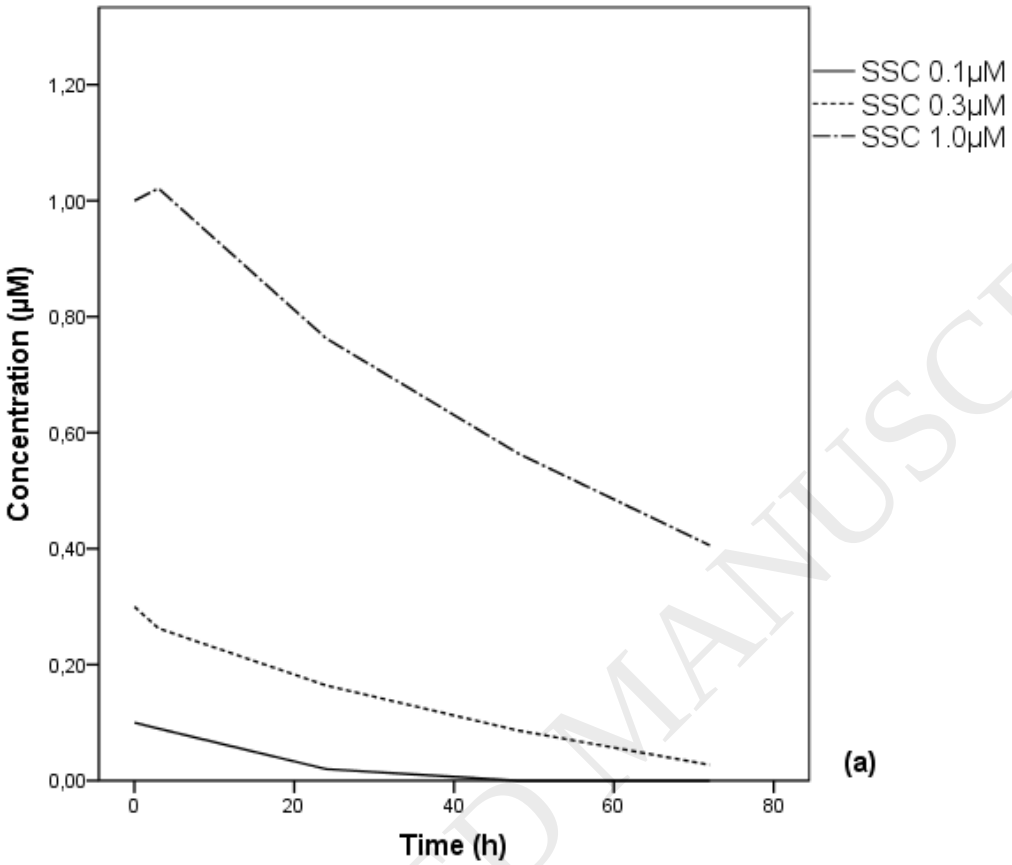
Both SSCs and MSCs were able to metabolize calcipotriol at a similar rate *in vitro* (Figure 5a-b). It should be noted that there were 2.0 million SSCs per flask, whereas the respective amount of MSCs was only 0.9 million (Table 2). The clearance rates of calcipotriol in these cells take the actual cell number into account. The clearance rates become higher at larger concentrations, which indicates that no saturation of metabolism was reached in either cell type. Some of the late concentrations in the 0.1µM calcipotriol solutions could not be assayed. The values may not be accurate near to the detection limit due to small peaks and the background noise.

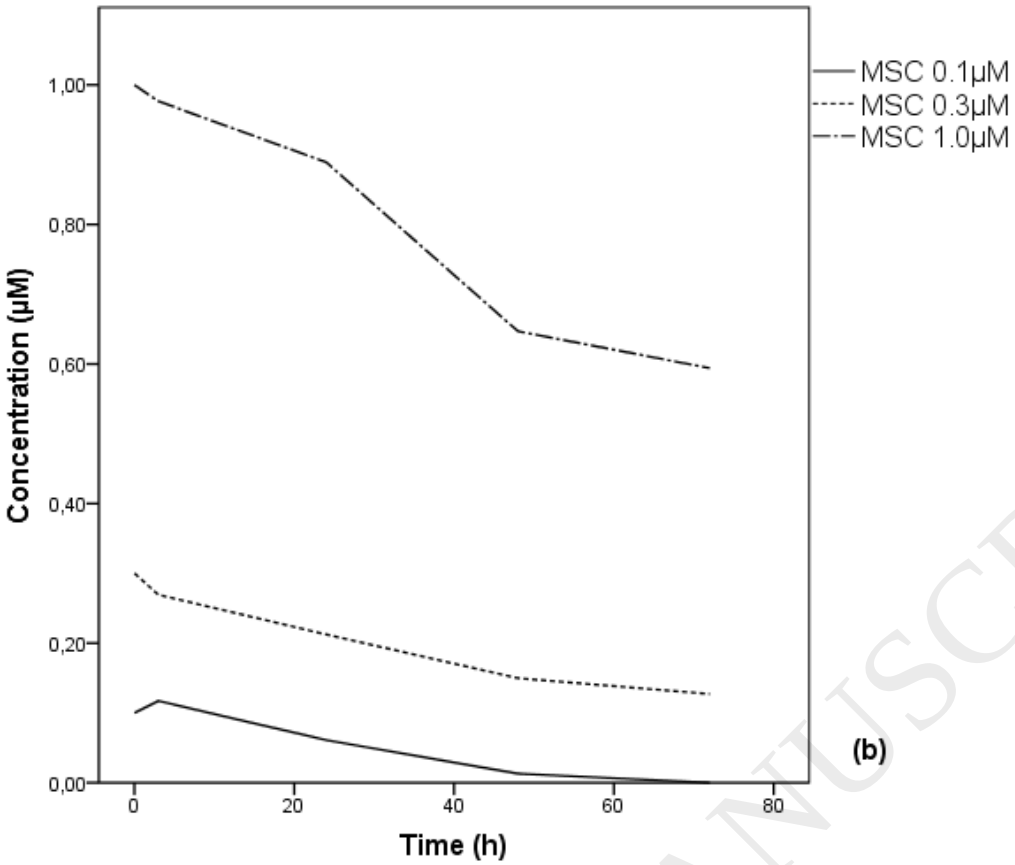
The no cell control indicated that spontaneous breakdown of calcipotriol did not occur (Figure 5c). In fact, the amount of calcipotriol in 1.0µM concentration seemed to increase over time (by 40% in total), which is probably a result of gradual dissolution of calcipotriol precipitates or detachment of calcipotriol from surface material with time (Figure 5c). In fact, taking this systematic error into account, the half-lives of calcipotriol using the largest concentration is somewhat smaller in reality than shown in Table 2.

Table 2. The observed half-life and clearance rates of calcipotriol in human synovial stromal cells (SSC) and in mesenchymal stromal cells (MSC) in *in vitro* cell cultures.

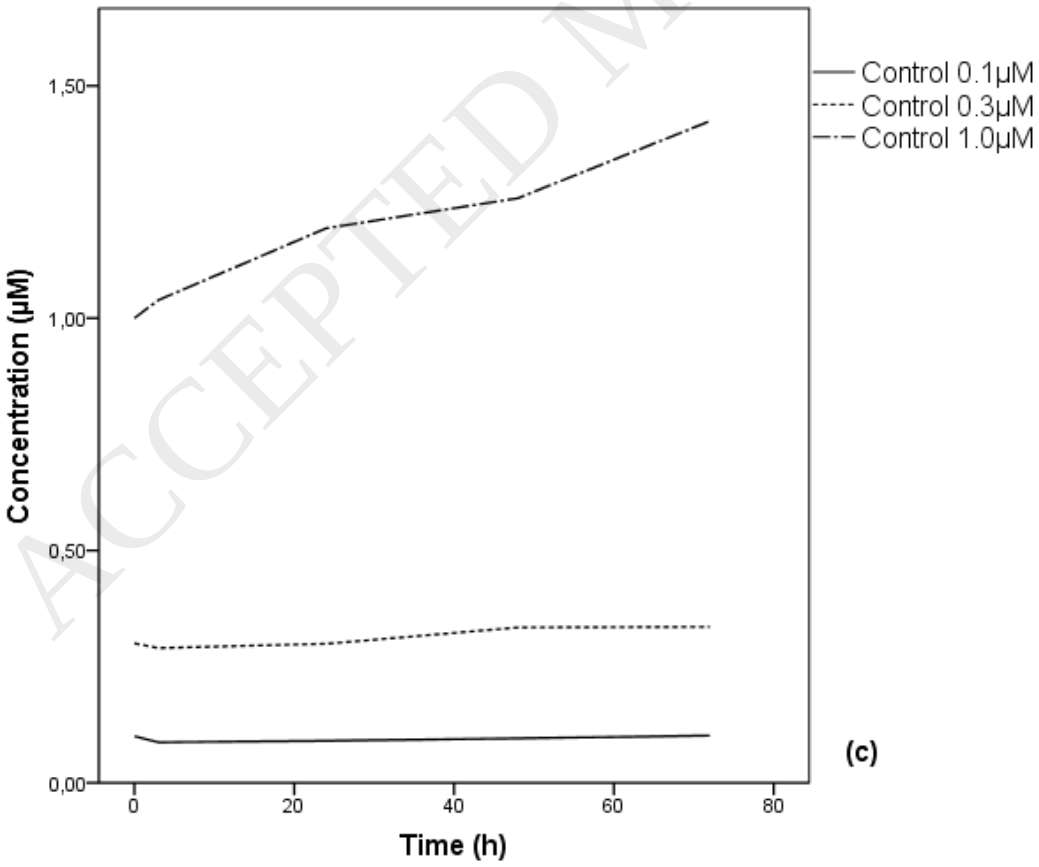
Cell type	Concentration	Half-life (h)	Clearance (µl/min/million cells)
SSC	0.1 µM	10.0	5.77
SSC	0.3 µM	26.6	2.17
SSC	1.0 µM	54.0	1.07
MSC	0.1 µM	15.2	8.43
MSC	0.3 µM	58.2	2.21
MSC	1.0 µM	89.6	1.43

Note that there were only 0.9 million MSCs compared to 2.0 million SSCs in a culture flask, explaining the simultaneous longer half-life and higher clearance in MSCs





(b)



(c)

Figure 5. Calcipotriol is metabolized by both synovial and mesenchymal stromal cells.

The concentration of calcipotriol over time in synovial stromal cells (a), in mesenchymal stromal cells (b) and without cells (c). The graphs represent the mean of two replicates. Note that the seemingly faster metabolism in SSCs is explained by their greater amount per flask (2.0 million) compared with MSCs (0.9 million). Trace amounts of calcipotriol were measured using 0.1µM at 48h and 72h (SSC) at 72h (MSC), hence the graphs go to zero. The amount of the calcipotriol concentration in the control flask appears to be increasing with the largest concentration (1.0 µM) which is due to physico-chemical interference: dissolution of calcipotriol precipitates or detachment of calcipotriol crystals from surfaces of flasks with time.

3.4.1 Identifying the metabolites of calcipotriol from synovial and mesenchymal stromal cells

The metabolite identifications and metabolite profile are presented in Tables 3 - 4. The detected metabolites were tentatively identified according to the accurate mass data, retention times, and high resolution fragment ion data. The metabolic profile was calculated as a percentual share of each compound from the LC/MS peak area of the calcipotriol amount at 0 h time point, with a mean value from two replicates. It is worth noting that all percentage values (metabolite profiles) are estimated assuming an identical LC/MS response between all metabolites and the parent compound, which is most probably not the case.

Calcipotriol was observed only as a dehydration fragment ion $[M+H-H_2O]^+$ and its metabolites lost water easily as well, which complicated their identification. A total of five calcipotriol metabolites (M1 – M5) were identified with LC/MS. M1 had the same m/z value as calcipotriol and was confirmed to be the metabolite MC1080 by comparing it against an authentic standard. M1 was the main metabolite in both SSC and MSC incubations with relative abundances of 19 and 21% at the 72 h time point, respectively. Metabolites M2 and M3 were formed via hydroxylation and are likely

to be the metabolites MC1439 and MC1441 reported earlier, but confirmatory MS/MS data was not obtained for M2 and M3 due to their low abundance. These had relative abundances of 3-5% at 72 h in both cell types. M4 was formed via dehydrogenation, and its identification was confirmed against an authentic standard of MC1046; *i.e.* their retention times, m/z values and MS/MS spectra were identical. M4 was the second most abundant metabolite with relative abundance of 13 - 14% at the 72h time point. Metabolite M5 was observed at m/z 411, but there is no certainty if this value is the $[M+H]^+$ ion of a dehydrogenation product, or if it is the dehydration product of an oxidized (hydroxylated) metabolite. However, the retention time of M5, which is longer than for calcipotriol, and much longer than for the other hydroxylation metabolites M2 – M3 and more close to the dehydrogenated metabolite M4, suggests that M5 is formed via dehydrogenation. The chemical structures of the metabolites are presented in figure 6.

Table 3. UPLC/ESI/QE-orbitrap-MS data obtained for calcipotriol and its metabolites.

	Identification	RT, min	Formula	Obs. m/z	Calc. m/z	Δ m/z, mDa
	Calcipotriol H2O fragment	**3.82**	**C27H40O3-H2O**	**395.2944**	**395.2945**	**0.0**
	Fragment (loss of H2O + H2O)		[C27H37O]+	377.2838	377.2839	-0.1
	Fragment (loss of H2O + H2O + H2O)		[C27H35]+	359.2727	359.2733	-0.6
	Fragment (loss of C8H16O)		[C19H25O2]+	285.1845	285.1849	-0.4
	Fragment (loss of C9H10O2 + H2O)		[C18H29]+	245.2261	245.2264	-0.3
	Fragment (loss of C18H30O)		[C9H11O2]+	151.0754	151.0754	0.1
	Fragment (loss of C18H30O + H2O)		[C9H9O]+	133.0649	133.0648	0.1
	Fragment (loss of C19H32O3)		[C8H9]+	105.0702	105.0699	0.4
M1	**Calcipotriol isomer (H2O fragment; MC1080)**	**3.90**	**C27H40O3-H2O**	**395.2944**	**395.2946**	**-0.2**
	Fragment (loss of H2O + H2O)		[C27H37O]+	377.2821	377.2839	-1.8
	Fragment (loss of C18H30O)		[C9H11O2]+	151.0752	151.0754	-0.2
	Fragment (loss of C18H30O + H2O)		[C9H9O]+	133.0649	133.0648	0.1
M2	**Hydroxylation (MC1439 / 1441?)**	**3.18**	**C27H40O4**	**429.3002**	**429.2999**	**0.3**
M3	**Hydroxylation (MC1439 / 1441?)**	**3.37**	**C27H40O4**	**429.2996**	**429.2999**	**-0.3**
M4	**Dehydrogenation (MC1046)**	**3.87**	**C27H40O4**	**411.2898**	**411.2894**	**0.4**
	Fragment (loss of C19H30O3)		[C8H9]+	105.0703	105.0699	0.4
M5	**Dehydrogenation**	**3.97**	**C27H38O3**	**411.2891**	**411.2894**	**-0.3**
	Fragment (loss of H2O)		[C27H37O2]+	393.2779	393.2788	-0.9
	Fragment (loss of H2O + H2O)		[C27H35O]+	375.2678	375.2682	-0.4
	Fragment (loss of C19H30O3)		[C8H9]+	105.0699	105.0699	0.0

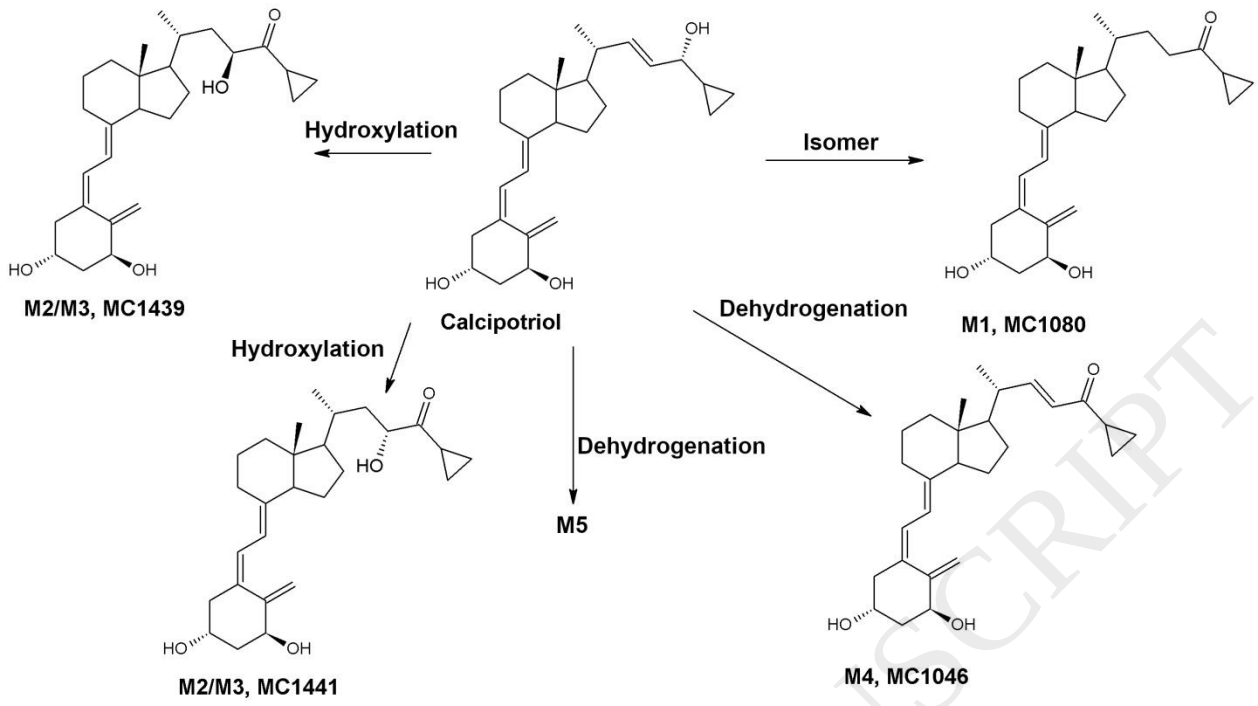
Table 4. The metabolic profile for calcipotriol in incubations with synovial stromal cells (SSC) and mesenchymal stromal cells (MSC).

		SSC		MSC	
	Name	**24 h**	**72 h**	**24 h**	**72 h**
	Calcipotriol	87.9	49.4	88.7	54.5
M1	Calcipotriol isomer (MC1080)	7.5	19.4	10.1	20.5
M2	Hydroxylation (MC1439 / 1441?)	1.8	4.9	2.0	5.3
M3	Hydroxylation (MC1439 / 1441?)	0.4	3.0	0.4	3.6
M4	Dehydrogenation (MC1046)	9.0	13.1	7.6	13.6
M5	Dehydrogenation/Hydroxylation	1.7	9.6	1.0	7.7

The values are calculated as a share (%) of each compound from the LC-MS peak area of calcipotriol at the 0 h time point. Values are the mean of two replicates.

Figure 6. The chemical structures of calcipotriol and its observed metabolites in synovial and mesenchymal stromal cell cultures.

The formerly known metabolites (MC1080 and MC1046) were verified by comparing against an authentic standard. M2 and M3 were formed via hydroxylation and were assumed to be metabolites MC1439 and MC1441. The structure of metabolite M5 could not be verified, but it was likely formed by dehydrogenation.



3.5 Expression of CYP24A1 gene in SSC and MSC cell cultures

The CYP24A1 transcripts were amplified in MSCs in the groups that were supplemented by calcipotriol with or without TNF-α compared to the control group. It was found that calcipotriol exposure elevates the expression of CYP24A1 to an easily measurable level in all 12 wells (triplicates from each four cell lines) with or without TNF-α, whereas without calcipotriol, there was hardly any expression, only 2 of 12 wells in both control and TNF-α groups showed any measurable expression at all.

In SSCs, there was no systematic effect with calcipotriol. The variability between different cell lines was large, while the overall expression of CYP24A1 in SSCs was low. The average Cq values were over 30 units in all groups (Table 5). One of the cell lines expressed CYP24A1 to same extent as MSCs when supplemented with calcipotriol or calcipotriol with TNF-α, another one a little lower expression, while two of the cell lines were not induced by calcipotriol at all. In fact, one of these non-induced cell lines showed high expression of CYP24A1 with TNF-α supplementation only and in control medium, which had an effect on the mean expressions.

The reference gene RPS20 was expressed quite evenly among different supplementation groups. The average Cq values of the supplementation groups ranged from approximately 22.8 to 23.7 in SSCs and from 23.2 to 23.9 in MSCs (Table 5). RPL30 (ribosomal protein L30) was initially tried as a reference gene, but its expression among different groups was not as stable as that of RPS20. However, the initial analysis with RPL30 showed similar results: the expression was enhanced with calcipotriol supplementation in MSCs (data not shown).

Table 5. The expression of CYP24A1 in synovial and mesenchymal stromal cells (SSCs and MSCs, respectively) measured with RT-qPCR.

Cells	n	Avg Cq (RPS20)	Avg Cq (CYP24A1)	Avg ΔCq (±SD)	Avg ΔΔCq (±SD)	Relative mRNA expression
SSCs						
Control	4	23.7	34.5	10.9 ± 4.74	0.0 ± 6.71	1.0
Calcipotriol	3	23.7	30.0	6.3 ± 2.82	4.6 ± 5.52	24.0
TNF-α	3	22.8	33.5	10.7 ± 6.76	0.1 ± 8.26	1.1
Calcipotriol + TNF-α	4	23.4	31.5	8.2 ± 3.63	2.7 ± 5.97	6.6
MSCs						
Control	2	23.2	36.1	12.6 ± 0.07	0.0 ± 0.10	1.0
Calcipotriol	4	23.8	25.9	2.1 ± 1.20	10.5 ± 1.20	1450.7
TNF-α	2	23.7	37.4	14.2 ± 2.10	-1.6 ± 2.10	0.3
Calcipotriol + TNF-α	4	23.8	26.8	3.0 ± 2.23	9.6 ± 2.23	782.9

The average ΔΔCq values and relative expression is calculated in relation to control within the same cell type. RPS20 is used as a reference gene. Calcipotriol enhances the expression of CYP24A1 in MSCs. In SSCs, the expression of CYP24A1 is quite low in all groups. Four cell lines were studied as technical triplicates in each group, but due to nonmeasurably low expression of CYP24A1, n<4 in some groups.

4 DISCUSSION

This preliminary pharmacokinetic study revealed that intra-articular calcipotriol crystals have a long retention time in the joint with a low systemic absorption. The low bioavailability (between 1 and 13%) and rapid clearance (approximate half-life of 1 hour after an IV administration) explain the good tolerability. There was neither hypercalcemia nor other side effects in any of the animals. Increasing concentration reduces the solubility of calcipotriol, slowing the systemic absorption and thus explaining the lowest plasma concentrations with the highest intra-articular dose. The pharmacokinetic and –dynamic profile of calcipotriol is favorable concerning its possible use in the local treatment of arthritis.

We observed that in one of the sheep the cartilage of calcipotriol-injected knee had higher cell density than the placebo-injected knee. The analysis of only one histological section per knee might explain this result, yet it indicates that calcipotriol does not appear to cause apoptosis or cell death in the cartilage.

One of the advantages of this pharmacokinetic study was the use of sheep as the animal model. The joint structure, size and weight are comparable to those of humans [13]. In particular, the stifle joint of sheep, where the intra-articular injections were given, is very similar to the human knee joint [19]. Sheep have strong jugular veins that are optimal for catheterization and sampling of blood. It has been noted the urinary rate of excretion of calcium and inorganic phosphate is quite constant in sheep due to the excretion of extra calcium into feces [20] and extra phosphorus into saliva [21]. Dietary restriction of inorganic phosphate does not change endogenous $1,25(OH)_2D_3$ levels but hypocalcemic diet raises $1,25(OH)_2D_3$ in sheep [22, 23].

Despite those differences between humans and sheep in calcium and phosphate metabolism and excretion, $1,25(OH)_2D_3$ administration causes elevation of calcium and inorganic phosphate in both

sheep [22] and humans. Taking all these aspects into account, the pharmacokinetics of intra-articularly dosed calcipotriol in sheep is likely to be similar to humans. However, there might be differences in the pharmacodynamics of calcipotriol between sheep and human, *i.e.* mild elevation of phosphate levels after calcipotriol dosing in this study could be related to different phosphate metabolism in sheep compared to humans [24].

In rats, calcipotriol disappears rapidly from circulation. Its half-life of elimination has been reported as 4 min [4], 0.2 h [25] and 0.9 h after an IV dose [11]. The half-life was 1.1 h in this study in one sheep, which is similar to the value measured in mini-pigs i.e. 1.0 h (originally conducted by LeoPharma, mentioned in [26]). The principal deactivation pathway of $1,25(OH)_2D_3$ and calcipotriol is the production of calcitroic acid in the liver and kidney and its excretion as bile acid conjugates to the gut [27]. No previous studies on the pharmacokinetics of calcipotriol after an intra-articular dose have been published.

After a topical administration of 150g-360g/week of calcipotriol ointment containing 50µg/g of calcipotriol, 5/16 patients developed serum and urine hypercalcemia and hyperphosphatemia. Furthermore, the serum $1,25(OH)_2D_3$ and PTH levels were suppressed dose-dependently [28]. The recommended dose of calcipotriol ointment 100g/week (50µg/g of calcipotriol) rarely causes hypercalcemia [29]. In children, topical calcipotriol administration with amounts not exceeding 100g/week does not cause significant hypercalcemia or hyperphosphatemia [30]. The systemic absorption after a topical dose is less than 1%, when calcipotriol was administered with betamethasone dipropionate in healthy skin for 12 hours [31].

In rats, calcipotriol had no effect on healthy bone with doses up to 10µg/kg/day p.o. or intraperitoneally (i.p.) for seven consecutive days, but the dose of 100 µg/kg/day with the same regimen produced a mild bone loss and hypercalcemia. [5] In contrast, $1,25(OH)_2D_3$ (0.5µg/kg/day i.p. or p.o. for seven days) decreased dry weight and calcium content of bone clearly and produced a

greater hypercalcemia and hypercalciuria than calcipotriol with the above-mentioned doses did. In the same study, rachitic mice were given p.o. calcipotriol (100 µg/kg/day for 14 days) with no healing impact, while $1,25(OH)_2D_3$ (0.5µg/kg/day p.o. for 14 days) alleviated the bone disease. [5] In this study, calcipotriol was 100-200 times less potent than 1,25(OH)2D3 when comparing vitamin D effects on bone and calcium metabolism.

We demonstrated for the first time that both SSCs and MSCs were able to metabolize calcipotriol *in vitro*. Metabolism of calcipotriol has been measured in rat and human hepatocytes *in vitro*, the mean clearances being 2.06 ml/min/million cells and 1.48 ml/min/million cells, respectively [25]. Here, it seemed that human SSCs and MSCs had a thousand-fold reduced capacity to metabolize calcipotriol compared to hepatocytes, even though the maximum capacity was not reached at calcipotriol concentrations between 0.1-1.0 µM during 0-3 days. No enhancement of metabolism (as indicated by CYP24A1 induction) was seen over time, perhaps since there were no time points for measurements between 3 h and 24 h.

The two main metabolites of calcipotriol in liver extracts of rats, mini-pigs and humans as well as in keratinocytes and osteosarcoma cells are MC1080 and MC1046. [1, 32] These metabolites have a biological activity of around 10% that of calcipotriol. [1, 4] These two compounds appear to be the prime metabolites observed in our MSC and SSC cell lines and were definitely verified by comparison with a reference molecule. The end product of this metabolic pathway, calcitroic acid, was not identified in this study, presumably due to its exceedingly low concentration.

$1,25(OH)_2D_3$ is known to be a strong regulator of the CYP24A1 gene, this effect being mediated by the VDR-RXR heterodimer and cofactors that bind to the vitamin D response element (VDRE) upstream of the gene [33]. Loss of function mutations in the CYP24A1 gene may lead to hypercalcemia, hypercalciuria and nephrolithiasis, highlighting the importance of CYP24A1 in vitamin D metabolism *in vivo* [34]. Elimination of $[1\beta\text{-}^3H]$-$1,25(OH)_2D_3$ was prolonged *in vivo* and

metabolism of [1β-^{3}H]-1,25(OH)$_2$D$_3$ was prevented in keratinocytes gathered from CYP24A1 null mice [35]. Furthermore, Chinese hamster lung V79 cells robustly increased production of calcipotriol metabolites after introduction of hCYP24 into cells [36].

CYP24A1 transcripts were lacking in the untreated MSCs in this study and in MSCs of 9/27 hip arthroplasty patients in a previous study [37], indicating that CYP24A1 expression is undetectably low in some cell lines. CYP24A1 was strongly induced by calcipotriol in our study in MSCs to a similar magnitude as in a previous study conducted in rat skin [38]. In agreement with our results, the natural metabolites 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ also promote the expression of CYP24A1 in MSCs [37]. Expression of CYP24A1 is also enhanced by 1,25(OH)$_2$D$_3$ in chondrocytes [39]. One of the natural products of CYP24A1 metabolism is 24R,25(OH)$_2$D$_3$ which has been shown to confer protection against the development of cartilage damage when delivered as intra-articular injections into joints of anterior cruciate ligament transection rats [40].

On basis of the previous literature and our results calcipotriol is likely to be metabolized through a CYP24A1-dependent pathway in MSCs. In SSC cells, our results are contradictory in a way that all SSCs did not show CYP24A1 expression upon calcipotriol induction but still the metabolic rate of calcipotriol in MSCs and SSCs was similar. This discrepancy may be explained by a small number of cells lines (individual variation shows bigger) and by the fact that different cells lines were used in the qPCR and metabolic analysis.

The high baseline 25(OH)D$_3$ levels in sheep did not affect the reliability of the pharmacokinetic results. Differences between binding affinities to vitamin D binding protein between calcipotriol and 1,25(OH)$_2$D$_3$ are 30-fold [24], and between 1,25(OH)$_2$D$_3$ and 25(OH)D$_3$, they are 10-fold [41], suggesting that the affinity of calcipotriol is approximately 300 times less than that of 25(OH)D$_3$. Also, the highest measured calcipotriol concentration was approximately 2.57 pmol/L, while the measured 25(OH)D$_3$ concentrations were invariably over 373 nmol/L (145,000 molar excess). This

suggests that calcipotriol was unable to occupy the binding sites in the vitamin D binding protein in this study. The high baseline levels of $25(OH)D_3$ are attributable to supplementation with the mineral feed that is widely used in Finland because of limited sunlight in the north. There is strong evidence that vitamin $25(OH)D_3$ should be maintained at an optimal level in sheep as it affects reproducibility and reduces the risk of parturient paresis due to hypocalcemia [42].

There were some limitations in this study. The main goal was to obtain preliminary knowledge on the safety and pharmacokinetics of calcipotriol after intra-articular dosing. Therefore only four sheep were examined and individual variation could not be assessed. In addition, the calcipotriol metabolites *in vivo* could not be analyzed due to low concentrations. Serum calcium-ion and phosphate concentrations remained the principal markers of excessive intake of calcipotriol, as calcipotriol interfered with the measurement of $1,25(OH)_2D_3$, preventing the detection of downregulation of $1,25(OH)_2D_3$ after calcipotriol injections. The human PTH assay could not be used for the measurement of sheep PTH levels due to the lack of crossreactivity of the human PTH antibody with its counterpart in sheep. The collection of 24-hour urine samples was omitted due to practical reasons. Calcipotriol was well tolerated as a single injection, but long-term side effects after local repeated calcipotriol injections cannot be estimated from this study.

This study was conducted following the principles of 3Rs. We favored the use of in vitro cell cultures in determining the intra-articular metabolism of calcipotriol, and used as few animals as possible (and one was rehomed) to obtain preliminary safety data on the intra-articular dosing of calcipotriol. The sheep were kept in a group whenever possible to minimize stress.

5 CONCLUSION

In sheep, calcipotriol remains in the knee joint for several days, and has a rapid systemic metabolism without causing any observable adverse effects. For these reasons, calcipotriol appears

to be a promising candidate for the local intra-articular treatment of arthritis. Nonetheless, its effectiveness in local treatment of arthritis will need to be verified in future studies.

Declarations of interest: none

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