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Effects of amniotic fluid mesenchymal stem cells in carboxymethyl cellulose gel on healing of spontaneous pressure sores: clinical outcome in seven hospitalized neonatal foals

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Abstract: In the present study, an innovative therapy for spontaneously arisen pressure sores in seven hospitalized neonatal foals is described, using mesenchymal stem cells obtained from equine amniotic fluid. Amniotic fluid mesenchymal stem cells (AFMSCs) were isolated from fluid samples recovered at delivery. Heterologous cells, at passage three of in vitro culture, were applied to sores twice a week for four consecutive times in a carboxymethylcellulose (CMC) gel. As a control, a commercial ointment was used. The results showed that the mean sore regression rate with AFMSCs in CMC gel was statistically higher than the mean value recorded in the control group. This was associated with a significant effect of the treatment used and a statistically significant effect of treatment over time. The results suggest that local application of mesenchymal stem cells isolated from amniotic fluid, using CMC as scaffold, could be considered as an effective treatment of deep sores in hospitalized neonatal foals.

Key words: Equine, amniotic fluid, mesenchymal stem cells, wound healing, carboxymethylcellulose

1. Introduction

The mechanical forces involved in the pathogenesis of pressure sores are locally exerted pressure, friction, and tension forces, which occur when the patient is lifted or put in decubitus (Kanj et al., 1998; Agrawal and Chauhan, 2012). Other predisposing factors are malnutrition and skin moisture (Fader et al., 2003; Hendrickson, 2011; Agrawal and Chauhan, 2012). Neonatal foals are particularly prone to this kind of injury, due to their thin skin and possible concomitant disorders, which force them into prolonged decubitus (Knottenbelt, 2009). Moreover, foals' sores could be a consequence of casts and rigid splints application for treating flexural deformities. After skin injury, a new epithelium has to form in order to close the wound and restore the skin's function. This requires the proliferation and directional migration of keratinocytes, fibroblasts, and mesenchymal stem cells (MSCs) (Balbino et al., 2005). MSCs are essential for skin homeostasis and repair by promoting cell differentiation, immunomodulation, secretion of growth factors to drive reepithelialization and neovascularization, and modulation of resident stem cells (Fuchs and Horsley, 2008; Balaji et al., 2012). In human medicine, different studies have also focused on the use of MSCs in skin care (Wu et al., 2007; Gianaroli et al., 2009; Sorrell et al., 2010).

In horses, despite the fact that extended wounds of the distal limbs are one of the most common dermatological lesions, which tend to have a long recovery period and poor response to conventional therapies, only a few papers exist on the use of regenerative therapy. Recently, Spaas et al. (2013) reported the successful use of peripheral blood stem cells for treating large wounds on the legs of four horses not responding to conventional therapies.

Amniotic fluid mesenchymal stem cells (AFMSCs), due to their differentiation potential, may be an exciting source of regenerative cells (Tsai et al., 2004; Perin et al., 2007). Our research group demonstrated their potential in skin wound repair, associated with platelet-rich plasma (PRP; Iacono et al., 2012b).

Due to the difficult healing of pressure sores in foals, in the present study the effectiveness of local application of allogenic equine AFMSCs, using carboxymethylcellulose (CMC) gel as a scaffold, was compared to an ointment based on formosulfathiazole, usually employed in our

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equine perinatology unit (EPU) in the treatment of pressure sores spontaneously arisen in neonatal foals.

2. Materials and methods

2.1. Animals

All experimental procedures were approved by the local ethics committee and the Italian Ministry of Health. All procedures were carried out with due care for animal welfare and oral consent was provided by the owners.

Foals between 10 and 15 days old and hospitalized at the EPU "Stefano Belluzzi" of the Department of Veterinary Medical Sciences, University of Bologna, during the 2012 breeding season were considered.

2.2. Experimental design

Before starting treatments, Sessing classification (Table 1; Ferrell et al., 1995) and National Pressure Ulcer Advisory Panel (NPUAP; Table 2; Bluestein and Javaheri, 2008) criteria, usually employed in human medicine, were applied to determine the severity of treated sores. In the literature, specific classifications for horse sores are not reported. In particular, Sessing classification was used to classify sores according to macroscopic appearance, while NPUAP criteria were used for the depth degree. After classification, sores were divided into two groups: Group 1, treated with local application of AFMSCs in CMC gel; Group 2 (control), treated with formosulfathiazole ointment. To evaluate the similar distribution of sores between groups and their homogeneity, a Student t-test for paired variable (SPSS 20.0) was performed before starting treatments.

In Group 1, treatment was applied twice a week for four consecutive times; in Group 2, ointment applications were performed every 48 h. In both groups, after day 30, limbs were no longer bandaged and no further treatments were applied.

2.3. Mesenchymal stem cell isolation and culture

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) and plastic dishes and tubes from Sarstedt Inc. (Newton, NC, USA), unless otherwise noted.

Mares between 6 and 15 years of age and housed at the EPU for delivery were enrolled. All mares had a normal pregnancy, followed by spontaneous delivery, and all

Table 1. Sessing classification of skin wounds (Ferrell et al., 1995).

Stage	Description
0	Normal skin, but at risk.
1	Intact skin, but hyperpigmented or reddened.
2	The bottom and edges of the ulcer are intact and not red.
3	The bottom and edges of the ulcer with pink granulation tissue, modest presence of exudate and odor.
4	Presence of modest granulation tissue, initial and modest necrotic tissue, moderate exudate and odor.
5	Abundant presence of foul smelling exudate, necrotic eschar, and whitish or reddish edges.
6	Further ulceration around the primary ulcer, purulent exudate, intense odor, presence of necrotic tissue and systemic symptoms of sepsis.

Table 2. Classification of the National Pressure Ulcer Advisory Panel (NPUAP; Bluestein and Javaheri, 2008).

Stage	Description
1	Intact skin with nonblanchable redness of a localized area usually over a bony prominence. Darkly pigmented skin may not have visible blanching; its color may differ from the surrounding area. The area may be painful, firm, soft, warmer, or cooler as compared to adjacent tissue.
2	Partial thickness loss of dermis presenting as a shallow open ulcer with a red pink wound bed, without slough. May also present as an intact or open/ruptured serum-filled or serosanguineous filled blister. Presents as a shiny or dry shallow ulcer without slough or bruising.
3	Full thickness tissue loss. Subcutaneous fat may be visible but bone, tendon, or muscle are not exposed. Slough may be present but does not obscure the depth of tissue loss. May include undermining and tunneling.
4	Full thickness tissue loss with exposed bone, tendon, or muscle. Slough or eschar may be present. Often includes undermining and tunneling. The ulcer can extend into muscle and/or supporting structures (e.g., fascia, tendon, or joint capsule), making osteomyelitis or osteitis likely to occur. Exposed bone/muscle is visible or directly palpable.

neonatal foals had an APGAR score between 9 and 10 (Vaala et al., 2002) within 10 min of birth.

Amniotic fluid samples were taken shortly after the foal or the amniotic membrane passed through the vulva, stored at 4 °C, and processed within 12 h, as previously described (Iacono et al., 2012a). Briefly, each sample was diluted 1:1 with Dulbecco's phosphate buffer solution containing antibiotics (100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin). The obtained solution was centrifuged for 15 min at 470 × *g* (Heraeus Megafuge 1.0R; rotor: Heraeus # 2704) and the obtained pellet was resuspended in 5 mL of culture medium containing Dulbecco's modified Eagle's medium (DMEM) and tissue culture medium (TCM199) (1:1), plus 10% (v/v) fetal bovine serum (GIBCO, Invitrogen Corporation, Carlsbad, CA, USA) and antibiotics. Mononuclear cells were isolated by centrifuging the sample on 5 mL of 70% Percoll solution (30 min at 1880 × *g*). The interphase was collected, diluted with 5 mL of culture medium, and centrifuged at 470 × *g* for 10 min. The supernatant was aspirated and cells were washed with culture medium a second and third time. Cells were then resuspended in 1 mL of culture medium and counted by hemocytometer. Primary cells were plated in a 25-cm^2 flask at a maximum density of 5×10^4 cells/cm^2 and incubated in a 5% CO_2 humidified atmosphere at 38.5 °C. The medium was completely replaced after 24 h in order to remove nonadherent cells. Afterwards, the medium was completely replaced every 3 days until the adherent cell population reached ~80% confluence. At this point, the adherent primary MSCs were passed by digestion with 0.05% (w/v) trypsin, counted with a hemocytometer, and reseeded as "Passage 1" (P1) at 25×10^3 cells/cm^2. Cells at P3 were used for clinical applications. The culture was maintained until the administration of the cell therapy (P3). Prior to the cellular use, an aliquot of cells (P3) was collected to perform immunophenotypic characterization, testing the following markers: CD44, CD90, and MHC Class I and II (Iacono et al., 2012a, 2014). Furthermore, the AFMSCs' negative expression for hematopoietic marker CD34 was assessed. Adipogenic, chondrogenic, and osteogenic differentiations were also performed to demonstrate the multipotent potential of the cells used.

2.4. Staging of preparations for MSC application

Immediately before application, under a laminar flow hood and in sterile conditions, AFMSCs were detached from the flask and cells were counted using a hemocytometer. For each application, approximately 5×10^5 cells/mL were used. A gel of CMC used as a scaffold was produced in a sterile petri dish under a laminar flow hood using 5 g of CMC and 5 mL of DMEM/TCM199. Once formed, the gel was placed for 30 min under a UV lamp to avoid bacterial contamination and to break the medium amino acid chains, which could affect skin healing. Immediately before treatment, cells were added to the gel.

Before applying MSCs, wounds were rinsed with isotonic sterile saline (0.9% NaCl) solution. The skin around the wounds was greased with Vaseline to prevent further damage at the time of the next dressing. Immediately after application, the limb was bandaged with cotton gauze, a bandage, and VetRap (3M, Milan, Italy).

2.5. Staging of preparations for ointment cutaneous application

As a control, formosulfathiazole, derived from sulfathiazole and formaldehyde condensation and usually employed in our unit for pressure sore treatment (unpublished data), was used (Socatil Cavallo, Acme Srl, Reggio Emilia, Italy). Due to its poor solubility in water, it is poorly absorbed, and it is therefore suitable for topical use. Before applying the ointment, sores were treated as described above, and after treatment application, the limb was bandaged.

2.6. Assessment of healing and statistical analysis

In Group 1 and 2 wound areas were photographed using a digital camera twice a week for at least 1 month (nine measurements: T0 = day starting treatments and T8 = end of follow-up). A ruler was placed next to the wound as a reference. Images of the wounds were analyzed using image analysis software (ImageJ, processing and analysis in Java, Version 1.6). The area (cm^2) was measured three times and the average was calculated to obtain a single measure. The line for measurement was drawn along the margin of the epithelium granulation, leaving scar tissue. The contributions of epithelialization and wound contraction were not quantified.

The mean percentage of regression was calculated as follows:

$$\% \text{ of regression} = (A - a \times 100)/A,$$

where A is the major area and a is the same area measured 48 h later.

The influence of time and treatment, and the interaction between them, were evaluated using a general linear model for repeated measures (SPSS 20.0). Descriptive statistical analysis results are expressed as mean ± standard deviation. $P < 0.05$ was considered statistically relevant.

3. Results

3.1. Confirmation of the equine AFMSCs' stem cell character

Adherent cells, derived from samples of equine amniotic fluid, showed elongated fusiform morphology with a central nucleus. Their multipotency was demonstrated by incubating cells in media promoting differentiation into the osteogenic, chondrogenic, or adipogenic lineage. AFMSCs differentiated into osteoblasts, chondrocytes, or adipocytes, as evidenced by calcium-rich mineralized matrix or glycosaminoglycans matrix deposits or by the presence of intracellular lipid droplets, respectively

(Figures 1A–1C). The analysis of cell phenotype by flow cytometry showed that they expressed CD90 (glycosylphosphatidylinositol-anchored glycoprotein) and CD44 (cell-surface glycoprotein) and were negative for hematopoietic marker CD34 (Figure 1D). According to the literature, the capacity of differentiation and self-

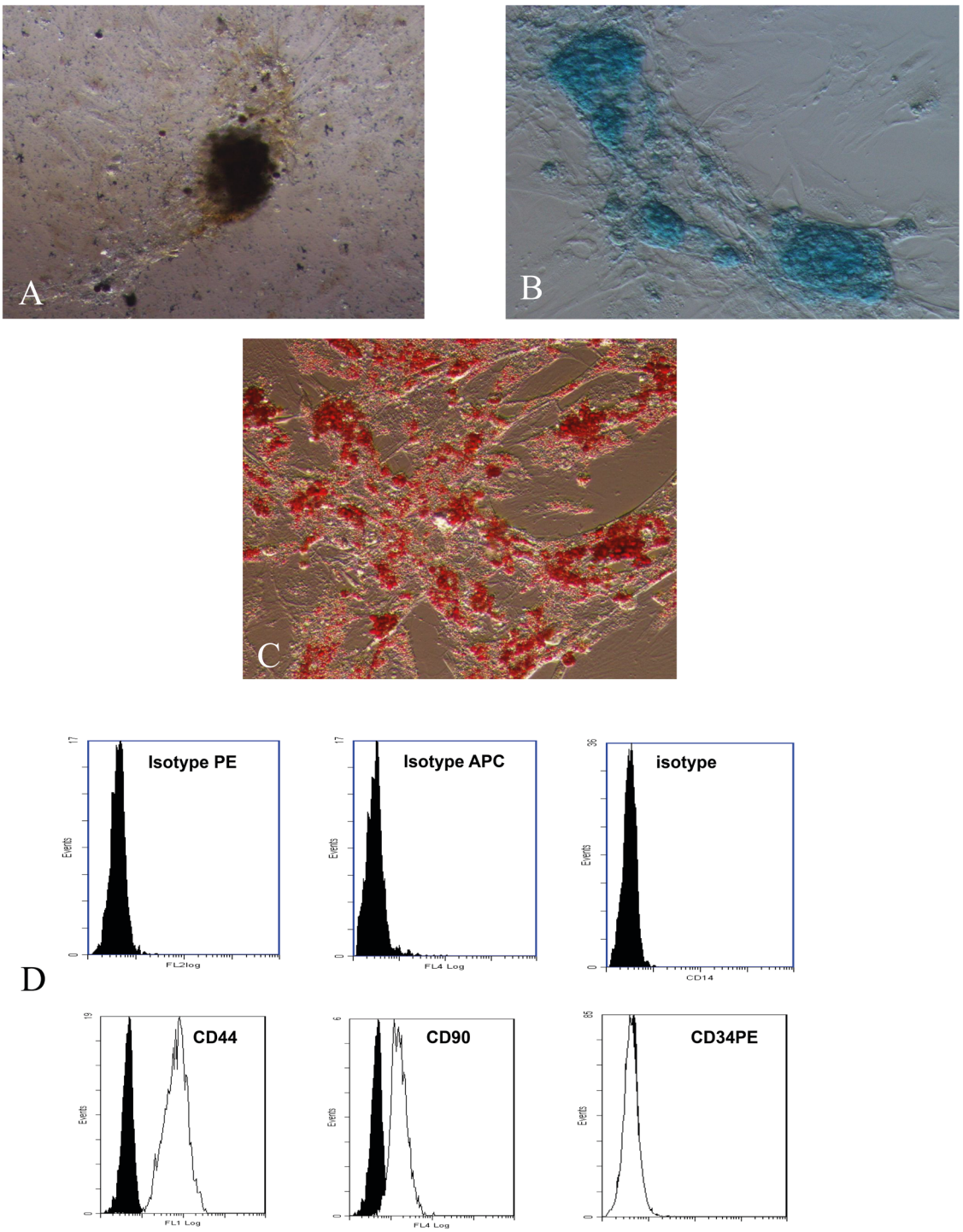

Figure 1. In vitro differentiation studies and flow cytometry analysis of equine AFMSCs at P3 of in vitro culture. A) Osteogenic induction: von Kossa staining of extensive extracellular calcium deposition. Magnification 10×. B) Chondrogenic induction: Alcian blue staining of glycosaminoglycans in cartilage matrix. Magnification 20×. C) Adipogenic induction: Oil red O staining of extensive intracellular lipid droplet accumulation. Magnification 10×. D) Overlay histograms of cytometry analysis. In black isotypic controls are represented. Empty histograms represent the analysis with mAbs on mesenchymal cell culture.

replication, as well as adherence on plastic, are parameters acceptable to confirm the identity of MSCs (Dominici et al., 2006). Furthermore, as previously reported by our research group (Iacono et al., 2014), by RT-PCR, isolated cells did not express major histocompatibility complex I and II, confirming the immunosuppressive function of the MSCs.

3.2. Healing

Seven foals between 10 and 15 days old and with severe skin sores were hospitalized at the EPU during the 2012 breeding season. Animals were affected by skin sores due to prolonged decubitus or the application of tube casts for treating limb flexural deformities. One foal was excluded from the study before starting treatments. This subject, affected by limb flexure deformities and septicemia, had sores on both the carpus and front fetlock of grade 5 and 4 of Sessing and NPUAP classifications, respectively. Due to the complete exposure of articular components, the owner decided on euthanasia. One foal was discharged before the end of the follow-up. Both of these foals were not considered for statistical analysis. A total of 9 pressure sores on the carpus (4), fetlock (2), and hock (3) of 5 hospitalized foals were considered. According to Sessing classification, 6/9 sores were grade 3, 1/9 grade 4, 1/9 grade 4.5, and 1/9 grade 5, while in the NPUAP classification they all corresponded to grade 3. Sores were divided into two groups: Group 1 (n = 6) was treated with local application of AFMSCs and Group 2 (control; n = 3) was treated with formosulfathiazole. Sores were uniformly distributed between the two groups. Due to the low number of cases, the location of the sores was not considered for treatment distribution and statistical analysis. Furthermore, given that the foals were hospitalized at an intensive care unit and that decubitus was changed by medical personnel every 1–2 h, the development side (left or right) of sores was not considered for statistical analysis. Finally, since in the present work naturally occurring sores were treated in owned foals, negative control groups (CMC gel scaffold only and placebo) were not used.

At the first measurement, the mean sore area in Group 1 was 24.86 ± 11.60 cm^2, while in Group 2 it was 17.27 ± 12.04 cm^2. While the mean area of sores in Group 1 decreased from T0 (24.86 ± 11.60 cm^2) to the last measurement (T8: 10.77 ± 7.42 cm^2; Figure 2), in Group 2, the mean sore area increased from T0 (17.27 ± 12.04 cm^2; Figure 3) to T2 (26.16 ± 17.50 cm^2) and then started to decrease until T8 (15.82 ± 14.63 cm^2; Figure 3). The mean regression rate in Group 1 was statistically higher than the value recorded in Group 2 (7.7 ± 5.3 vs. 3.0 ± 27.8; $P < 0.05$). This was associated with a significant effect of the treatment used ($P < 0.05$), while no time effect was demonstrated ($P > 0.05$). However, a statistically significant effect of treatment over time was found ($P < 0.05$; Figure 4).

4. Discussion

In the present study, conventional therapy of sores by application of local ointment was compared with local application of AFMSCs in CMC gel. Enrolled animals, unlike in the study recently performed by Kim et al. (2013) in dogs, were not experimental animals, but were rather subjects spontaneously referred to the EPU. This may have led to a greater variability in the severity and healing of treated sores, depending on subjects, concomitant diseases, and systemic medications administered during hospitalization; however, the failure to use experimental animals makes our results more useful to evaluate the feasibility of application of MSC treatment in the clinical field. Unfortunately, it was not possible to perform a biopsy after the last treatment, given the concern of the owners about a possible recurrence of the disease. This would have allowed us to evaluate the regenerated tissue in relation to different treatments.

In a recent study, Spaas et al. (2013) demonstrated that local injection of peripheral blood stem cells might be a useful treatment for large wounds in horses. In the present study, we chose to apply stem cells directly to the sores using a gel support, as intradermal administration would require the application of protective substances between the surface of the wound and the bandage, making it difficult to understand the results. In the equine species, AFMSCs were used for treating skin wounds in one septicemic foal using PRP as a scaffold (Iacono et al., 2012b). Since foals treated in the present study were sick and/or orphaned, it was not possible to produce PRP from either the foals or their mothers. To avoid an adverse reaction to any erythrocyte factor present in the PRP of a donor's blood, in this investigation, AFMSCs were applied using a CMC gel as a scaffold. It was obtained in our laboratory by mixing CMC as a jelly holder and tissue culture medium as metabolic support for cells. In sores treated with local application of AFMSCs in CMC gel regression was higher, compared to regression of sores treated with local application of formosulfathiazole-based ointment. Furthermore, sores treated with AFMSCs in CMC gel healed quicker, in spite of the persistence of the cause in most cases (i.e. the compression due to the application of splinting of the limbs and prolonged recumbence). CMC is a low-cost biomaterial that has been used as dressing for wounds, with the purpose of protection (Ramli and Wonga, 2011) and as an excipient for some drugs (van Zuijlen et al., 2001). It has a strong ability to absorb and transport fluids and protect from bacterial exposure from the external environment (Wonga and Ramli, 2014). These attributes are important both for human and equine wounds, which are considered to be pathologically similar (Cochrane et al., 2003).

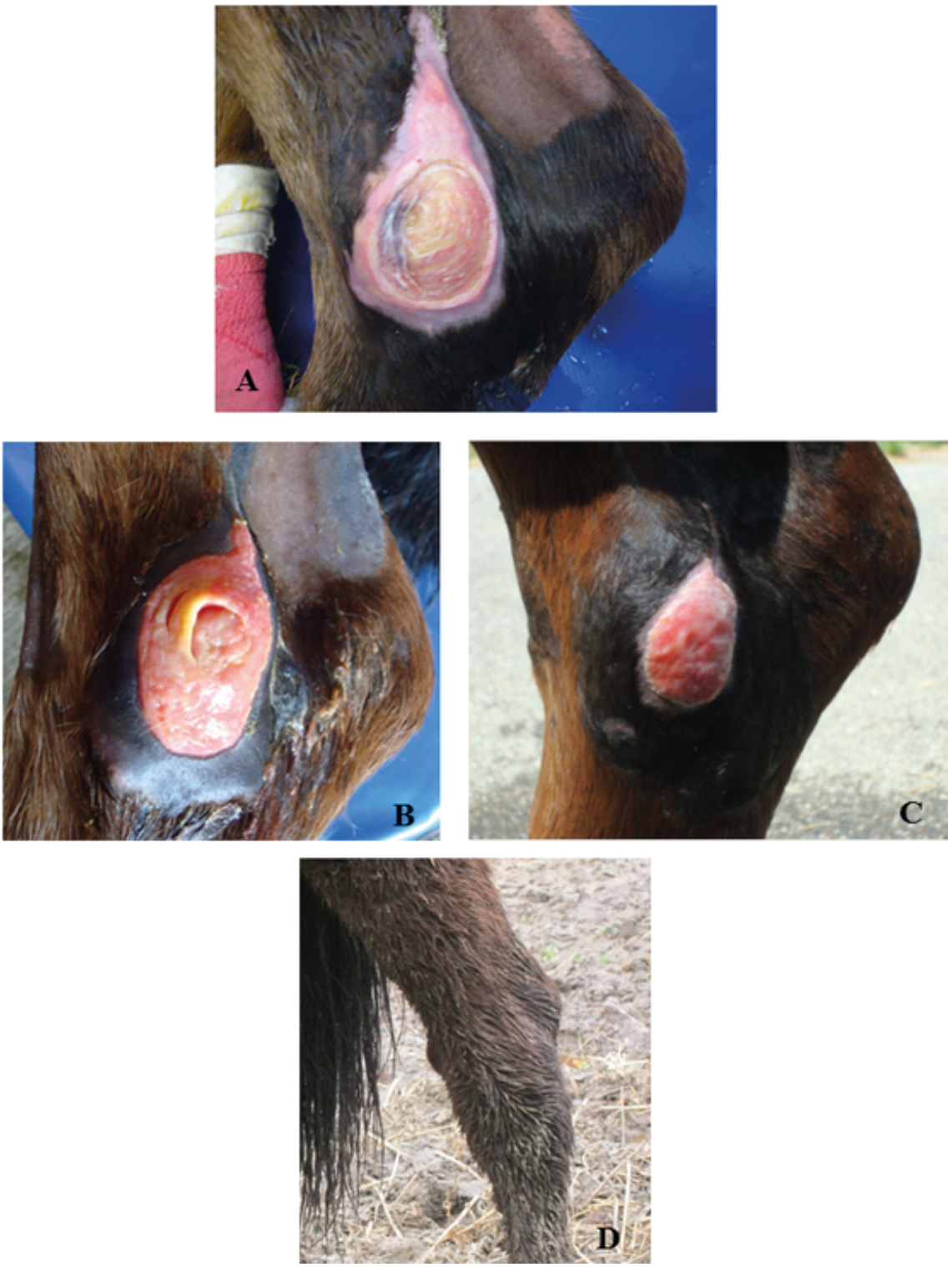

Figure 2. Pressure sore localized on the hock of an orphaned foal hospitalized at the EPU and treated with local application of AFMSCs in CMC gel. A) Before starting treatment; B) 2 weeks later; C) 4 weeks later; D) 6 months after hospitalization.

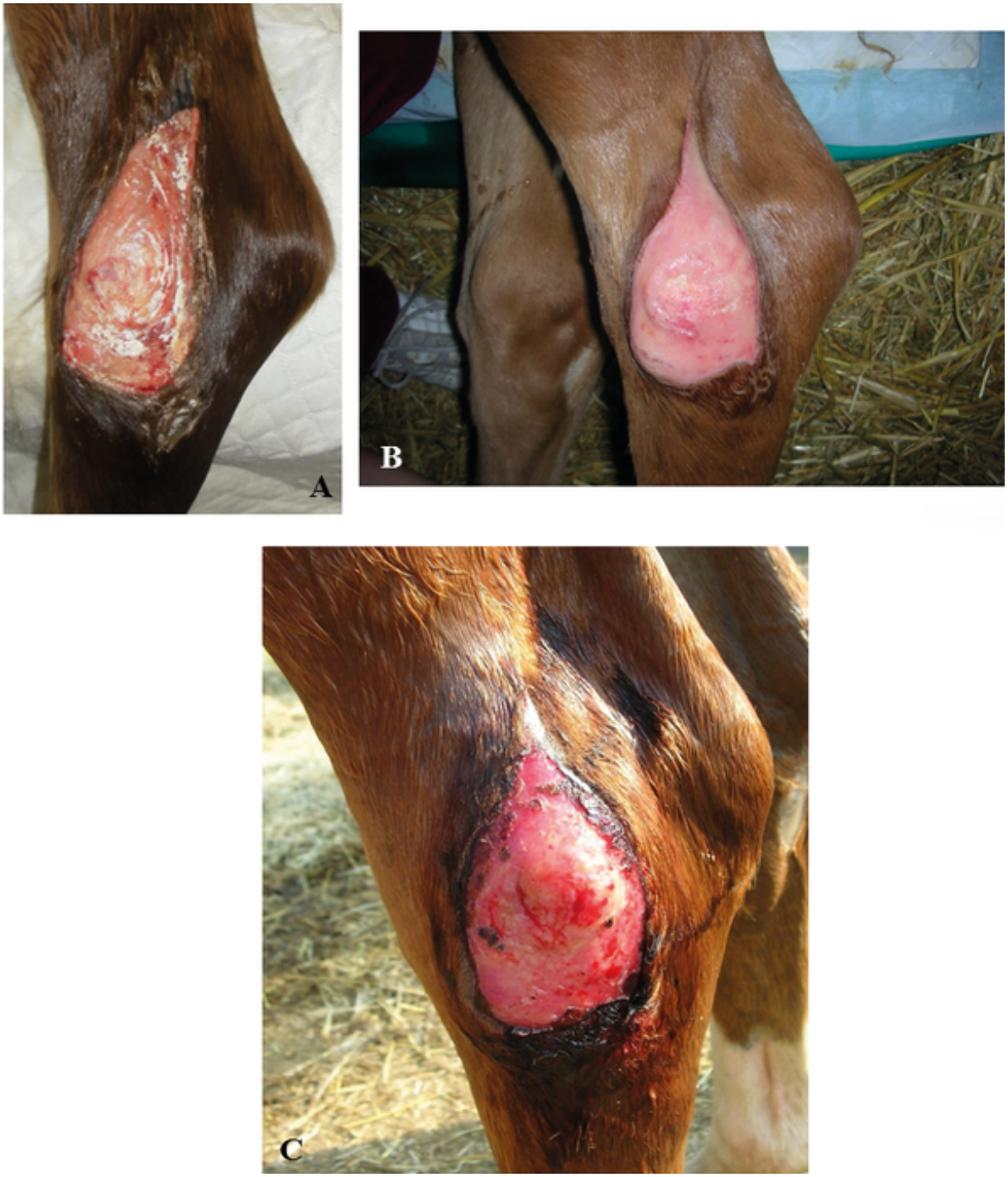

Figure 3. Pressure sore localized on the hock of an orphaned foal hospitalized at the EPU and treated with local application of formosulfathiazole. A) Before starting treatment; B) 2 weeks later; C) 1 month later.

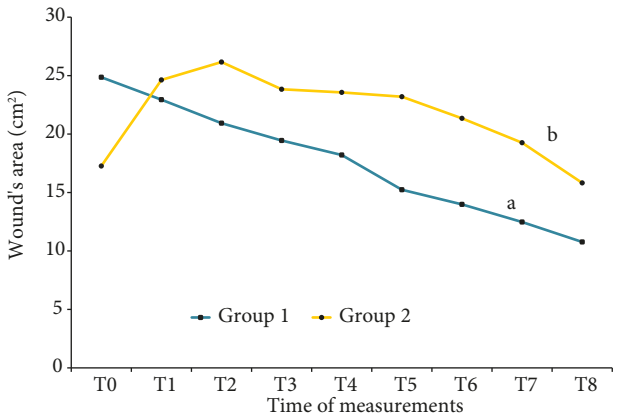

Figure 4. Trend of skin sores' regression in Group 1 (n = 6) and Group 2 (n = 3; control). a vs. b: $P < 0.05$.

Recently, an improvement of healing was observed at the histological level when CMC was used with adipose tissue MSCs in chemically induced wounds in mice (Rodrigues at al., 2014). The lack of systemic and local reaction (local inflammation, etc.), as pointed out by our study, confirms the safety of CMC gel (Rodrigues et al., 2014) and AFMSCs for heterologous use in horses, as already reported in previous studies both in humans (De Coppi et al., 2007) and equine medicine (Iacono et al., 2012a, 2012b). The results obtained in the present study could be ascribed to the CMC's action and to the production of bioactive substances by MSCs and their differentiation in skin cells, due to their demonstrated multipotency also in the horse (Iacono et al., 2012a). However, further studies are needed to evaluate the effectiveness of CMC gel alone in skin wounds' regression in horses.

Despite the low number of cases, the total absence of side effects and the wound regression in this study encourage the application of AFMSCs in clinical practice. The establishment of cell banks, like in human medicine, would allow the use of this treatment on a large scale.

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