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Intraperitoneal injection of microencapsulated Sertoli cells restores muscle morphology and performance in dystrophic mice

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ABSTRACT

Duchenne muscular dystrophy (DMD) is a genetic disease characterized by progressive muscle degeneration leading to impaired locomotion, respiratory failure and premature death. In DMD patients, inflammatory events secondary to dystrophin mutation play a major role in the progression of the pathology. Sertoli cells (SeC) have been largely used to protect xenogeneic engraftments or induce trophic effects thanks to their ability to secrete trophic, antiinflammatory, and immunomodulatory factors. Here we have purified SeC from specific pathogen-free (SPF)-certified neonatal pigs, and embedded them into clinical grade alginate microcapsules. We show that a single intraperitoneal injection of microencapsulated SPF SeC (SeC-MC) in an experimental model of DMD can rescue muscle morphology and performance in the absence of pharmacologic immunosuppressive treatments. Once i.p. injected, SeC-MC act as a drug delivery system that modulates the inflammatory response in muscle tissue, and upregulates the expression of the dystrophin paralogue, utrophin in muscles through systemic release of heregulin-β1, thus promoting sarcolemma stability. Analyses performed five months after single injection show high biocompatibility and long-term efficacy of SeC-MC. Our results might open new avenues for the treatment of patients with DMD and related diseases.

1. Introduction

Duchenne muscular dystrophy (DMD) is a lethal, recessive X-linked disease with an incidence of 1 in 3600 live male births caused by the absence of dystrophin following mutation of *DMD* gene [1,2]. Since dystrophin is involved in the structural stabilization of the sarcolemma, DMD patients experience membrane fragility of the myofibers which results in persistent degeneration/regeneration cycles causing progressive depletion of the muscle stem cell pool, and replacement of the skeletal muscle contractile components with fibrous and adipose tissue. The progressive muscle weakness leads to impaired locomotion in childhood, confinement to a wheelchair by 12 years of age, and premature death because of respiratory and/or cardiac failure [1,2]. Persistent compensative degenerative/regenerative cycles and the consequent chronic inflammation in muscle tissue are recognized as major factors in the outcome of DMD pathology [3,4]. DMD patients are currently being treated with antiinflammatory steroids, which are the only evidence-based effective treatment for these patients so far, albeit with limited efficacy and undesired side effects [5–7].

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Sertoli cells (SeC), a major component of the seminiferous tubules, are long known for their ability to secrete trophic, antiinflammatory, and immunomodulatory factors. In the testis SeC are responsible for a unique immune-privileged environment that protects developing germ cells from the host's immune system attack [8,9]. Because of their properties, SeC have been largely used to protect co-grafted allogeneic and xenogeneic tissues from the immune destruction in several settings [10,11]. Also, SeC induced trophic effects on neurons in experimental models of Parkinson's disease and amyotrophic lateral sclerosis, and showed beneficial antiinflammatory effects when grafted into an experimental model of Huntington's disease [12–14]. In the recent past we improved the use of SeC by encapsulating them in highly purified sodium alginate microcapsules [15]. Intraperitoneal injection of microencapsulated SeC resulted in a TGF-β-dependent restoration of systemic immune tolerance, diabetes prevention and reversion in spontaneous diabetic NOD mice [16], and promoted IGF-1-mediated body growth in a murine model of Laron syndrome (dwarfism) [17]. Here we have further improved SeC-based technology by isolating SeC from testes of specific pathogen-free (SPF)-certified neonatal pigs, obtaining pure, viable and functional SeC, and encapsulating them into clinical grade alginate-based microcapsules (SeC-MC) meeting the requirements of National Formulary and European Pharmacopoeia.

We show that intraperitoneal (i.p.) injection with SPF SeC-MC in acute-phase dystrophic *mdx* mice results in a dramatic reduction of infiltrating inflammatory cells, fibrotic areas, and necrotic myofibers in muscle tissue, and acquisition of resistance to exercise-induced muscle damage. SeC-MC restrain muscle inflammation by promoting the enrichment in antiinflammatory macrophages in the muscle mononuclear cell population, and upregulate utrophin at the sarcolemma of myofibers thus mimicking the function of dystrophin, through systemic delivery of heregulin β1. Notably, a single injection of SeC-MC remarkably reduces necrosis and fibrosis in the diaphragm (i.e., the chronically damaged muscle in *mdx* mice) in long-term analysis, and exerts beneficial effects in chronic-phase and presymptomatic dystrophic mice.

2. Results

2.1. Purified SPF SeC show high viability and functionality after encapsulation

SeC isolated and purified from testes of SPF-certified Large White neonatal pigs were composed of ~97% anti-Müllerian hormone-positive (Sertoli) cells [18], ~1.5% alpha-smooth muscle actin-positive (peritubular myoid) cells, ~1.0% insulin-like 3-positive (Leydig) cells, and ~0.5% protein gene product 9.5-positive (gonocytes and spermatogonial) cells, as assessed by FACS and immunofluorescence analysis (Fig. 1, A and B). Freshly purified SPF SeC were encapsulated using a newly-composed microdroplet generator in highly biocompatible, clinical grade (endotoxin levels ≤0.5 EU/g) alginate-based microcapsules. The obtained microcapsules were elastic and translucent, morphologically homogeneous with round shape and no tails or coalescence (Fig. 1C). The microcapsules were fairly monodispersed, with an extremely narrow size distribution (mean diameter, 600 ± 45 μm). Importantly, the current procedure permitted to increase the number of encapsulated cells from 1.0×10^7 [15,19] to 2.0×10^7 SeC/ml alginate. SeC viability and functionality proved unaffected by the encapsulation procedure since i) ethidium bromide/fluorescein diacetate double staining showed that ~96–97% SeC were viable before or after encapsulation (Fig. 1D), and ii) measurement of α-aromatase activity revealed similar levels of 17-β-estradiol production before and after encapsulation, in both the absence or presence of FSH (Fig. 1E).

2.2. SeC-MC treatment improves muscle morphology in mdx mice

We injected microencapsulated SPF SeC (SeC-MC; equivalent amount to 1.0×10^6 SeC per gram of body weight) or the same amount of empty microcapsules (E-MC) into the peritoneal cavity of 4-week-old *mdx* mice, i.e., dystrophic mice in the acute phase of the pathology [20]. Three weeks after injection, compared with muscles of mock-treated mice, the muscles of SeC-MC-treated mice showed reduced mononuclear cell infiltrate, and hallmarks of efficient muscle regeneration, that is a dramatic reduction of necrotic myofibers, a reduced percentage of regenerating (small and centrally-nucleated) myofibers, and increased percentages of regenerated (normally-sized and centrally-nucleated) and undamaged (normally-sized and with peripheral nuclei) myofibers (Fig. 2, A and B; Supplementary Fig. 1A). Accordingly, the frequency histograms of single-fiber area in TA muscles from SeC-MC-treated *vs* mock-treated mice showed a shift toward higher values (2641.28 *vs* 1894.62 μm^2 average cross-sectional area [CSA], respectively) which were more similar to those of untreated age-matched WT controls (average CSA, 2825.13 μm^2) (Fig. 2C), further pointing to the establishment of a microenvironment suitable for efficient regeneration. This was also evidenced by the marked reduction of the numbers of $PAX7^+$ cells (i.e., quiescent satellite cells/proliferating myoblasts), $MyoD^+$ cells (activated satellite cells/proliferating myoblasts), and $myogenin^+$ cells (differentiating myoblasts/myocytes) in TA, *Gastrocnemius* (GC) and diaphragm (DIA) muscles of SeC-MC-treated *vs* mock-treated mice (Supplementary Fig. 2).

Moreover, immunohistochemical analysis of the activated macrophage marker, MAC3, showed a dramatic reduction of the areas infiltrated with macrophages in the muscles of SeC-MC-treated *mdx* mice compared with mice injected with E-MC (Fig. 2D and E; Supplementary Fig. 1B) pointing to a local antiinflammatory effect exerted by the intraperitoneally located SeC. Finally, *mdx* mice treated with SeC-MC showed a dramatic reduction of muscle fibrosis (Fig. 2F and G; Supplementary Fig. 1C). This latter result appears relevant as fibrosis is a major issue in DMD patients; fibrous and adipose tissues progressively overtake the functional myofibers thus concurring to a progressive muscle wasting. Notably, DIA, the most affected muscle in *mdx* model [21], showed ~82% reduction of fat deposition following treatment with SeC-MC compared with DIA of *mdx* mice treated with E-MC (Supplementary Fig. 1D).

2.3. SeC-MC treatment rescues muscle performance in mdx mice

The overall morphological improvement observed in muscles of dystrophic mice after i.p. injection of SeC-MC translated into recovery of muscle performance, as assessed by treadmill running tests. SeC-MC-treated *mdx* mice stopped fewer times (4.5 ± 0.45 *vs* 9.5 ± 0.98 stops for SeC-MC- and mock-treated *mdx* mice, respectively, in a 30-min run) (Fig. 3A), and ran longer distances for longer times compared with the mock-treated counterpart (Fig. 3B), similar to untreated WT mice. Noteworthy, SeC-MC-treated *mdx* mice acquired resistance to exercise-induced muscle damage, since Evans blue dye (EBD) infiltration tests at the end of the exercise protocol revealed a marked reduction of the number of damaged myofibers in *Quadriceps femoris* (QF) muscles of SeC-MC- *vs* mock-treated *mdx* mice, which could be appreciated even at macroscopic inspection (Fig. 3C and D).

2.4. The antiinflammatory effect of SeC-MC treatment is an early event

Looking for a molecular mechanism underpinning the rapid recovery of muscle homeostasis in SeC-MC-treated dystrophic