http://dx.doi.org/10.1590/1678-4162-12571

Arq. Bras. Med. Vet. Zootec., v.75, n.1, p.147-152, 2023

Communication

[*Comunicação*]

Serum small extracellular vesicles in overweight and obese dogs before and after weight loss: preliminary observations

[*Vesículas extracelulares pequenas no soro de cães com sobrepeso e obesos, antes e depois da perda de peso: observações preliminares*]

P.C.S. *Nunes*, S.M. *Silva*, R. *Mazzarella*, C.G. *Lima*, J.C. *Silveira*, D.C.A. *Leite-Dellova*

Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, SP, Brasil

Excess weight and obesity are among the most common health problems in dogs and one of the main causes of veterinary clinic visits. Genetic predisposition and imbalance between intake and caloric expenditure are one of the main causes of canine obesity. In this sense, neutering also contributes to body weight gain, as well as the dog-tutor interaction, due to encouragement and motivation for food intake as a form of reward (Porsani *et al*., 2020).

Adipose tissue is a dynamic metabolic and endocrine organ that performs its functions through cell-to-cell communication by producing and releasing growth factors, hormones, and cytokines (Izaola *et al*., 2015). It has been recently found that adipose tissue can synthesize, release, and transfer small extracellular vesicles (sEVs) to target cells influencing its regulation (Pardo *et al*., 2018).

EVs are nanoparticles secreted by different cell types, including adipocytes, which can carry molecular and genetic information. Initially, these nanoparticles were classified according to their biogenesis, secretion, and size as exosomes (30 to 150nm), microvesicles (100 to 1000 nm), and apoptotic bodies (1 to 5µm). However, due to the difficulty in establishing the exact origin of EVs, they can be classified according to their size as small EVs (sEVs; <200nm) and large EVs (lEVs; >200nm) as recommended by the International Society of Extracellular Vesicles - ISEV (Théry *et al.*, 2018).

Among the distinct extracellular vesicles, sEVs can perform intercellular communication, genetic exchange, and antigen presentation. Additionally, the transport of their contents, such as proteins, lipids, and RNAs (including mRNAs and non-coding RNAs), allows sEVs to significantly affect their targets at cellular and biological level. Due to the presence of sEVs in different body fluids, the analysis and characterization of their contents define them as potential minimally invasive biomarkers and, as they are easily isolated from body fluids, they can be used as "liquid biopsy" (Aguilera-Rojas *et al.*, 2018).

Scientific evidence suggests that sEVs released into the circulation could reach various organ systems, enabling intercellular communication and thus, the systemic metabolic regulation, including inflammatory process, the function of pancreatic beta cells, tissue sensitivity to insulin, and lipid metabolism in obese humans and mice (Kim *et al*., 2018). Furthermore, in humans and mice, sEVs seem to play a critical role in obesity-related metabolic complications (Eguchi *et al*., 2016). However, there are no studies relating sEVs to canine obesity. Other studies have been carried out to determine the circulating concentration of sEVs in dogs with neoplasia, such as mammary adenocarcinoma and lymphoma (Sammarco *et al*., 2018; Garnica *et al*., 2020).

Corresponding author: paolacarolsilva@gmail.com
Submitted: October 6, 2021. Accepted: November 9, 2022.

In this way, this study aimed to evaluate the serum sEVs diameter and concentration in overweight and obese dogs before and after the weight loss program. The experimental protocol of this study was approved by the institution's Ethics Committee on Animal Use (protocol number CEUA 1940130519).

The experimental group consisted of 19 neutered dogs, nine females and 10 males, including mixed breed (n=3), Poodle (n=3), Maltese (n=2), Shih-tzu (n=2), Pug (n=2), and one of each of the following breeds: American cattle dog, Dalmatian, Golden retriever, Labrador retriever, Lhasa apso, Shetland shepherd, and Yorkshire. The age of dogs ranged from two to 13 years, and all dogs had body condition score (BCS) higher than seven before initiating the weight loss program (Laflamme, 1997).

Initially, dogs underwent clinical evaluation, including laboratory tests. Blood was collected from the jugular vein after a 12-h fasting period for hematological, biochemical, and sEVs examination. Blood count was performed using automatic blood analyzer, model BC-2800 Vet (Mindray BioMedical Electronics Co. Ltd., China). Serum glucose, cholesterol, triglycerides and albumin concentration and serum alkaline phosphatase (ALP) enzyme activity were assessed with specific kits (Labtest Diagnostica S.A., Brazil) using biochemical analyzer, model BS-120 (Mindray BioMedical Electronics Co. Ltd., China).

For sEVs analysis, blood samples (5 ml) were kept at room temperature for clotting and then at 4ºC for another 2h without agitation (Lacroix *et al*., 2012). Subsequently, blood samples were centrifuged (2,400 rpm, for 30 min) to obtain serum. After that, serum samples underwent three consecutive centrifugations at 4°C: 300 x g for 10 min to remove live cells, 2,000 x g for 10 min to remove cell debris, and 16,500 x g for 30 min to remove large vesicles, and then the supernatant was stored at -80º C until further use. For sEVs isolation, samples were thawed on the day of analysis, and supernatants were filtered using syringe filter with 0.20 µm pore polyethersulfone (PSE) membrane (Corning, USA), then centrifuged at 119,700 x g for 70 min at 4°C twice (Lässer *et al*., 2012; Garnica *et al*., 2020). The resulting pellet was resuspended in 50µL of phosphate-buffered saline (1X Ca^{2+}/Mg^{2+} free PBS).

Particle diameter and concentration were measured by nanoparticle tracking analysis (NTA) using NanoSight equipment, model NS300 (Malvern Panalytical, United Kingdom). Briefly, five 30-s videos were taken for each diluted sample (1:100 in PBS) and captured by a scientific complementary metal–oxide–semiconductor (sCMOS) camera at camera level 13, under controlled temperature of 38.5°C. Considering the threshold level of five, these captured images were tracked by the NanoSight NTA 3.4 Analytical Software, model NTA 3.4 Build 3.4.003 (Malvern Panalytical, United Kingdom), as described by Ávila *et al.* (2020).

Additionally, the presence of serum sEVs was confirmed by the expression of specific proteins by western blotting, according to a previously established protocol (Ávila *et al.*, 2020). For this, the primary antibodies used were goat polyclonal anti-Alix (1:1000; sc-49267; Santa Cruz, USA) and goat polyclonal anti-Cytochrome C (1:1000; sc-8385; Santa Cruz, USA). The secondary antibody used was anti-goat IgG (1:4000; sc-2020; Santa Cruz, USA).

After initial clinical evaluation and sEVs analysis, dogs started the weight loss program, being fed with commercial low-calorie diet (Pro Plan OM Overweight Management, Purina, Brazil) for four months. The resting energy requirement (RER) was calculated using the following equation: RER = [70 x (ideal body weight in Kg)]^0.75 (Brooks *et al*., 2014), with ideal body weight being equivalent to the dog's current weight minus 10%. The daily feed amount was calculated by dividing the RER value by the metabolizable energy of the commercial low-calorie diet (2,990 kcal/kg) and meals were divided into two or three daily servings. Dogs were evaluated every 15 days for weighing and general physical examination.

Blood count, serum albumin concentration and ALP activity were determined only in the recruitment and general evaluation phase of dogs. Serum glucose, cholesterol, triglyceride concentration, and sEVs analysis were performed before the beginning and at the end of the weight loss program.

Regarding the statistical analysis, the effect of sex, time, and sex / time interaction on body weight, BCS, serum glucose, cholesterol, triglycerides concentration, and serum sEVs diameter and concentration were evaluated by ANOVA, using the SAS software, version 9.3 (SAS Institute Inc., USA). Effects with $p \leq 0.10$ were considered statistically significant. ANOVA assumptions were evaluated by SAS/LAB. Results were presented as mean values with their respective standard errors of the mean (SEM).

Blood count, serum albumin concentration, and serum ALP activity were normal for all dogs. No differences were observed between female and male dogs before and after the weight loss program. Therefore, sex and sex / time interaction had no effect on body weight, BCS, serum glucose, cholesterol, triglycerides concentration, and serum sEVs diameter and concentration (Table 1). In contrast, time had a significant effect on body weight, BCS, serum sEVs diameter and concentration (Table 1). This result indicates a change in these parameters, comparing values obtained before and after the four-month weight loss program. In this sense, reduction on the mean body weight, BCS, serum sEVs diameter and concentration values after the weight loss program was observed (Table 2 and Fig. 1). None of the dogs showed changes in serum concentrations of glucose, cholesterol, and triglycerides. Additionally, these biochemical parameters did not differ before and after weight loss (Table 2).

Table 1. ANOVA – Effect of sex, time, and sex/time interaction on different parameters of dogs. Results correspond to the p-value

Parameters	Sex	Time	Sex / time interaction
Body weight (Kg)	0.8305	**0.0062***	0.2198
Body condition score	0.4555	**<0.0001***	0.2583
Glucose (md/dL)	0.2100	0.1290	0.2045
Cholesterol (mg/dL)	0.7926	0.3170	0.6077
Triglycerides (mg/dL)	0.1064	0.9322	0.1860
Serum sEVs diameter	0.8303	**0.0574***	0.3526
Serum sEVs concentration	0.8373	**0.0450***	0.7886

sEVs: small extracellular vesicles; * $p < 0.10$.

Table 2. Means body weight, body condition score, and serum biochemical parameter values in female and male dogs, before and after the weight loss program

Parameters	Before	After	p-value
Body weight (Kg)	15.50±2.48A	13.95±2.17B	**0.0062**
Body condition score	8.21±0.20A	6.83±0.20B	<**0.0001**
Glucose (md/dL)	96.06±3.00A	90.15±3.00A	0.1290
Cholesterol (mg/dL)	203.46±10.85A	192.11±10.85A	0.3170
Triglycerides (mg/dL)	91.73±12.82A	92.55±12.16A	0.9322

± SEM. Different letters on the same line represent difference between mean values.

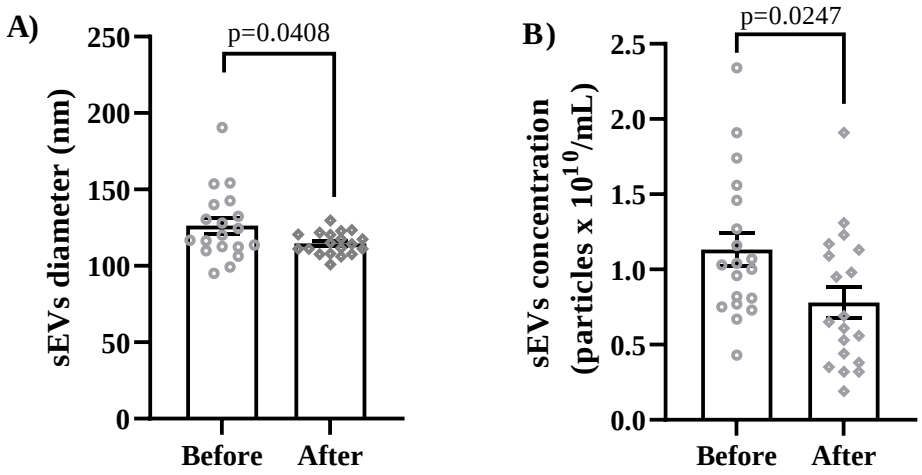

Figure 1. Small extracellular vesicle (sEVs) diameter and concentration in dog serum before and after the weight loss program. A diameter smaller than 200 nm is a characteristic of sEVs. After weight loss, there was a reduction in the mean (± SEM) diameter (126.30 ± 5.219 versus 114.60 ± 1.668 nm) **(A)** and concentration (1.13 ± 0.110 versus 0.78 ± 0.103 particles x 10^{10}/mL) **(B)** values of serum sEVs.

Analysis of protein expression is essential for the biochemical characterization of sEVs. Western blotting demonstrated that sEVs isolated from dog serum were positive for Alix and negative for Cytochrome C whereas the animal cells (cow oviduct cells) were positive for both Alix and Cytochrome C (Fig. 2). Alix is a multifunctional protein involved in the biogenesis of EVs and is commonly used as a marker for these nanoparticles (Iavello *et al.*, 2016). Cytochrome C is a mitochondrial membrane marker and therefore expressed only in cells and not in EVs. Animal cells express both proteins as they contain EVs and organelles, including mitochondria, within them. Western blotting result indicates that the isolated sEVs are not cells and that there was no contamination of the samples by cells either; therefore, the isolation protocol was performed correctly.

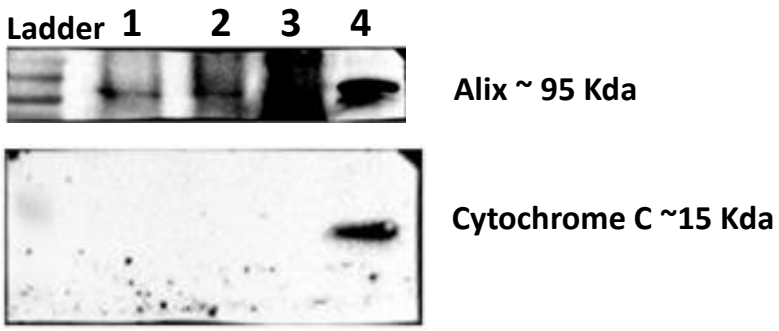

Figure 2. Analysis of protein Alix and Cytochrome C in serum small extracellular vesicles (sEVs) and cells. Lines 1-4: protein lysate from serum sEVs of obese female, obese male, and lean male dogs, and from bovine oviductal cells, respectively. Alix is an endosome pathway marker and was detected in both sEVs and animal cells. Cytochrome C is a mitochondrial membrane marker and was only detected in cells.

After four months, a 10% reduction in the body weight of dogs was observed (Table 2). Therefore, the initial goal of the weight loss program was reached (i.e., initial body weight minus 10%). In addition, 16.5% reduction in BCS was also observed (Table 2), indicating

reduction in body fat accumulation. The reduction in body weight and BCS of dogs resulted in a reduction of 9% in the diameter and of 31% in the concentration of serum sEVs (Fig. 1). Similar results have been described in obese humans. Eguchi *et al.* (2016) demonstrated that in obese humans, the number of circulating EVs (/µL of plasma) was reduced by 35% after 3 months of treatment with low-calorie diet. Furthermore, the greater amount of circulating EVs in obese humans has been linked to comorbidities caused by obesity, such as low-grade inflammatory state (Eguchi *et al*., 2016; Campello *et al*., 2016).

Even with overweight and obesity, no dog had fasting hyperglycemia or dyslipidemia, and comorbidities were not identified during the clinical evaluation. However, we consider that the weight loss, with the consequent reduction of the BCS, improved the physical condition of these dogs. A successful weight loss program is beneficial for dogs, as obesity control is directly related to an improvement in the animals' respiratory function and mobility, with direct impacts on their quality of life (German, 2016).

In the present study, we could not determine the benefits caused by reducing the diameter and concentration of serum sEVs in dogs after body weight loss, mainly because the content of the sEVs has not been determined. A study conducted by Campello *et al.* (2016) demonstrated that obesity control reduced both circulating levels of EVs and inflammatory markers in humans. This occured because circulating EVs increased simultaneously with body mass index, and EVs can carry pro-inflammatory adipokines (Amosse *et al.*, 2018).

Although our preliminary study demonstrated a reduction in the diameter and concentration of sEVs in overweight and obese dogs after a weight loss program, some limitations emerged. The cellular origin of the sEVs isolated from the serum of dogs was not examined. Therefore, we cannot say that the sEVs originate from adipocytes, even though the accumulation of body fat was the main alteration observed in the animals. The content of the sEVs needs to be determined in future studies.

In conclusion, weight loss promoted changes in the characteristics of sEVs isolated from the serum of overweight and obese dogs, such as diameter and concentration. This is the first study evaluating weight loss and circulating sEVs in dogs. The results generated expectations about the origin and content of these sEVs, and in the future, "liquid biopsy" could help in the diagnosis and monitoring of canine obesity.

RESUMO

As vesículas extracelulares (VEs) são nanopartículas circundadas por uma bicamada lipídica, que transportam lipídios, proteínas, ácidos nucleicos e metabólitos, e podem ser secretadas por diferentes tipos celulares. Os adipócitos são capazes de sintetizar e secretar VEs pequenas, que, uma vez na circulação, podem alcançar diferentes sistemas orgânicos, permitindo a comunicação intercelular. O objetivo deste estudo foi avaliar o diâmetro e a concentração de VEs pequenas no soro de cães com sobrepeso e obesidade, antes e após a perda de peso. Para isso, foram recrutados 19 cães, sendo nove fêmeas e 10 machos castrados, em bom estado geral e com escore de condição corporal (ECC) ≥ 7. A avaliação inicial dos cães incluiu exame físico e testes laboratoriais. Após avaliação inicial, os cães foram alimentados com uma dieta hipocalórica comercial por quatro meses, e, após esse período, os testes laboratoriais foram reavaliados. As VEs pequenas foram isoladas a partir do sangue total, por meio de centrifugações e ultracentrifugações seriadas, e o equipamento NanoSight foi utilizado para a determinação do diâmetro e da concentração das VEs pequenas presentes no soro antes e após a dieta. Houve uma redução de 10% do peso corporal e de 16,5% do ECC dos cães, além da diminuição de 9% do diâmetro e de 31% da concentração das VEs pequenas, após os quatro meses de alimentação com a dieta hipocalórica. Não foram observadas diferenças em relação às análises bioquímicas e entre os resultados de fêmeas e machos, antes e após a perda de peso. Em conclusão, a redução do peso corporal e do acúmulo de gordura foi capaz de modificar as características (diâmetro e concentração) das VEs pequenas, que foram isoladas a partir do soro de cães com sobrepeso e obesidade.

Palavras-chave: VEs pequenas, biópsia líquida, dieta hipocalórica, pequenos animais

ACKNOWLEDGEMENTS

The authors thank the Nestlé Purina for providing a low-calorie diet to the dogs recruited for this project. P.C.S.N was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP: 2018/26547-0). J.C.S was supported by FAPESP (2014/22887-0 e 2015/21829-9). R.M. was supported by FAPESP (2019/04981-2) and by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES – Finance code 001).

REFERENCES

AGUILERA-ROJAS, M.; BADEWIEN-RENTZSCH, B.; PLENDL, J. *et al.* Exploration of serum-and cell culture-derived exosomes from dogs. *BMC Vet. Res*., v.14, p.1-9, 2018.

AMOSSE, J.; DURCIN, M.; MALLOCI, M. *et al.* Phenotyping of circulating extracellular vesicles (EVs) in obesity identifies large EVs as functional conveyors of Macrophage Migration Inhibitory Factor. *Mol. Metab.*, v.18, p.134-142, 2018.

ÁVILA, A.C.F.C.M.; BRIDI, A.; ANDRADE, G.M. *et al*. Estrous cycle impacts microRNA content in extracellular vesicles that modulate bovine cumulus cell transcripts during in vitro maturation. *Biol. Reprod*., v.102, p.362-375, 2020.

BROOKS, D.; CHURCHILL, J.; FEIN, K. *et al.* AHA weight management guidelines for dogs and cats. *J. Am. Anim. Hosp. Assoc*., v.50, p.1-11, 2014.

CAMPELLO, E.; ZABEO, E.; RADU, C.M. *et al.* Dynamics of circulating microparticles in obesity after weight loss. *Intern. Emerg. Med.*, v.11, p.695-702, 2016.

EGUCHI, A.; LAZIC, M.; ARMANDO, A.M. *et al.* Circulating adipocyte-derived extracellular vesicles are novel markers of metabolic stress. *J. Mol. Med.*, v.94, p.1241-1253, 2016.

GARNICA, T.K.; LESBON, J.C.C; ÁVILA, A.C.F.C.M. *et al*. Liquid biopsy based on small extracellular vesicles predicts chemotherapy response of canine multicentric lymphomas. *Sci. Rep.*, v.10, p.1-11, 2020.

GERMAN, A.J. Weight management in obese pets: the tailoring concept and how it can improve results. *Acta Vet. Scand*., v. 58, p.3-9, 2016.

IAVELLO, A.; FRECH, V. S.; GAI, C. *et al*. Role of Alix in miRNA packaging during extracellular vesicle biogenesis. *Int. J. Mol. Med.*, v.37, p.958-966, 2016.

IZAOLA, O.; LUIS, D.; SAJOUX, I. *et al*. Inflamación y obesidad (lipoinflamación). *Nutr. Hosp.*, v.31, p.2352-2358, 2015.

KIM, A.; SHAH, A.S.; NAKAMURA, T. Extracellular vesicles: a potential novel regulator of obesity and its associated complications. *Children*, v.5, p.152, 2018.

LACROIX, R.; JUDICONE, C.; PONCELET, P. *et al*. Impact of pre-analytical parameters on the measurement of circulating microparticles: towards standardization of protocol. *J. Thromb. Haemost.*, v.10, p.437-446, 2012.

LAFLAMME, D. Development and validation of a body condition score system for dogs: a clinical tool. *Canine Pract.*, n.22, p.10-15, 1997.

LÄSSER, C.; ELDH, M.; LÖTVALL, J. Isolation and characterization of RNA-containing exosomes. *J. Vis. Exp.*, v.59, p.3037, 2012.

PARDO, F.; VILLALOBOS-LABRA, R.; SOBREVIA, B. *et al.* Extracellular vesicles in obesity and diabetes mellitus. *Mol. Aspects Med*., v.60, p.81-91, 2018.

PORSANI, M.Y.H.; TEIXEIRA, F.A.; OLIVEIRA, V.V. *et al*. Prevalence of canine obesity in the city of São Paulo, Brazil. *Sci. Rep.*, v.10, p.1-15, 2020.

SAMMARCO, A.; FINESSO, G.; CAVICCHIOLI, L. *et al*. Preliminary investigation of extracellular vesicles in mammary cancer of dogs and cats: Identification and characterization. *Vet. Comp. Oncol.*, v.16, p.489-496, 2018.

THÉRY, C.; WITWER, K.W; AIKAWA, E. *et al*. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J Extracell. Vesicles*, v.7, p.1535750, 2018.