

Original Article

The effect of hyaluronidase on stem cells yielding from infranatant fraction of lipoaspirate

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Abstract. The aim of our study was to evaluate the effect of adding hyaluronidase to the wetting solution used in liposuction on stem cell content and amount in the infranatant fluid of liposuction as a simple and easy way for stem cells isolation. This study was conducted on lipoaspirate obtained from patients asking for body contouring by liposuction. A hundred specimens from the infranatant fluid fraction of the lipoaspirate were included in our study. A high statistical significant difference with a $P < 0.05$ was found in the number of viable adipose-derived stem cells (ADSCs) isolated from infranatant of lipoaspirate by using hyaluronidase (33.5U/ml) compared with that without using it (11.47U/ml). The infranatant fluid of lipoaspirate being a rich source of ADSCs shouldn't be discarded. Time factor using infranatant fluid is much less than that consumed using digestive enzymes from fat layer and also it is more economic. Adding hyaluronidase to the wetting solution increases the yield of isolated stem cells compared to not adding it.

Keywords: Stem cell, ADSC, regenerative medicine

Introduction

Stem cells are a unique population of undifferentiated biological cells that have the capacity to self-renew and differentiate into different cell types. They play a central role in the field of regenerative medicine, aimed at the repair and replacement of diseased cells, tissues and organs through the transplantation of healthy cells and tissues; in particular, stem cells [1].

Human adipose-derived stem cells (ADSCs) are multipotent autologous mesenchymal stem cells. These multipotent cells are recognized as a potential regenerative tool that may be beneficial in a wide variety of medical therapies in reconstructive surgery and in a multitude of other medical disciplines [2].

In humans, ADSCs can be isolated from fat tissue wastes resulting from plastic surgery, i.e. liposuction aspirates and from reconstructive surgeries, through resection of a large tissue fragment. When the starting material is obtained from liposuction procedures, the isolation method is simplified, as the procedure generates finely minced tissue fragments that are more homogeneous, allowing a more efficient enzymatic digestion. When working with whole tissue pieces as starting material, the tissue is minced manually, requiring more time and effort for thorough enzymatic digestion [3].

In order to use stem cells for clinical treatments, there has to be a means to isolate cells in large enough quantities

[4]. The original, pioneering work on the isolation of ADSCs from liposuction waste, typically involves 8–10 hours of continuous intense effort [5,6] -making it a labor-intensive endeavor and increasing the risk of culture contaminations due to excessive handling. Based on recent reports [7] we originally postulated that the blood/saline portion of lipoaspirate waste would prove to be a rich source of ADSCs due to their association with the perivascular space [8].

The aim of our study was to evaluate the effect of adding hyaluronidase to the wetting solution used in liposuction on stem cell content and amount in the infranatant fluid of liposuction as a simple and easy way for stem cells isolation.

Materials and Methods

This study was conducted on lipoaspirate from patients asking for body contouring by liposuction. Written informed consent to participate was obtained from the patients before performing the procedures. A hundred specimens from the infranatant fluid fraction of the lipoaspirate were included in our study. In the sites to be treated with liposuction, we infiltrated the right side with normal wetting solution (epinephrine 1mg for each 500ml of lactated ringer's solution) while the left side was infiltrated with solution to which hyaluronidase was added (hyaluronidase 1IU/1ml of lactated ringer's solution).

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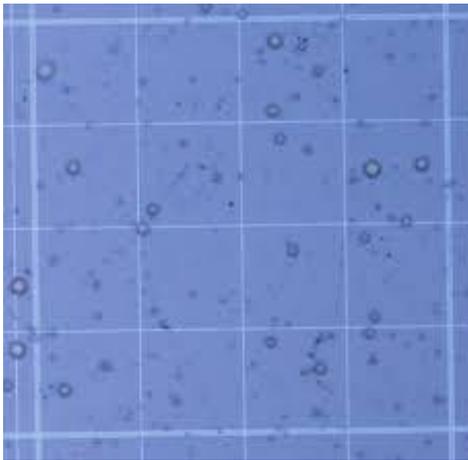


Figure 1: Hemocytometer grid under microscope showing viable unstained cells and stained non-viable cells

Liposuction began 20 minutes after infiltration. The lipo-aspirated specimens were collected in sterile jars, and centrifuged for 10 minutes at 400xg to separate the uppermost fat from the lowermost infranatant fluid; which was used to isolate Adipose derived stem cells (ADSCs).

Isolation of ADSCs from infranatant fluid

The collected specimens were classified into two subgroups:

Subgroup 1: It included 50 specimens, from which isolation of ADSCs from infranatant fluid without adding hyaluronidase enzyme to the wetting fluid. The infranatant fluid fraction was centrifuged at 400xg for 10 min, then Red Blood Cell lyses with 160 mM NH4Cl for 5 min. And final centrifugation for 10 min at 400xg was done to obtain the final pellet of stem cells.

Subgroup 2: It included 50 specimens, from which isolation of ADSCs from infranatant with adding hyaluronidase enzyme. The same steps were used for isolation of ADSCs.

Identification by immunophenotyping using flowcytometry

The isolated ADSCs were characterized for the expression of the markers CD90 and CD 45 by taking cells from the pellet for each method of isolation. The cells were washed in phosphate buffered saline (PBS) and stained using Fluorescein Isothiocyanate (FITC) conjugated CD90 and Phycoerythrin conjugated CD45. Analysis of data was carried out by Fluorescence Activated Cell Sorting caliber (FACS).

Counting of (ADSCs) using trypan blue stain

The glass hemocytometer was cleaned with alcohol before using and the coverslip was cleaned with alcohol and moistened with water and fixed to the hemocytometer. Before the cells have a chance to settle, we took out 0.5 mL of cell suspension using a 5 mL sterile pipette and placed in an Eppendorf tube and then we added 0.5ml of Trypan blue stain and mixed them together in this Eppendorf tube.

TABLE 1
COMPARISON BETWEEN THE NUMBERS OF VIABLE ADSCS

Variable	Isolation by hyaluronidase	No hyaluronidase	P-value
Mean ± SD	33.53 ± 6.23	11.47 ± 3.00	0.000*
Range	23.0 - 48.0	6.0 - 19.0	

Values are per 1 ml and multiplied by 10⁴. Wilcoxon Signed Ranks Test. * Statistical significant difference (P< 0.05).

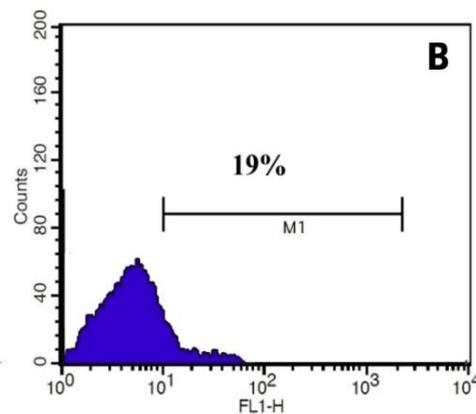
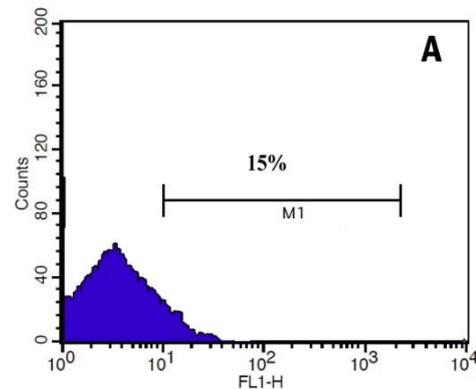


Figure 2. Histogram for flow cytometric expression of ADSCs marker CD90. A. Infranatant with hyaluronidase; B. Infranatant without hyaluronidase.

Using a pipette, we took 100 µL of trypan blue-treated cell suspension and applied to the hemocytometer very gently and filled both chambers underneath the coverslip, allowing the cell suspension to be drawn out by capillary action.

Using a microscope, we focused on the grid lines of the hemocytometer with a 10x objective lens. We counted the viable unstained cells (live cells do not take up Trypan Blue) in the 4 corners of the hemocytometer (Figure 1). We calculated the average from the count of four corners then multiplied by 10,000 multiply by 2 to correct for the 1:2 dilutions from the Trypan blue addition. The final value was the number of viable cells/ml in the original cell suspension.

Example: If the cell counts for each of the 16 squares

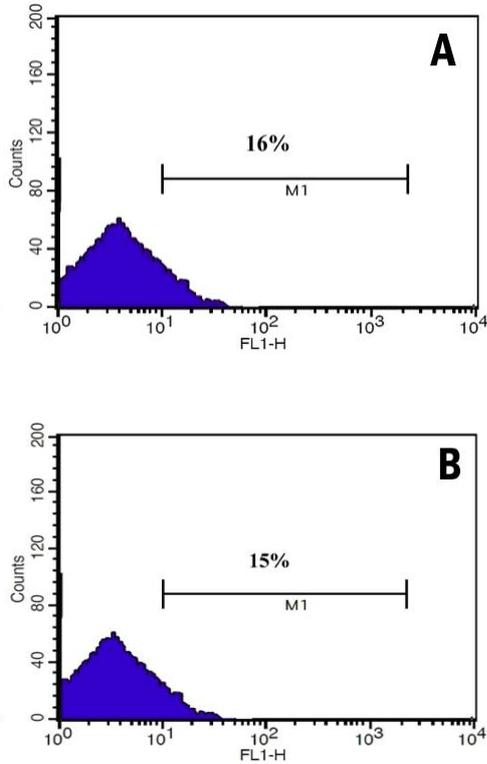


Figure 3. Histogram for flow cytometric expression of ADSCs marker CD44. A. Infranatant with hyaluronidase B. Infranatant without hyaluronidase.

TABLE 2
EXPRESSION OF ADSCS SURFACE MARKER CD90 WERE MEASURED BY FACS ANALYSIS IN ADSCS DERIVED FROM ADIPOSE CONNECTIVE TISSUE

Types	CD90 (%)	CD44 (%)
Unstained cells	2	2
Infranatant with hyaluronidase	19	16
Infranatant without hyaluronidase	15	15

were 50, 40, 45, 52, the average cell count would be:
 $(50 + 40 + 45 + 52) \div 4 = 46.75$
 $46.75 \times 10,000 = 467,500$
 $467,500 \times 2 = 935,000$ live cells/ml in original cell suspension.

Ultrastructural analysis using transmission electron microscopy (TEM)

The cell pellet of (ADSCs) from each method of isolation was taken and fixed with 2.5% glutaraldehyde in phosphate buffer 0.1 M, pH 7.4, for 24 hours at 4C and then post fixed with 1% OsO4 in the same buffer for 1 hour at room temperature. Samples were dehydrated in ascending grades of alcohol and embedded in epoxy resin.

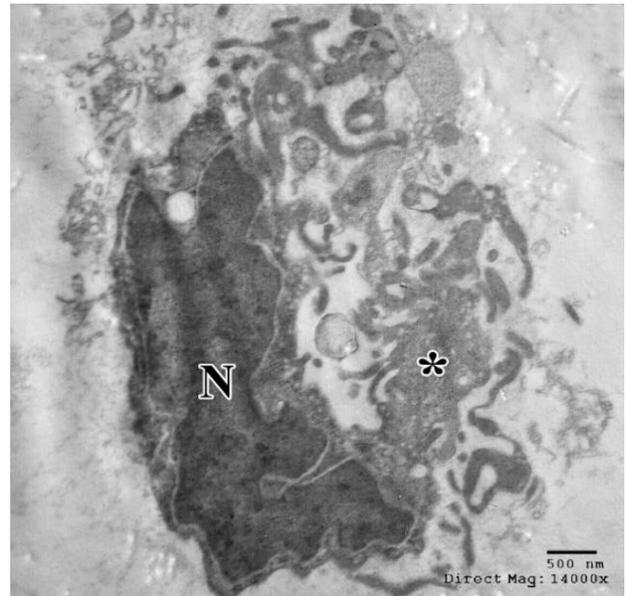


Figure 4. Electron microscopic image of isolated stem cell. N: Nucleus; *: Mitochondria.

Ultra-thin sections were counterstained with lead citrate and uranyl acetate and examined using transmission electron microscopy TEM [JEM – (1200EX) (JEOL Ltd. USA)].

Results

Cell viability assay count and its various relations

We found a high statistical significant deference; with a P value of 0.000 ($P < 0.05$) in the range and mean number of viable ADSC isolated from the infranatant of the lipoaspirate using hyaluronidase (33.5 / ml) compared to that without this enzyme (11.47 / ml). (Table 1, Fig. 2).

Flow cytometric expression of ADSCs

FACS analysis of isolated stem cells demonstrated that the isolated ADSCs were positive for the ADSCs markers; CD90, and CD44; being higher using Hyaluronidase (19%-16%) in comparison to not using it (Tables 2, Figs. 2, 3).

Transmission electron microscopic (TEM) evaluation of quality of enzyme dissociation

Under the TEM, the cytoplasm of the ADSCs isolated were moderately electron-dense. It contained numerous mitochondria and rough endoplasmic reticulums and electron-dense material. ADSCs isolated from the infranatant by both methods revealed large nuclei (N), located at one side of the cell and intact cellular membrane (Fig. 4).

Time factor

Time needed for isolation of ADSCs from the nfranatant fluid fraction of the lipoaspirate was around 1 hour which is the needed time for centrifugation and the use of red blood cell lyses buffer.

Calculation of cell yield in one liter of the infranatant fluid

With a simple multiplication equation; the 50cc specimen of the infranatant with or without hyaluronidase gives 2ml pellets and we have the mean of viable cells per ml of pellet for both. Considering that a liposuction procedure would result in 5 liter lipoaspirate 20% of which is fluid = 1 liter (1000cc) of infranatant fluid, thus each liposuction can obtain (1000/50) around 20 specimen. So, from 1 liter we can calculate the yield of viable cells as follows given that each specimen gives an average of 2ml pellet:

(The number of specimens in a liter * 2 * the number of viable cells in one milliliter) $20 \times 2 \times 33.5 \times 10^4 = 14 \times 10^6$. This is the yield from infranatant fluid fraction using hyaluronidase. While a liter of infranatant without using hyaluronidase can be calculated using the same equation:

(The number of specimens in a liter * 2 * the number of viable cells in one milliliter) $20 \times 2 \times 11.47 \times 10^4 = 4.5 \times 10^6$.

Discussion

Liposuction is a frequently performed cosmetic surgical procedure and the aspirated adipose tissue is usually discarded however, this tissue is a rich source of ADSCs and so can be used without culturing in treatments requiring ADSCs. Nevertheless, they still need to be enzymatically digested and centrifuged before transplantation to be more concentrated if more ADSCs are needed [9].

From the infranatant fluid of the lipoaspirate; we isolated ADSCs and had a mean ($9-11 \times 10^4/\text{ml}$) which is in accordance with the work of Francis et al. and Bowen [8, 10] who also isolated ADSCs from the infranatant fluid of lipoaspirate. The time consumed to get ADSCs from the infranatant fluid (1 hour to obtain the pellets), is much less than that consumed during using digestive enzymes with fat (about 8 hours). This method also requires less equipment, doesn't require specialized personnel and has lower costs. Although the yield is much lower than that from fat using collagenase, this method can yield millions of ADSCs using large amounts of the infranatant fluid, considering that it is an easier technique [8].

Conclusions

Hyaluronidase in vivo alone considered an addition to the variable enzymes used to increase the number of ADSCs as it breaks the glycosidic polysaccharide bondage without altering the ultrastructure of the ADSC isolated. Infranatant fluid of lipoaspirate is not a waste product and shouldn't be discarded, it is a rich source of ADSCs. To

enrich the stem cell content obtained from the infranatant fluid of lipoaspirate we can use hyaluronidase in the wetting solution used in the liposuction procedure. Time factor using infranatant fluid for stem cells is much less than that of fat using digestive enzymes and more economic.

Conflict of Interest

The authors declare no financial and conflicts of interest.

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