

Differences in exosome content of human adipose tissue processed by non-enzymatic and enzymatic methods

M. García-Contreras^{1,2,3}, F. Messaggio^{1,2}, O. Jimenez⁴, A. Mendez¹

¹Diabetes Research Institute, University of Miami, Miller School of Medicine, Miami, FL, USA.

²Ri.Med Foundation, Palermo, Italy.

³School of Medicine and Dentistry, Catholic University of Valencia, Valencia, Spain.

⁴Top Body Sculpting Rejuvenation Center, Weston, FL, USA.

M. García-Contreras and F. Messaggio contributed equally to this study

Corresponding Author: Armando Mendez, Ph.D.; e-mail: amendez2@miami.edu

ABSTRACT

Exosomes (EXOs) are membrane vesicles released by most of cell types. EXOs contain lipids, proteins, and RNA (mRNAs, micro RNAs, non-coding RNAs). EXOs have been shown to have diagnostic and therapeutic potential. Secreted EXOs transport to other cells and deliver their contents into recipient cells playing a role in cell-to-cell communication. Adipose tissue and adipose-derived stromal cells (ADSCs) represent a promising therapeutic option in regenerative medicine and tissue engineering applications. ADSCs effects on immunomodulation and tissue repair may be attributed to paracrine secreted factors, including EXOs. Here we examine whether human adipose tissue processing methods could affect the EXOs content of the final cell/tissue, comparing non-enzymatic and enzymatic methods.

Keywords: Exosomes, Lipogems[®], Lipoaspirate.

INTRODUCTION

Exosomes (EXOs) are membranous vesicles ranging between 30-200 nm. EXOs are released by most cells types and are found in most biological tissues and fluids¹⁻⁴. It is known that EXOs may play a role in cell-to-cell communication not only in neighbor cells but also over long distances modifying phenotype and functions of recipient cells⁵. EXOs carry lipids, proteins, and nucleic acids (e.g., mRNA, microRNA, non-coding RNA)⁶ and recently shown, genomic DNA^{7,8}.

Human adipose tissue is an abundant and accessible source of mesenchymal stromal cells which have potential therapeutic applications in regenerative medicine and tissue engineering⁹. These cells have been studied in tissue engineering because of

their potential to differentiate or expand into different cell types and promote tissue repair and regeneration at the site of tissue injury¹⁰⁻¹². In fact, it has been proposed that the therapeutic effect of adipose-derived cell products could be related to their paracrine potential and not to their engraftment or differentiation at target sites¹³⁻¹⁵. The paracrine potential of these cell products could be related to cytokine secretion, mitochondrial transfer and EXOs secretion at the site of injury¹⁵⁻¹⁹.

Lipoaspirated adipose tissue collected by liposuction surgeries are used for therapeutically either immediately or with minimally manipulated procedures or following isolation and expansion of adipose-derived stromal cells (ADSCs). The standard procedure to isolate ADSCs involves an enzymatic tissue digestion. Nevertheless, there are alternative novel enzyme-free technologies to process lipoaspirates^{20,21}.

In this present study, we hypothesized that adipose tissue processing methods could affect the amount of EXOs that are present in the final tissue/cell product used clinically for selected applications. To address our hypothesis, we characterized EXOs isolated from human lipoaspirate adipose tissue processed by enzymatic digestion and a non-enzymatic, mechanical method.

MATERIALS AND METHODS

ETHICS STATEMENT

This study was initiated upon approval by the Institutional Review Board (IRB) of the University of Miami. Subjects (females between the ages of 22 to 43 years) were enrolled at Top Body Sculpting Rejuvenation Center (Weston, FL, USA). All speci-

mens were negative for HIV (Human Immunodeficiency Virus 1 and 2), HCV (Hepatitis C Virus), HBV (Hepatitis B Virus) and cytomegalovirus. All subjects signed the IRB approved informed consent prior to adipose tissue collection by liposuction.

SAMPLES COLLECTION

Human adipose tissue samples were obtained from elective liposuction procedures under local anesthesia (n=5) (Table 1). The procedure involved an infiltration step, in which a solution of saline and the vasoconstrictor epinephrine (2 µg/ml) was infused into the adipose compartment to minimize blood loss and contamination of the tissue by peripheral blood cells. Subsequently, an inspiration step (lipoaspirate) was performed using a 10 cc luer lock syringe connected to a disposable 19 cm blunt cannula (3 mm OD), with 5 oval holes (1x2 mm). A few strokes using a standard liposuction technique were enough to harvest 6 to 10 ml of fat tissue.

PROCESSING OF ADIPOSE TISSUE SAMPLES AND PREPARATION FOR EXOs ISOLATION

Lipoaspirates obtained from abdominal subcutaneous fat were processed by either a mechanical fragmentation method or digestion with collagenase. EXOs were subsequently isolated from the processed tissues. The methods are described in detail below.

Mechanical method samples (LG): Non-enzymatic processing was performed using Lipogems® devices which are patented instruments (PCT/IB2011/052204) that allow processing of lipoaspirate adipose tissue, through a two-step tissue cell cluster reduction process. The first step was a cluster reduction obtained by passing the tissue through a 4 mm² mesh, follow by an emulsification step that removed of oil and blood residues using a saline solution counter-flow through the device. After the wash step, a second cluster size reduction step was obtained by passage through a 1 mm² mesh²⁰⁻²².

Table 1. Subjects information.

Sample	Age	Gender	Height (ft,in)	Weight (lbs)
Donor 1	33	Female	5'3"	162.3
Donor 2	43	Female	5'7"	233
Donor 3	34	Female	5'3"	170
Donor 4	32	Female	5'4"	143
Donor 5	22	Female	5'1"	152

Lipogems® fresh sample consisted of 5 ml of Lipogems® tissue product re-suspended in 10 ml of PBS and kept refrigerated overnight at 4°C. Lipogems® overnight sample consist in 5 ml of Lipogems® tissue product re-suspended in αMEM medium (Gibco, Thermo Scientific, Grand Island, NY, USA) supplemented with 1% Penicillin/Streptomycin and plated in 100 mm culture dish, kept overnight at 37°C in humid atmosphere containing 5% of CO₂.

Lipoaspirate + collagenase samples (EZ): The enzymatic protocol was performed according previously published method by Katz et al (23), to obtain cells from the stromal vascular fraction (SVF) the lipoaspirate was treated with collagenase I (Sigma) for 30 min at 37°C. Enzyme activity was neutralized with an equal volume of Dulbecco's modified eagles medium (DMEM Low Glucose) (Gibco) supplemented with 10% Fetal Bovine Serum (FBS, Hyclone) and the infranatant was collected after centrifugation for 10 min at 2000 rpm. The cellular pellet (consisting of the SVF) was washed three times in PBS in order to eliminate any enzyme residues. The EZ fresh samples consisted of the SVF pellet resuspended in 10 ml of PBS and kept at 4°C overnight. EZ overnight samples consisted of the SVF in 10 ml of αMEM supplemented with 1% Penicillin/Streptomycin and plated in a 100 mm culture dish, kept overnight at 37°C in humid atmosphere containing 5% of CO₂.

After the incubations the medium was collected and centrifuged at 300 g for 30 min at 4°C to remove cells and tissue and to obtain the EXOs containing fraction. The resulting samples were preserved at -20°C prior to processing²⁴.

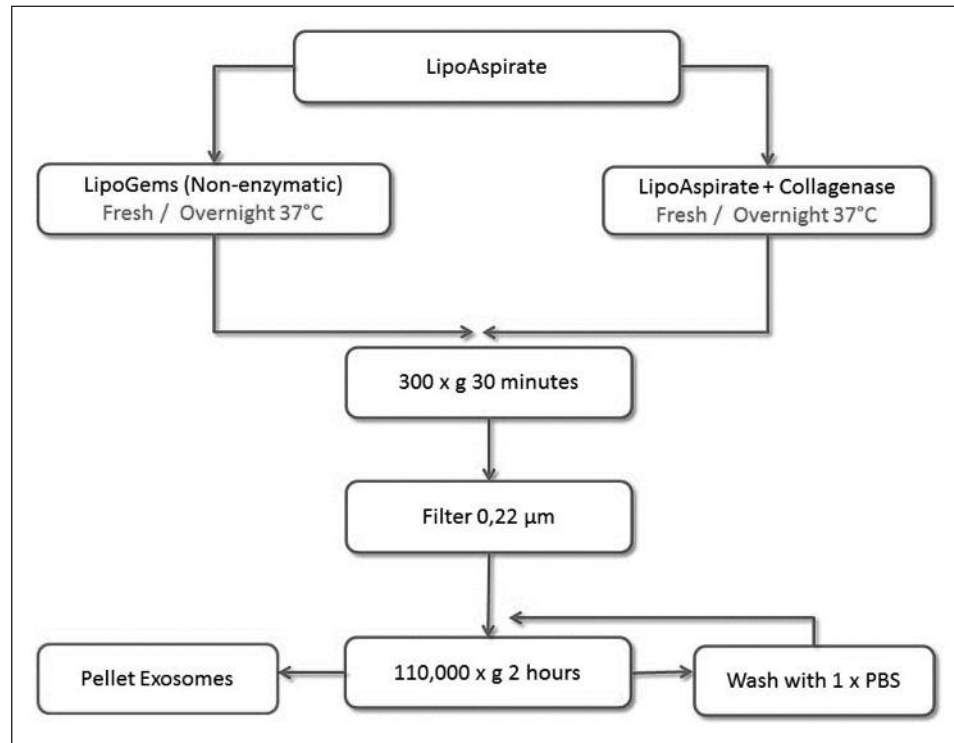
EXOSOME ISOLATION

EXOs from LG and EZ, were isolated by differential ultracentrifugation (Figure 1). First, samples were rapidly thawed in warm water, centrifuged at 300 g for 30 min at 4°C to remove cell remnants and debris then supernatants were filtered (0.22 µm pore). EXOs were pelleted by ultracentrifugation at 110,000 g for 2 hours at 4°C. Then EXOs were washed in PBS and centrifuged again at 110,000 g for 2 hours at 4°C, and finally resuspended in 200 µl PBS and stored at -20°C.

NANOPARTICLE TRACKING ANALYSIS (NTA)

Isolated EXOs were diluted in sterile PBS and analyzed by NanoSight NS300 and Nanosight NTA 2.3 Analytical Software (Malvern Instruments Company, Nanosight, and Malvern, United Kingdom). At least three measurement were performed for each sample.

Figure 1. Differential centrifugation procedure used for exosome isolation.



STATISTICAL ANALYSIS

Data are presented as mean \pm SD, with $n = 5$ samples per group. The effect of different treatments on EXOs concentration and size, were assessed by unpaired, two-tailed t -test. Statistical significance was defined as $p < 0.05$.

RESULTS

MORPHOLOGICAL OBSERVATION OF EXOSOME SAMPLES

EXOs morphological Nanosight NTA analysis was performed from human adipose tissue lipoaspirates processed by non-enzymatic ($n=5$) and enzymatic methods ($n=5$). Fresh samples and samples cultured overnight at 37°C samples from the same tissue products were analyzed. EXOs purification was confirmed in all samples by Nanosight NTA (Figure 2).

PARTICLE SIZE DISTRIBUTION OF EXOSOME SAMPLES

The size distribution of the EXOs was measured and results are shown in Figure 3 and Figure 4. The data show that the range of particles using Brownian motion have the expected EXOs size range of 30-200 nm (Figure 3). There were no significant differences between EXOs sizes in the different samples (Figure 4).

DETERMINATION OF EXOS CONCENTRATION

To determine the effect of non-enzymatic and enzymatic methods on the final EXOs content of adipose derived tissue/cell products, EXOs concentration was measured (Figure 5). The particle concentration demonstrated that LG final products had a significantly higher concentration of nanoparticles compared to exosome fractions from enzymatically treated final products (Figure 5). LG treated fresh samples demonstrated a significantly higher nanoparticle concentration compared to EZ fresh samples ($178.2 \times 10^8 \pm 74.0$ vs $36.2 \times 10^8 \pm 33.16$, $p < 0.0001$). Similar results were obtained in the samples cultured at 37°C overnight ($168.0 \times 10^8 \pm 99.0$ vs $26.90 \times 10^8 \pm 25.6$, $p < 0.0001$). EXOs isolated from either method had similar concentrations from fresh and overnight samples (Figure 5).

DISCUSSION

The data presented indicate that significant differences in the quantity of EXOs released or secreted were observed when lipoaspirate tissue samples were processed by different methods (LG or EZ). The LG (the non-enzymatic method) resulted in a significantly higher concentration of EXOs compared to the enzymatic method. These observed differences could arise for several reasons.

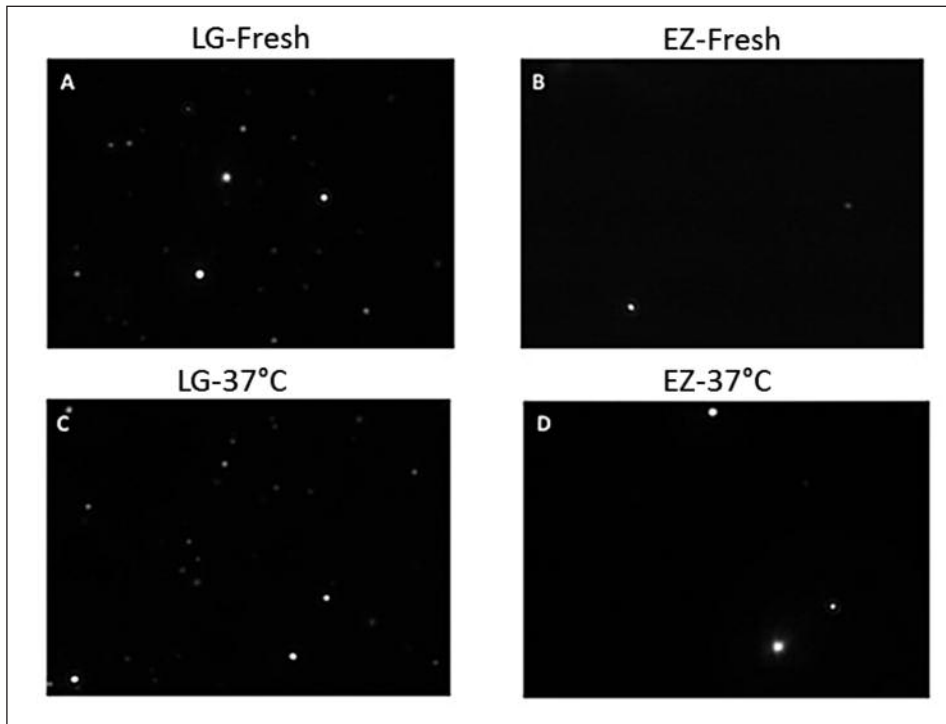


Figure 2. Images of EXOs identified by nanoparticle tracking analysis (NTA). Lipogems fresh sample (A), Lipoaspirate + Collagenase fresh sample (B), Lipogems cultured overnight at 37°C sample (C), Lipoaspirate + Collagenase cultured overnight at 37°C sample (D).

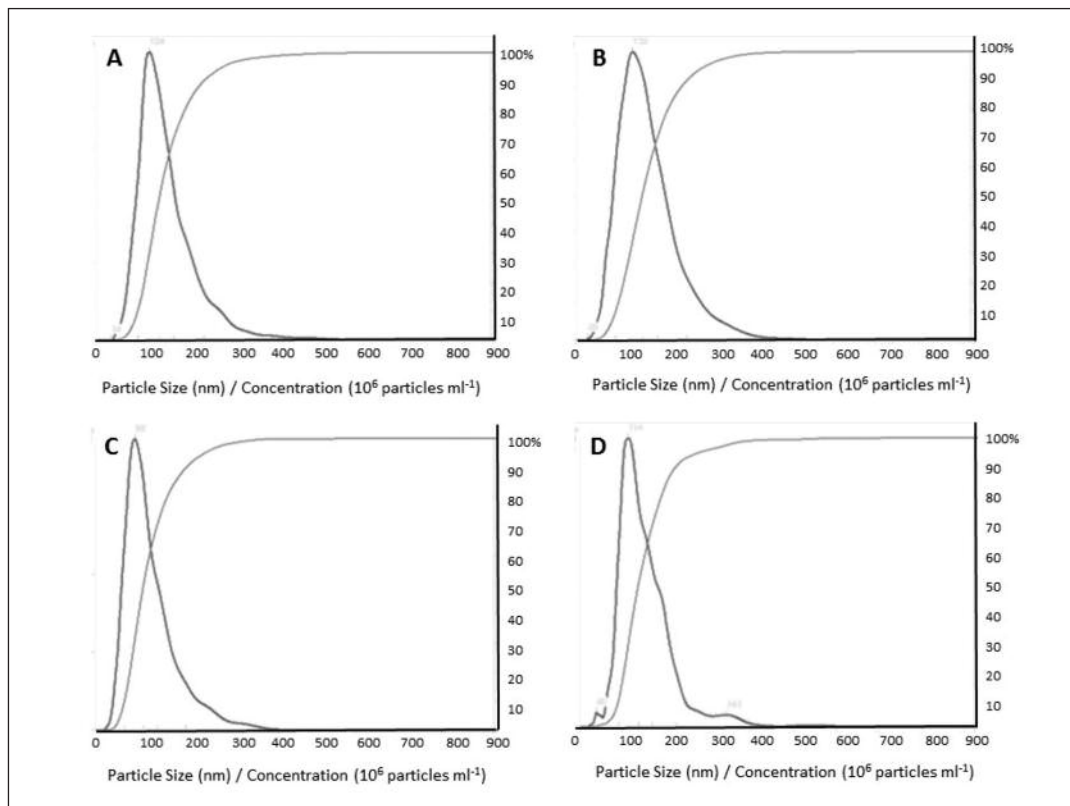


Figure 3. Representative cell-derived extracellular exosomes (EXOs) size analyzed by measurement with NanoSight. Three different preparation of each sample (n=5) were analyzed with similar results. EXOs maintain the same size distribution in all samples (A) LipoGems fresh (B) LipoGems overnight, (C) Lipoaspirate + collagenase fresh and (D) Lipoaspirate + collagenase overnight.

Figure 4. Size comparison of EXOs measured by Nanosight NTA. The size (nm) of EXOs is given as mean \pm standard deviation (n=5 samples per group).

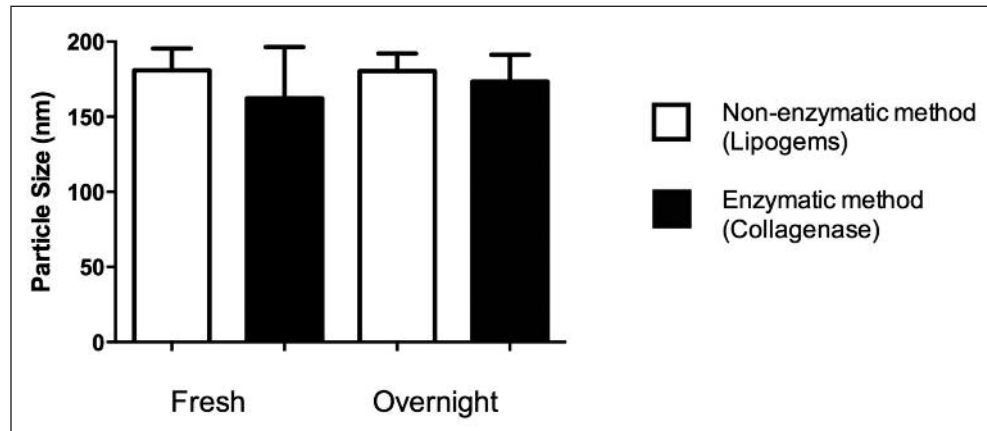
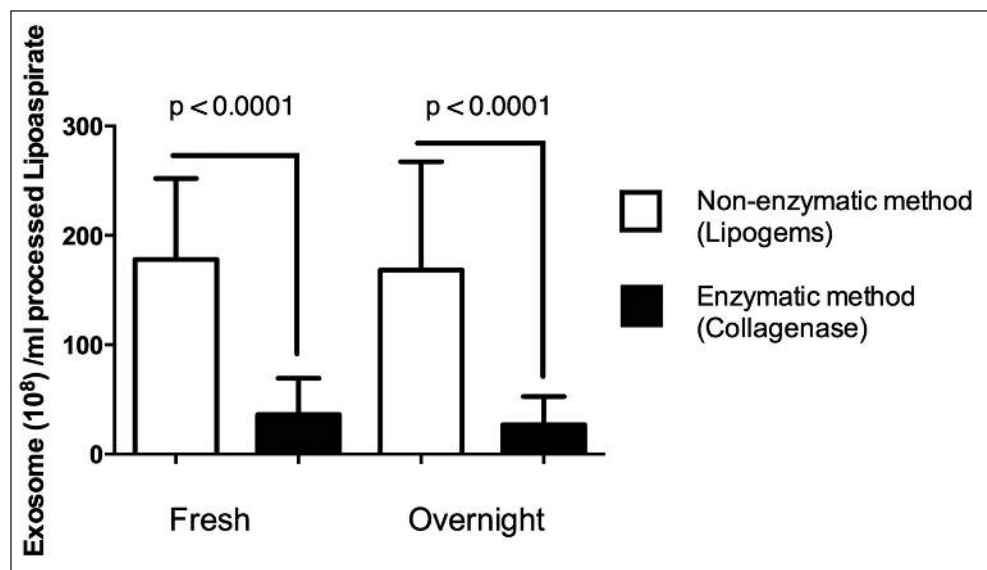


Figure 5. Exosome concentration data by nanoparticle tracking analysis (NTA). The exosome concentration (particles/ml) is given as mean \pm standard deviation (n=5).



First, the enzymatic treatment of cells will digest the extracellular matrix surrounding the cells possibly affecting cell secretory functions. Second, the digestion could also damage the cells, affecting cell function and viability. Third, the enzymatic method could be too aggressive and destroy exosomes during the processing. In contrast, the mechanical method is relatively gentle after the initial tissue cluster reduction and provides for removal of debris and single cells during the processing. The resulting tissue clusters maintain cells in a more “native” environment that may help to better support cell function including release and secretion of EXOs. These possibilities may account for the increased numbers of EXOs obtained by the LG method compared to the EZ method. Since EXOs have been reported to exhibit important paracrine factors affecting the therapeutic potential of tissue and cellular products, tissue processing methods that preserve the paracrine

potential of tissue/cell products could affect outcome in different cell therapy and regenerative medicine applications. Further studies will be necessary to better characterize the composition and function of EXOs present in the final tissue/cell products.

CONFLICT OF INTERESTS

All authors declare that there are no conflicts of interest regarding the publication of this paper.

REFERENCES

1. Dimov I, Jankovic Velickovic L, Stefanovic V. Urinary exosomes. *ScientificWorldJournal* 2009; 9: 1107-1118.
2. Huebner AR, Somparn P, Benjachat T, Leelahavanichkul A, Avihingsanon Y, Fenton RA, et al. Exosomes in urine biomarker discovery. *Adv Exp Med Biol* 2015; 845: 43-58.
3. Lasser C, Alikhani VS, Ekstrom K, Eldh M, Paredes PT, Bossios A, Sjöstrand M, Gabrielsson S, Lötvall J, Valadi H. Human saliva, plasma and breast milk exosomes contain RNA: uptake by macrophages. *J Transl Med* 2011; 9: 9.

4. Garcia-Contreras M, Ricordi C, Robbins PD, Oltra E. Exosomes in the pathogenesis, diagnosis and treatment of pancreatic diseases. *CellR4* 2014; 2: e807.
5. Lotvall J, Valadi H. Cell to cell signalling via exosomes through esRNA. *Cell Adh Migr* 2007; 1: 156-158.
6. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nature Cell Biol* 2007; 9: 654-659.
7. Lazaro-Ibanez E, Sanz-Garcia A, Visakorpi T, Escobedo-Lucea C, Siljander P, Ayuso-Sacido A, Yliperttula M. Different gDNA content in the subpopulations of prostate cancer extracellular vesicles: apoptotic bodies, microvesicles, and exosomes. *Prostate* 2014; 74: 1379-1390.
8. Thakur BK, Zhang H, Becker A, Matei I, Huang Y, Costa-Silva B, et al. Double-stranded DNA in exosomes: a novel biomarker in cancer detection. *Cell Res* 2014; 24: 766-769.
9. Konno M, Hamabe A, Hasegawa S, Ogawa H, Fukusumi T, Nishikawa S, et al. Adipose-derived mesenchymal stem cells and regenerative medicine. *Dev Growth Differ* 2013; 55: 309-318.
10. Liu Y, Yan X, Sun Z, Chen B, Han Q, Li J, Zhao RC. Flk-1+ adipose-derived mesenchymal stem cells differentiate into skeletal muscle satellite cells and ameliorate muscular dystrophy in mdx mice. *Stem Cells Dev* 2007; 16: 695-706.
11. Raffaini M, Tremolada C. Micro Fractured and Purified Adipose Tissue Graft (Lipogems®) Can Improve the Orthognathic Surgery Outcomes Both Aesthetically and in Postoperative Healing. *CellR4* 2014; 2: e1118.
12. Garcia-Contreras M, Vera-Donoso CD, Hernandez-Andreu JM, Garcia-Verdugo JM, Oltra E. Therapeutic potential of human adipose-derived stem cells (ADSCs) from cancer patients: a pilot study. *PLoS One* 2014; 9: e113288.
13. Lavoie JR, Rosu-Myles M. Uncovering the secretes of mesenchymal stem cells. *Biochimie* 2013; 95: 2212-2221.
14. Yu B, Zhang X, Li X. Exosomes derived from mesenchymal stem cells. *Int J Mol Sci* 2014; 15: 4142-4157.
15. Zhang B, Yin Y, Lai RC, Tan SS, Choo AB, Lim SK. Mesenchymal stem cells secrete immunologically active exosomes. *Stem Cells Dev* 2014; 23: 1233-1244.
16. Liang X, Ding Y, Zhang Y, Tse HF, Lian Q. Paracrine mechanisms of mesenchymal stem cell-based therapy: current status and perspectives. *Cell Transplant* 2014; 23: 1045-1059.
17. Katsuda T, Oki K, Ochiya T. Potential Application of Extracellular Vesicles of Human Adipose Tissue-Derived Mesenchymal Stem Cells in Alzheimer's Disease Therapeutics. *Methods Mol Biol* 2014 Aug 2. [Epub ahead of print].
18. Katsuda T, Tsuchiya R, Kosaka N, Yoshioka Y, Takagaki K, Oki K, Takeshita F, Sakai Y, Kuroda M, Ochiya T. Human adipose tissue-derived mesenchymal stem cells secrete functional neprilysin-bound exosomes. *Sci Rep* 2013; 3: 1197.
19. Pascucci L, Alessandri G, Dall'Aglio C, Mercati F, Coliolo P, Bazzucchi C, Dante S, Petrini S, Curina G, Ceccarelli P. Membrane vesicles mediate pro-angiogenic activity of equine adipose-derived mesenchymal stromal cells. *Veterinary J* 2014; 202:361-366.
20. Bianchi F, Maioli M, Leonardi E, Olivi E, Pasquinelli G, Valente S, Mendez AJ, Ricordi C, Raffaini M, Tremolada C, Ventura C. A new nonenzymatic method and device to obtain a fat tissue derivative highly enriched in pericyte-like elements by mild mechanical forces from human lipoaspirates. *Cell Transplant* 2013; 22(11): 2063-2077.
21. Carelli S, Messaggio F, Canazza A, Hebda DM, Caremoli F, Latorre E, Grimoldi MG, Colli M, Bulfamante G, Tremolada C, Giulio AM, Gorio A. Characteristics and Properties of Mesenchymal Stem Cells Derived from Micro-fragmented Adipose Tissue. *Cell Transplant* 2014 May 6. [Epub ahead of print].
22. Bianchi F, Olivi E, Baldassarre M, Giannone FA, Laggetta M, Valente S, Cavallini C, Tassinari R, Canaider S, Pasquinelli G, Tremolada C, Ventura C. Lipogems, a New Modality of Fat Tissue Handling to Enhance Tissue Repair in Chronic Hind Limb Ischemia. *CellR4* 2014; 2: e1289.
23. Katz AJ, Tholpady A, Tholpady SS, Shang H, Ogle RC. Cell surface and transcriptional characterization of human adipose-derived adherent stromal (hADAS) cells. *Stem Cells* 2005; 23: 412-423.
24. Sokolova V, Ludwig AK, Hornung S, Rotan O, Horn PA, Epple M, Giebel B. Characterisation of exosomes derived from human cells by nanoparticle tracking analysis and scanning electron microscopy. *Colloids Surf B Biointerfaces* 2011; 87: 146-150.