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Microvesicles from amniotic cells as potential novel therapeutics in regenerative medicine: first *in vitro* result in equine stressed tendon and endometrial cells

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INTRODUCTION: The unfavorable microenvironment of injured or degenerating tissues may result in the death or apoptosis of a large proportion of implanted MSCs in the short period immediately post-transplantation. Recovery of *in vivo* spontaneous equine tendon lesions by administration of horse amniotic mesenchymal cells conditioned medium (AMC-CM) suggests a paracrine mechanisms in the regeneration process [1]. It has recently been demonstrated that microvesicles (MVs) released from cells are an integral component of the paracrine cell-to-cell communication during tissue regeneration [2]. Aims of this study were to investigate the presence and type of MVs secreted by equine amniotic mesenchymal cells (AMCs), their incorporation in equine tendon and endometrial cells and their effect on these cell lines stressed *in vitro* by lipopolysaccharide (LPS).

METHODS: MVs were obtained by ultracentrifugation at 100.000g for 1h at 4°C of the media obtained culturing AMCs isolated from three different amnion. MVs size was evaluated by Nanosight technology and transmission electron microscopy (TEM). Tendon and endometrial cells were obtained from collagenase digestion for 17h and 3h respectively and cultured in HG-DMEM with 10% fetal calf serum. To study the ability of tendon and endometrial cells to incorporate MVs, a dose-response curve was performed adding 10-20-30-40-50x10^6 MVs/ml labeled with PKH-26 for 24h, 48h and 72h. The uptake of MVs was evaluated by an Olympus BX51 microscope equipped with software for image acquisition. A dose/response curve of LPS investigated by apoptotic and MTT tests showed that 100ng/ml at 48h on tendon cells and 10ng at 24h on endometrial cells were the doses and the times most effective in inducing cellular stress. RT-qPCR expression of pro-inflammatory genes such as tumor necrosis factor-α (*TNF-α*), metallopeptidase (*MMP*) 1 and 13, and of an anti-inflammatory gene such as transforming growth factor-ß (*TGF-ß*) was evaluated in the *in vitro* LPS stressed cells by Mann-Whitney U-test.

RESULTS: Nanosight showed that AMCs secrete MVs in the range of 100-200 nm. TEM revealed budding of AMCs membrane, proving that these MVs fall within the shedding vesicles category. The MVs uptake was gradual over time (Fig. 1). The same semi-quantitative fluorescence uptake signal was obtained when 50x10^6 MVs were incorporated at 24h, or 40x10^6 MVs at 48h and 30x10^6 MVs at 72h, suggesting that an inverse correlation between concentration and time was found in MVs uptake equally by tendon and endometrial cells. MVs induced a significant ($P<0.05$) down-regulation of *TNF-α*, *MMP1* and *MMP13* expression in both cellular lines after *in vitro* LPS stress, and up-regulation of *TGF-ß* expression.

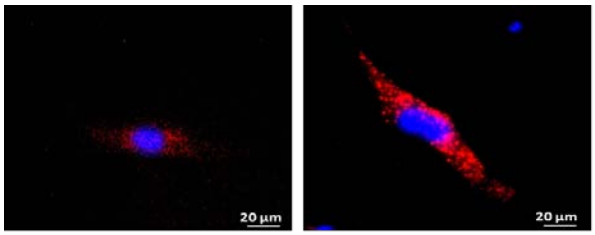

Fig. 1: Representative micrographs of internalization at 24h (A) and 72h (B) of MVs labeled with PKH-26 by tendon cells.

DISCUSSION & CONCLUSIONS: Our data suggest that MVs can be incorporated in tendon and endometrial cells and have a role in modulating inflammatory genes *in vitro*.

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