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HUMAN ADIPOSE-DERIVED STROMAL/STEM CELLS: USE OF ANIMAL FREE PRODUCTS AND EXTENDED STORAGE AT ROOM TEMPERATURE

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KEYWORDS
Adipose-derived stem cells; animal protein-free; current Good Manufacturing Practices; lipoaspirates; tissue storage.

ABSTRACT
Adipose-derived stromal/stem cells (ASCs) have been explored in recent pre-clinical trials to treat diseases in a broad range of tissues. The ultimate goal is to translate these findings to clinical trials to test safety and efficacy in human subjects. This will require the development of animal-free products suitable for the passage of the cells. Current protocols employ trypsin derived from porcine tissue (stomach) for this purpose. Likewise, the length of time between adipose tissue harvest and processing will need to be systematically evaluated with respect to cell yield, viability, and function. The objective of this study was to explore alternative, non-animal sources of trypsin-like enzymes as alternatives to porcine trypsin for the passage of ASCs and to determine the effect of time delays on the yield and function of ASCs after collagenase digestion. These outcomes have practical implications with respect to the development of Standard Operating Procedures for cGMP manufacture of clinical grade human ASCs, which is essential for allowing their future use in the clinical practice .

INTRODUCTION
Standard Operating Procedures (SOPs) that exclude animal protein products need to be developed and validated. There are commercially available substitutes for porcine trypsin. These are a bacterial-derived recombinant product (TrypLE) and a corn-derived product (TrypZean); both are animal protein-free alternatives for cell dissociation and passage.
When using adipose-derived stem cells, a delay is likely to occur between the time of liposuction surgery and tissue collection until the time of cell isolation. This period may extend from a few hours to several days to transfer the lipoaspirate tissue specimen from the operating room (OR) to the cGMP facility. The current study was designed to compare the efficacy of trypsin alternatives and determine the effect of extended lipoaspirate storage time at room temperature on the subsequent yield and differentiation potential of human ASCs.
These outcomes have practical implications with respect to the development of Standard Operating Procedures for cGMP manufacture of clinical grade human ASCs.

MATERIALS AND METHODS
Isolation and Culture of ASC: ASC were isolated from fresh human subcutaneous adipose lipoaspirate according to published methods with some minor modifications [2].
Enzimatic digestion: Digestion for 3, 5, 7, 10, 15 and 20 minutes was performed. Cell suspension was collected and analyzed on Cellometer for total cell count and cell viability.
Cell count and cell viability: After enzymatic digestion for each time point, 10 µl of cell suspension were resuspended in 10 µl of Trypan blue and pipetted to Cellometer slides to be analyzed.
Differentiation assay: Confluent cultures of ASC (P1) were induced with Adipogenic Differentiation Medium for 3 days before being converted to Adipocyte Maintenance Medium. Cells were maintained for 9 days before fixation and Oil Red O staining. Confluent cultures of ASCs were converted to Osteogenic Medium, maintained in culture for 9-12 days and stained with Alizarin Red.
Flow Cytometry: After 15 minutes of digestion, hASCs were incubated with phycoerythrin conjugated monoclonal antibodies directed against CD29, CD73, CD105 and IgG1κ control. hASCs isolated at days 0, 1, 2 and 3 were analyzed for: CD29, CD34, CD44, CD45,

CD73, CD90, CD105 and IgG1 control. This antibody panel was selected, in part, based on the ISCT position paper on the criteria for defining MSCs [1].

Statistical Methods: Values are presented as the mean ± S.D. Student t-test was used to evaluate significance ($p < 0.05$).

RESULTS

Trypsin alternatives

Results show that there is no significant difference between Trypsin and animal-free alternatives tested, TrypLE Express and TrypZean, in total cell recovered number and their viability; immunophenotype and differentiation capacity in adipogenic and osteogenic lineages is maintained. These results show that non-animal sources of trypsin perform as effectively as the porcine-derived product, suggesting that these reagents can be substituted into existing protocols without loss of yield or function.

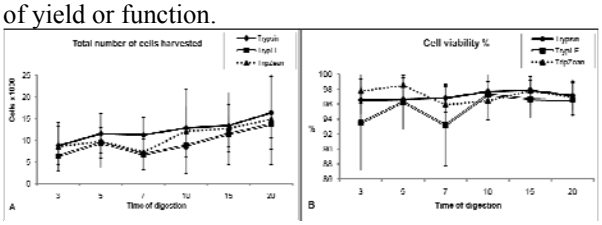

Figure 1 – Total number of cells and viability. One well of a 6 well plate was harvested for each time point of digestion with each product (Trypsin, Tryple and TrypZean). Cellometer analysis assessed **A)** total number of cells harvest per well and **B)** cell viability. A total of n=5 donors were assessed and no significant difference was found among the three trypsin protein sources (t Student test; $p<0.05$).

Effect of time delayed tissue storage

Results show significant differences between total number of nucleated cells obtained in stromal vascular fractions (SVF) harvested on day 0 relative to days 1, 2 and 3 for lipoaspirates stored at ambient (room) temperatures. Nevertheless, after seeding and expansion for an equivalent length of time in culture, there was no significant difference between ASC yields obtained on day 0 and day 1 of storage; however, ASC yields declined significantly on subsequent days.

Flow cytometric analysis showed no significant difference in the immunophenotype of ASCs obtained throughout the four day period.

	Day 0	Day 1	Day 2	Day 3
CD29	88.0 ± 20.9	96.5 ± 2.0	92.7 ± 6.6	89.9 ± 13.9
CD34	91.6 ± 3.4	91.7 ± 5.2	84.9 ± 6.9	84.1 ± 5.9 *
CD44	11.8 ± 4.9	20.3 ± 4.4	15.1 ± 11.5	8.5 ± 5.6
CD45	17.3 ± 7.4	17.5 ± 3.9	8.4 ± 4.2 *	6.0 ± 3.3 *
CD73	79.3 ± 9.2	76.1 ± 8.0	67.5 ± 21.9	75.1 ± 19.2
CD90	86.9 ± 5.5	80.9 ± 7.7	74.3 ± 26.5	68.8 ± 27.7
CD105	94.2 ± 5.5	93.9 ± 3.3	94.8 ± 6.2	96.7 ± 4.1

Table 1 – Flow Cytometry data. The percentage of positive cells for each marker is presented as an average value ± SD in a total of four different donors (n=4). Values with significant difference to day 0 are marked with an asterisk* ($p<0.05$).

Capacity for adipogenic and osteogenic differentiation remained present in cells harvested up to day 3 although a decrease in the intensity of the staining was evident. There were no visible differences in staining between days 0 and 1.

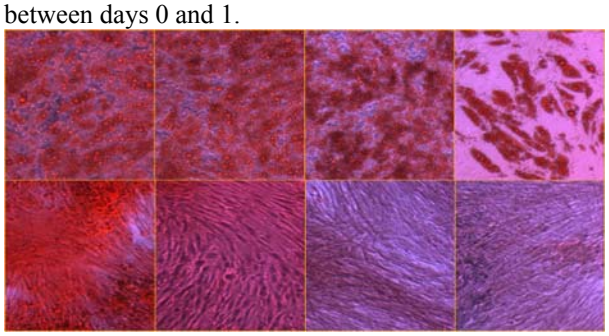

Figure 2 – Adipogenic and Osteogenic differentiation. Representative photomicrographs of adipogenic (up row) and osteogenic (bottom row)differentiation, through 4 consecutive days (left to right – Day 0 to Day 3) from one donor

These results indicate that adipose tissue samples can be successfully processed up to 24 hrs after surgery when stored at room temperature without great lost in total cells harvested.

CONCLUSIONS

We conclude that TrypLE Express and TrypZean can be used in cell culture protocols as viable and effective animal-free alternatives to Trypsin/EDTA. Cell yield, viability and phenotype will remain the same as cells treated with Trypsin/EDTA. This allows the gap between *in vitro* and *in vivo* use of ASCs to be reduced and the optimization of currently used protocols to obtain ASCs for tissue engineering and regenerative medicine.

Our findings indicate that one can obtain hASCs even 72hrs after surgical procedure but the cell yield and differentiation ability is optimal within the first 24hrs. These findings have implications on the ability to ship lipoaspirate from point of surgery to a cGMP processing center without compromising yield or quality of the final product. These studies optimizing GMP methods will have relevance as the use of ASCs in tissue engineering and regenerative medicine grows.

REFERENCES

1. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy.* 2006;8(4):315-317.

2. Dubois SG, Floyd EZ, Zvonic S, Kilroy G, Wu X, Carling S, Halvorsen YD, Ravussin E, Gimble JM. Isolation of Human Adipose-derived Stem Cells from Biopsies and Liposuction Specimens. *Methods Mol Biol.* 2008;449:69-79.

3. Matsumoto D, Shigeura T, Sato K, Inoue K, Suga H, Kato H, Aoi N, Murase S, Gonda K, Yoshimura K. Influences of preservation at various temperatures on liposuction aspirates. *Plast Reconstr Surg.* 2007;120(6):1510-1517.

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