Adipose-Derived Stem Cell Collection and Characterization in Bottlenose Dolphins (*Tursiops truncatus*)

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To assess the regenerative properties and potential therapeutic value of adipose-derived stem cells (ASCs) in the bottlenose dolphin, there is a need to determine whether an adequate adipose depot exists, in addition to the development of a standardized technique for minimally invasive adipose collection. In this study, an ultrasound-guided liposuction technique for adipose collection was assessed for its safety and efficacy. The ultrasound was utilized to identify and measure the postnuchal adipose depot and aid in the guidance of the liposuction cannula during aspiration. Liposuction procedures from 6 dolphins yielded 0.9–12.7 g of adipose. All samples yielded sufficient nucleated cells to initiate primary cell cultures, and at passage 2, were successfully differentiated into adipogenic, chondrogenic, neurogenic, and osteogenic cell lineages. The cultured dolphin cells expressed known stem-cell-associated CD markers, CD44 and CD90. Ultrasound-guided liposuction proved to be a safe and minimally invasive procedure that resulted in the successful isolation of ASCs in bottlenose dolphins. This is the first article that conclusively establishes the presence of stem cells in the dolphin.

Introduction

THE INHERENT ABILITY OF DOLPHINS to heal severe cutaneous wounds without surgical intervention or antibiotics is well documented [1–4]; but the unique mechanism that facilitates the restoration of soft tissue injuries in an aquatic environment is unknown. Recent observations of the wound-healing process in wild dolphins have led to the hypothesis that blubber should play an important function in the healing process of cutaneous wounds and brought the topic to the forefront of dermatology [5].

Dolphin blubber is a specialized hypodermis with a unique structure and lipid storage abilities [6]. We hypothesized that adult stem cells present in the dolphin blubber layer play a central role in facilitating the remarkable wound-healing abilities in dolphins. A better understanding of this regenerative cell population could lead to advancements in wound healing in terrestrial mammals and new therapies that are used to treat common disease of dolphins.

Regenerative medicine is a rapidly emerging field, and it may provide solutions for treating commonly occurring diseases in humans and veterinary patients. Success in numerous animal models of disease and emerging achievements in human clinical trials, along with hundreds of ongoing clinical trials, support the rationale for stem cell therapy [7]. Stem cells

have been either proposed or used as therapeutics for renal disease, hepatic disease, and diabetes, all of which are present in bottlenose dolphins (*Tursiops truncatus*) [8–10]. As such, there is interest in assessing the potential for stem cell therapies that treat diseases in dolphins.

Adipose has shown to be an excellent source of adult progenitor cells, most specifically, the multipotent adipose-derived stem cells (ASCs). ASCs have been harvested and characterized in many species other than humans, including rats, mice, rabbits, pigs, dogs, horses, and bears [7,11]. Autologous ASCs are currently used to treat traumatic and degenerative diseases, including bowed tendons, ligament injuries, osteoarthritis, and osteochondral defects in horses and dogs [12,13]. Harvesting adipose is typically achieved through the use of surgical biopsy, lipectomy, or liposuction-based techniques.

Dolphin blubber is composed of different strata, each of which is composed of varying amounts of structural fibers and adipocytes. The deep stratum of the blubber, referred to as subcutaneous adipose [14], contains the least amount of structural fibers [6] and is thought to be the most analogous to terrestrial animal adipose. Ultrasound techniques have been utilized in the bottlenose dolphin to differentiate the adipose layers, measure full-blubber thickness, and assess nutritional status [15].

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Conference Presentation: Collection of Adipose-Derived Stem Cells in Bottlenose Dolphins, International Association of Aquatic Animal Medicine Proceedings, Las Vegas, Nevada, 2011.

A traditional surgical lipectomy would require a large incision through the dolphin's epidermis and hypodermis due to the structure of the blubber. Tumescence liposuction has been demonstrated as being a safe and rapid technique for the collection of human adipose under local anesthesia without incisional lipectomy and is performed on an outpatient basis [16]. Since surgical biopsy is a less than ideal option for the collection of adipose in dolphins, the goals of this study were to identify a stratum of adipose from which ASCs could be harvested and to develop an ultrasound-guided, minimally invasive technique for adipose collection. Furthermore, cells released during the digestion of harvested adipose were cultured, characterized, and differentiated to ensure ASC isolation from the Atlantic bottlenose dolphin.

Materials and Methods

All procedures were conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee of the Navy Marine Mammal Program, Space and Naval Warfare Systems Center Pacific San Diego, CA, and The Office of Research Protections at the Bureau of Medicine and Surgery (IACUC Protocol No. 82-2009). Adipose tissue was collected opportunistically during a post-mortem examination from 1 dolphin and from 6 healthy living bottlenose dolphins under the care of the Navy.

Postmortem sampling and tissue digestion

Blubber, mandibular, and subcutaneous adipose tissue samples were collected during necropsy from a 46-year-old male dolphin. The samples were collected and processed within 6 h of death. Tissue samples were washed, minced, and digested using the procedure outlined next for *Isolation of stem cells from lipoaspirate* with the exceptions that tissue samples were neither initially centrifuged nor was a tissue sieve used to wash the tissue. The total digestion time for tissue samples was ~50 min with agitation. This opportunistic pilot sample was used to verify the presence of ASCs before collections were initiated in healthy living dolphins.

Liposuction technique (healthy dolphins)

In the surgical suite, mild sedation was administered by an intramuscular injection (midazolam, 0.08 mg/kg). Veterinary staff monitored heart and respiratory rates throughout the procedure. The postnuchal fat pad [17] was identified by digital palpation and outlined using transcutaneous ultrasound (M-turbo, Sonosite, 5-2 MHz, 60-mm broadband curved array). Subcutaneous adipose was measured at the center of the fat pad collection area, on the dorsum midline, ~15 cm caudal to the blowhole. The optimal location of the cannula entrance point, which allowed penetration through the blubber into the subcutaneous adipose, was on the lateral edge of the fat pad ~12 cm from dorsal midline. The skin entrance port site was aseptically prepared and desensitized by a circular local block with 2 mL of 2% lidocaine using a 25g $\times$ 1.5 in needle (BD, Franklin Lakes, NJ).

Infusion cannulation

Utilizing sterile procedures, a small skin incision was made with a scalpel blade to create an entrance port. A 15 cm

infusion cannula (2.4 mm diameter, Infiltrator, 60 cc hub; Tulip Medical, San Diego, CA) attached to a 60 cc Toomey syringe (Covidien, Mansfield, MA) was directed through the blubber into the postnuchal fat pad collection area. In order to expand the subcutaneous adipose space and provide local anesthetic, a tumescent solution consisting of 230 mL sterile normal saline (Vedco, Inc., St. Joseph, MO), 20 mL 2% lidocaine (AstraZeneca LP, Wilmington, DE), and 0.2 mL epinephrine (1:1,000; Vedco, Inc.) was prepared for infusion. With ultrasound guidance, the infusion cannula was directed through the epidermal entrance port into subcutaneous adipose, making multiple tunnels through the tissue while infusing the adipose harvest area with tumescent solution. The collection area was externally massaged by hand for 10 min to diffuse the tumescent solution into the adipose and break-up connective tissue.

Adipose harvest

Using techniques similar to those described in humans [18], a sterile 15 cm harvest cannula (4.6 mm diameter, Cobra Bibevel; Tulip Medical) attached to a 60 mL Toomey syringe was directed into the subcutaneous adipose through the same entrance port. Continuous negative pressure was created in the syringe by withdrawing the syringe plunger and locking it into place using a Johnnie Lok (Tulip Medical). Rapid movements were necessary to dislodge and aspirate the adipose into the syringe by partially withdrawing and redirecting the harvest cannula multiple times into the identified adipose collection area. Transcutaneous ultrasound imaging was used to monitor the cannula movements and determine when an area was depleted of subcutaneous adipose. The cannula was then directed to a new area for adipose harvest. When the syringe was filled with 40–45 mL of lipoaspirate, the harvesting cannula was removed from the entry port, and the lipoaspirate was emptied into a sterile transport tube (Vet-Stem, Inc., Poway, CA). The cannula was flushed with sterile normal saline before re-entry through the port into the collection area and re-initiating liposuction. Once liposuction was complete, sedation was reversed (fentanyl 0.005 mg/kg, IV), and the animal was returned to the water enclosure. Skin closure of the entrance port area was not necessary.

Isolation of stem cells from lipoaspirate

After harvest, tubes containing lipoaspirate were transported in Vet-Stem's cold storage shipping container for same-day processing at Vet-Stem, Inc. Harvested adipose was separated by centrifugation at 2°C–8°C, 500–600 rpm for 3 min. The floating adipose was then collected, placed in a fresh 50 mL conical tube (BD, Bedford, MA) with 45 mL of phosphate-buffered saline (PBS; Mediatech, Manassas, VA), and again centrifuged at 2°C–8°C, 500–600 rpm for 3 min. The floating adipose was again collected, placed in a sterilized tissue sieve (Collector, 40 mesh; BellCo Glass, Vineland, NJ), and washed with ~200 mL PBS until the rinse solution was visibly free of red blood cells. The adipose was then transferred to a fresh 50 mL conical tube and digested according to a previously described protocol (Black). After digestion with agitation for ~20 min at 37°C, the volume of the tube was brought up to 45 mL with PBS and centrifuged at 2°C–8°C, 1,500–1,700 rpm for 15 min. The supernatant was

discarded, and the cell pellet was resuspended in PBS before cell straining into a fresh 50 mL conical tube using a 70 μ m cell strainer (BD). The cell pellet was washed thrice by re-suspending with 50 mL of PBS and centrifugation at 2°C–8°C, 1,500–1,700 rpm for 10 min. After the third centrifugation, the cell pellet was re-suspended in 1–2 mL of PBS. A small aliquot was removed for counting nucleated cells using a NucleoCounter cell counter (Chemometec, Allerød, Denmark). Data are presented as the mean $\pm$ the standard deviation of adipose sample weight, viable cells per gram of adipose, percent viable cells, and total viable cell yield. The procedure used to digest the adipose tissue from Dolphin No. 1 was slightly different from that just mentioned and is not included in the calculations for the viable cells per gram, percent viable cells, and total viable cell yield. Briefly, the adipose was washed and rinsed as just described, then evenly split into multiple 1.8 mL microcentrifuge tubes. Digestion was performed as just described, and the tubes were incubated with agitation at 37°C until the adipose particles were visibly digested. The tubes were then vortexed for 10–15 s, 0.5 mL PBS was added, and the tubes were centrifuged in a mini-microfuge at 6,000 rpm for 1 min. The supernatant was removed, and the cell pellet was resuspended in 1 mL PBS. The tubes were again vortexed, centrifuged, cell strained, and an aliquot was used for cell counting.

Culture expansion of stem cells

Freshly isolated cells were placed directly in culture using mesenchymal stem cell media (MSCM; ScienCell, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS) and 1% antibiotics (penicillin and streptomycin) and seeded at a density between 13,333 and 20,000 cells/cm² in culture flasks (Corning, Lowell, MA). The flasks were placed in humidified cell culture incubators set to 37°C and 5% CO₂. On day 3, the cultures were supplemented with L-glutamine (Hyclone, Logan, UT). When the cultures were ~85%–90% confluent, cells were trypsinized (Hyclone) and plated at a density of ~4,000 cells/cm².

Colony-forming unit–fibroblastic assay

In the majority of the procedures, freshly isolated stromal vascular fraction cells were seeded in MSCM on 12-well plates (Corning) in triplicate serial dilutions at densities ranging from ~5 to ~13,000 cells/cm² and placed in a humidified cell culture incubator at 37°C with 5% CO₂. Media were replaced every 3 days for a period of 9 days. On day 9, the wells were washed with PBS, fixed in 10% formalin (Sigma-Aldrich, St. Louis, MO) for 30 min, rinsed twice with H₂O, and allowed to air dry. The cells were then stained with Giemsa solution (Sigma-Aldrich) for 5 min, washed with H₂O, and allowed to air dry. Colonies were counted using a microscope, and data were presented as the mean $\pm$ the standard deviation. Percent colony forming unit–fibroblastic (CFU-f) was calculated by dividing the number of colonies within a well by the total number of cells plated within the well and multiplying by 100.

Cell differentiation

After trypsinization at passage 2, cells were seeded in triplicate as per the manufacturer's specification in 12-well

plates and placed in humidified cell culture incubators set at 37°C with 5% CO₂. In all instances, the media were replaced every 2–3 days; control wells were maintained in MSCM. The cells were grown in MSCM until they reached the required confluence, at which time induction media were added. All induction media were obtained from Hyclone's AdvanceSTEM media catalog; all stain solutions were acquired from Electron Microscopy Sciences (Hatfield, PA). For adipogenic induction, the cells were seeded at ~10,000 cells/cm² until reaching 95% confluence. AdvanceSTEM adipogenic differentiation medium was then added to the treated wells for a period of 14 days. On day 14, the cells were fixed with 10% formalin for 30 min, rinsed twice with H₂O, and allowed to incubate in isopropanol for 5 min. The wells were then stained with Oil Red O solution (indicator of intracellular lipid accumulation) for 5 min, washed with H₂O, and photographed. For osteogenic induction, the cells were seeded at a density of 6,000–8,000 cells/cm² in MSCM until they were ~80% confluent. The media in treated wells were then replaced with AdvanceSTEM Osteogenic Differentiation medium for a period of 21–28 days. On the final day, the cells were rinsed with PBS, fixed with 10% formalin for 30 min, and washed twice with H₂O. The cells were then stained with Alizarin Red S solution for 3 min, washed twice with H₂O, and photographed. For chondrogenic induction, cultured cells were centrifuged at 2°C–8°C, 500 rpm for 10 min, and then resuspended in MSCM to create a cell suspension of 1.6×10^7 cells per milliliter. Five microliters of the cell suspension was placed in the center of the well and allowed to adhere for a period of 2 h in a cell culture incubator set at 37°C and 5% CO₂. MSCM was then added to the control wells, and AdvanceSTEM Chondrogenic Differentiation medium was added to the treated wells for a period of 14 days. On day 14, the wells were washed twice with PBS, fixed with 10% formalin for 30 min, and washed twice with H₂O. Alcian Blue stain was then added to the wells and allowed to incubate for 30 min. The stain solution was removed. The wells were washed twice with 0.1 N HCl, twice more with H₂O, and photographed when the wells were dry. For neurogenic induction, the cells were plated at 2,000–4,000 cells/cm² for 24 h, at which time the media were replaced with AdvanceSTEM Neurogenic Differentiation medium until the cells reached ~30%–35% confluence; typically around day 3 or 4. The medium was then aspirated; the cells were washed with PBS and fixed in 10% formalin for period of 30 min. The formalin was removed, the wells were washed with distilled water once, stained with Giemsa for a period of 5 min, and then washed twice with H₂O.

Cell surface (CD) marker analysis

Cultured cells were harvested at passage 2, cryopreserved in FBS/dimethyl sulfoxide, and stored in liquid nitrogen until the day of analysis. The cryovials were thawed in a 37°C water bath, placed in a conical tube containing 10 mL of Iscove's modification of Dulbecco's media (Mediatech), and allowed to recover for a period of 10 min before centrifugation at 300 g, 2–8°C, 10 min. For fresh blood, red blood cells were lysed in 1 mL of 1 $\times$ red blood cell lysis buffer (BioLegend, San Diego, CA) per 0.1 mL of whole blood for a period of 10 min and centrifuged at 300 g at 2°C–8°C for 10 min. The cell pellet was resuspended in cold PBS, the cells were

TABLE 1. STROMAL CELLS ISOLATED OPPORTUNISTICALLY FROM 3 SEPARATE ADIPOSE COLLECTION LOCATIONS DURING A BOTTLENOSE DOLPHIN (*Tursiops truncatus*) NECROPSY

Adipose collection location	Sample weight (g)	Viable cells per gram	Percent viable cells	Total viable cell yield	CFU% (mean $\pm$ SD)
Blubber	48.66	3,740	82.5	182,000	—
Subcutaneous	31.92	46,516	87.1	1,484,800	1.04 $\pm$ 0.24
Mandible	37.4	34,294	91.7	1,282,600	0.33 $\pm$ 0.28

CFU, colony forming unit; SD, standard deviation.

counted as just described, and a final suspension of 1×10^6 cells per milliliter in cold PBS was made. The cells were then incubated with LIVE/DEAD fixable stain (Life Technologies, Grand Island, NY) for 20 min at 2°C–8°C, protected from light. After a wash with antibody buffer [0.5% FBS, 2 mM ethylenediaminetetraacetic acid (EDTA), 0.1% NaN₃, in PBS] centrifugation, and resuspension, the cells were incubated with 10% combined mouse and rat serum (Sigma-Aldrich) for 20 min to block nonspecific binding before antibody addition. Antibodies and isotype controls were added according to the manufacturer's recommendation and incubated for 45 min, 2°C–8°C protected from light. An additional 2 mL of antibody buffer was added, the cells were centrifuged, and the supernatant was aspirated. Cells were fixed in formalin in PBS (1:10) for 15 min at room temperature. The antibodies tested were as follows with the clone number listed in parentheses: CD29 (MAR4), CD34 (1H6), CD44 (515), CD73 (AD2), CD90 (5E10), CD105 (266), CD271 (C40-1457) from BD Biosciences, CD45 (YKIX716.13) from AbDSerotec (Raleigh, NC), CD90 (DG3), CD105 (43A431), CD271 (ME20.4-1.H4) from MiltenyiBiotec (Auburn, CA), and CD105 (43A3) from BioLegend. All analyses were performed on a BD FACSCanto II using BD FACSDiva software version 6.1.3.

Results

Adipose samples were collected from 3 separate adipose depots during a dolphin necropsy: mandibular adipose, subcutaneous adipose, and blubber. After digestion, the blubber contained a large amount of ligamenture that could

not be digested, whereas the mandibular adipose and the subcutaneous adipose were readily digested with little residual fibrosities. In this single set of comparisons taken from postmortem samples, blubber contained the least number of nucleated cells per gram, whereas subcutaneous adipose contained the highest number of nucleated cells per gram (Table 1) and was chosen as the adipose depot for harvest.

Liposuction

Ultrasound-guided liposuction (Figs. 1 and 2) was performed without complication on 6 healthy bottlenose dolphins. The tumescent solution provided ~45 min of local anesthesia, allowing adequate time to perform liposuction. The liposuction procedures yielded 0.9–12.7 g of adipose. The dolphins showed very little negative response to the procedure and maintained normal heart rates, respiration rates, and behaviors during the liposuction procedures. Seroma formation at the site of liposuction was observed post procedure via ultrasound in 2 of the cases. Aspirated fluid from the seroma was serosanguineous, bacterial culture negative, and resolved without incidence. In all the dolphins, no significant hematologic or serum biochemical changes were observed after the liposuction procedure. The data for adipose digestion from the lipoaspirate collected from 6 dolphins are tabulated in Table 2. The average number of

FIG. 1. Ultrasound image of the blubber (A) and subcutaneous adipose (B) layers in the bottlenose dolphin.

FIG. 2. Photograph of the liposuction technique used to harvest adipose from the bottlenose dolphin. Ultrasound-guided ultrasound was used to monitor the entry and re-entry of the harvesting cannula into the postnuchal, subcutaneous adipose field. Note that the syringe is partially filled with harvested adipose.

TABLE 2. LIPOASPIRATE DATA FROM LIPOSUCTION PROCEDURES ON 6 BOTTLENOSE DOLPHINS (*Tursiops truncatus*)

Dolphin	Age (years)	Sex	Body weight (kg)	Sample weight (g)	Viable cells per gram	Percent viable cells	Total viable cell yield	CFU-f% (AVG $\pm$ SD)
1	22	M	218	6.30	86,667 ^a	86.7	546,000	—
2	30	M	213	12.76	257,053	88.2	3,280,000	0.17 $\pm$ 0.07
3	34	F	181	4.62	338,571	75.2	1,546,200	0.19 $\pm$ 0.16
4	29	M	210	5.89	695,705	83.1	4,097,700	0.11 $\pm$ 0.06
5	46	M	255	0.91	200,110	— ^b	182,700	—
6	49	F	224	5.79	566,805	83.0	3,281,800	0.03 $\pm$ 0.01
			AVG	6.05	411,649	82.4	2,477,680	0.13%
			SD	3.84	211,394	5.4	1,585,038	0.11%

^aNot included in AVG.^bCell count was at the limit of detection.

AVG, average; CFU-f, colony forming unit–fibroblastic.

viable cells per gram was 411,649 with an average percent viability of 82.4%.

Culture expansion

Typical growth and passage observation of the cultured stem cells are shown in Fig. 3. Throughout the culture process, the cells retained their characteristic spindle shape of cultured ASCs and remained as such throughout the culture expansions included in this study. The cells were expanded in MSCM, subcultured when the cells reached ~80%–90% confluence, and plated into new flasks at a seeding density $\geq 4,000$ cells/cm².

CFU-f analysis

To quantify the actual number of stem cells present in the dolphin adipose, processes that yielded cells of a sufficient quantity were plated for CFU-f analysis. Cells were seeded in culture wells using serial dilutions and expanded for a period of 9 days to determine the percentage of cells that can replicate and form colonies. The average percent CFU-f was found to be 0.13%, about 1 in 769 plated cells (Table 2). CFU-f assays for Dolphin No. 1 and No. 5 were not performed due to the limited number of cells available to ensure a successful culture expansion. CFU-f analyses were also successfully initiated for 2 of the 3 adipose depots from the pilot dolphin necropsy samples (subcutaneous and mandibular adipose) and are included in Table 1.

Cell differentiation

Dolphin-cultured cells expanded to passage 2 were successfully differentiated into multiple cell lineages. Photographs of the control and differentiated cells stained for the specific induction are shown in Fig. 4. The cultured cells responded to the respective induction media and differentiated into adipogenic, chondrogenic, osteogenic, and neurogenic cells as indicated by positive staining and/or cell morphology.

Cell characterization

Dolphin ASCs cultured to passage 2 were found to express high percentages of CD44 (97.5% $\pm$ 3.1%) and CD90 (99.3% $\pm$ 0.3%); see Table 3 and Fig. 5. Fresh dolphin blood

FIG. 3. Photographs of a representative bottlenose dolphin cell culture taken at (A) day 9—before trypsinization, and at (B) day 11 after replating. Cells were immediately plated after isolation from adipose and allowed to expand until colonies were formed (note large colony in center of A). Note the even redistribution of the cells after trypsinization and the spindle-shaped morphology of the cells that are characteristic of stem cells (B).

FIG. 4. Photographs from passage 2 bottlenose dolphin cultured cells expanded in culture media (control) or lineage-specific differentiation media. Note the difference in each comparison with regard to morphology and staining patterns. All direct comparisons were incubated for the same period of time and stained/fixed in parallel with the respective control group. Oil Red O stain for adipogenic differentiation in (A) control media and (B) adipogenic induction media. Alcian Blue stain for chondrogenic differentiation in (C) control media and (D) chondrogenic induction media. Note the distinctive nodule formation in the chondrogenic media. Alizarin Red S stain for osteogenic differentiation in (E) control media and (F) osteogenic induction media. Giemsa stain showing the morphologically distinct cells incubated in (G) control media and (H) neurogenic induction media. (I) A magnification of neurogenically differentiated cells *insert H*, showing cells with multiple processes *single arrow* (multipolar) and cells that have formed neuronal-like networks *triple arrow*.

TABLE 3. DOLPHIN EXPRESSION OF CD44 AND CD90

	<i>Dolphin 3</i>	<i>Dolphin 4</i>	<i>Dolphin 5</i>	<i>Average</i>	<i>SD</i>
CD44	93.9	99.0	99.6	97.5	3.1
CD90	99.2	99.1	99.7	99.3	0.3

was analyzed for the expression of CD markers to determine whether negative markers could be used for cell enumeration with dolphin-cultured cells expanded to culture passage 2. However, cells isolated after the lysis of red blood cells did not exhibit the expression of CD34 or CD45 (data not shown) and, therefore, were not used as negative expression markers in the dolphin.

Discussion

Dolphins have a highly adapted adipose storage structure referred to as blubber that is unique to marine mammals. The blubber has many functions beyond lipid storage, including providing buoyancy [19], thermoregulation [20], hearing [21], locomotion, and body streamlining [22]. In our study, we discovered the presence of stem cells in the different strata of the blubber in the Atlantic bottlenose dolphin. From necropsy samples, the total viable cells per gram for these tissues were very low (<50,000 cells per gram) and are most likely due to a combination of factors, which include the circumstance of the death, postmortem ischemia, and tissue degradation. Plastic adherent cells were isolated from all 3 tissue sites, indicating that these areas may be useful as potential extraction sites for isolating stem cells in future samples. Moreover, subcutaneous adipose had the highest cells per gram yield, prompting the use of deep subcutaneous adipose for isolating ASCs from live, healthy dolphins.

In the bottlenose dolphin, the greatest quantity of subcutaneous adipose accumulates in an area caudal to the nuchal crest of the skull. Observation of the postnuchal fat pad is often utilized as a visual indicator of a dolphin's body condition [23] and was used in our study as the source of subcutaneous adipose. An ultrasound-guided liposuction technique was successfully developed to harvest postnuchal adipose from dolphins in a minimally invasive manner with as little as 0.7 cm depth of subcutaneous adipose present. The mean total viable cells per gram of lipoaspirate from the postnuchal fat pad of $411,649 \pm 211,394$ is similar to the

$287,000 \pm 34,000$ reported from human lipoaspirate [24]. The cohort's percentage of CFUs is compared with internal results from equine liposuctions; likewise, lipectomies of subcutaneous adipose taken from equine display similar CFU-f percentages (data not shown) to the dolphin subcutaneous tissue sample. The differentiation of cells at passages 4 and 6 is currently underway; however, our progress thus far indicates that the ability of these cells to differentiate is not limited by the passage number. Numerous cell marker antibodies were tested with incubation in dolphin stem cells. Passage 2 ASCs expressed high levels of CD44 and CD90 markers that helped in identifying them as ASCs [25]. The low number of the antibodies found to bind dolphin epitopes is likely due to the fact that these antibodies are targeted to human or mouse epitopes.

Cells isolated from the dolphins postnuchal fat pad lipoaspirate meet all the criteria for stem cells as defined by The International Society for Cell Therapy [26]; (1) the cells are plastic adherent and have a spindle-shaped morphology; (2) can be differentiated into adipogenic, chondrogenic and osteogenic cell lineages; and (3) express key CD markers. In addition, the dolphin cells are also able to differentiate into neural cells and are morphologically comparable to human bone marrow and amnion-derived stem cells that are neuronally differentiated with the same media for a similar time course and that have been shown to express beta-tubulin III, microtubule-associated protein 2 (MAP-2), and glial fibrillary acidic protein (GFAP) [27], which are known neuronal markers.

The discovery of stem cells from human adipose that was able to differentiate in multiple cell types [28] led to an explosion of stem cell studies in multiple species. Most research conducted on non-human ASCs has been directed toward the development of stem cell therapy animal models for human diseases [29,30]. However, ASCs are now being incorporated into clinical veterinary medicine for the treatment of dog and horse orthopedic diseases [12,13], and Vet-Stem, Inc. has an ongoing clinical development program for diseases such as renal, hepatic, and immune-mediated diseases.

Our study demonstrates that stem cells are present in dolphin adipose and can be collected with a minimally invasive ultrasound-guided tumescent liposuction technique. Additional studies conducted on dolphin ASC could shed light on how dolphins and other marine mammals are capable of healing cutaneous wounds in a completely aqueous environment. Veterinarians can now also consider stem cell therapy when evaluating the treatment options for common dolphin diseases such as corneal trauma and liver disease, both of which have shown promising therapeutic benefits with stem cell treatment in humans [31,32]. Several dolphin diseases have similar human counterparts, including type 2 diabetes [8], urate nephrolithiasis with renal failure [33], and iron overload with liver disease [9,34]. The dolphin may serve as a human model for several disease processes, as was recently proposed for type 2 diabetes [10] and aging studies [35], where new treatment modalities using stem cells could be realized.

Acknowledgments

This work was supported by the Office of Naval Research Contract N00014-09-C-0378. The authors wish to thank the

FIG. 5. Representative CD marker analysis of dolphin passage 2 adipose-derived stem cells. (A) CD44 (white) compared with isotype control (gray). (B) CD90 (white) compared with isotype control (gray).

veterinarians, technicians, and staff at the U.S. Navy Marine Mammal Program and the National Marine Mammal Foundation for their assistance and support and Elizabeth Hoffman for initiating the partnership with Vet-Stem. Flow cytometry work was performed with the support of the Flow Cytometry Core at the UC San Diego Center for AIDS Research (AI36214), the VA San Diego Health Care System, and the San Diego Veterans Medical Research Foundation.

Author Disclosure Statement

No competing financial interests exist.

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Received for publication January 26, 2012

Accepted after revision April 24, 2012

Prepublished on Liebert Instant Online April 24, 2012