Canine colostrum exosomes: characterization and influence on canine mesenchymal stem cell secretory profile and fibroblast anti-oxidative capacity

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Abstract

Background: Canine colostrum milk (CCM) is a specific secretion of the mammary gland fundamental for the survival of the newborn. It has many described components (immunoglobulins, proteins or fat), but small vesicles named exosomes are largely unknown. Results: A characterization of the CCM exosomes has been performed. Exosome concentrations were abundant in CCM and appeared with characteristic cup-shaped morphology and well-defined round vesicles. Their size distribution was between 37–140 nm and western blot analysis showed positive expression of specific exosomal markers. Proteomic analysis revealed a total of 826 proteins in exosomes cargo. We also found that exosomes modified proliferation and secretory profiles in canine mesenchymal stem cells derived from bone marrow (cBM-MSCs) and adipose tissue (cAd-MSCs). Besides, CCM exosomes demonstrated a potent antioxidant effect on canine fibroblasts in culture. Conclusions: Our findings highlight, for the first time, the abundant presence of exosomes in CCM and their ability to interact with mesenchymal stem cells (MSCs). The addition of exosomes to the two types of MSCs in culture resulted in specific secretory profiles with functions related to angiogenesis, migration and chemotaxis of immune cells. In particular, the cAd-MSCs secretory profile showed a higher potential in adipose tissue development and neurogenesis, while cBM-MSCs production was associated with immunity, cell mobilization and hematopoiesis. Finally, exosomes also presented antioxidant capacity on fibroblasts against reactive oxygen species activity within the cell, demonstrating a fundamental role in the development and maturation of the puppy in the early stages of its life.

Background

The canine colostrum milk (CCM) is a specific secretion of the mammary gland produced during the first two days after labour and it is fundamental for the survival of the puppy during the first weeks after birth [1].

Next to its nutritional function, CCM plays a very significant role in passive immunity, the development of the immune system and the maturation of various organs, which improves the metabolism and vital functions of the neonate [2-8]. This secretion contains many described components (immunoglobulins, proteins or fat) and different biological membrane structures that transport bioactive molecules (cargo) related to signalling pathways and intercellular communication with the newborn tissues [8, 9]. Among these vesicular structures, exosomes stand out.

Exosomes are biological nanovesicles (30-200 nm), composed of a lipid bilayer and secreted by different cell types, whose cargo includes proteins, lipids and nucleic acids (mainly miRNA) [6, 10].

Thanks to its membrane, the exosomes of breast milk can survive harsh conditions, such as digestion, and are absorbed intact by intestinal epithelial cells [11–14] and incorporated into the circulatory system by endocytosis through vascular endothelial cells [15].

Breast milk exosomes are involved in the regulation of the neonate´s immune response, promoting the growth of the intestinal epithelium and microbiota development [16–18].

Exosomes have been described in human milk and some domestic species such as pig, cow, horse, buffalo, yak or camel [19–26]. Although exosomes have been isolated from different cell types in canine species [27-29], they have not been described in CCM.

The effect of CCM exosomes has been evaluated in some cell types [30], however, it has never been assessed in mesenchymal stem cells (MSCs). MSCs play a strategic role in the development, homeostasis and repair of different organs and tissues [31, 32] and have shown promising results in the treatment of different canine pathologies [33, 34]. Therefore, we believe that it is interesting to demonstrate the effect of canine colostrum exosomes on different types of MSCs and to help understand their role in the neonatal period in the canine species.

On the other hand, in the early stages of life after birth, there is an exponential increase in reactive oxygen species (ROS) [35], which may be responsible for serious alterations very well described in the human neonate [36, 37], calves [38] and canine newborn [35]. Among the components of colostrum, there are different essential antioxidants against oxidative damage [38, 39], however, the antioxidant potential of CCM exosomes has not been evaluated.

With these premises, the purpose of our study was, for the first time, to isolate exosomes by ultracentrifugation from CCM and their characterization by transmission electron microscopy (TEM), size distribution, electronegativity, exosome markers by western blot and their proteomic analysis.

Then, we demonstrated the effects of CCM exosomes evaluating their influence on canine MSCs proliferation and its secretory profile.

Finally, we have evaluated the antioxidant capacity of CCM exosomes on canine fibroblasts, given that in the early stages of the life of the canine neonate, a large number of ROS-mediated pathologies are related to the maturation of the respiratory system [35], where fibroblasts play a fundamental role.

Methods

All animal procedures were conducted by licensed veterinary surgeons and comply with both national and European legislation (Spanish Royal Decree RD1201/2005 and EU Directive 86/609/CEE as modified by 2003/65/CE, respectively) for the protection of animals used for research experimentation and other scientific purposes. Likewise, the protocols were approved by the Institutional Animal Care and Use Committee of BIONAND (Andalusian Center for Nanomedicine and Biotechnology) Málaga, Spain, and written consents were obtained from all dogs' owners.

Animals and colostrum sample collection

Eight client-owned healthy bitches of different breeds with a mean age of 3,87 ± 1,25 years and body weight of 16,5 +/- 10,97 kg were selected as colostrum donors. The average litter size was 4,75 ± 1,65 puppies. Animals were up to date with vaccinations and deworming and fed with a dry balanced diet for growing dogs *ad libitum*.

All animals were clinically examined previously, submitted to hematological and biochemical tests, and they did not manifest symptoms of infectious or parasitic diseases. No medication was administered during pregnancy. Colostrum was obtained in an interval that oscillates between parturition and 45 minutes after it, always before the suction by the puppies. The mammary glands were disinfected, a previous massage was performed, and 3 mL of colostrum was collected using a manual milk extraction syringe. It was collected in all mothers of the two inguinal glands (M-5). None of the animals required any type of anesthesia or sedation, and they were not sacrificed to obtain colostrum. The samples were stored at 4°C until analysis.

Refractive index

The colostrum refractive index was measured in thawed colostrum at room temperature (21°C) with a handheld refractometer on samples diluted 1:2 in distilled water (Atago, Japan; refractive scale from 1.333 to 1.360) as previously described [4, 40]. All samples were analyzed in the same session.

Colostrum exosomes isolation and characterization

CCM of each bitch was centrifuged separately at 13.000 g for 30 min to remove cellular debris and microvesicles. The supernatant was centrifuged once at 100.000 g for 60 min at 4°C and then exosome pellet was washed thrice with phosphate-buffered saline (PBS) at 135.000 g for 90 min at 4°C using 70 Ti rotor in an Optima LE-80K ultracentrifuge (Beckman Coulter). The isolated exosomes were resuspended in PBS and quantified by bicinchoninic acid (BCA) kit (Thermo Fisher Scientific) according to the manufacturer's instructions [41].

Pull of CCM exosomes from the eight bitches were used for all trials. Exosomal surface proteins were analysed by western blot (WB) as follows: 30 µg of CCM exosomes, previously quantified by BCA kit, were probed for mouse antibodies anti-ALIX (Abcam), anti-TSG101 (Abcam), anti-Hsp70 (Santa Cruz Biotechnology) and anti-Actin (Abcam). Appropriate secondary antibodies were used, and signal detection carried out using enhanced chemiluminescence reagent (ECL, Cell Signaling Technology) and visualized in ChemiDocTM XRS + system (BioRad) [29, 41]. A protein lysate of human adipose mesenchymal stem cells (hAd-MSC) was used as a positive control.

To determine the shape and size of the samples, they were analysed by Transmission Electron Microscopy (TEM, Morgagni 268D electron microscope). For this assay, an exosomal fraction was placed on a nickel grid (Aname) and allowed to dry overnight. Images were taken the next day [29].

The size distribution of purified exosomes was determined using a Zetasizer Nano ZS (Malvern Instruments). The Z potential parameters (electronegativity) and size distribution were analysed at 25 ºC

according to the instructions of the Central Research Support Services (SCAI) of the University of Málaga.

Proteomic analysis

CCM exosomes were analyzed by proteomics following the instructions provided by the SCAI. The Proteome Discoverer 2.2 software (Thermo Fisher Scientific) coupled to Sequest HT was used for the identification of proteins. The data of MS/MS^2 were matched against the TrEMBL and SwissProt protein sequence databases and with the biological processes provided by the Gene Ontology database. The following parameters were taken into account: (1) N-terminal acetylation and methionine oxidation as variable modifications, (2) Carbamidomethylation of the cysteines as a fixed modification, (3) Two missed cleavages by trypsin, (4) Significant threshold: 0.05, (5) Mass tolerance of 0.02 Da for precursors and fragmented masses, (6) Search in the same database with inverted sequences with identical search parameters ("Peptide decoy") to estimate the number of false positives using Percolator software [42, 43].

Canine MSCs culture and CCM exosomes proliferation effects

Canine bone marrow (cBM-MSCs) and adipose tissue (cAd-MSCs) mesenchymal stem cells from the same donor were isolated and characterized as previously described [29, 33, 34]. Cultures were carried out in standard culture conditions: Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) exosomes free, 2.5mM L-glutamine, 100U/mL penicillin, 100 µg/mL streptomycin, and 1.25 µg/mL fungizone (all from Sigma-Aldrich). Cells were trypsinized at the confluence and cryopreserved in liquid nitrogen. The experiments were carried out on culture passage 3. FBS exosome-free serum was obtained by ultra-centrifugation at 100.000 g for 60 min at 4 °C, using 70 Ti rotor in an Optima LE-80 K ultracentrifuge (Beckman Coulter). The supernatant was collected and precipitate (exosomes) was eliminated.

Cell proliferation was measured using MTS assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega) according to the manufacturer's instructions. cBM-MSCs and cAd-MSCs were seeded at a concentration of 3×10^3 cells per well in a 96 well plate. Two doses of CCM exosomes (25µg/mL) were administered on days 1 and 6, and the cell culture medium absorbance optical density was measured at 490 nm at 1, 2, 5, 7, 9, 12 days using a microplate reader (ELx800, BioTek instruments).

Colostrum exosomes effects on canine MSCs secretory profile

cBM-MSCs and cAd-MSCs were seeded at a density of 5×10^5 cells in a FT-25 flasks with standard culture conditions and incubated overnight. For the experimental group, CCM exosomes were added at a concentration of 25 µg/mL and secretome were collected and filtered after 24h of co-culture. The control group was performed under standard culture conditions for 24 hours. Concentrations of 11 analytes were determined by Luminex kit canine cytokine 11-plex assay (Thermo Fisher Scientific): chemokine (Monocyte Chemoattractant Protein-1, MCP-1); cytokines (Interleukins: IL-2, IL-6, IL-8, IL-10, IL-12p40,

Tumor Necrosis Factor alpha: TNF-α, Interferon gamma: IFN-γ); immune-mediator (Prostaglandin E2: PGE2) and growth factors (Beta-nerve grown factor: NGF-β, Stem Cell Factor: SCF, Transforming Growth Factor beta: TGF-β, Vascular Endothelial growth factor A: VEGF-A). All analytes concentrations were expressed in pg/10^6 cells.

Indoleamine 2, 3-dioxygenase (IDO) enzymatic activity and NO production were measured spectrophotometrically using kynurenine and Nitrite/Nitrate colorimetric assay kit (Roche) according to manufacturer protocol, respectively [29].

Canine fibroblasts viability assay

MTS assay was used to determine canine fibroblasts (Cellider Biotech) cell viability. Fibroblasts (3,000 per well) were seeded in a 96 well plate, incubated overnight and treated with different concentrations (50, 100, 200, and 500 µM) of hydrogen peroxide (H_2O_2) (Sigma) for 3h, 6h and 24h. Standard culture conditions were used for the control group. At the specified time points, 20 µL of MTS solution (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega) was added to the cells. After 3 hours of incubation, optical density values were determined at 490 nm using a microplate reader (ELx800, BioTek instruments). Each group was tested in quadruplicate. The cell proliferation rate of treated cells was calculated as relative values with the control group [44].

Reactive oxygen species measurement

ROS detection was performed using DCFDA / H2DCFDA - Cellular ROS Assay Kit (Abcam) according to the manufacturer's instructions. Canine fibroblasts were co-cultured with exosomes (25µg/ml) for 24h after being exposed to H_2O_2 (500 µM) for 3 h. Standard culture conditions were used after H_2O_2 treatment for the control group. Then, cells were incubated with 2', 7'-dichlorofluorescin diacetate (DCFDA, 25 µM, 100 µl/well) for 45 min at 37 °C in dark. DCFDA, a non-fluorescent compound, is oxidized by ROS into 2', 7'-dichlorofluorescin (DCF), a highly fluorescent compound. ROS signaling was detected by the fluorescence microplate reader (BioTek instruments) with excitation and emission wavelength at 485 nm and 535 nm. Results were analyzed by KC4 software (BioTek Instruments) [44].

Statistical analysis

Data analysis was performed by SigmaPlot 11.0 software and each test was repeated on three biological replicates. The data are presented as mean ± standard deviation (SD). The *student's t-test* was used for MSCs proliferation, canine fibroblast viability and ELISA assay results, and the *p*-value was adjusted using the Bonferroni method for multiple comparisons. The degree of significance was established in the following ranges: $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$(***).

Results

Colostrum refractive index

The values obtained from all colostrum samples were within the standard values described for this species. The average refractive index value was 1.343 ± 0.0014 (Table 1).

Table 1. Information about the donors.

Sample	Breed		Age (years)	Weight (Kg)	Puppies	RI
1	Spanish water dog		2	12	4	1.345
2	Yorkshire		4	6	3	1.343
3	Chihuahua		4	5	2	1.343
4	Mixed breed		5	14	6	1.342
5	French bulldog		4	29	7	1.343
6	Golden retriever		3	36	6	1.345
7	Pug		6	11	5	1.341
8	Boxer		3	19	7	1.342
		Mean	3.8	16.5	5	1.343
		SD	1.25	10.97	1.85	0.0014

The breed, age, weight, number of puppies and refractive index (RI) are indicated. Data presented as mean ± standard deviation (SD).

Canine colostrum exosomes characterization

The mean of exosome concentrations obtained in the eight CCM samples was 305.60 ± 46.7 µg/mL. CCM exosomes were visualized by TEM (Fig. 1a), and their size distribution was between 37–140 nm with a zeta potential of -11.40 ± 0.53 mV (Fig. 1c). The measurement of size is based on the Dynamic Light Scattering (DLS) technique.

CCM exosomes showed positive expression of ALIX, heat shock protein 70 (Hsp70) and TSG101 (Tumor Susceptibility Gene 101) exosomal markers (Fig. 1b).

Proteomic analysis

The total number of peptides was performed by mass spectrometry and analyzed using *Canis lupus familiaris* protein database. We found 826 proteins in CCM exosomes. Biological processes of characterized exosomes proteins were determined by *Gene Ontology* parameters (additional file 1). CCM

exosome proteins are involved in a variety of physiological functions such as cell differentiation, cell organization and biogenesis, cellular component movement, defense response, metabolic process, regulation of biological process, response to stimulus and transport. Proteins involved in cell communication and conjugation were not found. A list of specific proteins is shown in additional file 2. One protein can be related to different biological functions.

Colostrum exosomes increase significantly cAd- MSCs proliferation

Cell proliferation curve showed an increase in cAd-MSCs (Fig. 2a) proliferation for 12 days in the presence of CCM exosomes, whereas this effect was not observed in cBM-MSCs (Fig. 2b).

Secretory profile of canine MSCs in presence of colostrum exosomes

Secretory profile characterization in both MSC's sources is shown in figures 4 and 5. Canine colostrum exosomes increase significantly IL-12p40, IL-6, IL-8, MCP-1 and SCF production in cBM-MSCs and IFN-γ, IL-8, MCP-1, TNF-α and NGF-β in cAd-MSCs after incubation with such CCM exosomes. NO was only produced by cBM-MSCs whereas IDO activity was not observed in any case.

Canine colostrum exosomes demonstrate antioxidant capacity on canine fibroblast

Canine fibroblasts incubated with H_2O_2 were used as a model for ROS overproduction. Cell viability decreased with increasing concentration and exposure time to H_2O_2 (Fig. 5a). To the final assay, canine fibroblasts were incubated for 3h with 500μM H_2O_2, and CCM exosomes were added immediately after incubation. An important decrease in ROS levels was observed in cells treated with CCM exosomes, demonstrating their antioxidant effect (Fig. 5b).

Discussion

Colostrum plays a fundamental role in survival in the neonatal period of the puppy, as well as for its future development as adult [1, 3]. To date, despite knowledge of the nutritional and immunological components of colostrum in dogs [4, 5], there is no study on biological nanostructures, such as exosomes, their cargo and biological functions.

As far as we know, this is the first study that describes and characterizes the presence of exosomes from CCM and evaluates their interaction with canine MSCs and fibroblasts.

Ultracentrifugation techniques allowed the isolation of abundant exosomes from CCM, similar to that already described in the isolation of other canine species origins [27, 29]. The presence of CCM exosomes was confirmed by TEM, size determination and western blot analysis expressing ALIX, Hsp70 and TSG101 exosomal markers, according to the recommendation of the International Society for Extracellular Vesicles [45, 46].

Exosomes play a key role in cell-to-cell communication and contain different specific proteins depending on their cellular origin. Nevertheless, they share a subset of essential proteins for vesicular biogenesis, structure and distribution [34, 47, 48]. Through proteomic analysis, we identified 892 proteins mainly related to functions such as transport, metabolism, regulation of different biological functions, cell differentiation, organization and biogenesis. These results coincide with the colostrum milk exosomes of other species [7, 48], which suggest the evolutionary importance of these vesicles to regulate different cellular functions in the newborn [6, 13, 14, 25, 49], and it is shared between different species of mammals [10].

When we compared in the canine species the proteomic profile of colostrum exosomes with exosomes from different mesenchymal sources already described by our team [29], we found that they share 11 proteins with common functions, confirming that in their position, exosomes are carriers of certain proteins with basic functions within the same species. Among these proteins, those related to functions such as angiogenesis, growth, inflammation, metabolism and cell signaling stand out (additional file 3). Undoubtedly, more studies are needed to understand the functioning of exosomes in the canine species.

MSCs play a major role in homeostasis and tissue repair, however, very little is known about the factors that may influence them in the early neonatal stages. Evidence suggests that the loss or malfunctioning of stem/progenitor cells necessary for normal cell differentiation and tissue repair may underlie the pathobiology of some diseases [50].

Evaluating MSCs as the target of colostrum exosomes, we found interesting results, which depend on the cellular source. CCM exosomes co-culture with MSCs determined a statistically significant increase in cAd-MSCs proliferation, whereas this effect was not observed in cBM-MSCs.

We suggest that colostrum exosomes can play a very interesting role in the development of the puppy's fat reserves. The percentage of adipose tissue in newborns is low and increases rapidly during the first month of life, a critical process for avoiding the risk of neonatal mortality, which does not appear to be related to breeding size [1, 51, 52].

Adipose tissue, besides being an energy reservoir, represents a natural defense against hypothermia and fulfills metabolic, endocrine and regulatory functions, both with systemic and local effects [53,54]. They are exerted through a large diversity of adipokines secretions with complex autocrine and paracrine effects [55]. MSCs are multipotent postnatal progenitors, with adipose tissue being the main source of this cell type [29, 56]. MSCs fat residents are generally the principal source for adipocytes during postnatal growth and maintenance of adipose tissue [57]. So their increased proliferation would help increase fat reserves.

In this study, we demonstrated that canine colostrum exosomes determine changes in the secretory profile of both types of canine MSCs studied, but in a very different way. Of the 13 analytes evaluated, we found a significant increase in the production of 5 of them in cAd-MSCs (IL-8, MCP-1, IFN-γ, TNF-α and NGF-β), and 6 in cBM-MSCs (IL-12p40, IL-6, IL-8, MCP-1, SCF and NO).

Both cell types showed an increase in the secretion of IL-8 and MCP-1, factors related to migration, chemotaxis and angiogenesis.

IL-8, also known as CXCL8, has been shown to have potent pro-angiogenic properties, promoting vein endothelial cell proliferation, migration, tube formation and the ability to attract and activate neutrophils [58]. MCP-1, is one of the factors associated with the immunomodulatory effects of MSCs, reduces apoptosis and plays a direct mediating role for angiogenesis, which is manifested by the formation of new blood vessels [59], necessary for the development and growth process.

cBM-MSCs stimulated with CCM exosomes specifically increase the production of factors related to immunity (IL-6, IL-12p40, NO) and regulation and mobilization of hematopoiesis (SCF). IL-6 is a pleiotropic cytokine with a key role in different biological processes, such as regulation of the immune response, inflammation, hematopoiesis, apoptosis, cell survival and cell proliferation [60]. IL-12p40 has an important role in the development of T cells and enhances the production of immune factors [61]. NO is a highly immunosuppressive soluble factor that decreases the proliferation and modulation of T cells and promotes apoptosis of immune cells [62].

In contrast to cBM-MSCs, colostrum exosomes in cAd-MSCs, in addition to stimulating their proliferation, demonstrated a change in their secretory profile by increasing the release of proinflammatory cytokines (TNF-α and IFN-γ). TNF-α is a pleiotropic cytokine with important but sometimes contradictory functions in numerous physiological processes related to immunity and inflammation [63]. IFN-γ intervenes in the macrophage's activation, induces the expression of MHC class II molecules, and increases the cytotoxic potential and favors, together with TNF-α, the development of the fundamental Th1 cell responses to control viral infections [64, 65].

In addition, we found that colostrum exosomes increased the secretion of factors related to neurogenesis (NGF-β), most notably in cAd-MSCs. NGF plays a crucial role in the peripheral and central nervous systems that regulate the growth, differentiation and survival of neurocytes, improves cognitive functions and shows potential to induce angiogenesis in physiological and pathological conditions [66, 67].

Although both types demonstrate a secretory similarity in the functions related to angiogenesis, migration and chemotaxis of immune cells, the different behavior of each cell type would confirm the importance of the cellular niche in the different biological functions of individuals. Thus, while adipose tissue MSCs show an important endocrine and metabolic potential in adipose tissue development and neurogenesis, the response of BM-MSCs is more consistent with immunity, cell mobilization, angiogenesis and hematopoiesis.

Newborns, because of their immature antioxidant capacity, are more prone to oxidative stress than adults [35, 68-70], leading to an increase in the risk factors that trigger inflammation, infection and ischemia, resulting in damage to multiple organs, which plays a key role in the pathogenesis of several perinatal diseases [71–73].

Fibroblasts are abundant cell type in the body and their role is to produce the extracellular matrix necessary for the formation and maintenance of structural integrity at very important stages in the maturation of certain vital organs such as the lung [74, 75], and therefore they suffer the effect of free radicals. This is the justification for using this cell type to evaluate the antioxidant capacity of CCM exosomes.

Colostrum is known to be essential in the antioxidant mechanism of the neonate [76-79], however, to date the antioxidant potential of canine colostrum exosomes against fibroblasts has not been described. With our result, we demonstrate the important role that exosomes play in avoiding the effects of free radicals on them and intervene in the maturation and development of the puppy.

Therefore, the results presented in our study help the understanding of colostrum functions through its exosomes, its interrelationship with MSC and its antioxidant role [8, 16, 47].

Although our study obviously had limitations due to the small sample size of colostrum donors and the restriction posed by the lack of specific reagents available for the canine species, we believe that our work is the first step in this direction. However, a more in-depth investigation of exosomes' functions, the focus on miRNA cargos, gene regulation, immunity and metabolism may be an interesting line of research.

Conclusions

We described for the first time the isolation and characterization of exosomes from CCM. Our findings highlight their abundant presence in colostrum and their action on different biological functions. On the one hand, exosomes interact with MSCs by inducing proliferation and modulation of the secretory profile depending on their source.

For the two types of MSCs studied, the addition of exosomes resulted in a secretion profile with functions related to angiogenesis, migration and chemotaxis of immune cells. However, independently, mesenchymal stem cells from adipose tissue show a higher potential in adipose tissue development and neurogenesis, while mesenchymal stem cells from bone marrow show a higher potential in immunity, cell mobilization and hematopoiesis.

On the other hand, exosomes also present antioxidant capacity on fibroblasts against the negative effect of free radicals, demonstrating a fundamental role in the development and maturation of the puppy in the early stages of its life.

Abbreviations

BCA: bicinchoninic acid; cAd-MSCs: canine adipose-derived mesenchymal stem cells; cBM-MSCs: canine bone marrow-derived mesenchymal stem cells; CCM: canine colostrum milk; DCF: 2', 7'-dichlorofluorescin; DCFDA: 2', 7'-dichlorofluorescin diacetate; DLS: Dynamic Light Scattering; DMEM: Dulbecco's modified Eagle's medium; FBS: fetal bovine serum; Hsp70: heat shock protein 70; IDO: Indoleamine 2, 3-

dioxygenase; IFN-γ: Interferon gamma; IL-2, IL-6, IL-8, IL-10, IL-12p40: Interleukins; MCP-1: Monocyte Chemoattractant Protein-1; MSCs: mesenchymal stem cells; NGF-β: Beta-nerve grown factor; PGE2: Prostaglandin E2; ROS: Reactive oxygen species; SCF: Stem Cell Factor; SD: standard deviation; TEM: transmission electron microscope; TGF-β: Transforming Growth Factor beta; TNF-α: Tumor Necrosis Factor alpha; TSG101: Tumor Susceptibility Gene 101; VEGF-A: Vascular Endothelial growth factor A; WB: western blot.

Declarations

Ethics approval and consent to participate

All animal procedures were conducted by licensed veterinary surgeons and comply with both national and European legislation (Spanish Royal Decree RD1201/2005 and EU Directive 86/609/CEE as modified by 2003/65/CE, respectively) for the protection of animals used for research experimentation and other scientific purposes. Likewise, the protocols were approved by the Institutional Animal Care and Use Committee of BIONAND (Andalusian Center for Nanomedicine and Biotechnology) Málaga, Spain, and written consents were obtained from all dogs' owners.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article (and its supplementary information files).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

AJV: conceived the study, samples collection and drafted the manuscript; MCMA: participated in the design of the study, carried out the exosomes isolation and characterization, proteomics analysis and drafted the manuscript; CA: participated in the design of the study and ROS and ELISA assay and drafted

the manuscript; JB: conceived the study and participated in its coordination; helped to draft the manuscript and responded to the reviewers. All authors read and approved the final version of the manuscript.

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Additional Files Legend

Name and format: Additional file 1_Proteomic profile of CCM exosomes.pdf

Title and Description of data: Comparison of biological processes in characterized exosomes proteins determined by Gene Ontology parameters. One protein can be related to different biological functions.

Name and format: Additional file 2_ List of specific proteins in CCM exosomes.pdf

Title and Description of data: List of specific proteins in CCM exosomes.

Name and format: Additional file 3_ List of specific proteins in common between CCM exosomes and canine MSCs exosomes.

Title and Description of data: List of specific proteins in common between CCM exosomes and canine MSCs exosomes.

Figures

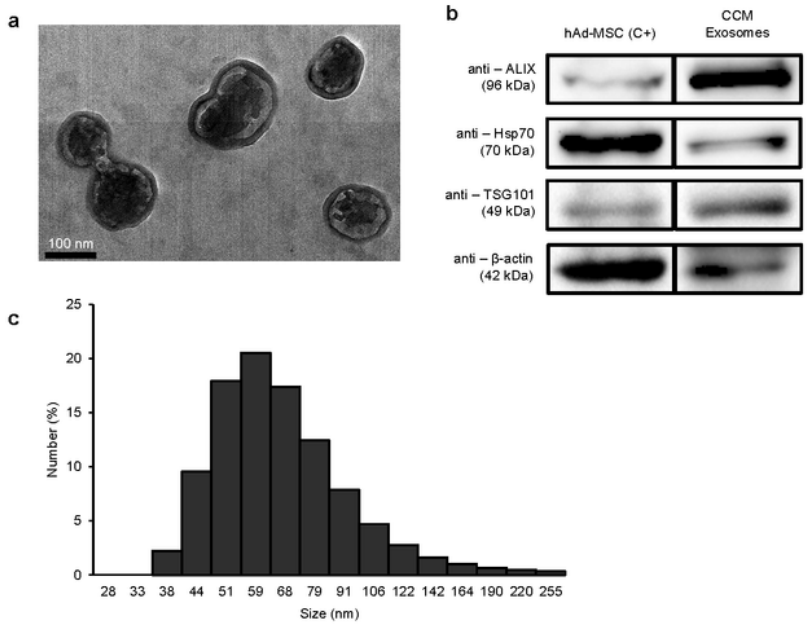

Fig. 1

Figure 1

Characterization of CCM exosomes. a Representative TEM images of exosomes isolated from CCM. Exosomes appear with a characteristic cup-shaped morphology and as round well delimited vesicles. Bars, 100 nm. b Western blot analysis showing positive expression of ALIX, Hsp-70 and TSG-101 specific surface exosomal markers. Positive control: protein lysate of human adipose mesenchymal stem cells (hAd-MSC). c Exosomal size distribution profile.

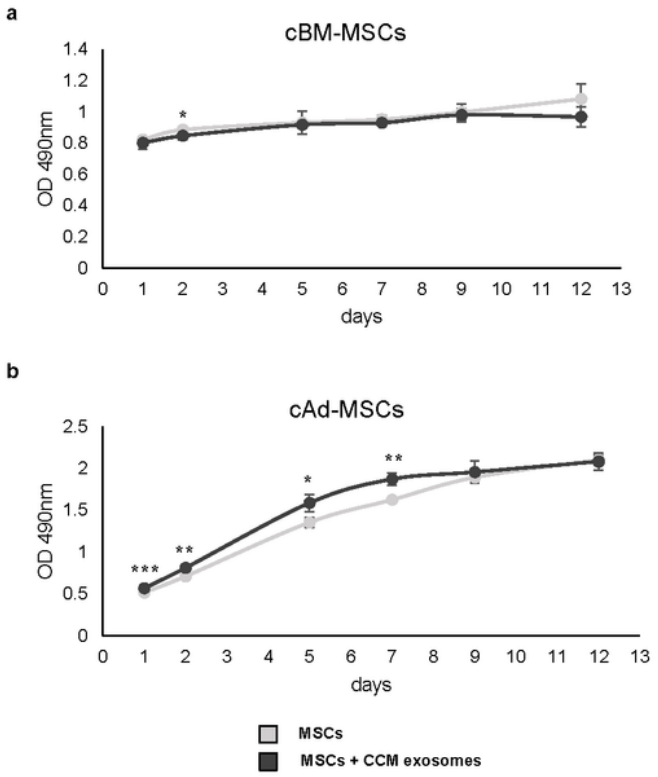

Fig. 2

Figure 2

cBM-MSCs and cAd-MSCs proliferation in presence of CCM exosomes. Comparison of cBM-MSCs (a) and cAd-MSCs (b) treated with CCM exosomes (dark grey) and their respective control (light grey). Data represent the mean ± SD. Asterisks indicate significant differences between compared values $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***).

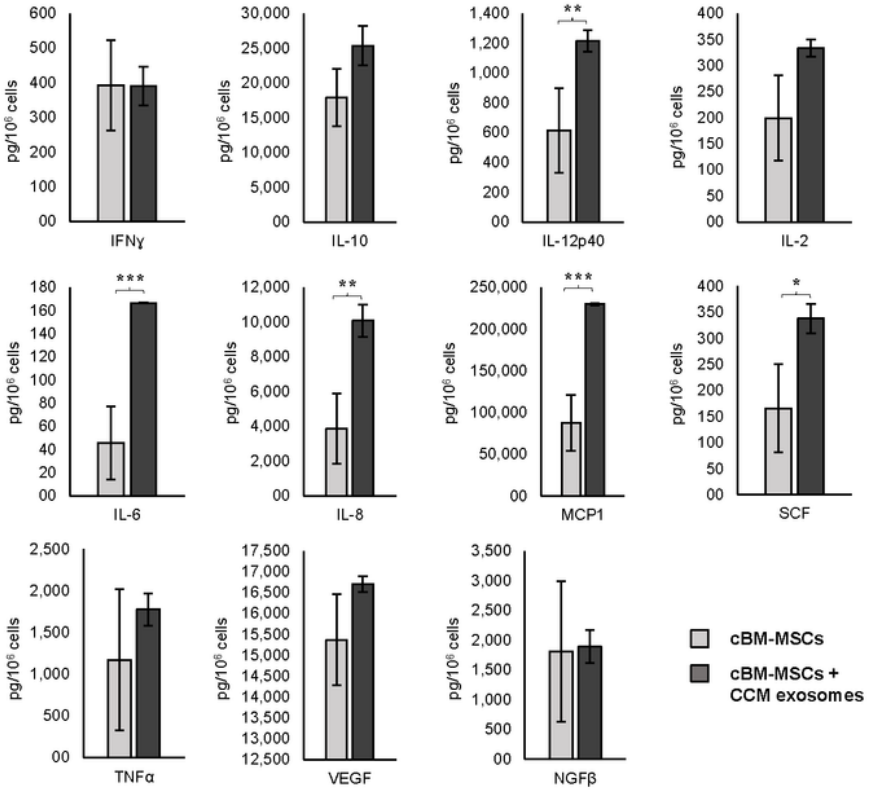

Fig. 3

Figure 3

Cytokines and growth factors secretory profile of cBM-MSCs. Controls are indicated in light grey and cells treated with CCM exosomes in dark grey. CCM exosomes increase significantly IL-12p40, IL-6, IL-8, MCP-1 and SCF production. Asterisks indicate significant differences between compared values $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***). Data presented as mean and standard deviation (n=3).

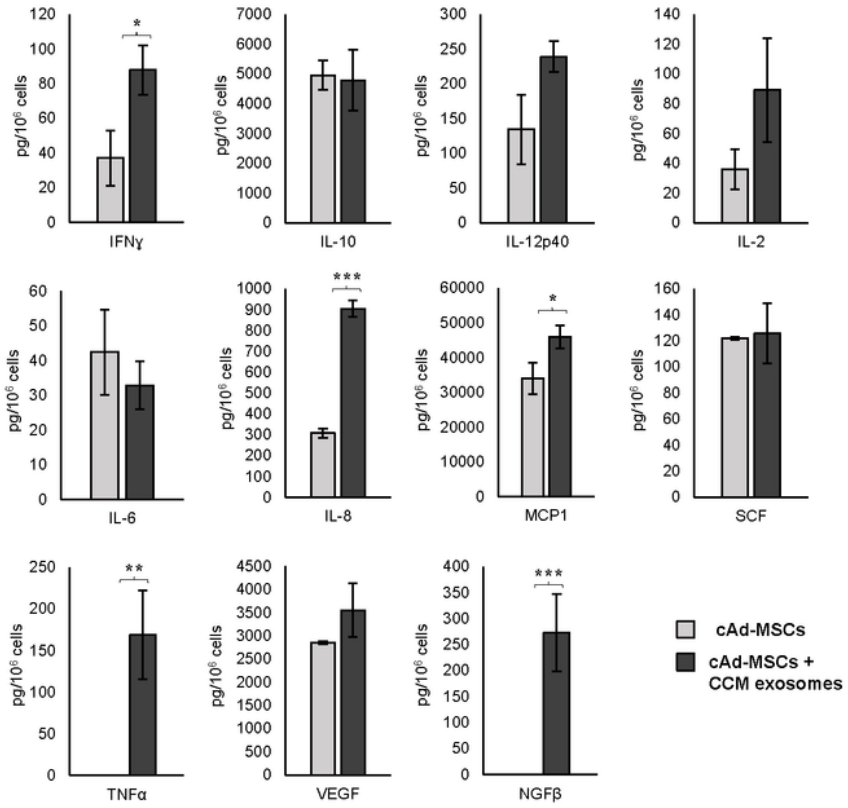

Fig. 4

Figure 4

Cytokines and growth factors secretory profile of cAd-MSCs. Controls are indicated in light grey and cells treated with CCM exosomes in dark grey. IFN-γ, IL-8, MCP-1, TNF-α and NGF-β production increase after incubation with CCM exosomes. Asterisks indicate significant differences between compared values $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***). Data presented as mean and standard deviation (n=3).

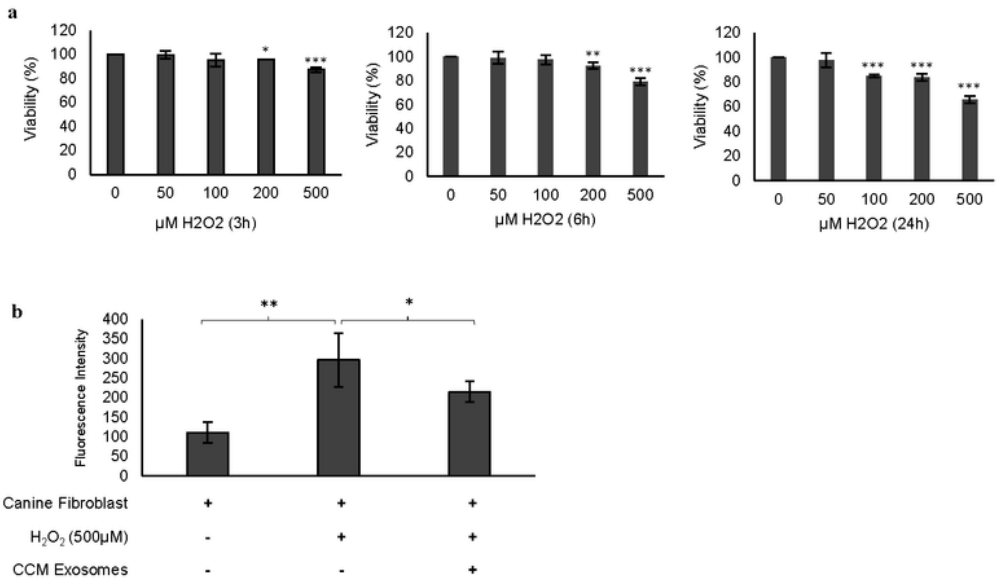

Fig. 5

Figure 5

CCM exosomes decrease ROS production in canine fibroblasts. a Canine fibroblast cell viability after exposure to 50,100,200 and 500 μM H2O2 for 3 h, 6 h and 24 h. b Fluorescence intensity equivalent to the ROS generation by the canine fibroblasts according to the applied treatment. Asterisks indicate significant differences between compared values P<0.05 (*), P<0.01 (**) and P<0.001 (***). Data are expressed as mean ± SD.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1ProteomicprofileofCCMexosomes.pdf
- NC3RsARRIVEGuidelinesChecklist.docx
- Additionalfile3ProteinsincommonCCMandMSCsexosomes.pdf
- Additionalfile2ListofspecificproteinsinCCMexosomes.pdf