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Developing optimized methods for cGMP compliance in the isolation of human adipose-derived stromal/stem cells.

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KEYWORDS

Adipose-derived stem cells; lipoaspirates; animal-free; trypsin.

ABSTRACT

Multiple steps are needed to make ASC available internationally. Tissue must be stable for up to 24 hrs during transit to cGMP laboratories (Matsumoto et al 2007). Second, isolation and expansion protocols must remove exposure to animal proteins. 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. The current study explores these technical challenges. These outcomes have practically implications with respect to the development of Standard Operating Procedures for cGMP manufacture of clinical grade human ASCs.

INTRODUCTION

This study aimed to explore 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. Standard Operating Procedures (SOPs) that exclude animal protein products need to be developed and validated.
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.

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 (Dubois et al 2008).
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 (Dominici et al 2006).
Statistical Methods: Values are presented as the mean ± S.D. Student t-test was used to evaluate significance ($p < 0.05$).

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RESULTS

Trypsin alternatives

There is no significant difference between Trypsin and animal-free alternatives tested, in total cell recovered number and their viability; immunophenotype and differentiation capacity in adipogenic and osteogenic lineages is maintained.

Lipoaspirate storage

Results show significant differences between total number of nucleated cells obtained in SVF harvested on day 0 relative to days 1, 2 and 3 (room temperature). There was no significant difference between ASC yields on day 0 and day 1. Flow cytometric analysis showed no significant difference in the immunophenotype of ASCs throughout the four day period.

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 in days 2 and 3.

CONCLUSIONS

We conclude that TrypLE Express and TrypZean can be used in cell culture protocols as effective animal-free alternatives to Trypsin/EDTA. Cell yield, viability and phenotype will remain the same as cells treated with Trypsin/EDTA.

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 studies have relevance to the optimization of GMP methods using ASCs in tissue engineering and regenerative medicine.

TABLES AND ILLUSTRATIONS

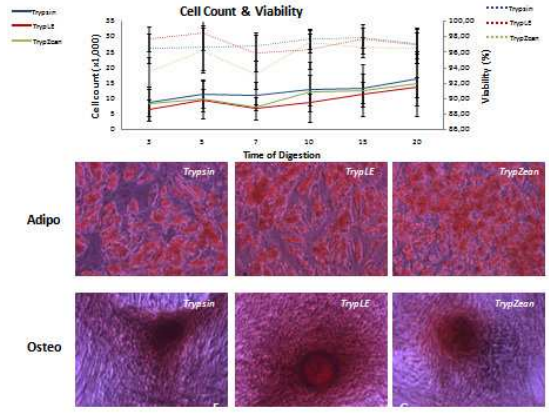

Figure 1 – Cell count, viability, and differentiation potential. (Top Panel) One well of a 6 well plate was harvested for each time point of digestion with each product (Trypsin, Tryple and TrypZean). Cellometer analysis assessed the number of ASCs harvested per well and viability for n = 5 donors (mean ± S.D.); no significant difference was observed across products (t Student test; $p<0.05$). (Bottom Panel) Representative photomicrographs (100x) of ASCs (n = 3 donors) passaged with Trypsin, TrypLE Express or TrypZean digestion for 15 minutes and differentiated under adipogenic (Adipo) conditions for 9 days (Oil Red O staining) or under osteogenic (Osteo) conditions for 14 days (Alizarin Red staining).

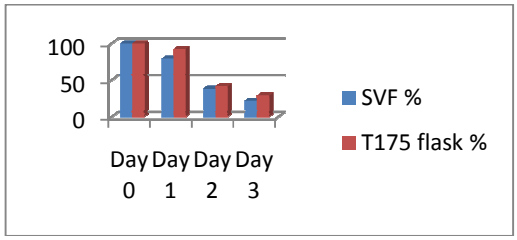

Table 1 – **Total cells harvested:** This table shows the number of cells obtained after each ASC isolation procedure on different days. Both freshly isolated SVF cells and culture expanded ASC per 175 cm^2flask (after 3.5 ±1 days in culture) were assessed. Values represent the average of four different donors (n=4).

REFERENCES

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.

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.

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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