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

Antonio J Villatoro  
Universidad de Malaga

María del Carmen Martín-Astorga  
Universidad de Malaga

Cristina Alcoholado  
Universidad de Malaga

José Becerra  (jbecerra@bionand.es) 
Universidad de Malaga

Keywords: Canine colostrum milk, Mesenchymal stem cells, Exosomes, Dog, Anti-oxidative capacity

Posted Date: September 25th, 2019

DOI: https://doi.org/10.21203/rs.2.15189/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License

Version of Record: A version of this preprint was published on November 2nd, 2020. See the published version at https://doi.org/10.1186/s12917-020-02623-w.
Abstract

Background: Colostrum is a specific secretion of the mammary gland fundamental for the survival of the puppy during the first weeks after birth. It contains important bioactive molecules involved in the passive immunity and the maturation of various organs, highlighting small vesicles named exosomes. Exosomes have not been described yet in canine colostrum milk, where its role is crucial in dam-newborn communication for the development of the neonate.

Results: Exosomes were abundant in canine colostrum milk and appeared with a characteristic cup-shaped morphology and well-defined round vesicles. Their size distribution was between 37−140 nm and western blot analysis showed an 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 profile in canine mesenchymal stem cells derived from bone marrow and adipose tissue, differently according to their origin. Besides, the exosomes of canine colostrum demonstrated a potent antioxidant effect.

Conclusions: We described for the first time the isolation and characterization of milk exosomes from canine colostrum. Our findings highlight the abundant presence of exosomes in the colostrum of the canine species and contribute to explain their important antioxidant capacity after parturition and their role in the modulation of cell development and tissue differentiation in the newborn, where mesenchymal stem cells seem to play a key role.

Background

The colostrum 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].

Colostrum is essential to provide important components in order to create a state of passive immunity and to develop the immune system. Since canine neonates are nearly agammaglobulinemic at birth [2–4], colostrum is fundamental to the organism’s defence against pathogens and other foreign molecules by promoting the closure of the intestinal barrier and local digestive immunity, limiting their penetration from the digestive tract into the newborn's bloodstream [4–5].

Colostrum also contains a significant amount of bioactive molecules (hormones and growth factors) involved in the development and maturation of various organs, such as the digestive tract, liver, pancreas and thyroid, which improves the metabolism and vital functions of the newborn [1, 6–8]. In addition to the nutrients and immune factors, this secretion contains different biological membrane structures that transport bioactive molecules (cargo) related to signalling pathways and intercellular communication in the newborn tissues. Among these vesicular structures, exosomes stand out.

Exosomes are biological nanovesicles (30−200 nm) composed by a lipid bilayer and secreted by different cell types, whose cargo includes proteins, lipids and nucleic acids (mainly miRNA) [6, 9]. These
nanovesicles have the ability to transfer selectively their molecular cargo being absorbed by specific cells, thus reprogramming the target cell by modulating different functions [8, 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]. In addition, vascular endothelial cells incorporate milk exosomes by endocytosis, allowing their distribution and delivery of their cargo in other tissues [15].

Breast milk exosomes play a fundamental role in dam-newborn communication, essential for the development of the neonate, intervening in the regulation of the immune response, promoting the growth of intestinal epithelium, the proliferation of different cells and the signal transduction [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, 28], they have not been described in canine colostrum milk (CCM), where its role is crucial.

Our group described for the first time the characterization of exosomes from canine mesenchymal stem cells (MSCs), which showed differences according to their tissue source [29]. MSCs play a strategic role in the development, homeostasis and repair of different organs and tissues [30, 31]. Its clinical use has shown promising results for different pathologies in canine species [32, 33]. Although colostrum exosomes' effect has been evaluated in different cell types [34], it has never been assessed on MSCs.

Likewise, recent findings point to the importance of oxidative stress during the early postnatal period in the newborn [35], which may be responsible for serious alterations very well described in the human neonate [36, 37] and in some domestic species [38]. Among the components of colostrum, there are different essential antioxidants against oxidative damage [38, 39]. However, the antioxidant role of colostrum exosomes has not been described enough. As far as we know, this is the first time that exosomes from canine colostrum have been isolated and characterized. In addition, their effects on canine MSCs have been evaluated in vitro, as well as their antioxidant effect.

The purpose of our study was to characterize canine colostrum exosomes and evaluate their antioxidative capacity and influence on canine mesenchymal stem cell proliferation capacity and modification of its secretory profile.

**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 consent was 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. Immediately after delivery, the mammary glands were disinfected, a previous massage was performed, and 3 mL of colostrum were collected using a manual milk extraction syringe. 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. Written consent was obtained from all dogs’ owners.

**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.**

Pull of CCM exosomes from eight bitches were used. Isolation and characterization protocols by transmission electron microscopy (TEM) (Morgagni 268D electron microscope, Philips), Western Blot (WB) and proteomics analysis, were performed as previously described [29].

Size determination of purified exosomes was carried out using a Zetasizer Nano ZS (Malvern Instruments). Exosomes were diluted in 1mL of 1X phosphate buffered saline (PBS) and the parameters of Z potential (electronegativity) and size distribution were analysed at 25ºC in accordance with the instructions provided by the Central Research Support Services (SCAI) at the University of Málaga. Exosomes were previously quantified by bicinchoninic acid (BCA) kit (Thermo Fisher Scientific), according to the manufacturer’s instructions.

**Proteomic analysis**

CCM exosomes were analyzed by high resolution proteomics in accordance with the instructions provided by the SCAI at the University of Málaga. The Proteome Discoverer 2.2 software (Thermo Fisher Scientific) coupled to Sequest HT was used for the identification of proteins. The data of MS/MS² were matched against the TrEMBL and SwissProt protein sequence databases and with the biological processes provided by 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 [41, 42].

**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, 32, 33]. 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 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). 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 supernatants’ 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 flask with standard culture conditions and incubated overnight. For the experimental group, CCM exosomes were added at a concentration of 25 µg/mL and supernatants were collected and filtered after 24h of co-culture. 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 was 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$_2$O$_2$) (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. Cell proliferation rate of treated cells was calculated as relative values with respect to the control group [44].

**Reactive oxygen species measurement**

Reactive oxygen species (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$_2$O$_2$ (500 μM) for 3 h. Standard culture conditions were used after H$_2$O$_2$ 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 three times. The data are presented as mean ± standard deviation (SD). *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 were 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 a 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 (Fig. 2). 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 are shown in additional file 1. One protein can be related with different biological functions.

**Colostrum exosomes increase significantly cAd- MSCs proliferation**

Cell proliferation curve showed an increase in cAd-MSCs (Fig. 3a) proliferation for 12 days in presence of CCM exosomes, whereas this effect was not observed in cBM-MSCs (Fig. 3b).

**Secretory profile of canine MSCs in presence of colostrum exosomes**

Secretory profile characterization in both MSCs 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**

Canine fibroblasts incubated with H$_2$O$_2$ were used as a model for ROS overproduction. Cell viability decreased with increasing concentration and exposure time to H$_2$O$_2$ (Fig. 6a). To the final assay, canine fibroblasts were incubated for 3h with 500µM H$_2$O$_2$, 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. 6b).

**Discussion**

Colostrum plays a fundamental role for 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 composition and biological functions in canine colostrum related to the modulation and maturation of the immune system of the newborn.
As far as we know, this is the first study that describes and characterizes the presence of exosomes from CCM and evaluates some *in vitro* functions in addition to demonstrate their interaction on canine MSCs. Our results reveal the existence of abundant exosomes in canine colostrum, which significantly promote the proliferation and viability of canine MSCs according to their origin, as well as modifying their secretory profile and their antioxidant potential.

Ultracentrifugation techniques allowed isolating abundant exosomes from all canine colostrum samples, in a similar manner to that already described in the isolation of other origins [27, 29].

The exosomes quantity obtained was like that described in the breast milk of other species [25, 45]. This concentration was between 40 and 350 times higher compared to the exosomes isolated by our group from canine MSCs [33], but very similar to the dog peripheral blood exosomes quantity [27].

According to the recommendation of the International Society for Extracellular Vesicles [46, 47], the identities of canine colostrum exosomes were confirmed by TEM, size determination and western blot analysis, expressing ALIX, Hsp70 and TSG101 exosomal markers.

Exosomes contain different specific proteins depending on their cellular origin. Nevertheless, they share a subset of essential proteins for vesicular biogenesis, structure and distribution [33, 48, 49]. Through proteomic analysis, our group identified 892 proteins mainly related to functions such as transport, metabolism and regulation of different biological functions, cell differentiation, organization and biogenesis. These results coincide with the colostrum milk exosomes of other species [7, 50], which suggest the evolutionary importance of these particles to regulate different cellular functions in the newborn, and it is shared between different species of mammals [10].

Exosomes play a key role in cell-to-cell communication. Despite the few studies of breast milk exosomes, they have been reported to act to elicit multiple regulatory functions in the recipient cells of the newborn [6]. Among them stand out those associated with the modulation of the immune response, promoting thymic regulatory T cell maturation [25], proliferation of intestinal cell and digestive tract development [13, 14], as well as contributing to the development of microbiota [51], crucial for the growth and health of newborns.

Oxidative stress is due to an imbalance between the formation of ROS and the capacity of the organism to eliminate reactive intermediates or to repair their resulting damage [52]. Newborns are more prone to suffer oxidative stress than adults [35, 53]. Inadequate balances determine an increase in the risk factors that trigger inflammation, infection and ischemia, resulting in multiple organs damage promoting ROS production, which plays a fundamental role in the pathogenesis of several pregnancy and perinatal diseases [54–56].

Colostrum is essential in the antioxidant mechanism of the newborn [57, 58]. Some factors such as their conservation or mother diseases can affect their ROS potential [59, 60].
Our results demonstrate for the first time the antioxidant potential of canine colostrum exosomes. We believe that the administration of antioxidant supplements in puppies fed with colostrum substitutes is important to counteract the generation of postpartum ROS [38].

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

The energy supply during the first days of life through colostrum plays a very important role in the growth rate of the puppy and affect the risk of neonatal mortality [1, 61]. Fat content was low in newborns and increased rapidly during the first month of life, this does not appear to be related to breed size [62].

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 [63, 64]. They are exerted through a large diversity of adipokines secretions with complex autocrine and paracrine effects [65]. MSCs are postnatal multipotential progenitors and can be found in adipose tissue, bone marrow and other connective tissues [29, 66]. MSCs fat residents are generally the principal source for adipocytes during postnatal growth and maintenance of adipose tissue [67].

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 [68]. 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 [69], necessary for the development and growth process.

cBM-MSCs stimulated with colostrum 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 [70]. IL–12p40 has an important role in the development of T cells and enhance the production of immune factors [71]. NO is a highly immunosuppressive soluble factor that decreases the proliferation and modulation of T cells and promotes apoptosis of immune cells [43].

In contrast to BM-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 [72]. IFN-γ intervenes in the macrophages activation, induces the expression of MHC class II molecules, increases the cytotoxic potential and favors, together with TNF-α, the development of the fundamental Th1 cell responses to control viral infections [73].

MSCs with a predominantly pro-inflammatory profile have been associated with early stage infections and infection processes, which stimulate the response of the innate immune system, fundamental in these early stages of life [74].

In addition, we found that colostrum exosomes increased the secretion of factors related to neurogenesis (NGF-β) by AD-MSCS in a very intense way. 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 [75, 76].

All these aspects would explain our results and describe for the first time the proliferative and modulatory effect produced by colostrum exosomes on the secretory profile of canine MSCs, playing an essential role in the development in the early stages of the puppies.

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 MSC 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.

Therefore, the results presented here open new ways for improving knowledge of colostrum functions and for a possible future use of these vesicles in the immune system modulation, the antioxidant response and the growth of puppies [8, 16, 48].

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 a 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 characterization of exosomes from canine colostrum milk. Our findings highlight the abundant presence of exosomes in the colostrum of canine species, with an important antioxidant capacity and responsible for the role in the proliferation and modulation of the secretory profile of canine MSCs. Depending on their source, the MSC on which the exosomes act may be
responsible for different fundamental biological functions in the development and maturation of the newborn.

**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 consent was 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.
Funding

This work was partially supported by the Spanish Network on Cell Therapy (Red TerCel, RD16/0011/0022), Junta de Andalucía and University of Málaga. CIBER-BBN is an initiative funded by the VI National R&D&I Plan 2008–2011, Iniciativa Ingenio 2010, Consolider Program, CIBER Actions and financed by the Instituto de Salud Carlos III with assistance from the European Regional Development Fund. The funders did not play any role in the design, conclusions or interpretation of the study.

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.

Acknowledgements

Not applicable.

References

1. Chastant-Maillard S, Aggouni C, Albaret A, Fournier A, Mila H. Canine and feline colostrum. Reprod Domest Anim. 2017;52,Suppl2:148–152.
2. Bouchard G, Plata-Madrid H, Youngquist RS, Buening GM, Ganjam VK, Krause GF, et al. Absorption of an alternate source of immunoglobulin in pups. Am J Vet Res. 1992;53:230–3.
3. Mila H, Feugier A, Grellet A, Anne J, Gonnier M, Martin M, et al. Inadequate passive immune transfer in puppies: definition, risk factors and prevention in a large multi-breed kennel. Prev Vet Med. 2014;116:209–13.
4. Mila H, Feugier A, Grellet A, Anne J, Gonnier M, Martin M, et al. Immunoglobulin G concentration in canine colostrum: Evaluation and variability. J Reprod Immunol. 2015;112:24–8.
5. Chastant-Maillard S, Freyburger L, Marcheteau E, Thoumire S, Ravier JF, Reynaud K. Timing of the intestinal barrier closure in puppies. Reprod Domest Anim. 2012; 47,Suppl6:190–3.
6. De la Torre Gomez C, Goreham RV, Bech Serra JJ, Nann T, Kussmann M. “Exosomics”-A Review of Biophysics, Biology and Biochemistry of Exosomes With a Focus on Human Breast Milk. Front Genet. 2018;9:92.
7. Zempleni J, Aguilar-Lozano A, Sadri M, Sukreet S, Manca S, Wu D, et al. Biological Activities of Extracellular Vesicles and Their Cargos from Bovine and Human Milk in Humans and Implications
for Infants. J Nutr. 2017;147:3–10.
8. Zempleni J, Sukreet S, Zhou F, Wu D, Mutai E. Milk-Derived Exosomes and Metabolic Regulation. Annu Rev Anim Biosci. 2018;7:245–262.
9. Kalra H, Drummen GP, Mathivanan S. Focus on Extracellular Vesicles: Introducing the Next Small Big Thing. Int J Mol Sci. 2016;17:170.
10. Van Herwijnen MJC, Driedonks TAP, Snoek BL, Kroon AMT, Kleinjan M, Jorritsma R, et al. Abundantly Present miRNAs in Milk-Derived Extracellular Vesicles Are Conserved Between Mammals. Front Nutr. 2018;5:81.
11. Liao Y, Du X, Li J, Lonnerdal B. Human milk exosomes and their microRNAs survive digestion in vitro and are taken up by human intestinal cells. Mol Nutr Food Res. 2017;61.
12. Benmoussa A, Lee CH, Laffont B, Savard P, Laugier L, Boilard E, et al. Commercial Dairy Cow Milk microRNAs Resist Digestion under Simulated Gastrointestinal Tract Conditions. J Nutr. 2016;146:2206–2215.
13. Chen T, Xie MY, Sun JJ, Ye RS, Cheng X, Sun RP, et al. Porcine milk-derived exosomes promote proliferation of intestinal epithelial cells. Sci Rep. 2016;6:33862.
14. Kahn S, Liao Y, Du X, Xu W, Li J, Lonnerdal B. Exosomal MicroRNAs in Milk from Mothers Delivering Preterm Infants Survive in Vitro Digestion and Are Taken Up by Human Intestinal Cells. Mol Nutr Food Res. 2018;62.
15. Kusuma RJ, Manca S, Friemel T, Sukreet S, Nguyen C, Zempleni J. Human vascular endothelial cells transport foreign exosomes from cow’s milk by endocytosis. Am J Physiol Cell Physiol. 2016;310.
16. Melnik BC, Schmitz G. MicroRNAs: Milk’s epigenetic regulators. Best Pract Res Clin Endocrinol Metab. 2017;431:427–442.
17. Melnik BC, Schmitz G. Exosomes of pasteurized milk: potential pathogens of Western diseases. J Transl Med. 2019;17:3.
18. Hock A, Miyake H, Li B, Lee C, Ermini L, Koike Y, et al. Breast milk-derived exosomes promote intestinal epithelial cell growth. J Pediatr Surg. 2017;52:755–759.
19. Ma J, Wang C, Long K, Zhang H, Zhang J, Jin L, et al. Exosomal microRNAs in giant panda (Ailuropoda melanoleuca) breast milk: potential maternal regulators for the development of newborn cubs. Sci Rep. 2017;7:3507.
20. Sedykh SE, Purvinish LV, Monogarov AS, Burkova EE, Grigor’eva AE, Bulgakov DV, et al. Purified horse milk exosomes contain an unpredictable small number of major proteins. Biochim Open. 2017;4:61–72.
21. Badawy AA, El-Magd MA, AlSadrah SA. Therapeutic Effect of Camel Milk and Its Exosomes on MCF7 Cells In Vitro and In Vivo. Integr Cancer Ther. 2018;17:1235–1246.
22. Pieters BC, Arntz OJ, Bennink MB, Broeren MG, van Caam AP, Koenders MI, et al. Commercial cow milk contains physically stable extracellular vesicles expressing immunoregulatory TGF-β. PLoS One. 2015;10.
23. Gu Y, Li M, Wang T, Liang Y, Zhong Z, Wang X, et al. Lactation-related microRNA expression profiles of porcine breast milk exosomes. PLoS One. 2012;7:e43691.

24. Baddela VS, Nayan V, Rani P, Onteru SK, Singh D. Physicochemical Biomolecular Insights into Buffalo Milk-Derived Nanovesicles. Appl Biochem Biotechnol. 2016;178:544–57.

25. Admyre C, Johansson SM, Qazi KR, Filen JJ, Lahesmaa R, Norman M, et al. Exosomes with immune modulatory features are present in human breast milk. J Immunol. 2007;179:1969–78.

26. Gao HN, Guo HY, Zhang H, Xie XL, Wen PC, Ren FZ. Yak-milk-derived exosomes promote proliferation of intestinal epithelial cells in an hypoxic environment. J Dairy Sci. 2019;102:985–996.

27. Aguilera-Rojas M, Badewien-Rentzsch B, Plendl J, Kohn B, Einspanier R. Exploration of serum- and cell culture-derived exosomes from dogs. BMC Vet Res. 2018;14:179.

28. Osamu I, Ohta H, Horino T, Nakamura T, Hosotani M, Mizoguchi T, et al. Urinary exosome-derived microRNAs reflecting the changes of renal function and histopathology in dogs. Scientific Reports. 2017;7:40340.

29. Villatoro AJ, Alcoholado C, Martin-Astorga MC, Fernandez V, Cifuentes M, Becerra J. Comparative analysis and characterization of soluble factors and exosomes from cultured adipose tissue and bone marrow mesenchymal stem cells in canine species. Vet Immunol Immunopathol. 2019;208:6–15.

30. Kang BJ, Lee SH, Kweon OK, Cho JY. Differentiation of canine adipose tissue-derived mesenchymal stem cells towards endothelial progenitor cells. Am J Vet Res. 2014;75:685–91.

31. Bearden RN, Huggins SS, Cummings KJ, Smith R, Gregory CA, Saunders WB. In-vitro characterization of canine multipotent stromal cells isolated from synovium, bone marrow, and adipose tissue: a donor-matched comparative study. Stem Cell Res Ther. 2017;8:218.

32. Villatoro AJ, Fernandez V, Claros S, Rico-Llanos GA, Becerra J, Andrades JA. Use of adipose-derived mesenchymal stem cells in keratoconjunctivitis sicca in a canine model. Biomed Res Int. 2015;527926.

33. Villatoro AJ, Hermida-Prieto M, Fernandez V, Farinas F, Alcoholado C, Rodriguez-Garcia MI, et al. Allogeneic adipose-derived mesenchymal stem cell therapy in dogs with refractory atopic dermatitis: clinical efficacy and safety. Vet Rec. 2018;183:654.

34. Casanas J, de la Torre J, Soler F, Garcia F, Rodellar C, Pumarola M, et al. Peripheral nerve regeneration after experimental section in ovine radial and tibial nerves using synthetic nerve grafts, including expanded bone marrow mesenchymal cells: morphological and neurophysiological results. Injury. 2014;45(Suppl 4:S2–6.

35. Vannucchi CI, Kishi D, Regazzi FM, Silva L, Veiga G, Angrimani D, et al. The oxidative stress, antioxidant profile and acid-base status in preterm and term canine neonates. Reprod Domest Anim. 2015; 50:240–246.

36. Beharry KD, Cai CL, Valencia GB, Valencia AM, Lazzaro DR, Bany-Mohammed F, et al. Neonatal Intermittent Hypoxia, Reactive Oxygen Species, and Oxygen-Induced Retinopathy. React Oxyg Species (Apex). 2017;3:12–25.
37. Al-Gubory KH, Fowler PA, Garrel C. The roles of cellular reactive oxygen species, oxidative stress and antioxidants in pregnancy outcomes. Int J Biochem Cell Biol. 2010;42:1634–50.

38. Abuelo A, Perez-Santos M, Hernandez J, Castillo C. Effect of colostrum redox balance on the oxidative status of calves during the first 3 months of life and the relationship with passive immune acquisition. Vet J. 2014;199:295–9.

39. Castillo-Castaneda PC, Garcia-Gonzalez A, Bencomo-Alvarez AE, Barros-Nunez P, Gaxiola-Robles R, Mendez-Rodriguez LC, et al. Micronutrient content and antioxidant enzyme activities in human breast milk. J Trace Elem Med Biol. 2019;51:36–41.

40. Morrill KM, Conrad E, Polo J, Lago A, Campbell J, Quigley J, Tyler H. Estimate of colostral immunoglobulin G concentration using refractometry without or with caprylic acid fractionation. J Dairy Sci. 2012;95:3987–96.

41. Haraszti RA, Didiot MC, Sapp E, Leszyk J, Shaffer SA, Rockwell HE, et al. High-resolution proteomic and lipidomic analysis of exosomes and microvesicles from different cell sources. J Extracell Vesicles. 2016;5:32570.

42. Schey KL, Luther JM, Rose KL. Proteomics characterization of exosome cargo. Methods. 2015;87:75–82.

43. Carrade DD, Lame MW, Kent MS, Clark KC, Walker NJ, Borjesson DL. Comparative Analysis of the Immunomodulatory Properties of Equine Adult-Derived Mesenchymal Stem Cells. Cell Med. 2012;4:1–11.

44. Liu L, Jin X, Hu CF, Li R, Zhou Z, Shen CX. Exosomes Derived from Mesenchymal Stem Cells Rescue Myocardial Ischaemia/Reperfusion Injury by Inducing Cardiomyocyte Autophagy Via AMPK and Akt Pathways. Cell Physiol Biochem. 2017;43:52–68.

45. Munagala R, Aqil F, Jeyabalan J, Gupta RC. Bovine milk-derived exosomes for drug delivery. Cancer Lett. 2016;371:48–61.

46. Lener T, Gimona M, Aigner L, Borger V, Buzas E, Camussi G, et al. Applying extracellular vesicles based therapeutics in clinical trials - an ISEV position paper. J Extracell Vesicles. 2015;4:30087.

47. Lotvall J, Hill AF, Hochberg F, Buzas EI, Di Vizio D, Gardiner C, et al. Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles. J Extracell Vesicles. 2014;3:26913.

48. Reiner AT, Witwer KW, van Balkom BWM, de Beer J, Brodie C, Corteling RL, et al. Concise Review: Developing Best-Practice Models for the Therapeutic Use of Extracellular Vesicles. Stem Cells Transl Med. 2017;6:1730–1739.

49. Simpson RJ, Lim JW, Moritz RL, Mathivanan S. Exosomes: proteomic insights and diagnostic potential.” Expert Rev Proteomics. 2009;6:267–83.

50. Yang M, Song D, Cao X, Wu R, Liu B, Ye W, et al. Comparative proteomic analysis of milk-derived exosomes in human and bovine colostrum and mature milk samples by iTRAQ-coupled LC-MS/MS. Food Res Int. 2017;92:17–25.
51. Le Doare K, Holder B, Bassett A, Pannaraj PS. Mother’s Milk: A Purposeful Contribution to the Development of the Infant Microbiota and Immunity. Front Immunol. 2018;28:9–361.

52. Birben E, Sahiner JM, Sackesen C, Erzurum S, Kalayci O. Oxidative Stress and Antioxidant Defense. World Allergy Organ J. 2012;5:9–19.

53. Kimura T, Kotani K. Perinatal veterinary medicine-related evaluation in hematological and serum biochemical profiles of experimental beagles throughout pregnancy and parturition. In Animal Model Exp Med. 2018;282–94.

54. Dani C, Poggi C, Fancelli C, Pratesi S. Changes in bilirubin in infants with hypoxic-ischemic encephalopathy. Eur J Pediatr. 2018;177,1795–1801

55. Mutinati M, Pantaleo M, Roncetti M, Piccino M, Rizzo A, Sciosci RL. Oxidative stress in neonatology: a review. Reprod Domest Anim. 2014;49:7–16.

56. Soni H, Yakimkova T, Matthews AT, Amartey PK, Read RW, Buddington RK, Adebiyi A. Early onset of renal oxidative stress in small for gestational age newborn pigs. Redox Rep. 2019;24:10–16.

57. Przybylska J, Albera E, Kankofer M. Antioxidants in bovine colostrum. Reprod Domest Anim. 2007;42:402–9.

58. Buescher ES, McIlheran SM. Colostral antioxidants: separation and characterization of two activities in human colostrum. J Pediatr Gastroenterol Nutr. 1992;14,47–56.

59. Marinković V, Ranković-Janevski M, Spasić S, Nikolić-Kokić A, Lugonja N, Djurović D, et al. Antioxidative Activity of Colostrum and Human Milk: Effects of Pasteurization and Storage. J Pediatr Gastroenterol Nutr. 2016;62:901–6.

60. Silberstein T, Hamou B, Cervil S, Barak T, Burg A, Saphier O. Colostrum of Preeclamptic Women Has a High Level of Polyphenols and Better Resistance to Oxidative Stress in Comparison to That of Healthy Women. Oxid Med Cell Longev. 2019;1380605:1–5.

61. Mila H, Grellet A, Mariani C, Feugier A, Guard B, Suchodolski J, et al. Natural and artificial hyperimmune solutions: Impact on health in puppies. Reprod Domest Anim. 2017;52,Suppl 2:163–169.

62. Kienzle E, Zentek J, Meyer H. Body composition of puppies and young dogs. J Nutr. 1998;128:2680s–2683s.

63. Rothwell NJ, Stock MJ. A role for brown adipose tissue in diet-induced thermogenesis. Obes Res. 1997;5:650–6.

64. Lee YH, Mottillo EP, Granneman JG. Adipose tissue plasticity from WAT to BAT and in between. Biochim Biophys Acta. 2014;1842:358–69.

65. Chen Y, Pan R, Pfeifer A. Regulation of brown and beige fat by microRNAs. Pharmacol Ther. 2017;170:1–7.

66. De Bakker E, Van Ryssen B, De Schauwer C, Meyer E. Canine mesenchymal stem cells: state of the art, perspectives as therapy for dogs and as a model for man. Vet Q. 2013;33:225–33.
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. c Exosomal size distribution profile.

Figure 2

Proteomic profile of CCM exosomes. Comparison of biological processes in characterized exosomes proteins determined by Gene Ontology parameters. One protein can be related with different biological functions.

a

![Graph a](image)

b

![Graph b](image)
**Figure 3**

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 (***)..

**Figure 4**

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).
Figure 5

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).

Figure 6

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.

- Additionalfile1Exosomeproteomicprofile.pdf