Research Article

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Regenerative Medicine

Extracellular vesicles derived from mesenchymal cells: perspective treatment for cutaneous wound healing in pediatrics

Gloria Pelizzo*[,1], Maria Antonietta Avanzini[2], Antonia Icaro Cornaglia[3], Annalisa De Silvestri[4], Melissa Mantelli[2], Paola Travaglino[2], Stefania Croce[2], Piero Romano[5], Luigi Avolio[5], Giulio Iacob[5], Massimo Dominici[6] & Valeria Calcaterra[7]

[1]Pediatric Surgery Unit, Children's Hospital, Istituto Mediterraneo di Eccellenza Pediatrica, Palermo, Italy
[2]Immunology & Transplantation Laboratory, Cell Factory, Pediatric Hematology Oncology Unit, Department of Maternal & Children's Health, Fondazione IRCCS Policlinico S. Matteo, Pavia, Italy
[3]Department of Public Health, Experimental Medicine & Forensic, Histology & Embryology Unit, University of Pavia, Italy
[4]Biometry & Clinical Epidemiology, Scientific Direction, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy
[5]Pediatric Surgery Unit, Department of Maternal & Children's Health, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy
[6]Laboratory of Cellular Therapies Department of Medical & Surgical Sciences for Children & Adults University Hospital of Modena & Reggio Emilia, Italy
[7]Pediatric Unit, Department of Internal Medicine, University of Pavia & Department of Maternal & Children's Health, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy
*Author for correspondence: Tel.: +39 091 666 6007; gloriapelizzo@gmail.com

Aim: We evaluated the effects of the intradermal injection of extracellular vesicles (EVs) derived from adipose stem cells (ASC-EVs) and bone marrow cells (BM-EVs) in an experimental cutaneous wound repair model. **Methods:** Mesenchymal stem cells (MSCs) were *in vitro* expanded from adipose (ASC) or BM tissues (BM-MSC) of rabbits. EVs were separated from the supernatants of confluent ASC and BM-MSCs. Two skin wounds were induced in each animal and treated with MSC or EV injections. Histological examination was performed postinoculation. **Results:** EV-treated wounds exhibited a better restoration compared with the counterpart MSC treatment. ASC-EV-treated wounds were significantly better than BM-EVs ($p = 0.036$). **Conclusion:** EV topical inoculation provides restored architecture during cutaneous wound healing and represents a promising solution for regenerative medicine in children.

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Keywords: animal model • children • cutaneous wound repair • extracellular vesicles • mesenchymal stem cells

Wound healing is a complex dynamic response to a physical trauma that comprises three overlapping phases: inflammatory, proliferative and remodeling. Phase progression depends on a well-coordinated interplay of cell-signaling events at the wound site and surrounding tissues, in which endogenous stem cells are vital players [1].

Children have specific characteristics of wound healing. Tissue insult or injury in the fetus can heal without scarring. After 2 years of age, wound healing is usually very rapid with an important remodeling phase and can be associated with more hypertrophic scars, both in duration and in intensity, compared with younger infants [2]. Skin thickness and the limited extension of tissue is a serious negative factor in case of trauma, extensive damage or loss of tissue in the pediatric age, even though efficient growth tissue mechanisms exist [2]. Cutaneous wound healing remains a challenge in children with disfiguring giant melanocytic nevi, where complete surgical removal is difficult to achieve because of the lack of available skin to graft over the resultant defects [3,4], or following serious burns or due to tissue surgical destruction or trauma in which skin damage usually never fully recovers [5,6]. Stem cells offer promising possibilities for improved wound repair and tissue regeneration.

Preclinical studies have shown that bone or adipose tissue-derived mesenchymal stem cells (MSCs) have a competitive advantage over other types of stem cells due to their better defined multipotent differentiating potential, paracrine effects, immunomodulatory properties and better safety profile [1,7–9]. However, large controlled clinical

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trials are needed to examine MSC capabilities in humans and further assess their safety profile to support their potential use in safe regenerative medical approaches [7–9], particularly in pediatric patients [10].

The therapeutic effect of MSCs is increasingly attributed to their capacity to secrete soluble factors that influence tissue regeneration by inhibiting cell apoptosis, stimulating cell proliferation and promoting tissue vascularization [7]. Recent studies have indicated that beside soluble factors, MSCs also secrete extracellular vesicles (EVs) which are small, spherical membrane-bound fragments involved in cell-to-cell communication and are capable of altering the cell fate and phenotype of recipient cells [8]. EVs derived from MSCs (EV-MSCs) have been shown to mimic the proregenerative effects of the origin cells in different experimental models [9].

We previously demonstrated that topical inoculation of adipose stem cells (ASCs) restored skin architecture during cutaneous wound healing, more rapidly than bone marrow-MSCs (BM-MSCs) [10].

In the present study, we evaluated the effects of an intradermal injection of EVs derived from ASCs (ASC-EVs) or from bone marrow-mesenchymal stem cells (BM-MSCs-EVs) in an experimental cutaneous wound repair model. Results of autologous and allogeneic ASCs and BM-MSCs were also compared.

Methods

Adipose tissue & bone marrow harvest

Healthy young female New Zealand rabbits (n = 18, 3 months old, median weight 3.5 kg) [11], were used as the animal model. The experimental protocol was approved by the National Animal Care and Ethics Committee (reference number: DGSAF0009484-A-13/04/2015) and conducted in accordance with Italian and European legislation (D.lgs. 116/92, European Directives 86/609/EE for the protection of animals used in scientific and experimental studies and 2010–63 UE).

After overnight fasting, eight experimental animals were premedicated with an intramuscular midazolam injection (1 mg/kg). Under general anesthesia with Zoletil 0.4 ml/kg (Virbac, Milano, Italy), and after local anesthesia with levo-bupivacaine 0.25% or ropivacaine 0.2% (2 ml/cm wound), a 2 cm longitudinal incision was made in the inguinal area in order to harvest the adipose panicle (lipectomy), while BM was harvested by aspiration from femoral medullary cavities [10].

Local anesthesia with levo-bupivacaine 0.25% or ropivacaine 0.2% (2 ml/cm wound) were administered for pain management. Subcutaneous Enrofloxacin (0.1 ml/2 kg/day for 3 days; Bayer, Milano, Italy) and Meloxicam (0.3 mg/kg/day for 3 days; Boehringer Ingelheim, Milano, Italy) were subsequently administered [10].

The animals were housed until cells and EVs were ready for autologous injection, another group of eight rabbits was then used for the allogeneic setting with the same MSCs and EVs previously expanded.

Isolation, culture & characterization of ASCs & BM-MSCs

ASCs and BM-MSCs and respective EVs from eight rabbits were isolated, cultured and characterized as previously described [10,12]. Briefly, to obtain ASCs, inguinal fat pads were placed in sterile phosphate-buffered saline (PBS, Euroclone, Milan, Italy) with gentamicin, minced manually and digested with 0.0075% type II collagenase (3 mg/ml, Sigma-Aldrich, MO, USA) in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Invitrogen, Monza, Italy) for 20 min at 37°C with gentle agitation. The stromal vascular fraction, containing ASCs, was suspended in DMEM +10% fetal bovine serum (FBS, Euroclone), in order to inhibit enzyme activity. The specimen was then filtered through a 100 mm sterile nylon mesh filter (Millipore, Darmstadt, Germany), and centrifuged at 1200 rpm for 10 min. The resultant pellet was suspended and counted with 0.4% Trypan blue (Sigma-Aldrich). Cells were subsequently plated in culture flasks (Corning Costar, Amsterdam, The Netherlands) at a density of 160,000/cm^2 in αMEM (Gibco, Invitrogen) containing 10% FBS (Euroclone) and 1% antibiotic–antimycotic (Sigma-Aldrich) at 37°C, 5% CO_2 in a humidified atmosphere.

To obtain BM-MSC, mononuclear cells were isolated from 1 ml of BM aspirate, by density gradient centrifugation (Ficoll 1.077 g/ml; Lympholyte, Cedarlane Laboratories Ltd, The Netherlands), counted and plated at the same density of 160,000/cm^2 in the same culture medium. Cultures were then maintained at 37°C, 5% CO_2 in a humidified atmosphere. After 48 h, nonadherent cells were removed and culture medium was replaced twice a week. After reaching ≥80% confluence, MSCs were harvested using Trypsin–EDTA (Lonza, Copenhagen, Denmark), and propagated at 4000 cells/cm^2. ASC were expanded until passage (P)4, while BM-MSC were expanded until P3 because of their lower rate of proliferation. At each passage, viable cells were counted using 0.1% eosin and culture supernatants were tested for sterility.

Table 1. Scheme of the injections.

Animal	First lesion	Second lesion
Rabbit 1	Allogeneic ASC	Control
Rabbit 2	Allogeneic BM-MSC	Control
Rabbit 3	Allogeneic ASC-EV	Control
Rabbit 4	Allogeneic BM-MSC-EV	Control
Rabbits 5 8	Autologous ASC-EV	Autologous ASC
Rabbits 9 12	Autologous BM-MSC-EV	Autologous BM-MSC
Rabbits 13 15	Allogeneic ASC-EV	Allogeneic ASC
Rabbits 16 18	Allogeneic BM-MSC-EV	Allogeneic BM-MSC

ASC: Adipose stem cell; ASC-EV: Adipose stem cell-extracellular vesicle; BM-MSC-EV: Bone marrow-mesenchymal stem cell-extracellular vesicle.

Isolation & characterization of ASC-EVs & BM-MSC-EVs

EVs were obtained from the supernatants of confluent ASC and BM-MSCs following a standard procedure, previously reported by Camussi *et al.* and validated in human MSC using specific exosome markers such as CD63, CD9 and CD81 [12]. Cells were cultured overnight in D-MEM deprived of FBS. To obtain EVs, the supernatants cells were centrifugated at 2000 $\times g$ for 20 min to remove debris, cell-free supernatants were then centrifuged at 100,000 g (Beckman Coulter Optima L-90K ultracentrifuge) for 1 h at 4°C, washed in serum-free medium and submitted to a second ultracentrifugation under the same conditions. EVs were characterized by flow cytometry by measuring their dimensions and expression of positive and negative MSC surface markers. Monoclonal antibodies specific for rabbit CD49e (as positive marker) and CD45 (as negative marker) were used [8,12].

Calibration beads (range 0.1–1 μm) were employed to gate MVs by dimension parameters (Mega Mix, Stago, Milan). Analysis of CD marker expression was performed by direct immunofluorescence with a Navios flow cytometer (Bekman Coulter) and data were calculated using the Kaluza software.

Rabbit cutaneous wound model

When an adequate number of MSCs and EVs were obtained, the skin wounds were surgically induced. After overnight fasting, experimental animals were premedicated with intramuscular midazolam (1 mg/kg). Under general anesthesia with Zoletil 0.4 ml/kg and after local anesthesia with levo-bupivacaine 0.25% or ropivacaine 0.2% (2 ml/cm wound), two identical full thickness 2 × 2 cm wounds were created on the back of each rabbit, at a distance of more than 2 cm from each other. After creation of the wounds, local anesthesia with levo-bupivacaine 0.25% or ropivacaine 0.2% (2 ml/cm wound) was administered. Subcutaneous enrofloxacin (0.1 ml/2 kg/day for 3 days) and meloxicam (0.3 mg/kg/day for 3 days) were subsequently administered [10].

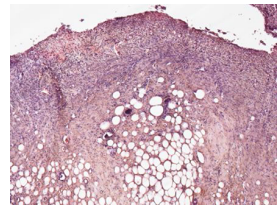
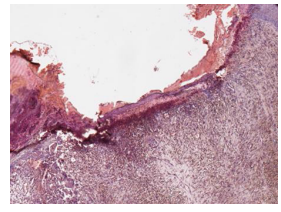
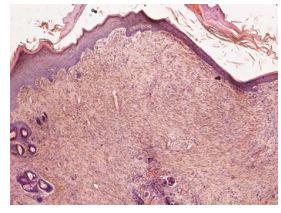
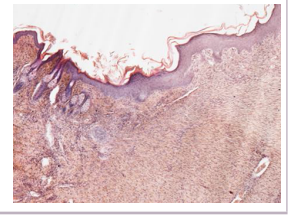
Intradermal injection

Rabbits were randomized as reported in Table 1. Within 5 min of wound lesion establishment, autologous or allogeneic ASC, BM-MSC, ASC-EVs or BM-MSC-EVs in 3 ml of saline + 2% rabbit albumin (Sigma-Aldrich) were injected (1.5 ml subcutaneously injected around the wounds at four injection sites and 1.5 ml directly applied to the wound beds of the first lesion). The protocol called for an injection of 10×10^6 cells into the wound bed [10]; while, an EVs volume obtained from the 10×10^6 of confluent ASC or BM-MSC was injected. As a control, 3 ml of saline, 2% rabbit albumin solution were injected. Rabbits did not receive any immune suppression. The general conditions of the animals and wound healing were monitored daily. The wounds were photographed using a digital camera.

Histological analyses

Rabbits were euthanized with a bolus of pentobarbital 100 mg/kg iv. For histological examination, wounds were harvested after 14 days of treatment (rabbits). The regenerated tissue biopsies were collected using dermal biopsy punches. The samples were bisected along the widest line of the wound, fixed in 4% neutral-buffered formalin for 48 h, dehydrated with a gradient alcohol series, cleared in xylene and eventually embedded in paraffin. Sections (8 μm) were obtained using a Leitz microtome and prepared for histology. All stained slides were examined under a Axiophot Zeiss light microscope (Oberkochen, Germany) equipped with a digital camera.

Table 2. Histological scoring system.

Score	Epidermal phenotypes	Dermal phenotypes	Histological phenotypes
1	Incomplete re-epithelialization; the surface is covered by fibrinous exudate infiltrated by polymorphs	High degree of inflammation in nonepithelized area; alterated collagen matrix organization, a layer of granulation tissue with dilated capillaries and edema, adipose tissue substitution	
2	Complete or nearly complete re-epithelialization; the epidermis has variable thickness and is linear	No dermal papillae. Mild infammatory cell infiltration, dilated vessels, granulated tissue	
3	Complete re-epithelialization; epidermis shows complete keratinization	Presence of dermal papillae. Remodeling of the granulation layer and collagen fibers	
4	Complete re-epithelialization; epidermis has normal thickness and is normally keratinized	Presence of dermal papillae and cutaneous annexes (roots of the hair, sebaceous glands). Well-formed connective matrix with thick collagen bundles in the reticular dermis and a network of thin fibers in the papillary dermis	

Histological scores were assigned as described by Galeano *et al.*, [13] considering the degree of epithelialization (absence or presence of epithelial covering, crusting and intraepithelial inflammatory cells), granulation tissue and collagen matrix organization (adipose tissue substitution as an index of impaired wound closure, dense eosinophilic collagen matrix, edema, hemorrhage and degree of inflammation), inflammatory infiltrates (neutrophils and lymphocytes) and angiogenesis. The histological scoring system ranged between 1 and 4 as described in Table 2.

The pathologist was blinded to the scheme of injections.

Statistical analysis

Quantitative data and the histological score were described as the median and interquartile range (IQR: 25–75th percentile) and compared by fitting multivariable ordinal logistic regression models. Groups (using controls as a reference) were included in the models as independent variables. Wald tests of simple and composite linear hypotheses regarding the parameters of the fitted model were performed to evaluate EV versus MSC and cells or EVs obtained from adipose tissue versus cells or EVs expanded from BM. Statistical significance was defined as a $p < 0.05$. Data analyses were performed with the STATA statistical package (release 14.2, 2012, Stata Corporation, TX, USA).

Results

ASC-EVs & BM-MSC-EVs

Following standard procedures, we were able to expand cells both from adipose tissue and BM in all eight animals. All of the MSC exhibited typical characteristics of plastic adherence, spindle-shape morphology and capacity to differentiate, as previously described [10]. We were also able to obtain and characterize EVs, in particular to precisely

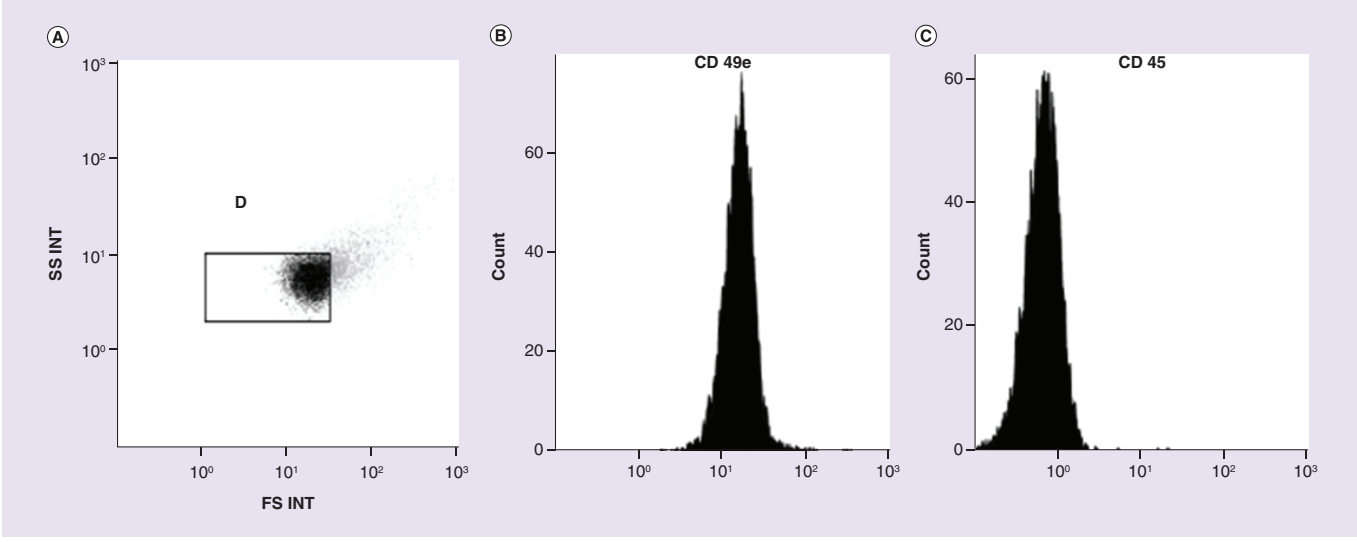


Figure 1. Morphological characterization of a representative extracellular vesicles (EVs) preparation (A), region D is defined using 0.1 μm calibration beads. EV characterization using a positive surface marker as anti-CD49e **(B)** and one negative as anti-CD45 **(C)**.

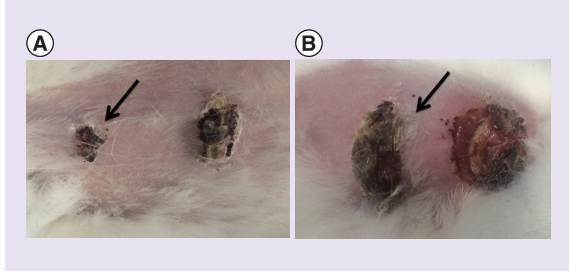


Figure 2. Skin regeneration at Day 14 after autologous ASC-EVs injection (arrow) vs ASCs (A), autologous BM-MSC-EVs (arrow) vs BM-MSCs (B).
ASC-EV: Adipose stem cell-derived extracellular vesicle; BM-EV: Bone marrow-derived extra-vesicle; BM-MSC: Bone marrow-derived mesenchymal stem cell.

gate them by morphological parameters, using 0.1–1 μm calibration beads. Cytofluorimetric analysis showed the presence of EVs positive for CD49e, a surface marker expressed on the rabbit MSC surface and negative for CD45 (Figure 1).

Macroscopic aspect of wound healing

No severe infections were reported for any of the skin wounds. Skin regeneration at Day 14, after EV injection was more rapid compared with MSCs- and/or saline-treated lesions. Lesions treated with cells or EVs derived from BM seem to exhibited slower wound restoration in comparison with wounds inoculated with sources from adipose tissue (Figure 2). ASC-EV treated wounds exhibited more complete wound healing when compared with the other experimental conditions.

Histological results

At 14 days, saline-treated wounds had extensive skin defects, necrosis and inflammatory cells in the cutaneous layer, as well as absence of hair follicles and sebaceous glands in the dermis.

Conversely, all of the treatment groups had accelerated reduction of inflammatory infiltration and partial restoration of the epidermal and dermal structure. The details of the hematoxylin and eosin-stained sections of wounds observed under the light microscopy are reported in Figure 3.

Histological scores for the different experimental conditions are provided in Figure 4. No differences were observed between the autologous or allogeneic setting (Figure 4). For this reason, data were statistically evaluated without this distinction.

EV-treated wounds exhibited better restoration compared with the MSC treatment ($p = 0.005$). In particular, BM-EVs achieved better regeneration than BM-MSC ($p = 0.048$) and ASC-EVs than ASC ($p = 0.017$) with

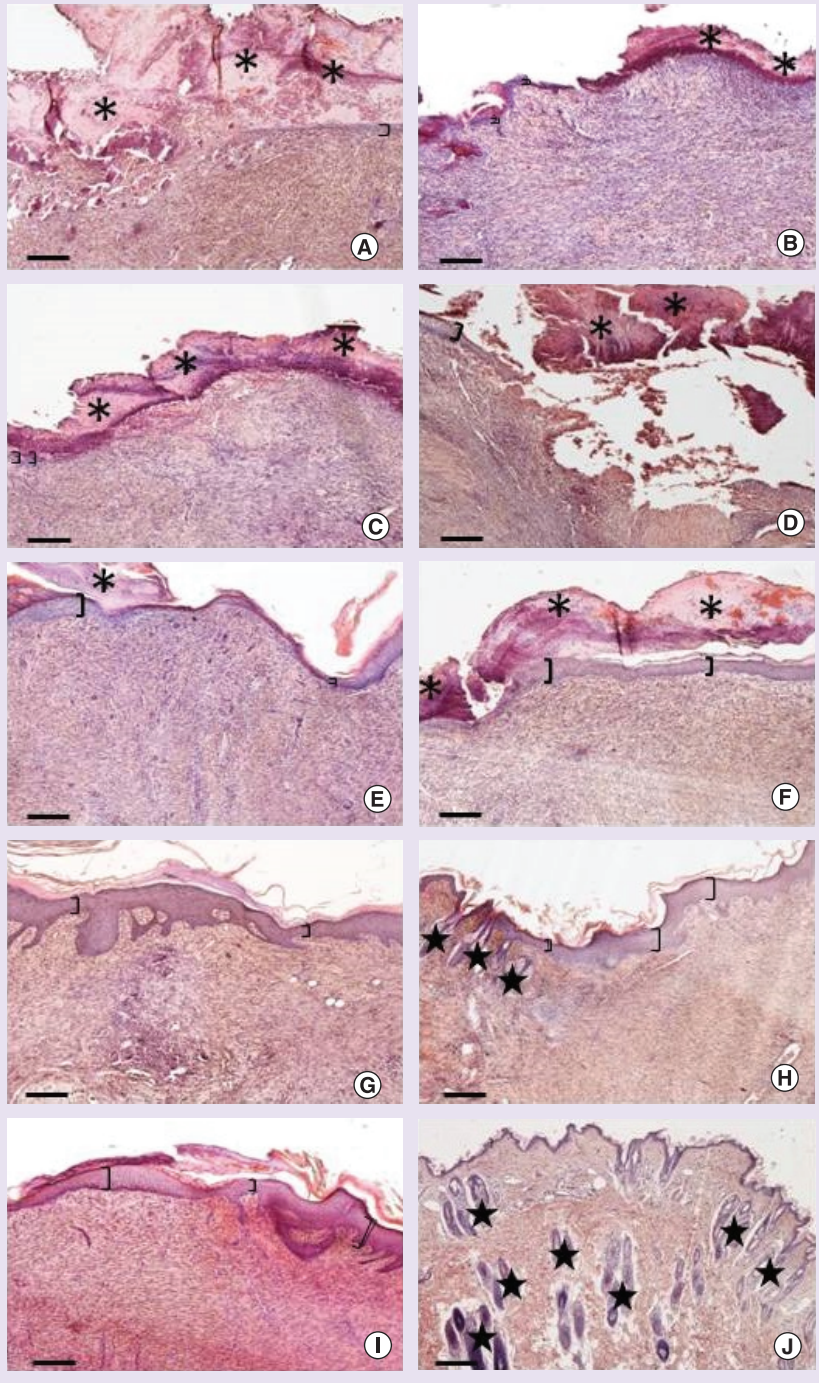


Figure 3. Haematoxylin and eosin stained sections of wounds at Day 14 observed under light microscopy. Original magnification 5× (Bar = 200 mm; Epidermis = Bracket; Necrotic material = Asterisk; Roots of the hair and sebaceous glands = Star; Empty dots = Allogeneic setting; Full dots = Autologous setting). Skin of saline-treated animals **(A)** shows extensive necrosis and inflammatory cells in the cutaneous layer and absence of hair follicles and skin glands. Autologous BM-MSCs **(B)** as well as allogenic BM-MSCs **(C)** treated wounds have an incomplete epithelial layer with mild inflammatory cell infiltration. The skin of autologous ASC-infused rabbits **(D)** exhibit nearly complete re-epithelialization, while in allogenic ASCs **(E)** and autologous BM-MSC-EVs **(F)** a complete epithelial layer as well as a partially organized derma is visible. Animals treated with allogenic BM-MSC-EVs **(G)** exhibit almost completely restored skin, with a keratin layer and reorganized dermal structures. Autologous **(H)** and allogenic ASC-EV **(I)** treated wounds exhibit well regenerated tissue with the presence of a complete epithelial layer, similar to that present in healthy skin **(J)**. Dermal papillae and cutaneous annexes (roots of the hair, sebaceous glands) are present. The connective matrix is also well restored, with thick collagen bundles in the reticular dermis and a network of thin fibers in the papillary dermis.
ASC: Adipose stem cell; ASC-EVs: Adipose stem cell-derived extracellular vesicles; BM-EV: Bone marrow-derived extra-vesicle; BM-MSC: Bone marrow-derived mesenchymal stem cell.

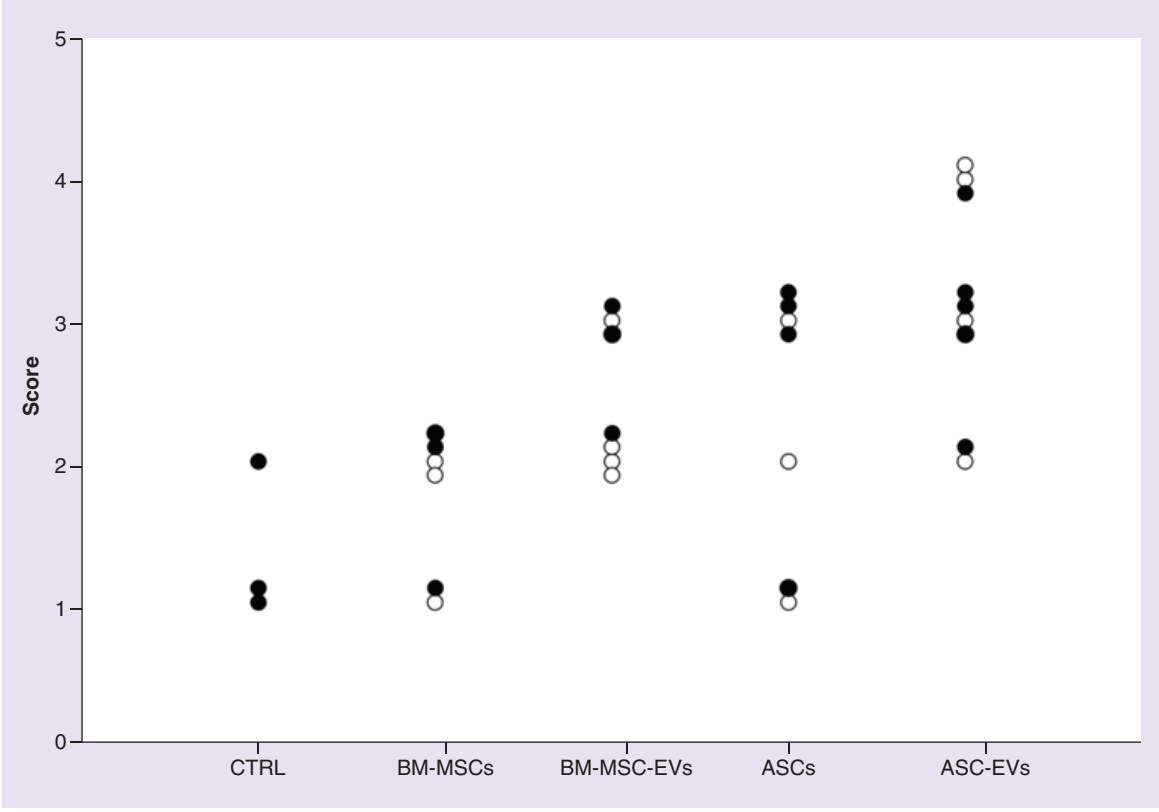


Figure 4. Histological scores of wounds according to Galeano's criteria [13], for the different experimental groups.
Empty dots = Allogeneic setting; Full dots = Autologous setting.
ASCs: Adipose stem cells; ASC-EVs: Adipose stem cell-derived extracellular vesicles; BM-MSCs: Bone marrow-derived mesenchymal stem cells; BM-MSC-EVs: Bone marrow-mesenchymal stem cell-derived extra-vesicles; CTRL: Saline treated control.

complete re-epithelialization and restored architecture between the dermis and epidermis with the presence of dermal papillae. Moreover, as previously reported, wound restoration was better when cells or EVs obtained from adipose tissue were used with respect to cells or EVs expanded from BM ($p = 0.036$).

Discussion

In the present study, we investigated whether the intradermal injection of ASC-EVs or BM-MSC-EVs exerts therapeutic effects in a cutaneous injury model. Our findings demonstrated for the first time that MSC-EV treated wounds promote tissue regeneration with the presence of a complete epithelial layer. Further, the results indicated that EVs obtained from adipose tissue promote better regeneration than EVs expanded from BM.

The integrity of healthy skin plays a crucial role in the maintenance of physiological homeostasis. Moreover, it provides protection against mechanical forces and infections, fluid imbalance and thermal dysregulation. At the same time, it allows for flexibility enabling joint function in some areas of the body and more rigid fixation to hinder shifting of the palm or foot sole.

Inadequate wound healing necessitates medical intervention; tissue destruction following surgery or acute trauma are followed by a loss of skin organ function rendering the organism vulnerable to infections, thermal dysregulation and fluid loss [14–18,20]. Currently, treatment outcomes are not always ideal because of the failure to achieve complete wound closure in around 60% of cases, as well as high rates of recurrence and scarring [19]. Therefore, there is a need for more effective therapies. Stem cells offer promising possibilities and they have been considered as an excellent material for regenerative medicine. Preclinical studies have shown that bone- or adipose tissue-derived MSCs have a competitive advantage over other types of stem cells due to their better defined multipotent differentiating potential, paracrine effects, immunomodulatory properties and safety profile [7–9].

Wound healing requires coordinated interplay among cells, growth factors and extracellular matrix proteins. Central to this process are endogenous MSC, which coordinate the repair response by recruiting other host cells and secreting growth factors and matrix proteins [9,14–18]. The beneficial effect of exogenous MSCs on wound healing has been observed in a variety of animal models and in reported clinical cases [7–10].

The therapeutic effects of MSCs might also be attributed to the secretion of a wide array of paracrine factors in order to stimulate the survival and functional recovery of resident cells, or to regulate the local microenviroment or niche and immune response [19,21]. In fact, stem cells are able to communicate with nearby and distant cells through soluble factors and direct cell–cell contact by long and thin tubular appendages such as cytonemes and cilia, as well as via detached, EVs.

Recently, on the basis of paracrine or endocrine MSC mechanisms, a novel strategy of 'cell-free therapy' which uses cell-derived EVs for tissue repair has been proposed [27–32]. EVs are membranous structures released by various cells; they can transfer bioactive molecular contents including proteins, mRNAs and miRNAs sequences to target injured tissue cells [12]. EVs are released from many cell types and recent studies have shown that EVs are one of the key secretory products of MSCs mediating cell-to-cell communication to enhance wound healing [28]. Hu *et al.* [33] reported that exosomes secreted by ASCs have a positive role in the promotion of skin tissue wound repair and can facilitate cutaneous wound healing by optimizing the characteristics of fibroblasts.

In the present study, we obtained EVs from adipose tissue and BM and confirmed the potential use of EVs in skin regeneration. The high abundance of MSCs found in adipose tissue makes it a very attractive source of adult stem cells. Additionally, adipose tissue-derived MSCs are expected to be a more reliable cell source for regenerative medicine because they can be isolated using minimally invasive techniques compared with other MSCs. We showed that MSC-EVs are superior in their ability to support wound healing and that the adipose tissue source induced better wound restoration compared with BM [10,33]. These data suggest that different therapeutic potentials of MSCs from various sources [20] may also correlate with different paracrine effects. Even though the autologous setting is generally recommended, the absence of differences in therapeutic effects between autologous or allogeneic sources may be considered a relevant factor in the translation of results to the pediatric population, particularly in neonates or young infants, in which technical difficulties in obtaining adipose tissue can occur.

Children with abnormal, extended or pathological wound healing should benefit from complementary treatments to minimize tissue scarring and to prevent specific complications related to their growth body rate, such as contractures, alopecia and scar intussusceptions [2,22]. Several reports support the promise of MSCs in dermal wound healing, nevertheless clinical translation to the pediatric age remains limited [21]. The translation of MSCs to the clinic should be cautiously considered, because of immune-mediated rejection, senescence-induced genetic instability or loss of function, limited cell survival and possibility of malignant MSC transformation [34]. Our preclinical study, using young animals, showed that EVs could be a promising alternative to treat wounds in children. After ASC-EV inoculation, well-regenerated tissue with the presence of a complete epithelial layer, dermal papillae and cutaneous annexes and restored connective matrix was obtained. The complete reparative properties observed supports the therapeutic role of EVs in both superficial and in full-thickness cutaneous lesions, such as third-degree burns, in which all layers of skin are involved and hair follicles and sebaceous glands are destroyed. Thus, MSC-EVs represent a novel 'cell-free therapy' for pediatric skin regeneration, which might overcome the obstacles and risks associated with the use of native or engineered stem cells. Moreover, further studies are mandatory to optimize the exact dose of MSC-EVs necessary to obtain the best results.

The evolutionary character of wound healing in children implies a need for further dedicated experimental and preclinical studies focusing on cell free extracellular vesicle approaches in order to validate their skin regeneration efficacy in the pediatric area.

Conclusion

Rabbit EVs can be obtained from *in vitro* ASCs and BM-MSCs. Topical inoculation of EVs restored skin architecture during cutaneous wound healing, with better results after ASC-EV treatment as compared with BM-MSC-EVs. The use of EVs may improve regenerative medicine in pediatric surgery.

Translational perspective

Our preclinical study, using young animals, showed that EVs could be a promising alternative to treat wounds in children. After ASC-EV inoculation, well-regenerated tissue with the presence of a complete epithelial layer, dermal papillae and cutaneous annexes and restored connective matrix was obtained. The complete reparative properties

observed support the therapeutic role of EVs in both superficial and in full thickness cutaneous lesions, such as third degree burns, in which all layers of the skin are involved and hair follicles and sebaceous glands are destroyed. These experimental results could also be translated to regenerative medicine for the treatment of congenital and acquired skin lesions occurring in the pediatric age, particularly in neonates or young infants with disfiguring lesions, in which the technical difficulties in obtaining adipose tissue preclude their use.

Thus, MSC-EVs represent a novel 'cell-free therapy' for pediatric skin regeneration, which might overcome the obstacles and risks associated with the use of native or engineered stem cells.

Summary points

- Based on the paracrine and endocrine mechanisms of mesenchymal stem cells (MSCs), extracellular vesicles (EVs) derived from MSCs have been used for tissue repair.
- We evaluated the effects of the intradermal injection of EVs derived from adipose stem cells (ASC-EVs) and bone marrow (BM-MSCs-EVs) in an experimental cutaneous wound repair model. A comparison of autologous and allogeneic ASCs versus BM-MSCs is also reported.
- MSCs were *in vitro* expanded from adipose (ASC) or BM tissues (BM-MSC) of young female New Zealand rabbits. EVs were obtained from the supernatants of confluent ASC and BM-MSCs.
- All treatments accelerated inflammatory infiltrate reduction and promoted partial epidermal and dermal restoration.
- EV treated wounds, exhibited better restoration compared with the counterpart MSC treatment.
- Better wound restoration was observed with cells or EVs obtained from adipose tissue in comparison with BM.
- EV topical inoculation provides restoration of the skin architecture during cutaneous wound healing, and significantly better results in comparison with those obtained from ASC.
- EVs represent a promising solution for regenerative medicine in pediatric surgery.

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Financial & competing interests disclosure

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Cerqueira MT, Pirraco RP, Marques AP. Stem cells in skin wound healing: are we there yet? *Adv. Wound Care* 5(4), 164–175 (2016).
2. Sanchez J, Antonicelli F, Tuton D, Mazouz Dorval S, François C. Specificities in children wound healing. *Ann. Chir. Plast. Esthet.* 61(5), 341–347 (2016).
3. Arneja JS, Gosain AK. Giant congenital melanocytic nevi. *Plast. Reconstr. Surg.* 124(1 Suppl), 1E–13E (2009).
4. Liem PH, Morimoto N, Ito R, Kawai K, Suzuki S. Autologous skin reconstruction by combining epidermis and acellular dermal matrix tissue derived from the skin of giant congenital melanocytic nevi. *J. Artif. Organs.* 16(3), 332–342 (2013).
5. Sheridan RL, Greenhalgh D. Special problems in burns. *Surg. Clin. North Am.* 94(4), 781–791 (2014).
6. Brown M, Coffee T, Adenuga P, Yowler CJ. Outcomes of outpatient management of pediatric burns. *J. Burn. Care Res.* 35(5), 388–394 (2014).
7. Stappenbeck TS, Miyoshi H. The role of stromal stem cells in tissue regeneration and wound repair. *Science* 324(5935), 1666–1669 (2009).

8. Phinney DG, Prockop DJ. Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair – current views. *Stem Cells* 25(11), 2896–2902 (2007).
9. Bruno S, Camussi G. Role of mesenchymal stem cell-derived microvesicles in tissue repair. *Pediatr. Nephrol.* 28(12), 2249–2254 (2013).
10. Pelizzo G, Avanzini MA, Icaro Cornaglia A *et al.* Mesenchymal stromal cells for cutaneous wound healing in a rabbit model. Pre-clinical study applicable in the pediatric surgical setting. *J. Transl. Med.* 8(13), 219 (2015).
11. Daubs MD, Tyser A, Lawrence BD *et al.* The effect of aging on posterior intertransverse lumbar fusion: a New Zealand white rabbit model. *J. Spinal Disord. Tech.* 28(2), E115–E120 (2015).
12. Camussi G, Deregibus MC, Bruno S, Cantaluppi V, Biancone L. Exosomes/microvesicles as a mechanism of cell-to-cell communication. *Kidney Int.* 78(9), 838–848 (2010).
13. Galeano M, Altavilla D, Cucinotta D *et al.* Recombinant human erythropoietin stimulates angiogenesis and wound healing in the genetically diabetic mouse. *Diabetes* 53(9), 2509–2517 (2004).
14. Buganza-Tepole A, Kuhl E. Systems-based approaches toward wound healing. *Pediatr. Res.* 73(4Pt2), 553–563 (2013).
15. Gurtner GC, Werner S, Barrandon Y, Longaker MT. Wound repair and regeneration. *Nature* 453(7193), 314–321 (2008).
16. Werner S, Grose R. Regulation of wound healing by growth factors and cytokines. *Physiol. Rev.* 83(3), 835–870 (2003).
17. Singer AJ, Clark RA. Cutaneous wound healing. *N. Engl. J. Med.* 341(10), 738–746 (1999).
18. Diegelmann RF, Evans MC. Wound healing: an overview of acute, fibrotic and delayed healing. *Front. Biosci. J.* 1(9), 283–289 (2004).
19. Grada A, Falanga V. Novel stem cell therapies for applications to wound healing and tissue repair. *Surg. Technol. Int.* 26(29), 29–37 (2016).
20. Strioga M, Viswanathan S, Darinskas A, Slaby O, Michalek J. Same or not the same? Comparison of adipose tissue-derived versus bone marrow-derived mesenchymal stem and stromal cells. *Stem Cells Dev.* 21(14), 2724–2752 (2012).
21. Maxson S, Lopez EA, Yoo D, Danilkovitch-Miagkova A, Leroux MA. Concise review: role of mesenchymal stem cells in wound repair. *Stem Cells Transl. Med.* 1(2), 142–149 (2012).
22. Salibian AA, Widgerow AD, Abrouk M, Evans GR. Stem cells in plastic surgery: a review of current clinical and translational applications. *Arch. Plast. Surg.* 40(6), 666–675 (2013).
23. Motegi SI, Ishikawa O. Mesenchymal stem cells. The roles and functions in cutaneous wound healing and tumor growth. *J. Dermatol. Sci.* 86(2), 83–89 (2017).
24. Kim KH, Blasco-Morente G, Cuende N, Arias-Santiago S. Mesenchymal stromal cells: properties and role in management of cutaneous diseases. *J. Eur. Acad. Dermatol. Venereol.* 31(3), 414–423 (2017).
25. Otero-Viñas M, Falanga V. Mesenchymal stem cells in chronic wounds: the spectrum from basic to advanced therapy. *Adv. Wound Care (New Rochelle).* 5(4), 149–163 (2016).
26. Lee DE, Ayoub N, Agrawal DK. Mesenchymal stem cells and cutaneous wound healing: novel methods to increase cell delivery and therapeutic efficacy. *Stem Cell Res. Ther.* 9(7), 37 (2016).
27. Baglio SR, Pegtel DM, Baldini N. Mesenchymal stem cell secreted vesicles provide novel opportunities in (stem) cell-free therapy. *Front. Physiol.* 6(3), 359 (2012).
28. Rani S, Ryan AE, Griffin MD, Ritter T. Mesenchymal stem cell-derived extracellular vesicles: toward cell-free therapeutic applications. *Mol. Ther.* 23(5), 812–823 (2015).
29. Keshtkar S, Azarpira N, Ghahremani MH. Mesenchymal stem cell-derived extracellular vesicles: novel frontiers in regenerative medicine. *Stem Cell Res. Ther.* 9(1), 63 (2018).
30. Cabral J, Ryan AE, Griffin MD, Ritter T. Extracellular vesicles as modulators of wound healing. *Adv. Drug Deliv. Rev.* doi:10.1016/j.addr.2018.01.018 (2018) (Epub ahead of print).
31. Nooshabadi VT, Mardpour S, Yousefi-Ahmadipour A *et al.* The extracellular vesicles-derived from mesenchymal stromal cells: a new therapeutic option in regenerative medicine. *J. Cell Biochem.* doi:10.1002/jcb.26726 (2018) (Epub ahead of print).
32. Simons M, Raposo G. Exosomes-vesicular carriers for intercellular communication. *Cur. Opin. Cell Biol.* 21(4), 575–581 (2009).
33. Hu L, Wang J, Zhou X, *et al.* Exosomes derived from human adipose mensenchymal stem cells accelerates cutaneous wound healing via optimizing the characteristics of fibroblasts. *Sci. Rep.* 6, 32993 (2016).
34. Lim P, Patel SA, Rameshwar P. Effective tissue repair and immunomodulation by mesenchymal stem cells within a milieu of cytokines. In: *Stem Cell-Based Tissue Repair*. Gorodetsky R, Schaĺfer R (Eds). RSC Publishing, Cambridge, UK, 346–365 (2011).