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Chondrogenic potential of canine articular cartilage derived cells (cACCs)

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Abstract: In the present paper, the potential of canine articular cartilage-derived cells (cACCs) for chondrogenic differentiation was evaluated. The effectiveness of cACCs' lineage commitment was analyzed after 14 days of culture in chondrogenic and non-chondrogenic conditions. Formation of proteoglycan-rich extracellular matrix was assessed using histochemical staining – Alcian Blue and Safranin-O, while elemental composition was determined by means of SEM-EDX. Additionally, ultrastructure of cACCs was evaluated using TEM. The expression of genes involved in chondrogenesis was monitored with quantitative Real Time PCR. Results obtained indicate that the potential of cACCs for cartilaginous extracellular matrix formation may be maintained only in chondrogenic cultures. The formation of specific chondro-nodules was not observed in a non-chondrogenic culture environment. The analysis of cACCs' ultrastructure, both in non-chondrogenic and chondrogenic cultures, revealed well-developed rough endoplasmic reticulum and presence of mitochondria. The cACCs in chondrogenic medium shed an increased number of microvesicles. Furthermore, it was shown that the extracellular matrix of cACCs in chondrogenic cultures is rich in potassium and molybdenum. Additionally, it was determined that gene expression of collagen type II, aggrecan and SOX-9 was significantly increased during chondrogenic differentiation of cACCs. Results obtained indicate that the culture environment may significantly

influence the cartilage phenotype of cACCs during long term culture.

Keywords: canine articular cartilage derived cells, chondrogenic differentiation, morphology, ultrastructure, gene expression, chondrogenic markers

1 Introduction

Osteochondral defects are a serious clinical problem both in human as well as in veterinary medicine. The avascular nature of articular cartilage and its limited capacity for spontaneous regeneration are the reasons why cartilage injuries have poor prognosis for repair [1].

Currently, cell-based therapies provide real promise for the treatment of musculoskeletal diseases involving osteochondrotic articular cartilage defects [2]. Due to the fact that articular cartilage lesions in dogs are very common, the treatment of these is a great challenge for orthopaedics. The naturally-occurring pathology of articular cartilage in dogs means that these animals represent a clinically important group, and the canine model is often used in translational research. Current studies in canine cartilage regenerative medicine are focusing on the application of multipotent stromal cells for treatment of defects, however the clinical application of chondrocytes in autologous transplantation (ACT) is also noted [1–4].

Nevertheless, a small percentage of the transplanted chondrocytes have sufficient ability to produce components of the extracellular matrix. Therefore, in considering autologous transplantation of chondrocytes, studies regarding optimal methods for the *in vitro* expansion of chondrocytes are essential, due to the fact that chondrocyte culture is an integral part of the ACT procedure [3].

The extracellular matrix (ECM) of functional articular cartilage is rich in proteoglycans and type II collagen (COL2A), constituting respectively 40% and 60% of the dry weight of cartilage. The main proteoglycan in

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cartilage tissue is aggrecan (ACAN). Both collagen type II network and proteoglycan aggregated complexes play a crucial function in protecting cartilage from mechanical deformation. Articular cartilage also contains small cellular proteoglycans, one of which is decorin (DCN), which responds in fibrillogenesis and interfibril interactions [5,6]. In the regulation of proteoglycan metabolism a number of growth factors are involved, especially those belonging to the transforming growth factor- β (TGF- β) superfamily [6]. The most important unique group of proteins within the TGF- β superfamily are bone morphogenetic proteins, including isoform 7 (BMP7), also known as a osteogenic protein-1 (OP-1). The BMP7 proteins play a crucial role in cartilage development, homeostasis and repair [7,8]. This is achieved by BMP7-dependent up-regulation of chondrocyte metabolism and cartilage-specific extracellular proteins synthesis. It has been shown that BMP7 influences the synthesis of collagen type II, aggrecan and decorin [9]. Chondrocytes participating in ECM synthesis constitute only 2% of the total volume of articular cartilage [6,10,11]. The chondrogenic phenotype is characterized by an increased expression level of specific genes, coding expression of collagen type II, aggrecan and also SOX-9 [12]. During long-term, monolayer *in vitro* culture, chondrocytes undergo de-differentiation which is manifested by changes in cell morphology and alteration of the collagen expression phenotype. As a consequence of de-differentiation, *in vitro* cultures of chondrocytes are characterized by the presence of fibroblastic-like cells expressing collagen type I (COL1A) instead of collagen type II [13,14] and these features are typical for fibrotic tissues. Additionally, the chondrocytes are cells of mesenchymal origin, therefore the expression of vimentin is noted. Vimentin (VIM) belongs to the intermediate filaments that are responsible for maintaining the mechanical integrity of mesenchymal cells and tissue, also assisting in intracellular mRNA transport [15].

There are several ways to prevent the process of de-differentiation, one of which is to culture cells on specific chondrogenic medium [14,16].

The aim of this study was to evaluate the chondrogenic differentiation potential of canine articular cartilage derived cells (cACCs) during *in vitro* culture. In order to determine the influence of the culture environment on cells' morphology, ultrastructure and gene expression profile, the cACCs were maintained in chondrogenic and non-chondrogenic conditions. Cultures of cACCs were evaluated using histochemical staining, scanning electron microscopy with an energy dispersive X-ray analytical system (SEM-EDX), transmission electron

microscopy (TEM), while changes in cartilage specific genes were monitored using quantitative Real Time PCR.

2 Methods

All reagents used in this experiment were purchased from Sigma-Aldrich (Poland), unless indicated otherwise.

All experimental procedures were approved by the II Local Ethics Committee of Environmental and Life Science University (Dec. No. 177/2010 of 11.15.2010).

2.1 Tissue sampling

The cartilage was harvested in an aseptic manner, with owner consent, from dogs euthanized for reasons unrelated to this study. The dogs were skeletally mature, male and aged 4 years old. Samples were placed in Hank's Balanced Salt Solution (HBSS) with addition of 1% antibiotic/antimycotic solution.

2.2 The isolation and propagation of canine articular cartilage derived cells (cACCs)

After surgical harvesting, cartilage was cut into small fragments and washed extensively in HBSS. Biopsies were pre-digested using 0.1% of hyaluronidase for 40 minutes at 37°C. Subsequently, tissue fragments were washed with HBSS and digested using 0.2% collagenase type I for 6 hours at 37°C. The cells were pelleted at $1200 \times g$ for 10 minutes and re-suspended in Dulbecco's modified Eagle's medium (DMEM) with Nutrient F-12 Ham. Cells were maintained in a humidified incubator with 5% CO₂ at 37°C. Subsequent cultures, after first passage, were propagated in DMEM. Media were supplemented with 10% of fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution. The culture medium was changed every two days. Cultures at 70% of confluency were detached from culture dishes using TrypLE Express solution (Life Technologies).

2.3 Analysis of morphology of primary cACC cultures

Morphology of primary cACC cultures and cultures at P1 were evaluated using the inverted microscope (Axio Observer A1). The $50 \times$ and $100 \times$ magnifications were used.

2.4 The chondrogenic cultures of cACCs

The cACC cultures, established at passage three (P3) were inoculated into 24-well plates. Chondrogenic differentiation (CH) of cACCs was induced using the commercial kit - StemPro® Mesenchymal Chondrogenic Medium (Life Technologies). In order to determine the effectiveness of chondrogenic stimulation, cultures were also maintained in non-chondrogenic conditions (NonCH; DMEM, 10% FBS and 1% antibiotic/antimycotic solution). Culture media were changed every two days. Evaluation of matrix assembly was performed after 14 day of the culture. The experiment was conducted in twelve independent repetitions.

2.5 Proliferation of cACCs in chondrogenic and non-chondrogenic cultures

Cells were cultured in 24-well plates at a concentration equal to 3×10^4 per 0.5 mL of proper growth medium. Cell proliferation was evaluated after 48, 96, 148, 172, 220 and 244 hours using a metabolic assay - Alamar Blue (resazurin – resorufin system). To perform the assay, culture media were removed and replaced with medium containing 10% dye. Cells were incubated with the dye for 2 hours in a CO₂ incubator. Following incubation, supernatants were transferred into a 96-well microplate. The absorbance of the supernatants was measured by spectrophotometry (BMG Labtech, Germany) at a wavelength of 600 nm for resazurin and 690 nm as a reference wavelength. All measurements were verified according to spectrophotometric measurement of blank samples (complete growth medium with dye and without cells). Cell number was counted in a hemocytometer after trypan blue staining. For the assay, cells were trypsinized and re-suspended in equal volumes of medium and trypan blue (0.02% solution). Each assay was repeated three times.

2.6 The effectiveness of cACCs chondrogenic stimulation

2.6.1 Specific staining of chondrogenic matrix

Alcian blue staining included the following steps: (i) rinsing of cells using HBSS, (ii) fixation of cells in 10% formalin for 1 h, (iii) aspirating the formalin and washing twice with distilled water, (iv) staining the of the cACC cultures with Alcian Staining Solution (5 mg Alcian Blue

in 50 mL ethyl acetate) overnight at room temperature in the dark, (v) washing twice with 25 mL ethyl acetate for 20 min at room temperature, (vi) rinsing with distilled water. Final washing was performed using HBSS. Stained cultures were examined under a light microscope. Images were taken using a Canon PowerShot Camera.

For Safranin-O staining cultures were: (i) washed three times with HBSS, (ii) rinsed with 1% acetic acid solution for 10 sec, (iii) stained with 0.1% Safranin-O solution for 5 min, (iv) washed using HBSS and (v) examined under light microscope. Images were taken using Canon PowerShot Camera.

2.6.2 Analysis of chondrogenic nodules formation and extracellular matrix elemental composition (SEM/SEM-EDX)

The morphology of cACCs in culture was assessed using a scanning electron microscope (SEM, Zeiss Evo LS 15), inverted microscope and epifluorescent microscope (EpiFM); (Zeiss, Axio Observer A.1). The cultures analyzed under epiFM were prepared as previously described [17,18]. Briefly, the samples were (i) washed three times using HBSS, (ii) fixed with 4% paraformaldehyde for 45 min at room temperature, (iii) rinsed again as described above, (iv) permeabilized for 15 min using Triton X-100 at room temperature, (v) washed again, (vi) stained with atto-488-labeled phalloidin (1 : 800) for 40 min in the dark at room temperature, (vii) counterstained with diamidino-2-phenylindole (DAPI; 1:1000) for 5 min in the dark at room temperature. Observations were performed under inverted and epifluorescent microscope at magnification 100 × and 50 ×, respectively. For SEM observations, cultures were prepared as described previously [17,19]. Briefly, the procedure included (i) fixation in 2.5% glutaraldehyde in DMEM; (ii) washing using HBSS; (iii) dehydrated in a graded ethanol series (50% - 100%); (iv) air-drying for 30 min at room temperature and (v) coating with gold using 300-second program (Edwards, Scancoat six). Cultures of cACCs were imaged with SE1 detector at 10 kV filament tension (SEM, Zeiss Evo LS 15) under 1000 ×, 3000 × and 20 000 × magnification.

2.6.3 Ultrastructure of chondrogenic and non-chondrogenic cACC cultures

The ultrastructure of cACC cultures was assessed using a scanning transmission electron microscope (TEM, Zeiss Evo LS 15). The cACC cultures for TEM analysis

were prepared accordingly to the procedure described previously [17]. The preparations were: (i) fixed in 2.5% glutaraldehyde in DMEM overnight at 4°C, (ii) centrifuged at 2000 × g for 10 min, (iii) rinsed with 0.1 M PBS (pH = 7.0) for 30 min at room temperature, (iv) centrifuged as described above, (v) incubated with 1% osmium tetroxide in PBS for 2 hours, (vi) washed using 0.1 M PBS and centrifuged again, (vii) dehydrated in a graded acetone series (30 - 100%) and (viii) embedded using Agar Low Viscosity Resin Kit (Agar Scientific Ltd., Stansted). Finally, the ultrathin sections (80 nm) of the specimens were collected on copper grids, contrasted with uranyl acetate (30 min incubation) and lead citrate (15 min incubation). Images of cACCs were captured using TEM detector, at 10 kV filament tension.

2.6.4 Analysis of gene expression in chondrogenic cACC cultures - RT-qPCR

Total RNA was isolated from cACCs using phenol-guanidine isothiocyanate reagent (TriReagent®). All reagents used for total RNA extraction were of molecular biology grade.

The RNA extraction was performed according to the manufacturer's instructions. Purity of total RNA samples was measured in a spectrophotometer (VPA Biowave II).

The material obtained was characterized by a A260/A280 ratio of between 1.8-2.0. For reverse transcription reaction, 500 ng of total RNA was used. The reaction was performed using a Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) and oligo(dT)15 primers (Verte KIT oligo(dT)15, Novazym). Synthesis of cDNA was completed according to the manufacturer's protocol using T100 Thermo Cycler (Bio-Rad). The sequences of specific oligonucleotides, with characteristics, are presented in Table 1. The qRT-PCR mixtures were prepared using SensiFast SYBR & Fluorescein Kit (Bioline). The total reaction volume was 20 µL. The concentration of primers in each reaction was 0.5 µM. The thermal profile of qRT-PCR included the following steps: (i) 95°C for 2 min; (ii) 95°C denaturation for 30 s, annealing temperature gradient for 30 s, 72°C measurement of single fluorescence for 30 s. Each cycle was repeated 45 times. A dissociation curve of amplicons was used to validate the specificity of the PCR products. Analysis of the melting curve was performed after each amplification at 65°C - 95°C with a heating rate of 0.2°C/s and continuous fluorescence measurement. The matrices investigated were derived from three cultures that were propagated simultaneously. All samples were amplified in three repetitions. The values of the threshold cycle (Ct) acquired in each test were used to calculate the fold change in relation to the expression of housekeeping gene glyceraldehyde-3-phosphate dehydrogenase.

Table 1. Sequences of primers used in qPCR.

Genes	GenBank accession number	Primer pairs	Annealing temperature (°C)	Amplicon size (bp)
ACAN	NM_001113455.2	F: GAGCCTGAAAACACAGACGGA R: TCTCCTCTGTTGCTGTGCTG	58.1	109
BMP-7	NM_001197052	F: GATCCTCTCTATCCTGGGCTTG R: GGTGGTGGTAACGTGGATAGAA	58	290
COL1A	NM_001003090.1	F: ACCGACCAAGAAACACAGG R: GCACGGAGATTCCTCCAGTT	58.1	226
COL2A	NM_001006951.1	F: CAGGACGGGCAGAGGTATAATG R: TTGATGTCTCCGGGTCTCC	58.7	233
DCN	NM_001003228.1	F: GGAGCCTTCCAGGGAATGAA R: CACCGGGTACTCTGATGAGC	58.5	280
GAPDH	NM_001003142.2	F: GATTGTGAGCAATGCCTCCT R: GTGGAAGCAGGGATGATGTT	57.9	198
SOX-9	NM_001002978.1	F: AGCAAGACGCTGGGCAAG R: GCGGGCCCTGGGATT	58.9	299
TGFβ-1	NM_001003309.1	F: CAAGAAAAGTCCGCACAGCA R: CTGAGGTAGCGCCAGGAATC	58.3	177
VIM	NM_001287023.1	F: CTACGAGGAGGAGATGCGGGA R: GCTCAAGGTCAAGACGTGCC	58.9	218

3 Results

3.1 Morphology of cACCs

The cACCs in primary cultures resembled fibroblasts, predominantly of bipolar shape, however polygonal cells were also noted. The nuclei were centrally localized with round or oval shape. The presence of undigested tissue in primary cultures was noted. After first passage, cACCs appeared as single multipolar cells. After 72 hours of culture homogeneous a population of fibroblast-like cells, forming dense monolayer was observed (Fig 1).

3.2 Proliferation and metabolic activity of cACCs *in vitro*

The number of cells in differentiated cACC cultures slightly decreased, when compared to the control culture, however the difference was not statistically significant. In both cultures the percentage of dead cells did not exceed 12%. The metabolic activity of cACCs was evaluated using Alamar Blue test (Fig. 2). The course of growth curve of cACCs in non-chondrogenic and chondrogenic could be divided into phases typical for sub-cultured cells. The adaptation stage of cACC growth lasted 24 hours. The proliferation activity of chondrogenic cultures in this stage decreased in comparison with non-chondrogenic cultures. The exponential growth of cACCs in both culture environments was noted between 48 and 172 h of culture. Subsequently, cACCs entered into stationary phase of cell growth and after 220 h the growth rate declined, regardless of culture conditions.

3.3 The effectiveness of chondrogenic stimulation of cACCs

The results of the morphology evaluation are shown in Figure 3. To investigate the effectiveness of chondrogenic potential of cACCs the analysis was performed in comparison to the cACCs in non-chondrogenic culture environment. The extracellular matrix of cultures was stained with Safranin-O (3a) and Alcian Blue (3b). Results of staining showed a lack of chondrogenic nodules in non-stimulated cultures of cACCs, whereas formation of nodules rich in glycosaminoglycans was evident under chondrogenic conditions. The chondrogenic nodules were visualized under inverted microscope and epiFM (3c and 3d). The DAPI-derived fluorescence was intense within nodules formed by aggregated cells.

3.4 Analysis of extracellular matrix composition

The SEM analysis revealed differences in morphology of cACC cultures in chondrogenic conditions compared with the control cultures. The analysis confirmed observations obtained under light microscopy. The chondrogenic nodules were evident only in cultures where chondrogenesis was induced (Fig. 4). Additionally, analysis of the elemental composition of the extracellular matrix showed that chondrogenic cultures are rich in potassium ($1.66\% \pm 0.20$) and molybdenum ($3.42\% \pm 0.58$), whereas the level of carbon, oxygen and nitrogen decreased in chondrogenic cultures (Table 2).

3.5 Analysis of ultrastructure of chondrogenic cACC cultures

The TEM analysis revealed euchromatic nuclei of cACCs cultured in non-chondrogenic conditions. The mitochondria observed in the cytoplasm had regular size and shape. Moreover, fragments of *plasma membrane* were formed into *microvesicles* (Fig. 5a). The analysis of ultrastructure of cACCs showed that cells derived from chondrogenic cultures had a number of elongated mitochondria and centrally localized, enlarge nuclei with visible nucleoli. The enhanced production and shedding of microvesicles in differentiated cACCs was visualized with TEM images (Fig. 5b).

3.6 Analysis of gene expression

The results of qRT PCR analysis of cACC culture showed that expression of specific markers (*COL2A*, *SOX-9*, *ACAN*) of chondrogenesis significantly increased in chondrogenic cultures (Fig. 6a, b). The expression of *COL1A* was elevated in chondrogenic cultures, however the difference noted was not statistically significant (Fig. 6a). The mRNA level of *BMP-7* increased in chondrogenic stimulated cACCs (not statistically significant), while the expression of another growth factor, *TGF- β* , was not altered in chondrogenic cultures of cACCs (Fig. 6b). The increase of decorin transcription was also noted in chondrogenic cultures, nevertheless the observed difference was not statistically significant. Moreover, the expression of vimentin mesenchymal marker was constitutive in both cultures of cACCs (Fig. 6c).

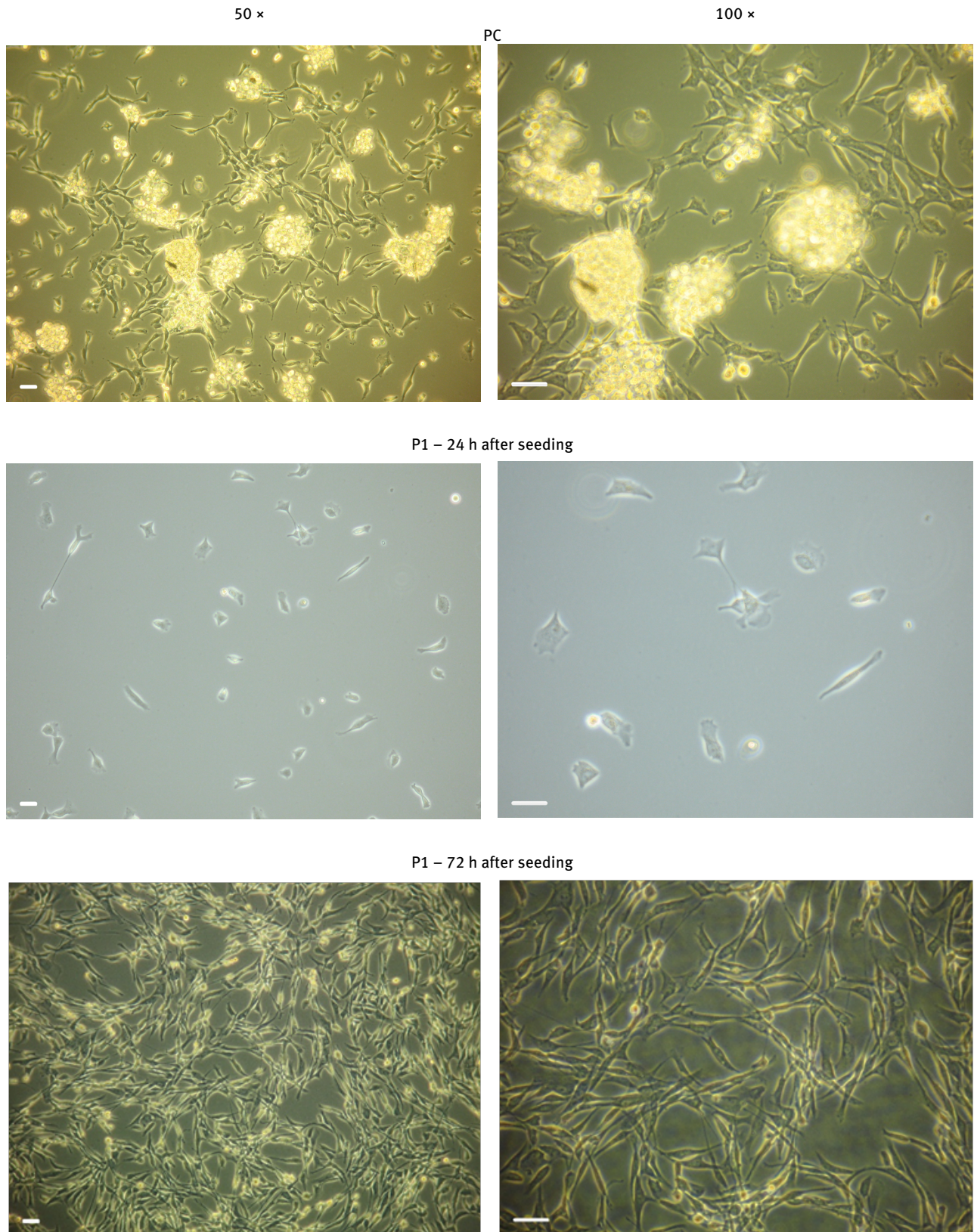


Figure 1 The morphology of canine articular cartilage derived cells in primary cultures (PC) and at passage 1 (P1). Magnification used is 50 × and 100 ×. Scale bar is 100 μm.

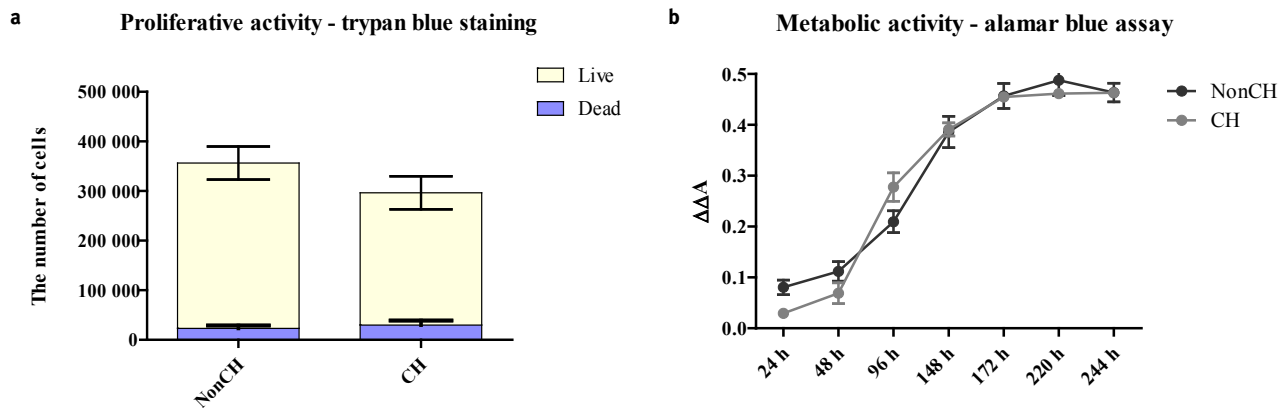


Figure 2 Proliferation activity of cACCs under chondrogenic conditions (CH) in comparison to the control culture (NonCH) (a) results of trypan blue staining showing the number of dead and living cells (b) results of the Alamar Blue assay - the X-axis refers to the time of cells' propagation. The difference between absorbance read at 600nm and 690 nm, including absorbance of blank sample, was indicated as $\Delta\Delta A$ mark (Y-axis). Error bars represent standard deviation from the mean value calculated from three separated measurements.

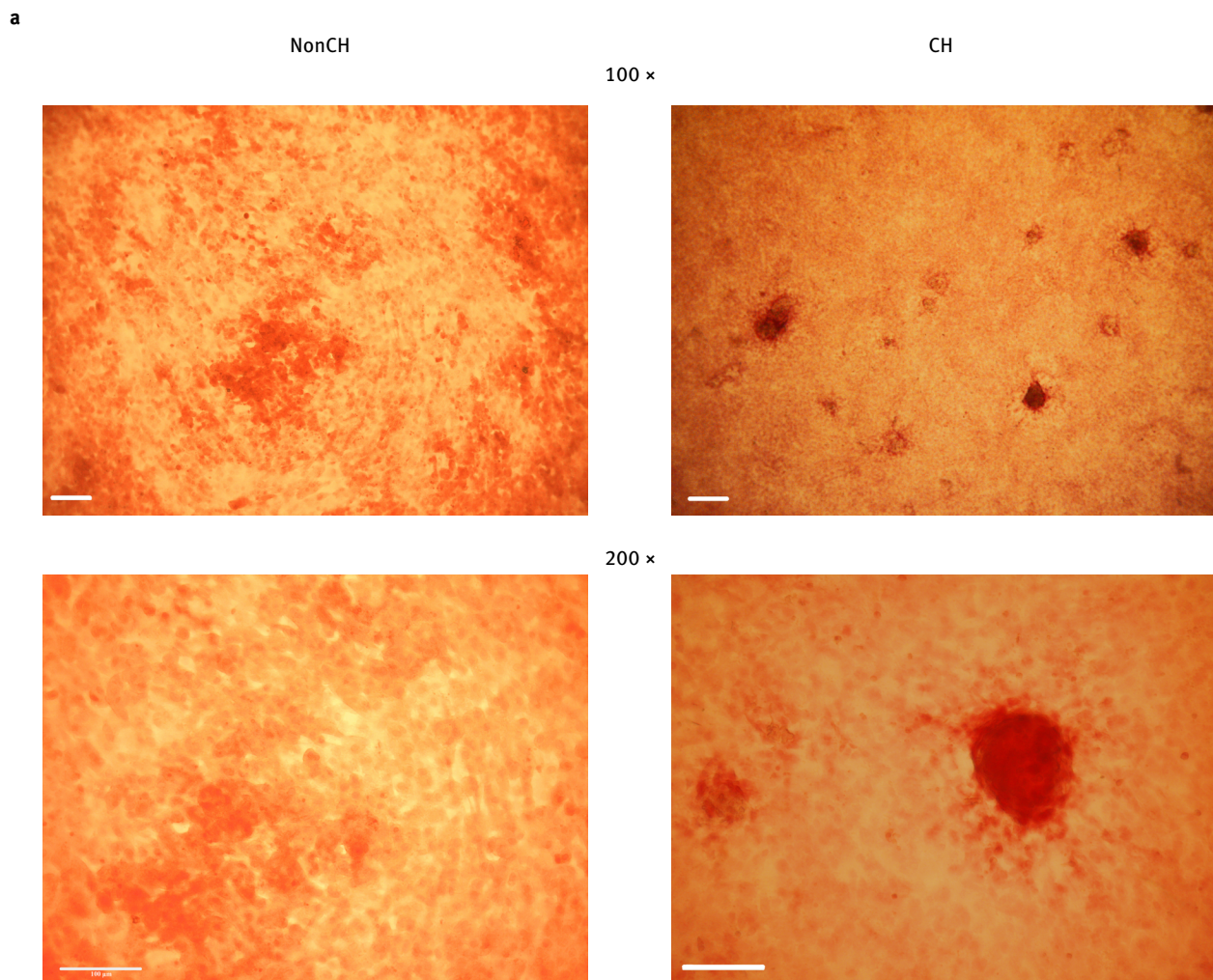


Figure 3 Morphology of cACCs under non-chondrogenic (NonCH) and chondrogenic (CH) conditions. For visualization of glycosaminoglycans deposits specific Safranin-O (a) and Alcian Blue (b) staining were used. Additionally, the morphology of NonCH and CH cultures of cACCs was showed using inverted microscope (c) and epifluorescent microscope – the actin filaments were stained using atto-488-labeled phalloidin, while nuclei were counterstained using DAPI (d). Magnification used is 100 × and 200 ×. Scale bar is 100 μm

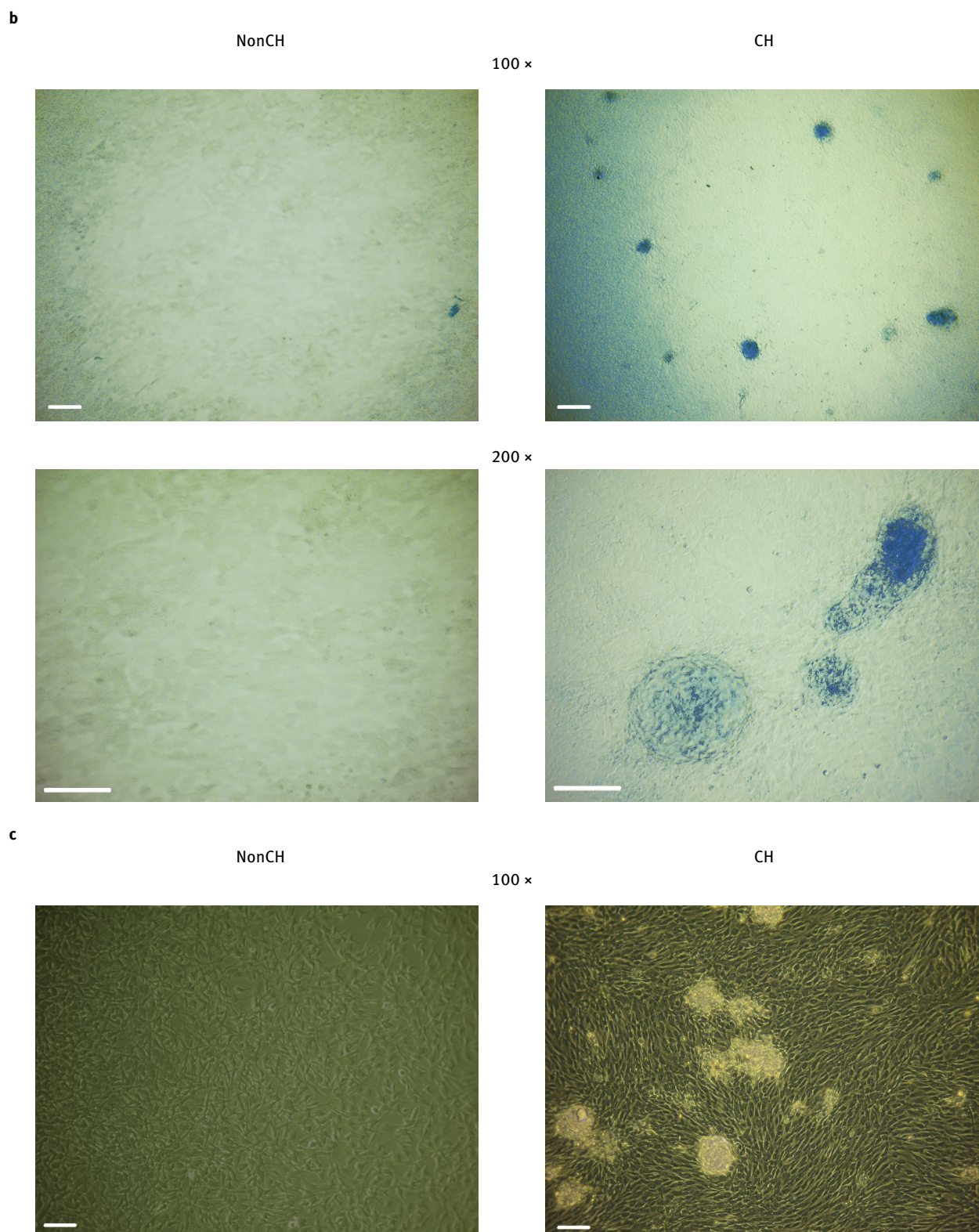


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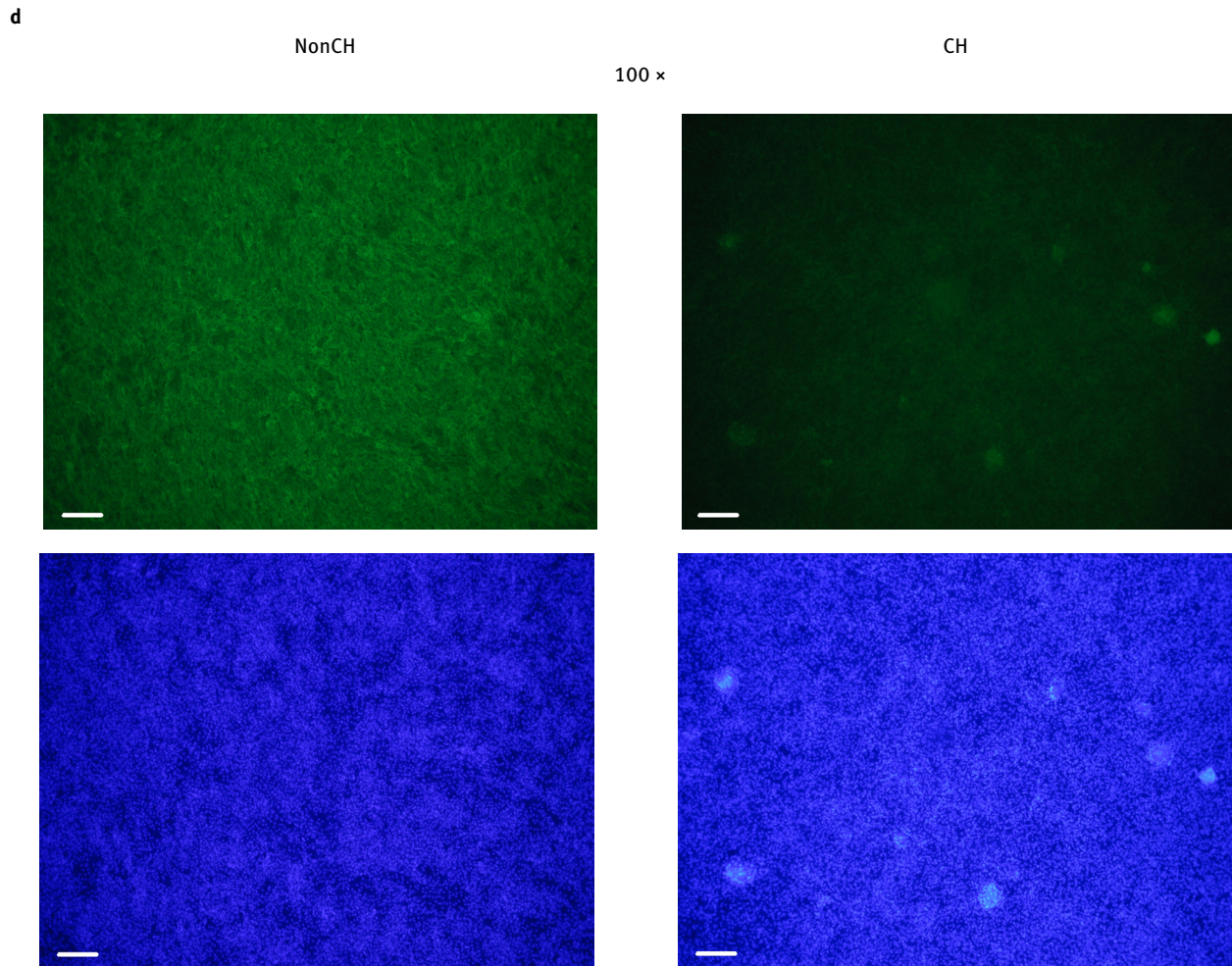


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4 Discussion

In the recent years, much attention has been paid to the development of regenerative medicine [2, 20, 21]. The search for new cell-based methods of treatment can help in repair of cartilage injuries and osteoarthritis in dogs [22]. Articular cartilage can be used as a source of chondrocytes, used for autologous transplantation [1,2,21,22], however their potential for differentiation decreases during *in vitro* expansion.

In the present study, we investigated the influence of culture microenvironment on differentiation potential of canine articular cartilage derived cells. We have focused on examination of cell morphology, ultrastructure and gene expression of cACCs in long-term monolayer cultures under non-chondrogenic conditions and chondrogenic-promoting conditions. Our results revealed that cACCs

may recover their chondrogenic potential *in vitro*, when propagated in specific differentiation medium.

The cells isolated from articular cartilage in monolayer, eventually lose their phenotype and become fibroblastic. This phenomenon is known as de-differentiation [23]. Our research confirmed that cACCs undergo de-differentiation manifested by changes in morphology and chondrogenic phenotype. The cells' morphology was monitored in primary and subsequent cultures. In the primary cultures we noted both polygonal and round cells of chondrogenic morphotype, as well as bipolar cells resembling fibroblasts. In the subsequent cultures only fibroblast cells were noted. The same observations were made previously in cultures of human [24,25], bovine [26] and rabbit [27] chondrocytes growing in monolayer. It is worth mentioning that the proliferative activity of cACCs was not altered by chondrogenic stimulation: only in the

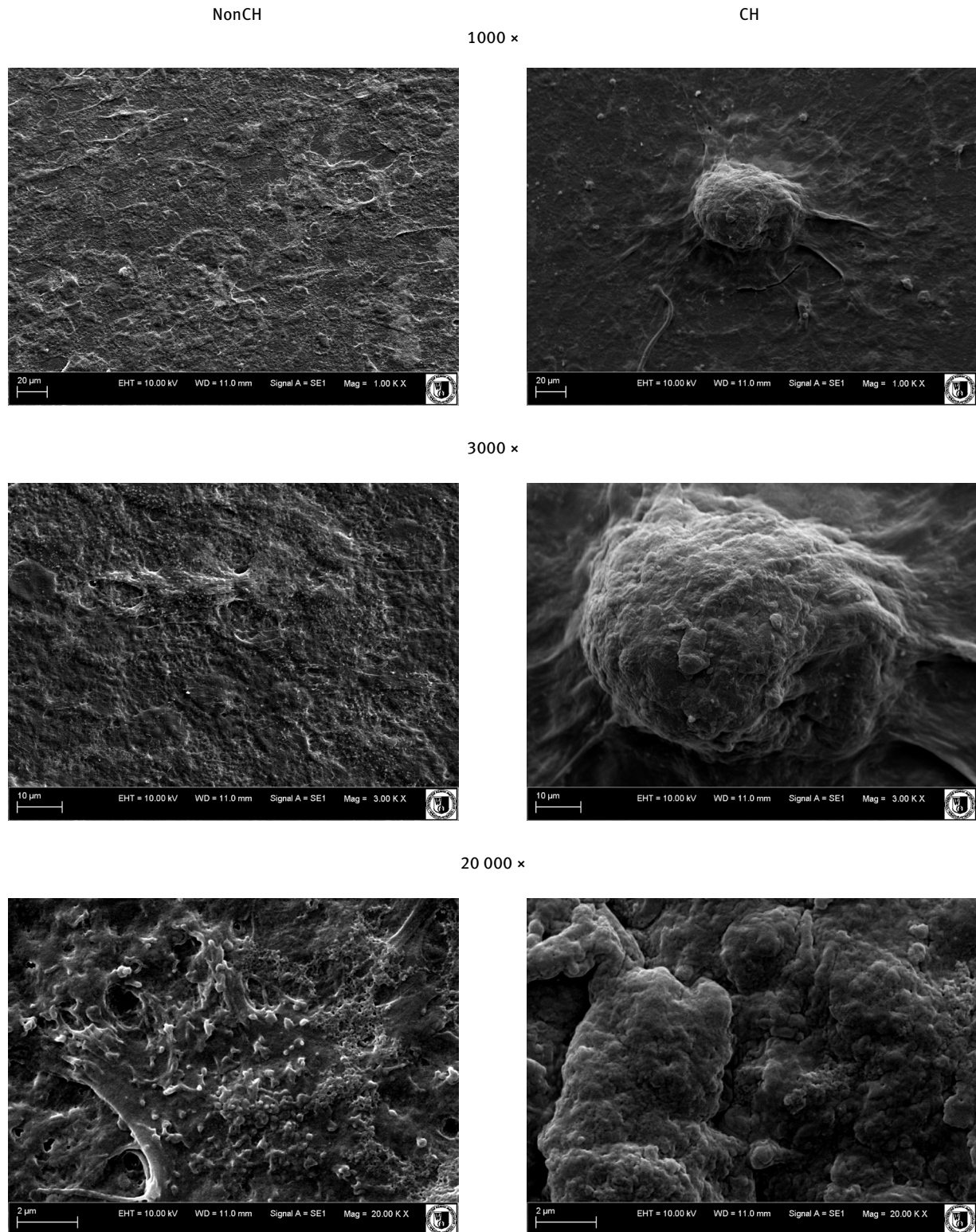
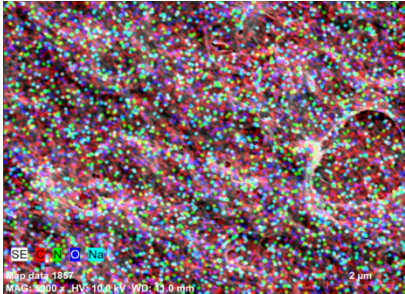
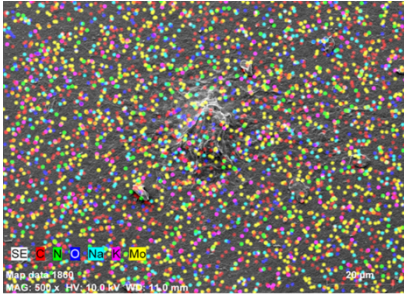


Figure 4 SEM analysis of extracellular matrix of cACCs under non-chondrogenic and chondrogenic conditions. Images were captured at magnification 1000-, 3000-, 20 000-fold, scale bar = 20, 10, 2 μm .

Table 2. Analysis of the elemental composition of cells.

Element	Culture conditions			
	NonCH		CH	
	C norm [wt%]	SD +/- [wt%]	C norm [wt%]	SD +/- [wt%]
C - carbon	24.61	0.87	12.37	2.28
O - oxygen	7.51	1.17	6.36	2.70
N - nitrogen	12.61	0.65	6.82	1.84
K - potassium	undetectable	undetectable	1.66	0.20
Mo - molybdenum	undetectable	undetectable	3.42	0.58
SEM-EDX mapping				

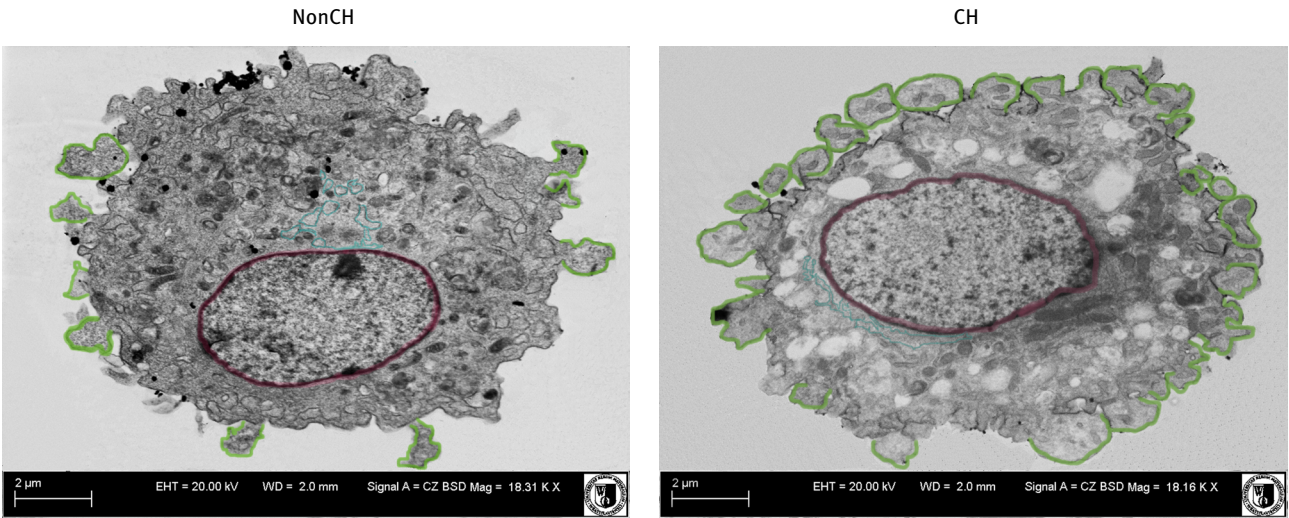


Figure 5 Ultrastructure of cACCs in non-chondrogenic and chondrogenic conditons. Typical ultrastructural features were indicated with proper colors: violet – nucleus, blue – ER and green – microvesicles. Images were captured at magnification 18 000-fold, scale bar = 2 µm.

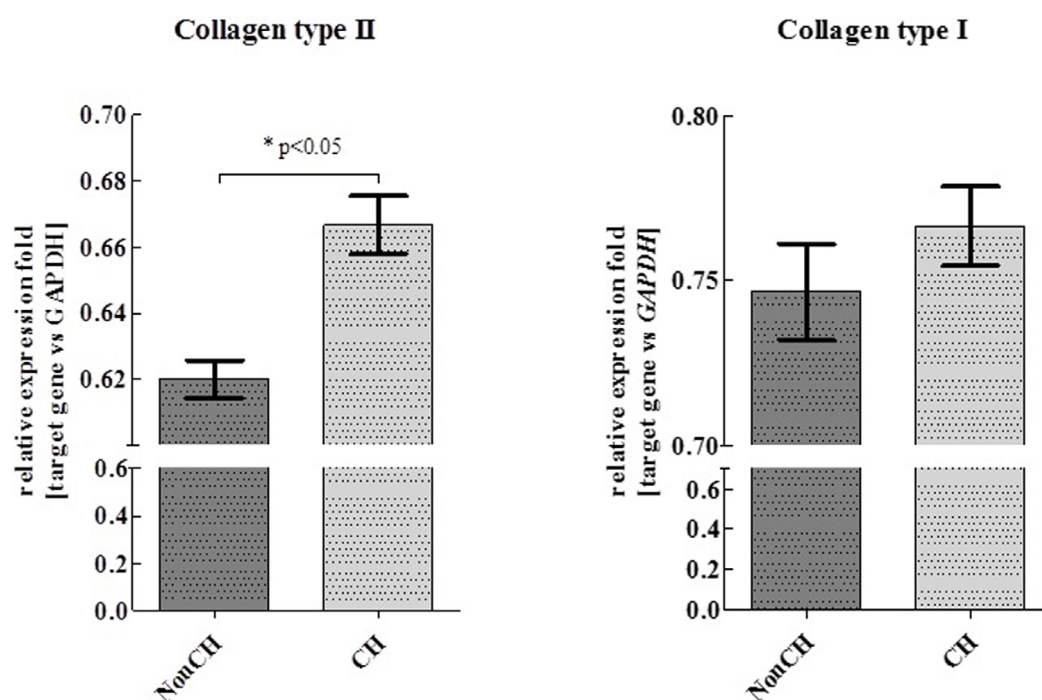
characteristic lag phase of cell adaption was reduced cell activity noted.

The phenotypic stability of chondrocytes during *in vitro* cultures is a very important issue in the context of autologous transplantation. To reduce occurrence of de-differentiation of cartilage derived cells and to restore the chondrogenic potential in cACCs, it's possible to use specific chondrogenic medium what was shown in pellet cultures [28]. Our results indicate that chondrogenic stimulation may also maintain proper differentiation

potential of cACCs in monolayer cultures. The Alcian Blue and Safranin-O staining specific for cartilage matrix rich in glycosaminoglycans were positive only in chondrogenic cultures of cACCs. Cultures of cACCs under chondrogenic conditions were characterized by formation of glycosaminoglycan-rich nodules that were confirmed with Alcian blue and Safranin-O staining.

Furthermore, examination of extracellular matrix composition by SEM-EDX revealed that cACCs, maintained in chondrogenic medium, contained deposits

a



b

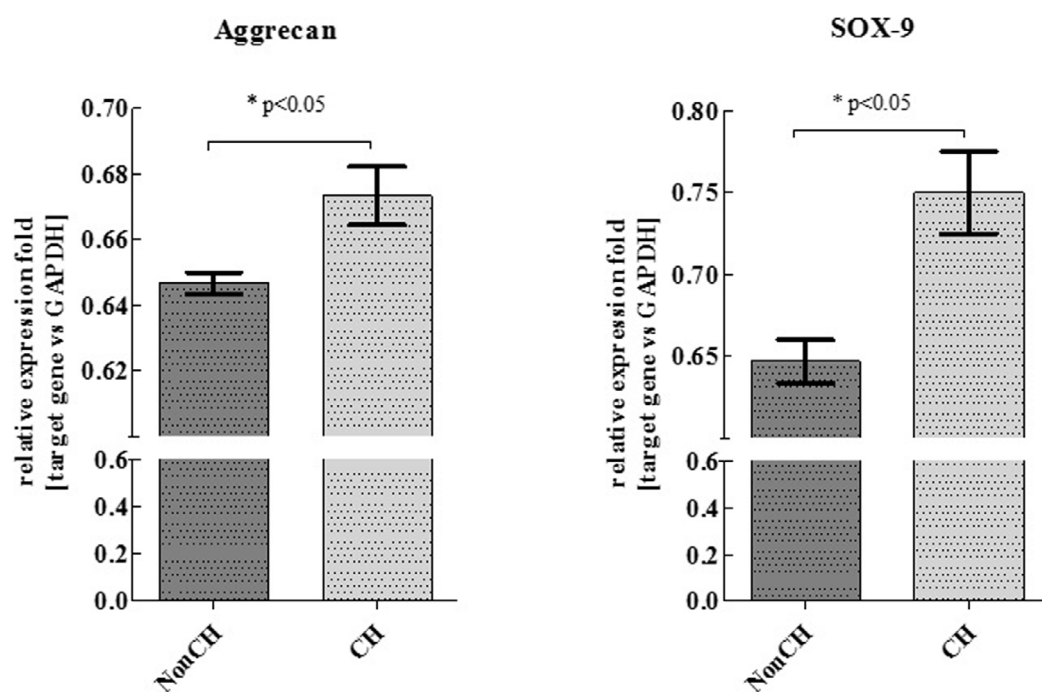
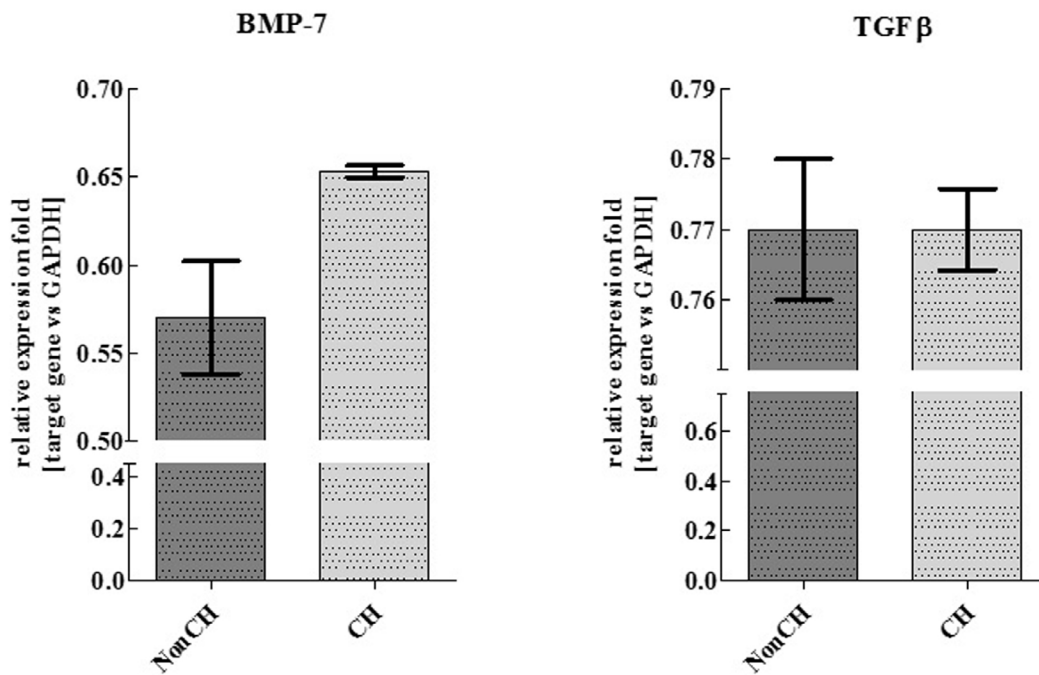


Figure 6 Expression of mRNA for a) COL2A, COL1A, b) ACAN, SOX-9, c) BMP-7, TGF- β , d) DEC, VIM in chondrogenic and non-chondrogenic cultures of cACCs. Quantification of relative values was performed using $2^{-\Delta\Delta CT}$ method, normalizing data to control culture and including expression of reference gene; error bars represent standard deviation from the mean calculated for normalized values obtained in three separate reactions; asterisks represent statistically significant differences ($p < 0.05$).

c



d

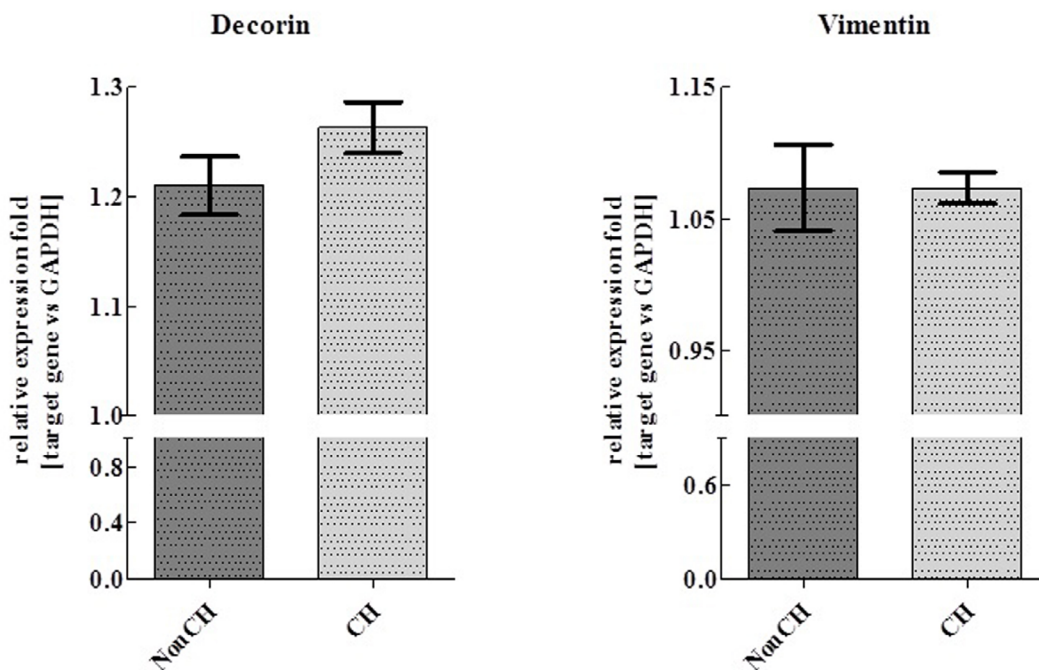


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of molybdenum and potassium. Molybdenum plays a significant role as a cofactor for enzymes involved in the reconstruction of the cartilage [29], whereas, potassium channels are crucial for chondrocyte biosynthetic activity [30].

The results of TEM analysis indeed showed that cACCs in chondrogenic cultures are metabolically active cells, releasing an increased number of microvesicles. This observation is consistent with the results of Roy [31] and Roy *et al.* [32].

Gene expression alterations are also evident during the de-differentiation process, especially in the context of the main chondrogenic markers. The de-differentiated chondrocytes are characterized by the replacement of collagen type II by collagen type I [33] and decreased expression of aggrecan [34]. Moreover, SOX-9, which plays crucial role by regulating the expression of collagen II-type [11,35,36], is another determinant of chondrogenesis, that decreased during chondrocyte culture. In our experiments, cACCs cultured in chondrogenic conditions expressed a higher level of COL2A, ACAN and SOX-9 transcripts, when compared with cells derived from non-chondrogenic cultures. Burton-Wurster *et al.* [37] showed that synthesis and accumulation of DCN in articular cartilage is made as a response to TGF β -1. The expression of DCN in the cACC chondrogenic cultures increased, while TGF- β transcript level was comparable both in chondrogenic and non-chondrogenic conditions. However, similarly to the increased expression of DCN we noted elevated mRNA levels for BMP7. The mesenchymal origin of cACCs was confirmed by expression of vimentin. The expression of this gene was constitutive and did not depend on culture condition [15].

In conclusion, we have shown that canine articular cartilage derived cells cultured under chondrogenic conditions maintain their characteristic phenotype and form chondrogenic nodules rich in proteoglycans. The chondrogenic stimulation of cACCs prevent these cultures from de-differentiating. Propagation of cACCs in chondrogenic promoting conditions may influence the effectiveness and regeneration potential of chondrocytes in autologous transplantation.

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

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