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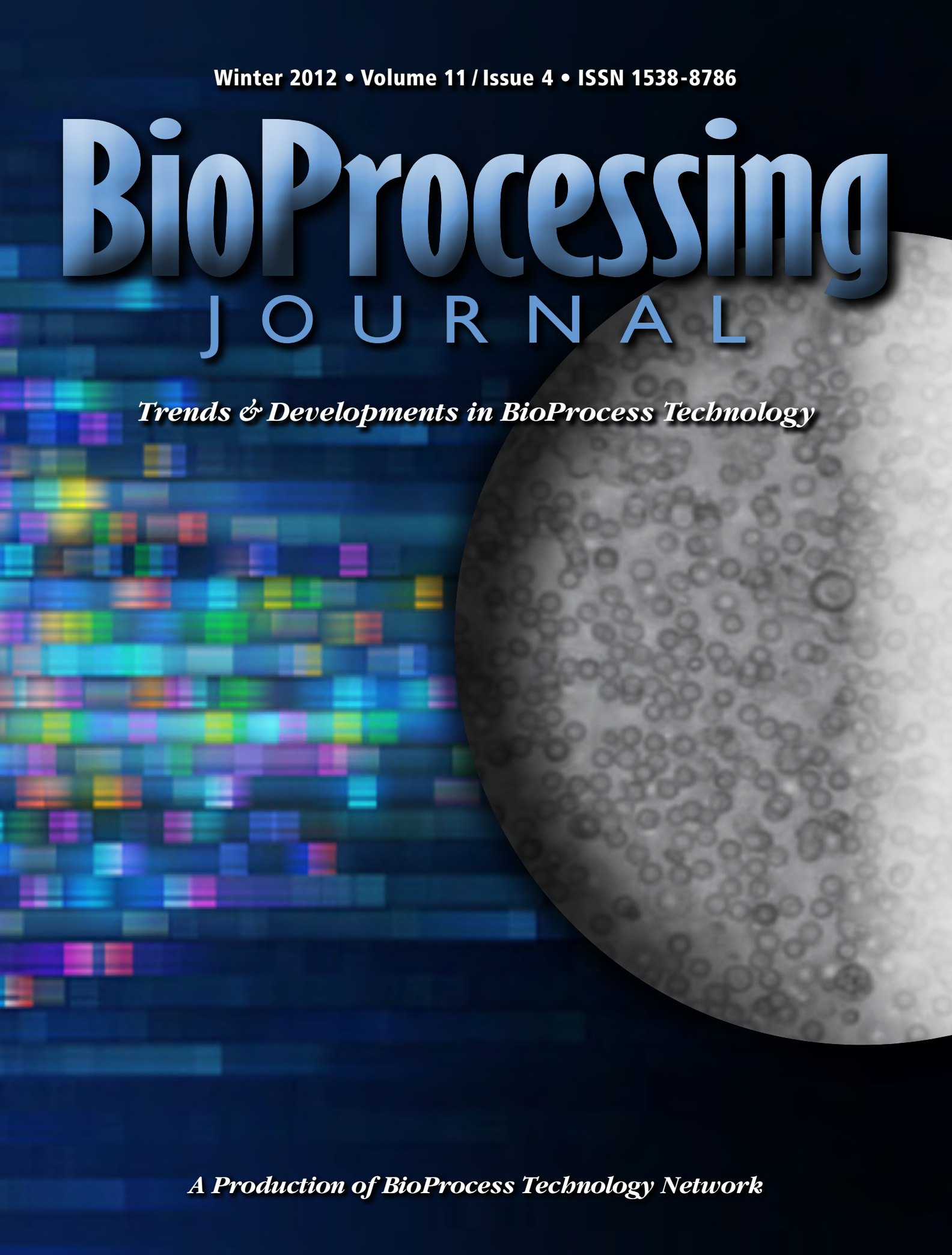

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Counting and Processing Methods Impact Accuracy of Adipose Stem Cell Doses

By DAVID G. MORRISON, DIRK A HUNT, ISAAC GARZA, ROBBIE A. JOHNSON, and MARY PAT MOYER*

Cell therapy products derived from adipose tissue have some unique processing issues with regard to obtaining accurate cell counts. This is because processing methods may not only show us the nucleated stromal vascular fraction (SVF) cells but also the micellular and microvesicle particles. This is true for both veterinary and human clinical products, and poses special concerns for in-clinic processing where the cell therapy dose is correlated with cell numbers and other QC data is not especially useful.

In this study, multiple cell counting methods were compared for SVF cell preparation that were derived from canine adipose tissue using commercially-available processing kits. The data clearly showed that many non-nucleated particles appear cell-like by size and shape, and can lead to counting errors with automated counters. In addition, certain reagents important to processing can have properties wherein the reagents alone (*e.g.*, lecithin) may be counted as cells. The most accurate cell numbers were from hemocytometer-counting of cells stained with 4′,6-diamidino-2-phenylindole (DAPI) which shows the nuclei in concert with a viability stain such as trypan blue. The data clearly showed that care must be taken when counting cells used as a therapeutic dose.

Introduction

Adipose tissue contains blood elements, adipocytes, vascular cells, and stromal cells. Multi-potent, regenerative cells are found in the stromal vascular fraction of adipose tissue, and SVF cells are the subject of intense basic and clinical research.[1-9] These SVF cells can replicate and differentiate into vascular, bony, neural, and cartilaginous components when isolated and put into the correct microenvironment.[10-12] Currently, SVF cells separated from surgically-resected fat are used by veterinarians for injection into animals afflicted with arthritis and hip dysplasia.[13] Results show that stem cells hold great promise to treat human osteoarthritis.[14] As summarized in a recently posted white paper[15], the dose of therapeutic SVF cells is based on cell numbers, and the accuracy of cell counts can vary widely depending on the processing and counting methods used.

Adipose tissue "point-of-care" harvesting equipment and systems to release SVF for autologous use are of significant clinical and business interest.[16] Although Cytori and others have CE-mark systems (conforming

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to EU standards) used in Europe, and there are FDA-approved collection devices and ongoing clinical trials with various collection devices and methods, no complete harvest and cell isolation systems have been approved by the FDA for autologous SVF harvest for immediate use. In animals, such "point-of-care" harvesting products to release cells for autologous use (*e.g.*, for arthritis) are currently being marketed to veterinary offices. One such kit, the Adipose Stem Cell Procedure Kit sold by MediVet America (the "MediVet Kit"), was chosen for this study. The rationale was that it would allow parallel testing of multiple counting methods with very high cell yields expected (*e.g.*, 5–20 and up to 60 million cells per gram of fat tissue) based on claims made by the creators of the MediVet Kit process.[17] It is generally recognized that MediVet's numbers are 10 to 20-fold higher than those reported for a variety of mammalian species in peer-reviewed scientific literature (*e.g.*, [18-20]), and also by product competitors (*e.g.*, Vet-Stem). The concern by researchers was that SVF cells extracted from processed fat tissue weren't necessarily the only things being counted. Also included were artifacts from the tissue dissociation, processing methods, and/or the reagents used. For example, micellular and fat droplets that might have a cell-like size and appearance (*i.e.*, round, refractile, and/or autofluorescent) might be mistaken for cells.

One common method for counting cells and determining viability in a population is a dye exclusion assay in which viable cells exclude the entry of trypan blue whereas dead or dying cells do not. Micellular structures, if present in such a cell preparation, would not take up the dye since they would not have active exclusion functions like live cells. Thus, they would be clear and refractile and would appear to be viable. In other methods, such as acridine orange (AO) staining, non-specific fluorescence could easily be misinterpreted, leading to: (1) incorrect and oftentimes over-estimated readings by automated cell counting analyzers; and (2) invalid calculations of the true cell numbers. The final result would be erroneous treatment dosing and inaccurate numbers for cell banking (before and after thawing).

The purpose of this study was to evaluate the cell numbers obtained using the MediVet Kit processing method, implement the recommended equipment, and assess the relative accuracy of various counting methods in evaluating the isolated adipose-derived SVF cells. In an effort to identify potential sources for cell counting errors, this study also included analyses of the emulsifying Solution E (part of the MediVet Kit) and platelet-rich plasma (PRP) which is prepared from donor blood as part of the tissue processing protocol.

The concern about inaccurate counting is further emphasized by data obtained from Peter Hanson's lab at Monash University. They used different equipment (the guava® easyCyte™ flow cytometer [EMD Millipore]) than in this study but found similar counting errors in processed dog SVFs from fat tissue, especially when emulsifying agents were used for isolation.[21] Likewise, Barbara Krutchkoff, an independent consultant, recognized similar problems when human cells were isolated using lecithin methods.[21]

Materials and Methods

Tissue Donors and Blood

Adipose tissue was surgically removed from each donor. Blood was collected and shipped in acid citrate dextrose (ACD) vacutainer tubes. All refrigerated adipose tissue and blood (materials) were received with Chain-of-Custody transfer documentation and standard procedures for receipt of clinical source samples. Materials were transferred to the laboratory and prepared for processing.

MediVet Kit Methods

SVF cells were isolated from approximately 20 g of adipose tissue from three dogs (dog #1: 19.17 g; dog #2: 20.0 g; dog #3: 20.7 g) using MediVet Kit procedures[17] with no deviations from the manufacturer's instructions. Blood was processed to prepare PRP as described in the kit protocol. As part of the MediVet protocol, the cells were light-activated with their three-color Medi-Light device, included in the kit. Because this was a novel process among SVF cell processing methods, part of the purified SVF cells were excluded from the light activation to evaluate the effect that the light had on the vitality and proliferation ability of the cells.

Vital Dyes and Fluorescent Probes

Trypan blue solution, neutral red (NR), and hematoxylin solution were purchased from Sigma-Aldrich. DAPI, acridine orange, CellMask™ orange (CM), and propidium iodide (PI) were purchased from Life Technologies/Molecular Probes™. The Cellometer® ViaStain™ was supplied by Nexcelom, and the Giemsa Diff-Quick Stain Set® was purchased from Siemens Healthcare Diagnostics. The unique features of these

reagents have been detailed elsewhere[22], but Tables 1 and 2 briefly describe their detection and use in this study.

Microscopy

Fluorescence microscopy was done using an EVOS® microscope (Advanced Microscopy Group [AMG]) equipped with planar non-fluorescence light microscopy objectives, digital image capture, fluorescence objectives light cubes, and wavelengths appropriate to the dye used (Table 1). Evaluation of samples was done by capturing images with either transmitted light or fluorescence using EVOS software. The transmitted light and fluorescent captured images were used alone, or multiple images taken of the same frame of material captured at multiple wavelengths were overlaid to demonstrate light and/or fluorescent staining overlap.

Light magnification of stained or unstained cells was done using various microscopes and imaging methods, including: (1) the EVOS, with planar objectives; (2) a Nikon Diaphot inverted microscope equipped with a CCD camera; or (3) a Wetzlar Hund microscope equipped with a Nikon camera. Selected representative individual and composite photographs were taken.

Cell Counting Methods and Visualization

Cells were prepared for counting as SVF using MediVet Kit methods. PRP and the 1:10 dilution of Solution E were also included in the assessment of cell counting methods. Cell numbers and viability percentages were assessed by the methods shown in Table 2. Values were back-calculated to original grams of tissue weight to determine cell numbers per gram of adipose tissue.

TABLE 1. Fluorescent filters and dyes used

Fluorescent Filter	Wavelengths Ex/Em	Dyes Used
Blue	360 nm Ex, 447 nm Em	DAPI
Green	470 nm Ex, 525 nm Em	AO and autofluor
Red	530 nm Ex, 593 nm Em	PI, CM, AO, and autofluor

TABLE 2. Methods used to count and visualize cells

Equipment*	Description of Method	Comments
Cellometer	Automated cell counting using AO/PI method	Analyzed with default settings as per MediVet Kit guidelines; other studies with gating
NucleoCounter	Automated cell counting using propidium iodide method	Used company-provided specifications for analyses
HemaTrue	Automated cell counting using the Coulter Principle	Clinical equipment; done as contract service at local veterinary clinic
Countess	Automated cell counting using trypan blue (0.5%) dye exclusion	A routine-use tool in the cell culture lab
Hemocytometer and Microscopy	Manual cell counting using:	
	Trypan blue (0.5%) dye exclusion	Standard cell viability assay; viable cells clear and refractile; dead or dying cells blue
	DAPI-stained nuclei	Live and dead cells take up DAPI into nuclei; visualized by fluorescence microscopy
	Trypan blue + DAPI	Uses overlaid photomicrographic images
	Neutral red + DAPI	NR is taken up by live cells but not dead cells
	Trypan blue + NR + DAPI	Blue = dead; red = live; DAPI = bright blue nuclei
	Cell mask + DAPI	CM = labels membranes and lipids red; DAPI = bright blue nuclei
	Acridine orange + DAPI	AO = green nuclei, red cytoplasm; DAPI = bright blue nuclei
	Giemsa Quick-Diff stain	Light microscopy of SVF cells stained dark blue after blood-smear-like preparation

**Equipment details are provided on pages 6–8).*

To better assess the automated counting methods, samples were evaluated by direct visualization with light and fluorescent microscopy. The samples were stained with the same dye combinations that were used in the automated counting methods as well as evaluated for autofluorescence that would interfere with the staining methods. The automated cell counting done using the AO/PI (live/dead) Cellometer method was reanalyzed with gating to eliminate erroneous counting of subcellular sized particles that were interfering with an accurate count.

Direct visualization and automated imaging data are important to compare the counting methods for donor SVF cells, and testing of the Solution E and PRP preparations. The fluorescence parameters, staining methods, and colors of the test dyes by fluorescence or light microscopy have been described in Tables 1 and 2, and are shown in Figure 1 (and later in Figure 4).

Hemocytometer Counts

SVF cells, PRP, and Solution E were all evaluated. Trypan blue, DAPI, and neutral red were utilized to ascertain dead versus living cells and cells versus debris, respectively. Green autofluorescence was also evaluated to help distinguish debris from adipose tissue and ascertain potential sources of counting errors. The technique for use of a hemocytometer has been described elsewhere.[23]

HemaTrue Cell Counts

Samples were done on a Heska HemaTrue® veterinary hematology analyzer by a local veterinarian. The location was less than 0.2 miles from INCELL and samples were read immediately upon receipt at the clinic. The machine, which incorporates the Coulter Principle, was used as per manufacturer's instructions by trained personnel.

NucleoCounter Counts

An on-site NucleoCounter® (ChemoMetec) was utilized for one set of SVF enumerations. The machine was used according to the manufacturer's instructions.

FIGURE 1.

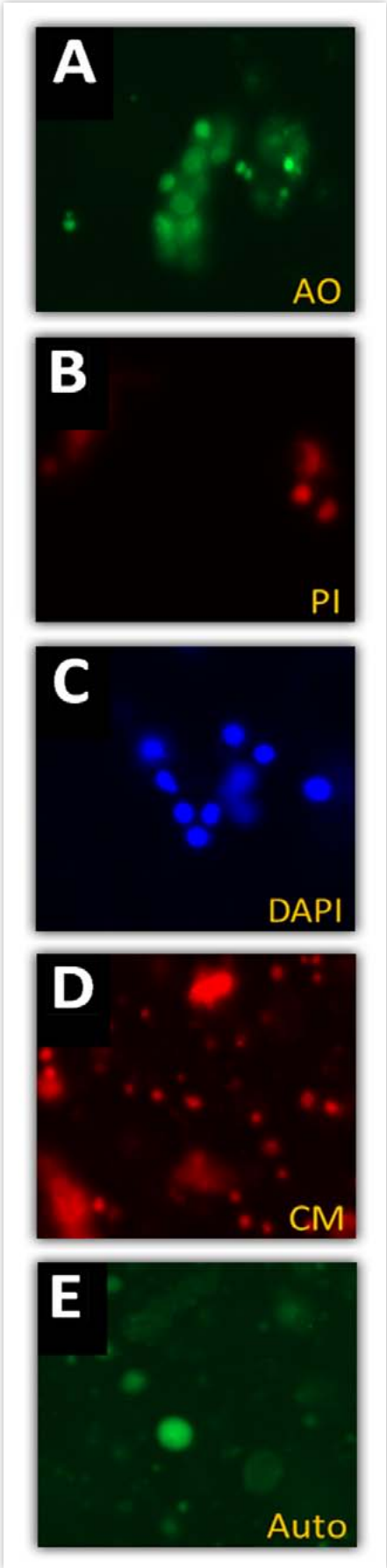
(A) AO stains RNA orange and DNA green. It is used as a counterstain of all cells in the Cellometer device;

(B) PI stains DNA red and can only penetrate non-viable cells. Used as a non-viable indicator in both the NucleoCounter and the Cellometer. Once the cells are treated with a lysis buffer, it is also used for the total cell count for the NucleoCounter;

(C) DAPI stains DNA bright blue and can penetrate living or dead cells;

(D) CM, a red fluorescent dye, is picked up and stays with lipids and fatty acids; and

(E) Auto provides an example of green autofluorescence. This is a natural property of a protein or lipid that fluoresces when exposed to specific wavelengths of light.



Cellometer Counts

A Cellometer Vision® (NC-100) from Nexcelom Biosciences, LLC was used. Staff training was done by a company representative who was familiar with the MediVet method and use of the counter. The training was documented for all staff members who performed the cell counting in this study. The counting was performed with the default settings for the dual-fluorescent AO/PI assay for cell concentration and viability. The machine and analysis software were utilized according to the MediVet Kit and manufacturer's instructions.

The images captured for the initial counting were reanalyzed after setting the machine to ignore any particle below 10 µm in diameter. The 10 µm cutoff was derived from light and fluorescent observations of various size ranges of adipose-derived SVF cells.

Countess Counts

A Countess® automated cell counting device (Life Technologies/Invitrogen) was utilized for one set of SVF cell enumerations. The device was used according to the manufacturer's instructions.

Giemsa Stained Smears

Samples of SVF cells, PRP and Solution E were smeared on slides and allowed to air dry. These slides were stained with a differential Giemsa stain according to manufacturer's instructions. Standard light microscopy was used for viewing and evaluating the stained slides.

Ethanol Clearance of Micelles

Seventy percent denatured ethanol (Ricca Chemical Co.) was 0.22 µm filtered and mixed 4:1 v:v with SVF cells and incubated at −20 °C for 15 minutes. The cells were centrifuged and re-suspended in ZSol-F™ (INCELL) and transferred to a standard slide for imaging by fluorescent methods. In parallel, samples were mixed 1:1 v:v and smeared on glass slides for Diff-Quick staining.

Cell Culture and Colony-Forming Unit Fibroblast (CFU-F) Assays

Following protocol methods, NucleoCounter and Cellometer viable cell counts were used to perform parallel CFU-F assays of each batch of SVF to compare the viable cell counts with corresponding colony-forming efficiency.

Briefly, SVF cells were re-suspended to a concentration of 200,000 viable cells based on the cell counting method used. A series of dilutions were made such that cells could be seeded in triplicate and the wells had 24, 108, 487, or various thousands of cells per assay as set by the study design. Cells were cultured in 12-well plates as triplicate replicas. The cells were seeded in mesenchymal stem cell (MSC) medium (STEMCELL Technologies) which is formulated to support multiple sources of MSC populations in primary cultures, and for colony-forming assays.

Cultures were seeded at "time 0" and observed daily to verify growth and the health of the cells. Supplements were added to the culture about four days after seeding. Cells formed individual colonies that were fixed at eight to nine days after seeding. The colonies were visualized by staining the cells with hematoxylin for three minutes and rinsed before covering the wells with crystal-mount wet-mount sealant. Individual, well-defined growth foci were counted as colonies. For each replicate set (N = 3) as a group, the mean and standard deviation (SD) values were calculated. The CFU-F and percent colony formation were calculated based on the original 200,000 cells/mL calculated from each of the counting methodologies.

Technical Staff

All technical staff members were experienced with processing human and animal adipose tissue from surgically-removed tissues by multiple published methods, and adipose tissue processing by INCELL proprietary methods and other published techniques. Each of the staff observers performing light and fluorescent microscopic evaluations of the slide preparations by the hemocytometer had no less than 15 years of experience with hemocytometer-based cell counts. Staff performing the cell counting, cell culture, and CFU assays had several years of experience and were supervised by managers who had 25 or more years of cell culture experience.

Results

Cell Counting Methods and Visualization

SVF cells obtained with the MediVet Kit process were counted using five different methods: (1) Cellometer; (2) Coulter Principle; (3) NucleoCounter; (4) Hemocytometer with DAPI staining of nuclei; and the (5) Countess automated counter. For counting methods comparison, the CFU-F assays were performed to assess the relative percentages of colonies from a known number of cells. Mean values were determined for data from all three donor dog fat samples. All values were back-calculated to reflect SVF cells per gram adipose tissue for each of the three donor dogs. Counts were averaged for the same

counting method and mean, plus SD values were graphed as shown in Figure 2.

Cells were counted by the Cellometer according to the guidance by MediVet for use of the kit. The procedure stains the cells with AO which results in fluorescent green nuclei in live cells and PI that stains dead cell nuclei red when the default settings for the instrument are used for cell detection. In each of the fat donor animals, the raw data indicated that 30–64 million cells were obtained at >90% viability. Only the live cell counts determined by this method and the other test methods are shown in Figure 2 as cells per gram of adipose tissue. When samples were sent to a local veterinary group for Coulter-type counting on a Heska counter, the live cell values were about 75% of those seen with the Cellometer. Cells were also counted by a NucleoCounter, which only counts cell nuclei based on PI staining before and after cell lysis. Those live cell counts were less than 25% of those with the Cellometer.

The cell counts from the NucleoCounter are more in line with the manual hemocytometer counts and more closely reflected the outgrowth of a subset of live cells in culture as CFU. The NucleoCounter method has been reported in the literature by human and veterinary adipose stem cell companies (*e.g.*, Cytori and Vet-Stem) as being an accurate automated counting method for mammalian SVF cells.

Cells were also counted manually on a standard hemocytometer with and without various staining methods. SVF cells were counted with a combination of DAPI (nuclear stain) and trypan blue (dye exclusion by viable cells) staining by overlaying the images on a fluorescent microscope and counting the DAPI-stained nuclei in cells that excluded trypan blue. This provides an approximation of the true nucleated, live cells in a population. It is important to include DAPI staining of nuclei since artifacts of cell-like particles can be found in SVF preparations from fat tissue. This is particularly problematic when there is an emulsifying solution in the processing steps such as one containing lecithin. Examples from the MediVet Kit process are shown in Figure 3 (and later in Figures 7–9).

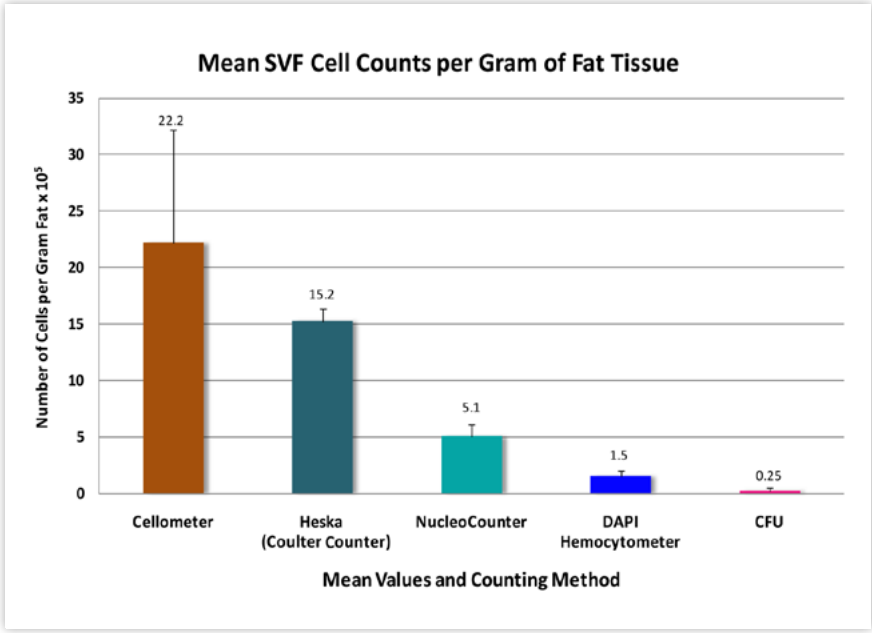


FIGURE 2. Dog SVF cells were isolated using the MediVet adipose processing system and then counted by the recommended Cellometer method and other standard counting methods. Mean values were normalized as numbers of live cells/g of fat based on the separate amounts from the three donor dogs. Each graph represents the mean for that method, which is also indicated by the number above the graph. The bars are the SD for the indicated group.

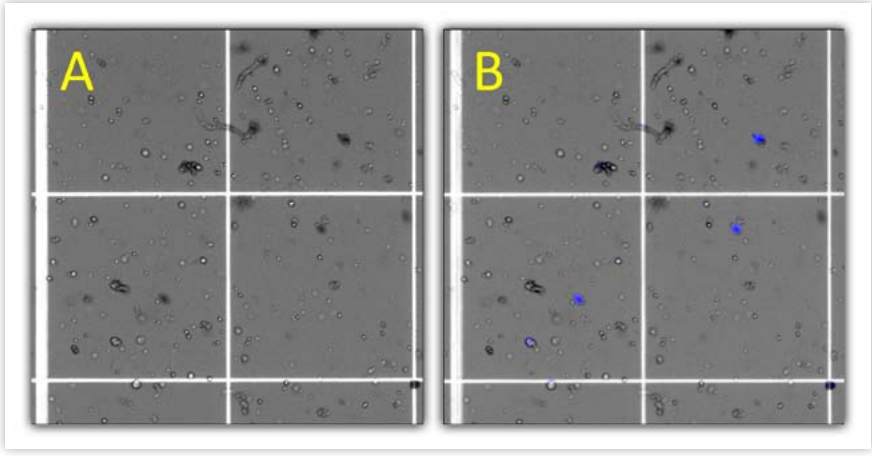


FIGURE 3. (A) A light micrograph image of trypan blue and DAPI stained SVF from animal #2. Note all the small droplets that make sample reading difficult. (B) The same image as A but visualized first under blue fluorescence for DAPI staining then overlaid onto the light image to generate a composite. The DAPI-stained nuclei clearly help to differentiate the debris and artifacts from actual cells. (Image taken on a standard hemocytometer.)

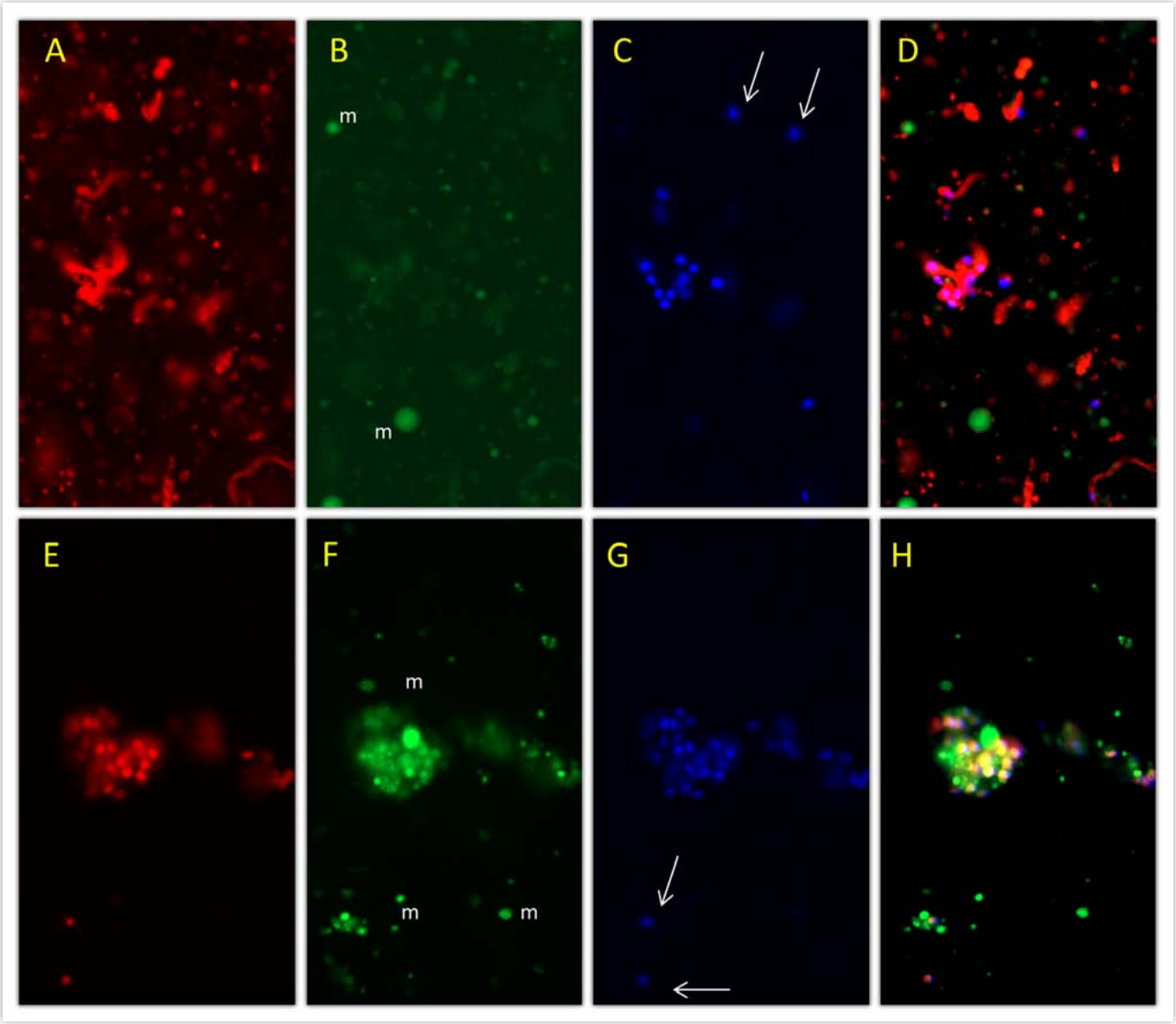


FIGURE 4. SVF from dog donor fat was stained with CM-DAPI. A–D are matched views of the same slide (unfixed) and E–H are matched views of another slide (ethanol fixed) under red (A and E), green (B and F), or blue (C and G) fluorescence light. Note that the ethanol-fixed slide panels E–H are clearer than A–D. Panels C and G show DAPI-stained blue nuclei and individual cells highlighted with arrows. D and H panels are overlay composites of all colors in their respective series, and convey the complexity of cellular and non-cellular components in the SVF samples.

Microscopic visualization of the SVF cells was done under a variety of staining and fluorescent light conditions. Representative photographs were taken and areas of the same field of view were merged as composite figures. In addition, some studies involved clearing the slides by using ethanol as a fixative that would clear the micellular background but still allow visualization of the cells. An example of SVF cells under these types of varying conditions is shown in Figure 4. Images in Figure 4 A–D are CM and DAPI-stained SVF preparations that have not been cleared with ethanol. There is no stain that would induce a green fluorescence in these samples. All of the green on the images is due to autofluorescence or some bleed-through red fluorescence. Clearing the lipids (introduced by Solution E) by fixing the sample with cold ethanol, as seen in Figures 4 E–H, greatly reduced the amount of background clutter and debris. In all SVF samples, the DAPI stain clearly showed the cell nuclei to confirm the presence of cells.

Giemsa Diff-Quick-stained slides of the SVF cell fractions from the processed fat showed that debris and artifacts were present in a large amount of the MediVet

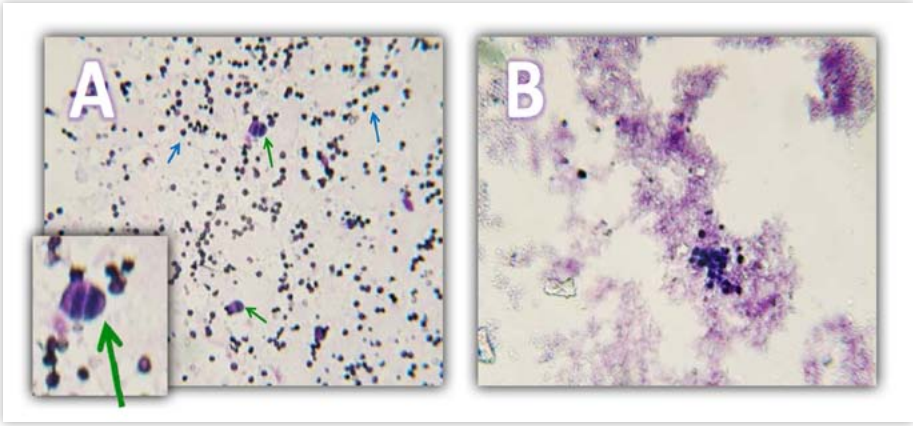


FIGURE 5. (A) Diff-Quick slide of SVF fraction demonstrating that debris and artifacts are present. The blue arrows are pointing to micellular artifacts. Green arrows indicate SVF cells. (Magnification 400 x). Lower corner inset shows a higher magnification of two SVF cells from the central upper part of the photo. (B) Diff-Quick-stained SVF cell suspension shown in panel A but cleared with alcohol. Note the various dark purple single cells and the cluster of SVF cells near the center of the slide. There is cellular debris evident (light purple), but the micellular structures that were present in uncleared slides are now no longer present. (Magnification 400 x).

Kit-prepared SVF suspensions. This is shown in Figure 5A, which had extensive micellular artifacts in addition to SVF cells. When SVF suspensions shown in panel A were cleared with alcohol and then stained, the SVF single cells and clusters could be seen. Cellular debris was evident (light purple), but the micellular structures that were present in the uncleared slides were no longer present.

For all three test sets, the Countess reader gave an error message of "cell size being greater than 60 µm" likely due to background interference from the micelles present in the samples. Even though the Countess works very well for counting cultured cells, it was an unsatisfactory method for samples prepared with the MediVet Kit. Thus, no data for the Countess method was included.

Assessment of Cell Counting Artifacts from Processing Reagents

Potential sources of the counting artifacts from using the automated counters, or misinterpretations of visual observations, were investigated. This work included an assessment of Solution E from the MediVet Kit, and PRP processed by hemocytometric methods using standard light and fluorescence microscopy, and Coulter Counting.

Solution E

Microscopic visualization was done under a variety of staining and fluorescent light conditions similar to those done with the SVF cells (Figure 4). Representative photographs were taken and areas of the same field of view were merged as composite figures. In addition, some studies involved clearing the slides by using a fixative such as ethanol that would clear

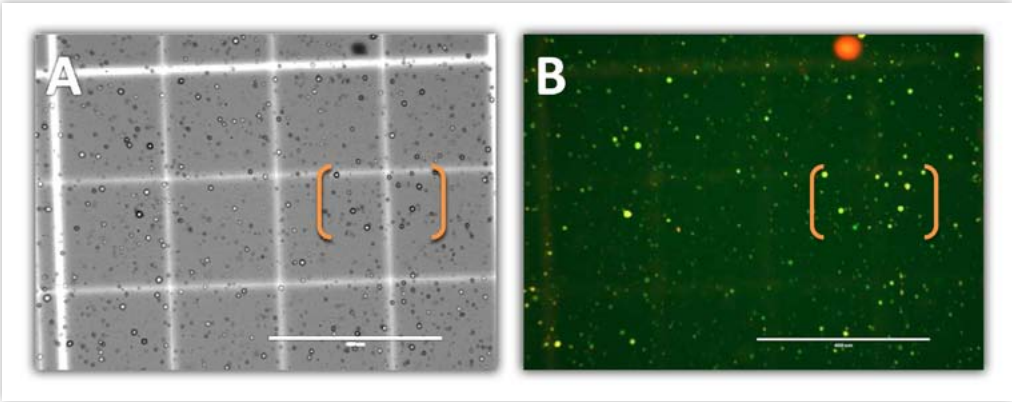


FIGURE 6. (A) Light micrograph of Solution E on hemocytometer. (B) Trypan blue in composite photo of red-green-blue fluorescence. Orange brackets show cell-like micelles of the same field. Notice the lack of blue staining and extensive green autofluorescence.

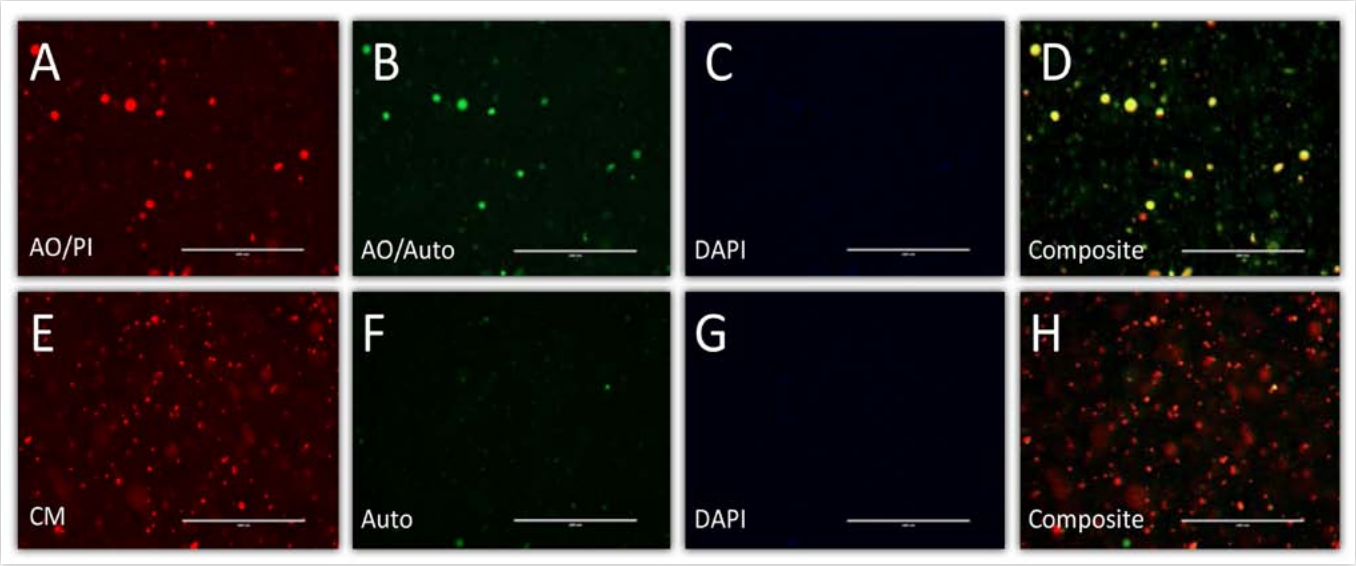


FIGURE 7. Frames A–D were AO/PI/DAPI and imaged after fixing/clearing with ethanol. Frames E–H were CM/DAPI and were fixed/cleared with ethanol. Clearing samples with ethanol removes most lipids and oils from the sample while the density of the cells remains the same based on the DAPI staining of the cell nuclei. Note that the background is reduced by clearing the samples of oils and lipids with ethanol. However, as expected, because there are no cells and therefore no nuclei, there is no positive DAPI blue staining.

the micellular background but still allow visualization of the cells. Some examples of Solution E under these types of varying conditions are shown in Figures 6 and 7. Clearly, there were many cell-like particles and some of them either appeared to stain with AO and PI (but this is actually autofluorescence) or to stain with dyes like CM used for cell counting. However, because no actual cells were present, no nuclear DAPI staining (*e.g.*, Figures 7C and 7G) was seen for the Solution E samples under any of the test conditions.

Solution E was also counted with the Cellometer to obtain cell counts even though there are no cells in the sample. The data was collected and recorded as viable and non-viable cells as shown in Figure 8. These data clearly showed that Solution E can contribute false counts or artifacts to SVF cell counts. In this series, the viable cell counts from the Solution E reagent ranged between 15 and 25 million (Figure 8A).

When used with the default settings, the Cellometer was fooled into counting micelle bodies as cells, since

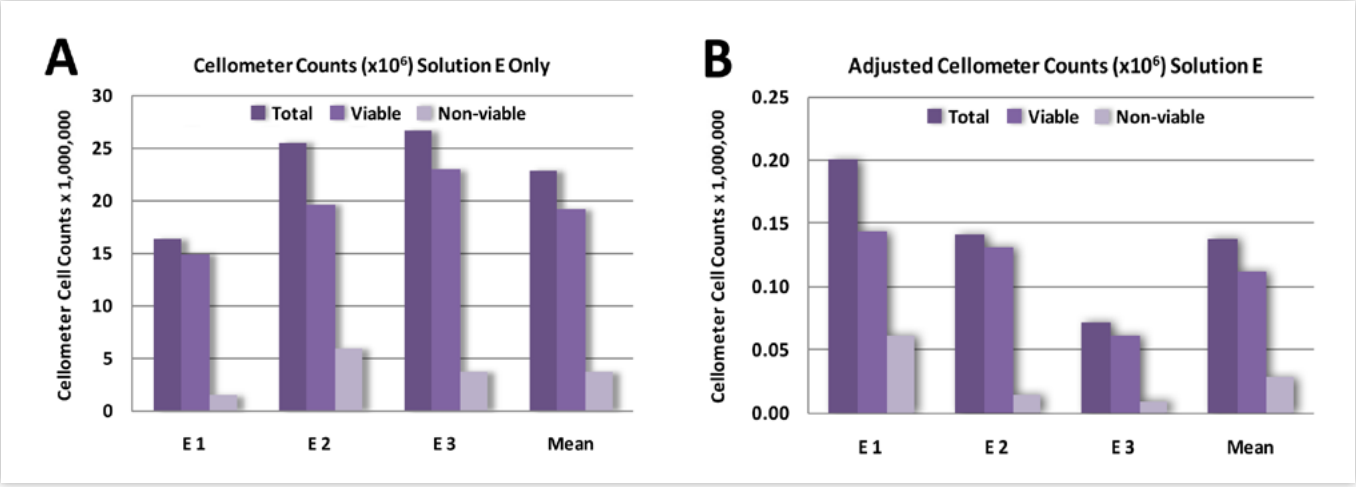


FIGURE 8. (A) Solution E samples that came with each of the three MediVet Kits used with the three donor dog fat samples (E1, E2, E3 for dogs 1, 2, 3, respectively) were counted with the Cellometer using standard protocols for cell counting. The data generated from the counts and mean values for those counts were each separately plotted as total, viable, and non-viable cell counts in the cell-free Solution E reagent for comparisons. (B) The Cellometer was adjusted to record counts above 10 µm and values were plotted as described for (A). Please note the 2 log differences in the y axis.

it was unable to differentiate between the green autofluorescence of the micelles and the acridine orange staining that would have been present if there were cells in the sample. Once the Cellometer was gated to exclude micelle bodies below 10 µm in diameter, the counts caused by mini-micelle autofluorescence were decreased by 2 logs, but the micelles in solution were still counted as cells (Figures 8B and Figure 9).

In contrast, when Solution E was counted using the NucleoCounter, no cells were found because of the lack of any nuclei to stain with PI (Figure 9).

Platelet-Rich Plasma

To assess whether PRP contributed numbers to the cell counts, microscopic cell counting and visualization were done under various staining and fluorescent light conditions similar to those done with the SVF cells and Solution E analyses. Sets of representative photos were taken and graphs of the data were plotted. Figure 10A shows that there were some detectable counts with the Cellometer, and these were possibly due to residual white blood cells and/or autofluorescence. The count was lowered when the Cellometer was adjusted to >10 µm. The counts in the PRP were below the counting threshold for the NucleoCounter. Variable-sized particles could be seen microscopically in PRP (Figure 10B), but they were not bright and did not resemble viable cells, as might be counted with a hemocytometer. Thus, the overall contribution of the PRP to higher cell numbers would generally be negligible.

Colony-Forming Unit Assay

Comparative CFU assays based on SVF cell counts reported by the NucleoCounter and Cellometer methods were done as a relative measure of the number of potential stem or renewable cells in the SVF population. In this assay, the SVF cells were diluted to known numbers of cells based on the Cellometer and NucleoCounter counts and placed in plastic culture plate wells (in triplicate) and allowed to attach and grow into visible colonies. While only a subset of viable cells in the SVF attached to the wells and formed colonies, the CFU assay provided a good index for growth capacity. In this study, the three lowest numbers of cells seeded were 24, 108, and 487.

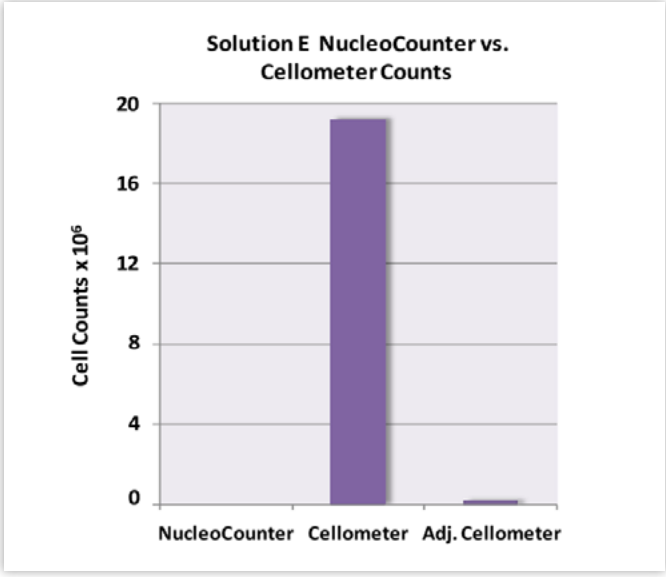


FIGURE 9. All NucleoCounter and Cellometer counts >10 µm were compared.

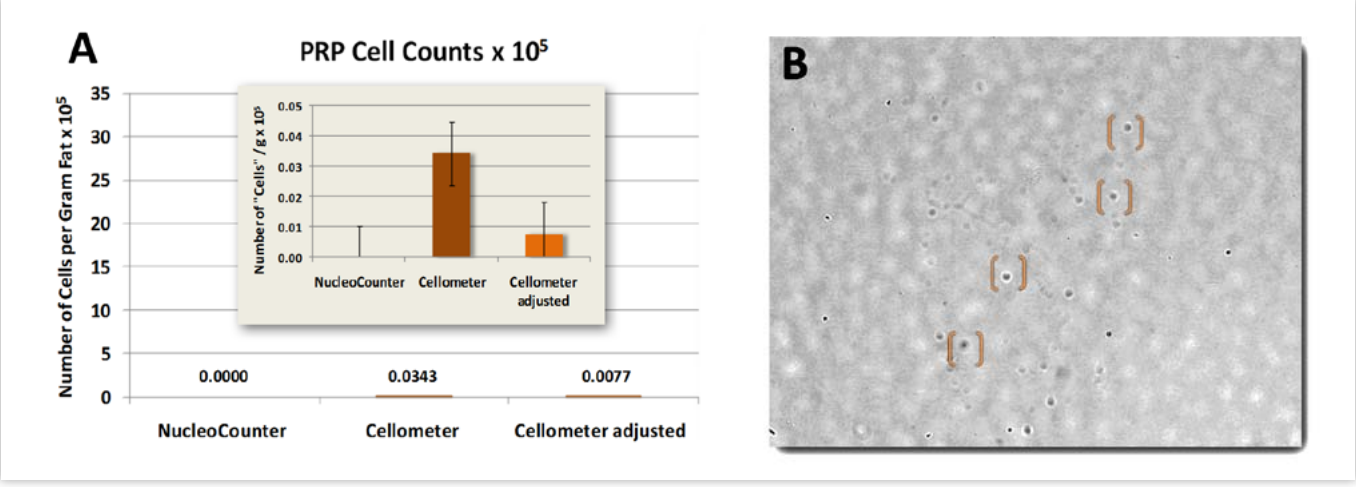


FIGURE 10. PRP cell counts by: (A) automated counting numbers ranging from zero or undetectable (NucleoCounter) to low but reproducibly detectable (Cellometer) with numbers depending on the gating methods used; and (B) microscopy with barely visible particles (shown in the brackets) that would not be counted as cells by a trained observer.

As summarized in Figure 2 and shown in Figure 11, the outgrowth of renewable stem cells, measured by CFUs, was different for the NucleoCounter and the Cellometer. There were far fewer CFU colonies when the cell number was based on the counts from the Cellometer. This was statistically significant for all groups ($p < 0.05$) and clearly demonstrated that the Cellometer cell counts were greatly exaggerated, also shown in Figure 2. Thus, the MediVet-recommended Cellometer counting method significantly over-estimated actual, renewable cell numbers versus counting methods based on the presence of cell nuclei.

Another variable, when using the MediVet Kit, was the light activation step. This particular step was supposed to activate cells to a more proliferative state. If this was correct, then light activation should have increased the percentages of CFU-F derived from the cell population, or increased the size of the colonies when compared to the cells that were not activated. However, when the identical donor SVF cell suspensions were plated before and after light activation (Figure 12) the percent CFU-F from the SVF suspensions decreased for two of the three donors (dogs 1 and 3), and was not different for the samples from dog 2. For all donors, there was no apparent difference in colony sizes or morphology between the cell populations or individual cells that were treated with light, and those that were not.

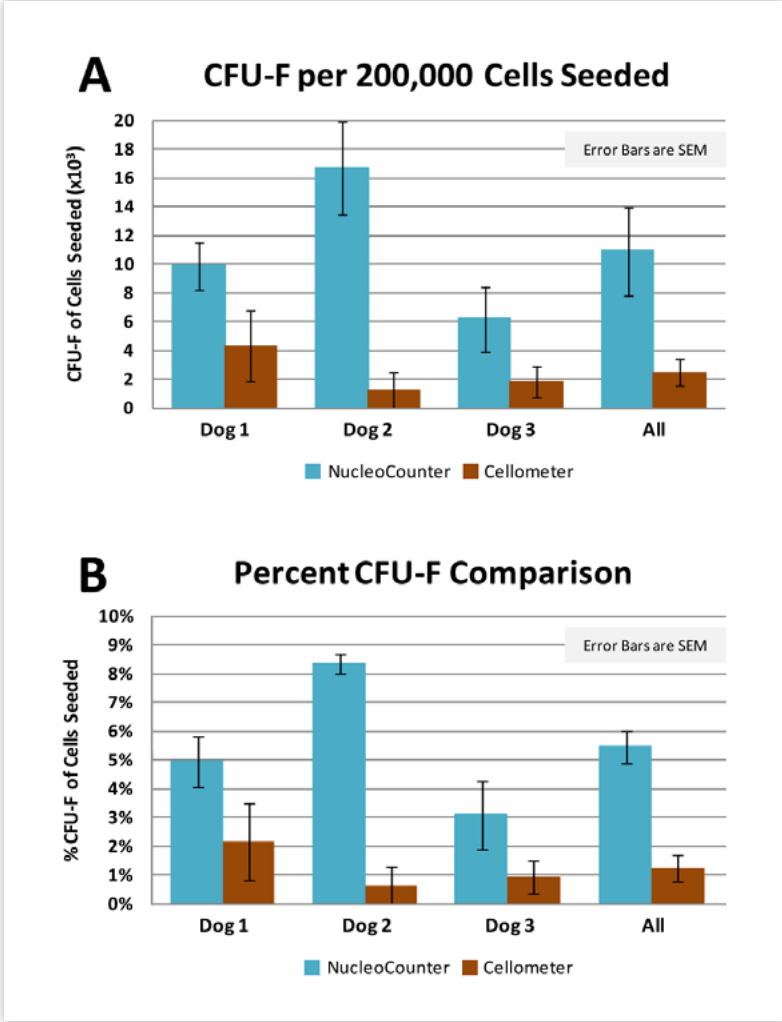


FIGURE 11 (above). SVF cells were obtained for dog donor fat samples (N = 3) processed according to MediVet instructions. The resultant SVF cells were counted by a variety of methods. Cells were seeded into CFU studies with cell numbers designated according to two of the counting methods used: NucleoCounter and Cellometer. After the colonies formed, they were counted and calculations were done to determine: (A) the mean +/– SD numbers of CFU-F per 200,000 cells seeded; and (B) the percentage of CFU-F for cells seeded and compared with the counting method. These data were calculated for each individual dog and for all data with a specific counting method. Statistical analyses (Student's *t*-tests for NucleoCounter vs. Cellometer) between the two counting methods showed statistical significance at $p < 0.05$ for the individual dogs and overall.

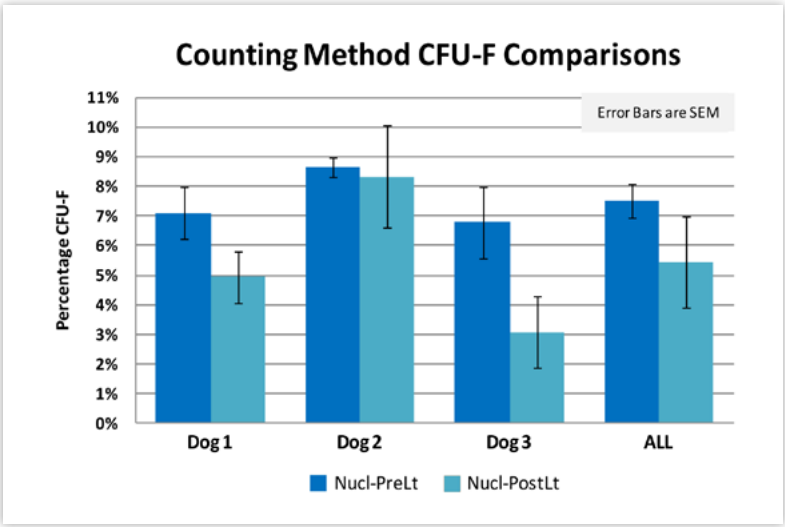


FIGURE 12 (left). Relative percentages of CFU-F were compared between SVF cell suspensions before and after the recommended light activation step for each dog, and overall for all data for each group. Nucl-Pre-LT and Nucl-Post-Lt bars are, respectively, the NucleoCounter counts pre-light and post-light treatments.

Discussion

Data obtained from all three donor dogs were used for comparative analyses to assess the MediVet adipose tissue processing kit and associated methods. Of particular interest was the fact that MediVet reports much higher cell numbers from its preparations of processed fat than most other methods. Specifically, while in agreement with their advertising and marketing materials, the cell counts obtained with the Cellometer at the MediVet default settings were much higher than the actual cell numbers in all groups using the: (1) hemocytometer, trypan blue-DAPI (HTB-DAPI) method; (2) NucleoCounter; or (3) Cellometer, when adjusted to ignore all particles smaller than the average SVF cell. Because of the over-estimated cell counts, the MediVet method also led to fewer CFU-F counts which further demonstrated that the inaccurate stem or renewable cell counts were exaggerated.

The micelles and tissue breakdown components in SVF cell suspensions from the processed fat required careful interpretation. The HTB-DAPI counting method was considered the most accurate of those used in this study. However, it required the visualization of actual cells through the background clutter, use of fluorescence microscopy, and well-trained, highly-skilled personnel. The ability to see the DAPI-stained nuclei through the clutter is important since inaccurate readings can come from the use of other common methods. For example, in standard trypan blue dye exclusion assays, the round refractile micelles may appear to be live cells since they do not take up trypan blue.

The various photographs showing the SVF cell suspensions under different light or fluorescence microscopy conditions clearly showed how important interpretation could be. They also revealed that treatment with a reagent such as ethanol clears residual fat or micelles and helped show the difference between background/debris and viable cells. Giemsa-stained slides allowed visualization of the SVF cells in a sea of micelle materials but did not facilitate the counting. Alcohol treatment improved clarity of the slides and visualization of cells in SVF suspensions. The alcohol treatment also helped show reagent-based, false-positive cell counts in the preparations, and particularly those resulting from Solution E.

The NucleoCounter is considered by many to be the most user-friendly and accurate of the automated counting devices, but it still over-estimated the cell counts in the samples when compared to the HTB-DAPI counts. This can be explained by the small number of larger micelles that fluoresce red, and may be confused for PI-stained nuclei. This was also reflected in the Cellometer counting of Solution E that produced viable and non-viable cell counts (Figure 8). Coulter counting, which relies on the impedance of a current when a cell or particle breaks the path as it passes between two electrodes, is a commonly-used clinical method. However, the Heska counter could not tell the difference between a micelle and a nucleated stem cell. This led to higher counts than the NucleoCounter, but still less than the Cellometer. Interestingly, the Countess cell counter, which is very useful for trypan blue exclusion-counting of healthy suspensions of *in vitro*-grown cell cultures, would not count the samples and indicated an oversize error, likely due to the large amount of background material that blended with the cells.

A gating study was done with the Cellometer. By restricting the size of the counted "cells" to greater than 10 µm and less than 30 µm (default upper size limit), the counts seemed to more accurately estimate the actual number of cells in a sample. However, there were still micelles in that size range that interfered with producing final, accurate counts. For samples without autofluorescent background, the default settings on the Cellometer may be useful for most cell types but they should still be gated for the size range of the cells. However, the Cellometer default settings were not appropriate because of the micellular component in the background of cells that were derived from the MediVet Kit emulsifying agent.

Because of the demonstrated possibility for errors among the different automated counter methods, the NucleoCounter is recommended over the Cellometer and Coulter-type equipment for counting SVF adipose tissue-derived cells.

Investigation of the background material and potential artifacts in counting with the MediVet Kit revealed that autofluorescence of fat-derived samples is a key contributing factor when red or green fluorescence signals are part of an automated or visual analysis. The reagents for processing were evaluated for appearance and potential interference with counting, and only Solution E appeared to have fluorescent, cell-like particles which gave very high, false cell counts of 20–30 million, and included counts for viable and non-viable cells. PRP had some particles, but in very low numbers, so it was not likely that it was contributing to the high cell counts and over-estimated numbers.

The use of light to activate stem cells is loosely based on medical work involving low laser light (LLL) irradiation to activate cells.[24] Photobiostimulation of stem cells in tissue, while an old concept[25], uses

low intensity laser light to stimulate cells. The main differences between what is talked about in the Lin review[24] and what is done in this kit are light intensity and time. LLL is generally used in short time courses of a minute or less at very specific intensities and wavelengths whereas the Medi-Light device is a broad spectrum, multi-color, LED-lined tube with light intensities at the very upper limit of what would be called low intensity, and with a preset 20-minute exposure. The light-activated SVF cells, when compared with cells that were not light-activated, showed no increase in proliferative ability. Overall, the cells tended to have been damaged by the light treatment as indicated by the CFU-F data showing fewer colonies, but the limited number of samples and the individual variance of the samples failed to demonstrate this trend statistically (Figure 12).

Failure to obtain an accurate cell count is a failure to know the dose of cells being injected. As shown in this study, the SVF cell counts obtained with the Cellometer, and when used with default settings according to the MediVet Kit's recommended methods, were gross over-estimates of the actual cell numbers and the renewable stem cell population as reflected by CFU-F data. The reduced CFU-F percentage from the Cellometer counts, as compared to the NucleoCounter, is a direct reflection of that error. This is extremely important because it shows that manufacturers' claims of higher cell yields than those reported in peer-reviewed scientific literature are not only misleading but incorrect. Practitioners are cautioned to better understand the methods of cell isolation, including the introduction of substances such as emulsifiers, and how cells are counted so that they can better evaluate and monitor therapeutic outcomes. Research groups are encouraged to design further studies that tackle the issues with enumeration of SVF, as well as stem or progenitor cells, from different types of processing so that accurate processing, characterization, and dosing are better defined for patients.

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AUTHOR DISCLOSURE STATEMENT

Robbie A. Johnson and Mary Pat Moyer hold shares in INCELL. There are no competing financial interests in this business sector since INCELL does not sell products or direct services to veterinary practices.

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