

# Evaluation of TransFix® Mediated Stabilisation of Adipose-Derived Stromal Vascular Fraction for Delayed Flow Cytometry Analysis

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# Abstract

The increasing implementation of multicentre studies has led to a need for the optimization of a method that allows for accurate post-hoc analysis of patient biological samples. Assessment of total cell number, viability and immunophenotype can present logistical challenges which can be aided by batch processing. The increased sample storage time that this requires necessitates the use of reagents to preserve cellular integrity, viability and immunophenotype. TransFix® is a stabilising reagent that has been developed for the preservation of cell numbers and cell marker expression in peripheral whole blood for up to ten days. This study investigated the use of TransFix® reagent for the preservation of the stromal vascular fraction (SVF) of collagenase digested adipose tissue. It was demonstrated that TransFix® was suitable for accurately measuring nucleated SVF cell numbers for up to seven days as well as back calculating original cell viability. It also stabilised three CD markers commonly used to identify populations within SVF (CD90, CD31 and CD45) for up to seven days. There was no significant difference between the number of CD90, CD31 and CD45 positive cells after stabilisation at Day 7 compared to Day 0 unstabilised samples. The results suggest that TransFix® can be used to preserve a biological mixed cell population from human adipose-derived SVF for up to seven days for accurate post-hoc analysis.

# **Keywords**

TransFix®, Adipose Tissue, Stromal Vascular Fraction, Immunophenotyping

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## **1. Introduction**

Mesenchymal stem cells (MSCs) have been investigated for the treatment of a wide range of medical conditions including graft vs host disease [1] and corneal ulceration [2]. MSCs are present in high numbers in the stromal vascular fraction (SVF) of digested adipose tissue which is comprised of a mixed population of cells including erythrocytes, leukocytes, endothelial cells and MSCs [3] [4]. The SVF could be administered immediately for the treatment of a range of conditions [5]-[7] (as opposed to after MSC expansion in culture), allowing for the treatment to be delivered on the same day that the adipose tissue was collected and prepared. However, for use in multicentre studies where analysis is conducted at a core facility, accurate post-hoc analysis of SVF samples can be impeded. For example, shipping and storage can lead to time-dependant alteration of cell makers and cell viability [8]. For this reason, a method of cellular stabilisation would be highly advantageous to allow for delayed evaluation of total cell number, cell viability and population immunophenotyping by flow cytometry.

TransFix<sup>®</sup> reagent is a stabilising solution that was designed as a fixative to maintain cellular integrity of whole blood for 7 - 10 days at 2°C - 22°C [9]-[11]. Barnett *et al.* [11] reported that there was no loss in antigenicity for approximately 20 cell makers including CD45, CD13, CD34 and CD79b over a 10-day period. Ng *et al.* [12] compared the efficacy of three fixatives and reported that TransFix<sup>®</sup> was the most effective at preserving extracellular markers in whole blood. On this basis, it was hypothesised that the immunophenotype and perhaps the viability of adipose-derived cells could also be maintained for up to 10 days with the use of TransFix<sup>®</sup> reagent.

This study was performed to evaluate the use of TransFix<sup>®</sup> reagent for the stabilisation of the SVF of digested adipose tissue for up to 9 days. This was achieved through the comparison of the total nucleated cell count and viability and the analysis of the immunophenotype of CD31, CD45 and CD90 positive cells in the SVF of unstabilised and stabilised patient samples.

# 2. Materials and Methods

#### 2.1. Reagents

TransFix<sup>®</sup> reagent (Cytomark, Buckingham UK) was used as a stabilising agent. The monoclonal antibodies used for immunophenotyping are outlined in **Table 1**. The cellular stains propidium iodide (PI) and Syto 11 were purchased from Sigma Aldrich (NSW, Australia) and Life Technologies (Australia) respectively. Tru-COUNT<sup>TM</sup> tubes and FACS lysing solution were purchased from Becton Dickinson (Australia) and flow cytometer staining buffer was purchased from eBioscience (SA, Australia).

### **2.2. Patient Samples**

Aliquots of SVF were collected from patients receiving a commercial adipose-derived stem cell procedure prior to administration of the therapy. The patients receiving the commercial therapy provided informed consent to provide an aliquot of their SVF for analysis. This study presents the quality control findings from 77 patients. Patient information was kept restricted and confidential at all times. All samples were deidentified and were labelled in a coded format.

#### 2.3. Adipose-Tissue Processing

The SVF was isolated after collagenase digestion and centrifugation as previously reported [13]. Briefly, the lipoaspirate was digested with 0.5 mg·mL<sup>-1</sup> collagenase (human grade, NB6 GMP, SERVA Electrophoresis

Table 1. Monocional antibodies used id	or immunolluorescence cell	staining.	
Antibody	Flurochrome	Clone	Manufacturer
Mouse IgG1 $\kappa$ iso control	FITC	P3.6.2.8.1	eBioscience
Anti-human CD31	FITC	WM-59	eBioscience
Anti-human CD45	FITC	HI30	eBioscience
Anti-human CD90	FITC	eBio5E10 (5E10)	eBioscience

GmbH, Heidelberg, Germany) for 20 minutes. The adipose tissue was subsequently washed and the SVF was isolated by centrifugation (1500 g, 5 min, 23°C) and resuspended in saline. The quantity of adipose tissue collected from each patient was dependent upon the individual and varied between 100 - 300 g.

## 2.4. Sample Preparation

Directly after SVF isolation and resuspension in saline, two or more aliquots (one or more unstablised samples and one or more TransFix<sup>®</sup> samples) were taken. The unstabilised samples consisted of the prepared SVF resuspended in saline for a total of 1 mL. For the TransFix<sup>®</sup>-treated samples, 500  $\mu$ L of the SVF patient sample was combined with 600  $\mu$ L of fetal bovine serum (FBS) and 100  $\mu$ L of TransFix<sup>®</sup> reagent. The chosen concentration of TransFix<sup>®</sup> in the stabilised samples were optimised for adipose-derived cells (data not shown). All samples were stored at 4°C for zero to ten days until required for analysis.

### 2.5. Flow Cytometry

Immunophenotyping, cell count and cell viability of nucleated cells were performed on the samples (**Table 2**) by flow cytometry on a FACScan flow cytometer (Becton Dickinson) with a 488 nm argon laser. The analysis was carried out using CELLQuest software (ver. 3.3). Before analysis, the control and TransFix<sup>®</sup>-treated samples were passed through a 35  $\mu$ m cell strainer (Becton Dickinson) to attain a single cellular suspension. The Day 0 control samples were stored at 4°C and were assessed within 24 hrs of the adipose digestion. The other unstabilised samples and the TransFix<sup>®</sup> samples were stored at 4°C for analysis at various time points up to ten days after initial isolation. Daily QC was performed on the flow cytometer to standardise instrument settings and fluidics using flow-check fluorospheres (Beckman Coulter, Australia). The fluorospheres were used according to manufacturer's instructions.

Subjects	Group 1	Group 2
Type of analysis	Cell count and viability	Immunophenotyping
Subjects (n)	$48^*$	$29^{*}$
Samples		
Control		
Unstabilised (n)	Day 0 (48)	Day 0 (29)
Experimental		
Unstabilised (n)	Day 2 (3)	Day 2 (3)
	Day 4 (3)	Day 4 (3)
	Day 6 (3)	Day 6 (3)
	Day 8 (3)	Day 8 (3)
	Day 10 (3)	Day 10 (3)
Experimental		
Stabilisd ( <i>n</i> )	Day 0 (3)	Day 0 (3)
	Day 1 (9)	Day 1 (3)
	Day 2 (8)	Day 2 (5)
	Day 3 (4)	n/a
	Day 4 (8)	n/a
	Day 5 (18)	Day 5 (3)
	Day 6 (5)	Day 6 (3)
	Day 7 (25)	Day 7 (8)
	Day 8 (6)	n/a
	Day 9 (8)	Day 9 (4)

\*There was no overlap between samples used for immunophenotyping and cell count/viability.

#### **Cell Count and Viability**

Briefly, 100  $\mu$ L of the samples were added to individual TruCOUNT<sup>TM</sup> tubes containing 600  $\mu$ L of Isoflow, 100  $\mu$ L of Syto 11 (0.005 mM) and 200  $\mu$ L of PI (0.015 mM) directly prior to flow cytometry analysis. These prepared tubes were then run on the flow cytometer which was set-up as previously reported [14]. Cells were gated according to FSC characteristics. Live/dead counts were measured by PI exclusion, live cells were denoted by no or little PI staining, whilst dead cells were denoted by high levels of PI staining (**Figure 1**). Cell counts were calculated to the total nucleated cell count in the original patient sample.

#### Monoclonal Antibody Labelling (Immunophenotyping)

Samples were divided into four 50  $\mu$ L aliquots. These were then pelleted (2000 rcf, 5 min) and resuspended in 50  $\mu$ L of staining buffer. The cellular samples were incubated for 30 min at 4°C with 5  $\mu$ L FITC-coupled monoclonal antibodies for CD31, CD45, and CD90 or with 5  $\mu$ L of an isotype control (Mouse IgG1 $\kappa$  IsoControl) to assess the level of background staining. All samples were washed in a staining buffer, and were fixed in 180  $\mu$ L of a 10% formaldehyde solution (FACS lysing buffer). The cells were then stained with PI to achieve a final stain concentration of 0.0015 mM. The proportion of cells in each sample that were CD31, CD45 and CD90 positive was determined according to quadrant analysis using flow cytometry (Figure 2) [15].

#### 2.6. Statistical Analysis

Stabilised and/or unstabilised samples from one patient was compared only to the unstabilised Day 0 controls from that same patient. Data from each pair-wise (control vs experimental sample) comparison was collated, averaged (±standard deviation) and assessed for consistency between patients. Two-tailed, paired t-tests were performed at each time point (control vs stabilised and/or unstabilised samples) to assess statistical significance.



**Figure 1.** Primary data and gating strategies for flow cytometric analysis of cell number and viability. (A) Dot plots prepared to identify the cell population of (i) unstabilised Day 0 samples and (ii) samples stabilised for 1 day or more. Analysis was concluded when 500 beads were counted. (B) Dot plots were prepared from the gated cell population to observe level of propidium iodide (PI) staining. Live cells were denoted by little to no PI staining. Note that cell number appears lower in the stabilised sample due to differences in dilution factor.



**Figure 2.** Primary data and gating strategies for immunophenotyping analysis. (A) Dot plots, (B) dot plots with quadrant analysis gated off the cell population in (A), and (C) overlay histograms prepared to analyse the immunophenotype of unstabilised Day 0 samples (subject 105). Samples were stained with FITC conjugated,  $IgG\kappa 1$  antibodies: (i) a mouse  $IgG\kappa 1$  Isocontrol (negative control), (ii) CD31, (iii) CD45, and (iv) CD90. A comparison of the isocontrol (black) and CD31, CD45 or CD90 (red) was performed using overlay histograms (B).

A p < 0.05 was considered statistically significant. Statistical analysis and graphing of data was performed using GraphPad Prism 6 (vers. 6.01, 2010, California, United States) and Flowing Software 2 (vers. 2.5.1, Finland).

# 3. Results

## 3.1. Cell Count and Viability

The cell counts and cell viability values attained from the Day 0 unstablised controls were normalised to 100% to present as the baseline. A significant increase in total nucleated cell count after TransFix<sup>®</sup> stabilisation was observed (**Figure 3**). After seven days of storage in TransFix<sup>®</sup> reagent, the mean nucleated cell count for each sample was  $127\% \pm 50.2\%$  of the corresponding Day 0 unstabilised sample.

Total cell viability was  $66\% \pm 11.7\%$  at Day 0, which then decreased and plateaued to  $43\% \pm 13.4\%$  after stabilisation for nine days. Normalisation of this data (Day 0 samples normalised to 100%) demonstrated a declining trend of approximately 40% of the initial viability after stabilisation for seven days at 4°C (Figure 3). A significantly lower viability was observed for each of the TransFix<sup>®</sup> stabilised samples at each storage time point when compared to the Day 0 controls.

Whilst there are some significant differences between the Day 1 to 10 samples, a clear trend can be observed for each condition (Figure 3).



**Figure 3.** Cell number and viability of stabilised and unstabilised samples after extended storage. (A) Viable cell numbers, (B) Total cell numbers and, (C) Cell viability of TransFix<sup>®</sup> stabilised and unstabilised stromal vascular fraction samples after extended storage. Data was normalised to the experimental control (unstabilised, Day 0 samples) which were represented as 100%. Values are mean  $\pm$  SEM, signifcant difference (\*) to Day 0 unstabilised samples if p < 0.05.

## 3.2. Monoclonal Antibody Staining for CD Surface Markers (Immunophenotyping)

There was minimal variation between the proportional presence of CD90 cellular markers before and after stabilisation for up to seven days, and no significant difference for CD31 cellular markers (Figure 4). There was, however, a significant difference observed between the corresponding Day 0 control samples and the samples stabilised for nine days for both CD90 and CD31 markers (Figure 4). The proportion of CD31 and CD90 posi-



**Figure 4.** Immunophenotype of stabilised and unstabilised samples after extended storage. Difference between the percentage of (A) CD90+ cells, (B) CD31+ cells and (C) CD45+ cells in the Day 0 unstabilised samples and the TransFix<sup>®</sup> stabilised samples (control minus experimental) at multiple time points as measured by immunophenotyping. (D) Analysis of the immunophenotype of stabilised (7 days) and unstabilised (8 days) samples after extended storage was performed. Mouse anti-human CD90, CD31 or CD45 IgG $\kappa$ 1 antibody staining and flow cytometric analysis were used. The plots document the median, min and max value for each data set, significant difference (<sup>\*</sup>) to Day 0 control if p < 0.05. Positive y-axis values indicate that the CD90+, CD31+ and CD45+ population was found to be higher in the Day 0 control than the experimental sample(s) from the same patient at each time point.

tive cells in the Day 9 samples was significantly lower than that observed in the original unstabilised Day 0 samples, indicating a notable discrepancy at this time point between the Day 0 unstabilised and the Day 9 stabilised samples for each patient. The variation that is observed in the Day 1 data for CD90 (Figure 4) is a result of one sample that displayed a 20% increase of CD90+ cells after one day of stabilisation. Considerable biological variation in the proportion of CD31+ cells for each patient was evidenced in all samples.

There was no significant difference in the presence of the CD45 marker between the unstabilised and the stabilised samples for Day 1, 2, 6 and 7 (**Figure 4**). Interestingly, each patient sample stabilised for five days (n = 3) had a significantly higher proportion of CD45+ cells than its Day 0 unstabilised counterpart. In addition, considerable biological variation in the proportion of CD45+ cells was observed for every time point except for the Day 5 samples. The significant variation observed at the Day 1 stabilised comparison is a result of one sample, which appeared to increase the percentage of CD45+ cells by 35% after one day of stabilisation. Although this difference is quite large, the results for the stabilised counterpart are closer to the average proportion of CD45+ cells in adipose tissue ( $42.4\% \pm 10.4\%$ ) than the Day 0 samples.

Likewise to the results observed for the CD90 and CD31 markers, there was a significant difference in the amount of CD45+ cells observed after nine days of stabilisation when compared to the Day 0 unstabilised counterparts (**Figure 4**). The biological variation observed for the presence of CD45+ cells in the Day 0 unstabilised samples was notable, with the presence of CD45+ varying between 9% and 73% of the total population.

After extended storage without a stabilising reagent, there was a significant loss of CD90 and CD45 positive cells when compared to the stabilised counterpart, however the proportion of CD31 positive cells remained relatively consistent (Figure 4).

#### 4. Discussion

Biological variation can be a confounding factor in patient to patient comparisons, especially so at the cellular level. For this reason, each patient served as their own control, wherein the results of all the cell samples were compared to the results of the corresponding Day 0, unstabilised aliquot. The biological variation of cell types in Day 0 samples was demonstrated by the widely varying proportion of CD45+ cells in the samples (between 9% and 73%, n = 29). The proportion of how many CD45+ cells (leukocytes) in blood, and likewise in a sample of SVF, can be affected by a range of factors such as the immune status of the patient on the day of the procedure, any exposure to pro-inflammatory stimuli from diet or environment, or the overall weight of a patient [16]-[18].

It was anticipated that the total nucleated cell count of the Day 0 samples would be the maximal cell count of all samples (unstabilised and stabilised) as storage time before analysis was minimal (up to 24 hrs, as opposed to one to nine days). However, the opposite proved to be true (**Figure 3**). There was a statistically significant increase in total cell number after stabilisation across all storage times compared to the Day 0 controls. The increased nucleated cell count after stabilisation is potentially a result of the disruption of cell clumping in the samples, which was not actively inhibited in the unstabilised samples. The impact of TransFix<sup>®</sup> on cell aggregation has not been previously reported in the literature. The fixative properties of TransFix<sup>®</sup> reagent also appear to be able to stabilise cellular protein structures for immunophenotyping in white blood cells [11], and so it is likely that it also inhibits processes involved in cell division and protein synthesis.

We hypothesise that the stabilised cell counts in this data set are likely to be more accurate in the quantification of single cells in suspension because of the inhibition of cellular processes that lead to cell loss such as cell clumping. Filtration of the sample to remove cell clumps prior to flow cytometry is essential, but this can result in a significant discrepancy between the total cell number that is counted and the actual cell number that is present in the sample. The use of TransFix<sup>®</sup> stabilisation may prevent cell aggregation, explaining the significant typical increase in total cell number in the samples after stabilisation and storage. Additionally, the TransFix<sup>®</sup> treated samples were stabilised immediately after SVF sample preparation, whilst unstabilised samples were stored at 4°C for up to 24 hrs before assessment without the addition of inhibitors of cell division, apoptosis, the formation of clumps or cell-matrix aggregation. Storage of whole blood at 4°C has been previously demonstrated to negatively impact cell viability [19], cell population yield [20], and function [21]. The cell viability dropped consistently after stabilisation for seven days, in that the TransFix<sup>®</sup> samples are likely to have a viability that is  $62.8\% \pm 16.5\%$  of the Day 0 samples (Figure 3). As a result of this, an estimate of the original cell viability can be back-calculated from the stabilised data.

For immunophenotyping, CD90, CD31 and CD45 cell markers were largely preserved for use up to seven

days post-collection in TransFix<sup>®</sup> reagent, with no statistical difference between the results attained post and pre-stabilisation (with the exception of the CD45 marker at five days) (Figure 4). The results at Day 5 do not appear to demonstrate the biological variation observed at each other time point, however further analysis would be required to test this hypothesis.

## **5.** Conclusion

For post-hoc analysis of cell count and viability of nucleated cells in the stromal vascular fraction of digested adipose tissue, it is concluded that the sample remains stable in TransFix<sup>®</sup> reagent for up to seven days with the ability to provide an accurate total nucleated cell count and to back calculate the viability of the original sample. TransFix<sup>®</sup> reagent also appears to capable of preserving the CD90, CD31 and CD45 cellular marker on cells within an adipose-derived mixed cell population for up to seven days after stabilisation.

#### **References**

- [1] Herrmann, R., Sturm, M., Shaw, K., Purtill, D., Cooney, J., Wright, M., et al. (2102) Mesenchymal Stromal Cell Therapy for Steroid-Refractory Acute and Chronic Graft versus Host Disease: A Phase 1 Study. International Journal of Hematology, 95, 182-188. <u>http://dx.doi.org/10.1007/s12185-011-0989-2</u>
- [2] Oh, J.Y., Kim, M.K., Shin, M.S., Lee, H.J., Ko, J.H., Wee, W.R., et al. (2008) The Anti-Inflammatory and Anti-Angiogenic Role of Mesenchymal Stem Cells in Corneal Wound Healing Following Chemical Injury. Stem Cells, 26, 1047-1055. <u>http://dx.doi.org/10.1634/stemcells.2007-0737</u>
- [3] Varma, M.J.O., Breuls, R.G., Schouten, T.E., Jurgens, W.J., Bontkes, H.J., Schuurhuis, G.J., et al. (2007) Phenotypical and Functional Characterization of Freshly Isolated Adipose Tissue-Derived Stem Cells. Stem Cells and Development, 16, 91-104. <u>http://dx.doi.org/10.1089/scd.2006.0026</u>
- [4] De Ugarte, D., Morizono, K., Elbarbary, A., Alfonso, Z., Zuk, P., Zhu, M., et al. (2003) Comparison of Multi-Lineage Cells from Human Adipose Tissue and Bone Marrow. Cells, Tissues, Organs, 174, 101-109. <u>http://dx.doi.org/10.1159/000071150</u>
- [5] Riordan, N.H., Ichim, T.E., Min, W.P., Wang, H., Solano, F., Lara, F., et al. (2009) Non-Expanded Adipose Stromal Vascular Fraction Cell Therapy for Multiple Sclerosis. *Journal of Translational Medicine*, 7, 29. http://dx.doi.org/10.1186/1479-5876-7-29
- [6] Zimmerlin, L., Rubin, J.P., Pfeifer, M.E., Moore, L.R., Donnenberg, V.S. and Donnenberg, A.D. (2013) Human Adipose Stromal Vascular Cell Delivery in a Fibrin Spray. *Cytotherapy*, **15**, 102-108. <u>http://dx.doi.org/10.1016/j.jcyt.2012.10.009</u>
- [7] Premaratne, G.U., Ma, L.P., Fujita, M., Lin, X., Bollano, E. and Fu, M. (2011) Stromal Vascular Fraction Transplantation as an Alternative Therapy for Ischemic Heart Failure: Anti-Inflammatory Role. *Journal of Cardiothoracic Surgery*, 6, 43. <u>http://dx.doi.org/10.1186/1749-8090-6-43</u>
- [8] Olson, W.C., Smolkin, M.E., Farris, E.M., Fink, R.J., Czarkowski, A.R., Fink, J.H., et al. (2011) Shipping Blood to a Central Laboratory in Multicenter Clinical Trials: Affect of Ambient Temperature on Specimen Temperature, and Effects of Temperature on Mononuclear Cell Yield, Viability and Immunologic Function. *Journal of Translational Medicine*, 9, 26. <u>http://dx.doi.org/10.1186/1479-5876-9-26</u>
- [9] Canonico, B., Zamai, L., Burattini, S., Granger, V., Mannello, F., Gobbi, P., et al. (2004) Evaluation of Leukocyte Stabilisation in TransFix-Treated Blood Samples by Flow Cytometry and Transmission Electron Microscopy. Journal of Immunological Methods, 295, 67-78. <u>http://dx.doi.org/10.1016/j.jim.2004.09.013</u>
- [10] Jani, V., Janossy, G., Iqbal, A., Mhalu, F., Lyamuya, E., Biberfeld, G., et al. (2001) Affordable CD4+ T Cell Counts by Flow Cytometry. II. The Use of Fixed Whole Blood in Resource-Poor Settings. *Journal of Immunological Methods*, 257, 145-154. <u>http://dx.doi.org/10.1016/S0022-1759(01)00458-6</u>
- [11] Barnett, D., Granger, V., Pockley, A., Saxton, J., Storie, I., Whitby, L., et al. (1999) TransFix: A Clinical Sample Stabilising Fluid for Use in Cellular Haematology and Immunology. Cytometry, 38, 88.
- [12] Ng, A.A., Lee, B.T., Teo, T.S., Poidinger, M. and Connolly, J.E. (2012) Optimal Cellular Preservation for High Dimensional Flow Cytometric Analysis of Multicentre Trials. *Journal of Immunological Methods*, 385, 79-89. http://dx.doi.org/10.1016/j.jim.2012.08.010
- [13] Blaber, S.P., Webster, R.A., Hill, C.J., Breen, E.J., Kuah, D., Vesey, G. and Herbert, B.R. (2012) Analysis of *in Vitro* Secretion Profiles from Adipose-Derived Cell Populations. *Journal of Translational Medicine*, 10, 172. http://dx.doi.org/10.1186/1479-5876-10-172
- [14] Sasaki, D.T., Dumas, S.E. and Engleman, E.G. (1987) Discrimination of Viable and Non-Viable Cells Using Propidium Iodide in Two Color Immunofluorescence. *Cytometry*, 8, 413-420.

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http://dx.doi.org/10.1002/cyto.990080411
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- [15] Oldaker, T.A. (2007) Quality Control in Clinical Flow Cytometry. *Clinics in Laboratory Medicine*, 27, 671-685. <u>http://dx.doi.org/10.1016/j.cll.2007.05.009</u>
- [16] Field, J., Gougeon, R. and Marliss, E.B. (1991) Changes in Circulating Leukocytes and Mitogen Responses during Very-Low-Energy All-Protein Reducing Diets. *American Journal of Clinical Nutrition*, 54, 123-129.
- [17] Kondo, T., Hayashi, M., Takeshita, K., Numaguchi, Y., Kobayashi, K., Iino, S., Inden, Y. and Murohara, T. (2004) Smoking Cessation Rapidly Increases Circulating Progenitor Cells in Peripheral Blood in Chronic Smokers. *Arterio-sclerosis, Thrombosis, and Vascular Biology*, 24, 1442-1447. <u>http://dx.doi.org/10.1161/01.ATV.0000135655.52088.c5</u>
- [18] Potter, C.G., Potter, A.C., Hatton, C.S., Chapel, H.M., Anderson, M.J., Pattison, J.R., *et al.* (1987) Variation of Erythroid and Myeloid Precursors in the Marrow and Peripheral Blood of Volunteer Subjects Infected with Human Parvovirus (B19). *Journal of Clinical Investigation*, **79**, 1486-1492. <u>http://dx.doi.org/10.1172/JCI112978</u>
- [19] Ashmore, L.M., Shopp, G.M. and Edwards, B.S. (1989) Lymphocyte Subset Analysis by Flow Cytometry Comparison of Three Different Staining Techniques and Effects of Blood Storage. *Journal of Immunological Methods*, **118**, 209-215. http://dx.doi.org/10.1016/0022-1759(89)90008-2
- [20] Garraud, O. and Moreau, T. (1984) Effect of Blood Storage on Lymphocyte Subpopulations. *Journal of Immunological Methods*, 75, 95-98. <u>http://dx.doi.org/10.1016/0022-1759(84)90228-X</u>
- [21] McKenna, K.C., Beatty, K.M., Vicetti, M.R. and Bilonick, R.A. (2009) Delayed Processing of Blood Increases the Frequency of Activated CD11b+ CD15+ Granulocytes Which Inhibit T Cell Function. *Journal of Immunological Methods*, 341, 68-75. <u>http://dx.doi.org/10.1016/j.jim.2008.10.019</u>



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