UNDERSTANDING THE TOXICOKINETICS OF PCBS IN NORTHERN ELEPHANT SEALS (MIROUNGA ANGUSTIROSTRIS): DEVELOPMENT OF PRIMARY CULTURES OF ADIPOCYTES

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Introduction

Marine top predators are highly informative in understanding the quality and health of ocean habitats. Through bioamplification, marine top predators face a very large risk of exposure to toxic, persistent, fat-soluble molecules such as polychlorinated biphenyls (PCBs). The northern elephant seal (NES) (*Mirounga angustirostris*) is an apex predator that forages in the pelagic Pacific Ocean[1,2]. The species is characterized by extended terrestrial fasting durations (up to 3 months) corresponding to breeding, lactation, moulting and a post-weaning period[3]. During this post-weaning fast, pups mobilize primarily lipids from their large adipose tissue stores, in order to prevent protein catabolism and extend the developmental fast[4]. This mobilisation of blubber lipids presents a risk through the release of environmental pollutants into circulation[5-7]. A previous study elucidated the mobilization dynamics of PCBs from blubber to blood during NES post-weaning fast[7]. The authors clearly showed that the mobilization patterns of PCBs were not correlated to those of lipids. PCBs remained preferentially in the adipose tissue stores during the first part of the fast before starting to be released in high amounts at the end of the fasting duration. Young animals being particularly sensitive to environmental pollutants, a release of such toxic molecules into the circulation could be at the origin of physiological disruptions[8]. So far, the mechanisms associated with the release of fat-soluble pollutants remain unknown. In order to study the biochemical aspects of the mobilization of PCBs and lipids from the adipose tissue of NES, we investigated the development of primary cultures of adipocytes. Preliminary results concerning the *in vitro* differentiation of NES progenitor cells into late adipocytes are presented here.

Materials and methods

Samples collection. This study took place at Año Nuevo State Reserve, CA, USA (37°06'30''N, 122°20'10''W) during 2011 (January – April). All work was conducted under the National Marine Fisheries Service Marine Mammal Permit #14636. Blubber biopsies were collected under sterile conditions on chemically-immobilized NES weaned pups. Animals were initially immobilized with an intramuscular injection of Telazol (Fort Dodge Animal Health, Fort Dodge, IA, USA) at a rate of 1 mg/kg of estimated body mass. Sedation was maintained through subsequent intravenous injections of 50 mg of Ketamine (Ketaset, Fort Dodge Animal Health) as needed. Blubber biopsies were collected in the lateral pelvic area using biopsy punches (Acu-punch, Acuderm Help Medical, France) and stored in sterile phosphate buffered saline (PBS) kept at 4°C. Two pups were sampled for each primary culture in order to get 4.5g of adipose tissue. Samples were immediately placed on ice and transported to the laboratory for isolation of progenitor cells.

Isolation and adipogenic differentiation of progenitor cells. Blubber was minced with scissors under sterile conditions and added to Dubbelcco's modified Eagle medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v:v) heat-inactivated Foetal Bovine Serum (FBS) (PAA Laboratories Inc., Darthmouth, MA, USA), 10mM Hepes Buffer solution (Invitrogen), antibiotic and antifungal mixture (0.5K/ml Penicillin-0.5K/ml Streptomycin-1.25µg/ml Amphotericin B Mixture (Lonza, Walkersville, MD, USA), 1mg/ml Gentamicin (Invitrogen), 40U/ml Nystatin (Lonza)) and collagenase (Type II) (Sigma-Aldrich, Atlanta, GA, USA). The minced fat was then incubated in the medium at 37°C for over 35 min to allow tissue digestion. The details of tissue digestion, centrifugations and filtrations are described elsewhere[9]. After those steps, ACK lysis buffer composed of 150mM NH_4Cl, 1mM $NaHCO_3$ and 0.001mM EDTA was added to the pellet at a ratio of 180:1 (v:v) and stored on ice for 30 min with gentle and regular stirring. The solution was then centrifuged at 200 x g for 10 min at 4°C and the supernatant was then removed. This step was repeated twice. Finally, the isolated progenitor cells were suspended in DMEM supplemented with 10% (v:v) heat-inactivated FBS and

antibiotic and antifungal mixture as described above and seeded at a density of 21x10³ cells/cm² on 6-well plastic culture plates. Plates were stored in an incubator at 37°C and 5% CO_2 for 24 hours to allow for the sedimentation and adhesion of the cells. Twenty-four hours after the precursor isolation, the medium was removed and replaced by a differentiation medium containing DMEM, 10% (v:v) heat-inactivated NES serum, 0.1K/ml Penicillin-0.1K/ml Streptomycin-0.25µg/ml Amphotericin B Mixture, 10nM Dexamethasone (Sigma-Aldrich), 0.5mM 3-Isobutyl-1-methylxanthine (Sigma-Aldrich), 10nM Ciglitizone (Sigma-Aldrich) and 8.6µM Insulin (Sigma-Aldrich). This medium was removed and replaced by fresh differentiation medium every 2-3 days until the end of the experimental period. The first day with differentiation medium was considered as "Day 1".

Oil red O (ORO) staining. After medium removal, cells were washed twice with warm PBS before being fixed with 4% paraformaldehyde (Sigma-Aldrich) for 40 min. Cells were rinsed once with water and incubated with working ORO solution (Sigma-Aldrich) for 15min at room temperature. Fixed cells were stored in water and the stained fat droplets were visualized by light microscopy.

Results and discussion:

Role of NES serum in the culture medium

After birth, pups are nursed with fat-rich milk. During this period, their body mass increases from 2 to 4 times (from 30 to 125 kg, on average)[10]. Triacylglycerol concentrations measured in the serum of NES suckling pups are 1.9 ± 0.3 g/l at early and 2.2 ± 0.4 g/l at late lactation (unpublished results). Circulating triacylglycerols packed in lipoproteins (chylomicrons and VLDL) are hydrolysed by lipoprotein lipase and the fatty acids are picked up and stored as triacylglycerols into the adipose tissue. In order to provide NES progenitor cells with triacylglycerols and get as close as possible to the *in vivo* situation, 10% of NES serum were added to the culture medium from Day 1.

Role of differentiation cocktail

The differentiation cocktail used here is a classical mixture for adipogenic differentiation of progenitor cells from rodents[11]. However, several *in vivo* studies revealed that NES present physiological specificities. Among others, different levels of insulin resistance have been noticed in fasting individuals[12,13]. It is thus possible that the differentiation cocktail used for rodents does not have the same impact on NES progenitor cells. The purpose of the present test was to investigate the effect of the presence/absence of this cocktail on the differentiation of the cells. Fig.1 clearly shows that, in absence of the cocktail, there was not accumulation of lipids inside the cells. The differentiation cocktail is thus necessary to differentiate NES progenitor cells into adipocytes.

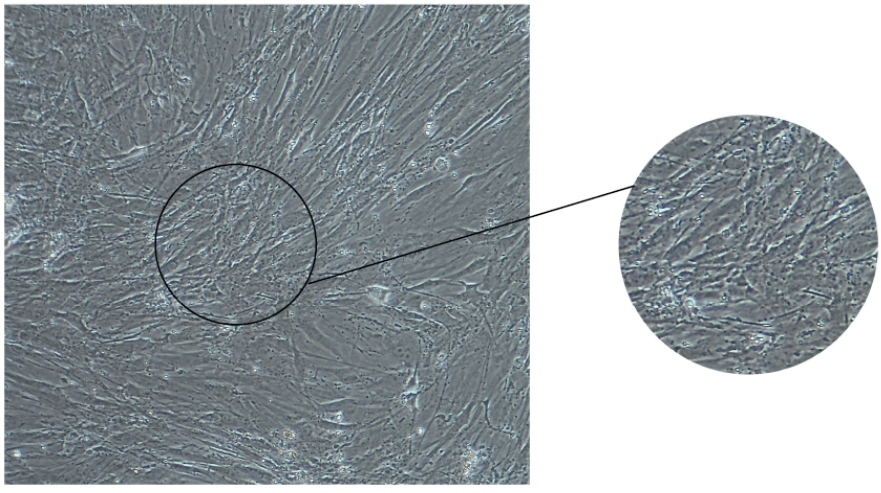

Fig. 1 Progenitor cells from NES at Day 20 (light miscroscopy). Cells were cultivated without the differentiation cocktail. The potential accumulation of cytoplasmic triacylglycerols was investigated by ORO staining.

Isolation and adipogenic differentiation: first results

The NES progenitor cells presented a typical fibroblastoid shape from Day 3 (Fig.2A). Cells were filled with small fat droplets from around Day 10. After 20 days of culture, around 10% of the cells were considered as adipocytes, while around 80% of the cells kept an elongated shape but accumulated very small fat droplets as evidenced by ORO staining on Fig.2B. The cells considered as late adipocytes exhibited various forms as shown in Fig. 2B. Biomolecular experiments will be conducted in order to confirm the presence of *in vitro* adipocytes.

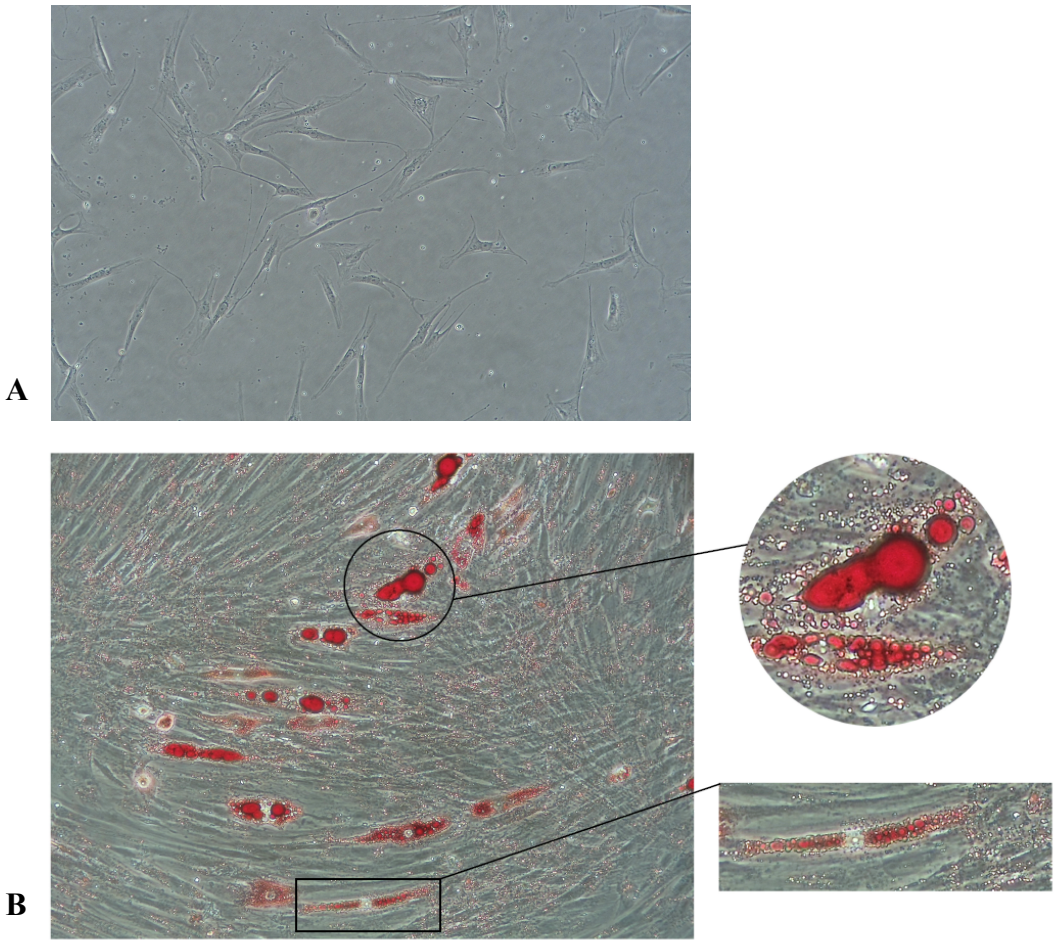

Fig. 2 Isolation and adipogenic differentiation of progenitor cells from NES during 20 days of culture with differentiation cocktail. Cells exhibited a typical fibroblastoid shape at Day 3 (A– light miscroscopy). The accumulation of cytoplasmic triacylglycerols was detected by ORO staining at Day 20 (B – light miscroscopy).

Conclusions and perspectives

This study represents the first attempt to differentiate NES progenitor cells into adipocytes *in vitro*. The microscopic observations presented here are encouraging. The purpose of the next experiments is to confirm the presence of adipocytes. Among others, we plan to follow some adipogenic genes by RT-PCR, to evaluate the lipid content of the cells and to trigger the adipocyte lipolysis by adding isoproterenol to the medium. This *in vitro* model will then be used to study the biochemical aspects of the mobilization of PCBs from adipocytes.

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