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Albumin Supplementation to Cold Injection Solution increases Viability of Endothelial and Smooth Muscle Cells

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Abstract

Cell-based therapies have become one of the leading fields of human genetics, where administration of certain cell suspensions now constitutes a potential treatment for various diseases. However, common injection solutions may not sufficiently preserve cell stability and functionality beyond certain times. The present study investigates whether albumin improves viability of endothelial and smooth muscle cells suspended within injection solution. Cells were extracted from vein segments, cultured and suspended in injection solutions supplemented with 1%, 2.5% or 5% albumin and incubated at 4°C for 24 and 48 hours. The viability of both cell lines at 24 hours was found to be significantly higher ($P<0.001$) when cultured with any of the three albumin concentrations, compared to the control albumin-free solution. Furthermore, the 2.5% albumin-containing solution promoted cell adherence, compared to the control, after 24 hours in culture. These results imply the potential of albumin in preserving cell viability for clinical purposes.

Keywords: Endothelial cells; Smooth muscle cells; Injection solution; Human serum albumin; and Cell viability

Introduction

The evolving field of cell therapy has posed both enormous potential and extraordinary challenges. New technologies have infused vast potential into cultured tissue, enabling them to replace diseased and damaged tissues in the body, eliminating the risk of rejection and side effects. There are, however, many technical obstacles to overcome in order to render such cells useful for therapeutic treatments. The development, handling, and administration of such cell-based therapies are characterized by equivalence. Therefore, the preservation of cells for certain periods of time while maintaining their cellular functionality postimplantation, is central to the efficacy of the therapy [1-3]. Extended shelf life of cellular-based therapies is highly advantageous when serving large and remote populations. Within the framework of cell therapy, autologous endothelial cells (ECs) and smooth muscle cells (SMCs) have been employed in order to provide treatment to patients with occluded blood vessels [4]. These cells, isolated from vein segments, were genetically modified with angiogenic genes, to restore blood flow by enhancing the endogenous process of new blood vessel formation [4,5]. In early studies, it has been demonstrated that injection of autologous cell suspensions into occluded femoral arteries in miniature pigs produces new collaterals and restores blood flow in the ischemic hind limbs [4,5]. The viable and functional cells promoting blood vessel formation in these studies must be administered within a short time of preparation, while cell damage occurring while in the injection solution and during the transport from the cell culture laboratory to the operating animal house must be avoided. Thus, administration of cell-based therapies in humans presents certain logistic restrictions, namely, processing and transportation, which can involve lengthy periods in the cold injection solution in a nonadherent and unnatural state. Sustained exposure of cells to the cold injection solution can cause histological changes, and even intracellular injuries due to free radical generation, which eventually result in cell death [6,7]. It has been demonstrated that cellular damage resulting from oxidative stress is related to the activation of stress-activated protein kinases in response to certain cellular changes [8]. A number of studies have suggested that cellular damages are associated with the activation of c-Jun N-terminal protein kinase (JNK) and p38 signaling pathway [9,10], whereas the mitogen-activated protein kinase (MAPKs), has been proved to be important for cell survival [11]. Cellular damage can be minimized by certain antioxidants, which may potentially extend cell endurance in cold injection solution. Evidence indicate that albumin, the most abundant blood protein (3-5 gr/100 ml (namely 3%-5%)), may provide antioxidative protection to cell suspensions by functioning as a serum peroxidase [12]. Albumin has been shown to act as an extracellular transition metal ion-binding and radical-scavenging antioxidant [13,14]. In addition, albumin may regulate the colloidal osmotic pressure and help stabilize cells in suspension [13,14]. It fulfills several other biological functions, particularly as a ligand binder within blood, shuttling a broad range of endogenous and exogenous ligands, including more than 70% of drugs [6,13,14]. Moreover, albumin acts as an extracellular transition metal ion-binding and radical-scavenging antioxidant [13,14]. Studies have demonstrated that low albumin levels are associated with an increased risk of mortality from both cardiovascular disease and cancer, whereas highest life expectancy correlated with high or normal albumin levels [15,16]. The abundance of blood albumin may reflect its important role in protecting blood cells, and in maintaining the cells suspended in the bloodstream. A

similar abundance of albumin was found in colostrum [17], where albumin has been proposed to assist in maintaining higher levels of white blood cells viability [18]. Furthermore, albumin has been found to be an effective cryoprotectant for spermatozoa [19], as well as a preservative for oral keratinocyte in cold storage medium [20]. These features of albumin may be of benefit to cells in injection solutions and may prolong their survival. The albumin dissolved in the injection solution is neither toxic to the cells nor to the recipient and is clinically approved for intravenous injection [21]. However, it has been indicated that similar to other therapies, albumin has some potential risks or shortcomings. Injudicious use of albumin, can lead to fluid overload, myocardial depression, and some allergic reactions [22]. In the present study, we investigated the effectiveness of much lower concentration of albumin, at least 5-fold less of the clinical albumin solutions [23]. We tested preserving the viability of endothelial and smooth muscle cells suspended in cold injection solution and assessed the potential use of albumin protein toward improving and prolonging cell viability.

Materials and Methods

Primary culture of endothelial and smooth muscle cells

For in vitro experiments, endothelial and smooth muscle cells were isolated from saphenous vein segments from patients undergoing coronary artery bypass grafting. The use of vein segments not utilized in surgery was approved by the Lady Davis Carmel Medical Center Review Board. Informed consent was obtained from the patients scheduled to undergo a bypass surgery. SMCs were isolated using the modified ex-plant method [24]. ECs were isolated from vein segments according to the vein isolation protocol [4]. When the isolated ECs formed large colonies that covered half the plate surface (2-4 days after isolation), the cells were trypsinized and transferred to a tissue culture plate pre-coated with gelatin 0.2%. Once confluent, typically within 48-96 hours, cells were passaged several times until a sufficient number of cells were obtained.

Smooth muscle cell isolation and expansion procedures

SMC isolation was performed upon completion of the endothelial cell isolation procedure. The remaining vein segment was cut, opened longitudinally and incubated in a 15 ml tube at 37°C, 5% CO_2 for 60 min, in a solution mixture of elastase (0.65 unit/ml) and collagenase (277 unit/ml) (Serva Electrophoresis, Germany). The entire vein was then sectioned, using surgical scissors, into 2×2 mm segments. SMC isolation from these segments corresponded was performed as previously described [4].

Human serum albumin and injection solution

Human albumin was isolated from pooled human venous blood (Kamada Ltd., Israel) and heat-treated for virus inactivation at 60°C for 10 hours. The product consists of units of human plasma which have been tested and found non-reactive for hepatitis B surface antigen (HBsAg), and include no antibodies to Hepatitis C virus (anti-HCV) and HIV I/II, as determined in FDA and/or EC-approved tests. The injection solution consisted of Ringer lactate supplemented with glucose 0.1%, heparin 100 U/ml, sodium bi-carbonate 20 mM and human serum albumin (albumin, 20% USP solution). Cold (4°C) injection solution was used in all cell experiments. The FDA approved-Lactated Ringer's solution is a sterile, nonpyrogenic isotonic solution. Aside from the serum albumin, this injection solution is supplemented with heparin, to reduce the aggregation of cells, and with glucose, that contributes to ATP synthesis and to generation of compounds useful in biosynthetic pathways. The addition of sodium carbonate helps stabilize the pH of the injection solution.

Viability assay

All viability tests were conducted on trypsinized cells that had been washed and incubated for 30 minutes with the tested injection solution. This is considered as time zero of the viability tests. The acceptance criterion for cell viability was set at >70%; suspension of cells with viability above 70% can be used for treatment of patients [25]. Cell viability was tested by sampling triplicates of 20 µl of the cell suspension and gently mixing with 20 µl 0.04% Trypan blue reagent (Sigma). Cells were counted using a hemacytometer and a light microscopy. Cell viability is expressed as the ratio of live (unstained cells) and total cell number. Viability was determined at 0, 24, 48 hours after cell incubation in the final product formulation.

Guava ViaCount Assay

The Guava ViaCount (Guava Technologies, Millipore) application is a rapid and reliable method for determining cell count and viability. Results from each testing cycle are stored in a data file which may be viewed using the CytoSoft software (Millipore) and in the corresponding spreadsheet file. Acquired data are displayed in two dot plots: Viability (PM1) vs. Nucleated Cells (PM2) and forward scatter (FSC) vs. Viability (PM1). Each plot has moveable cursors in order to allow gating of events. The first dot plot includes a marker that enables gated acquisition of events which contain a nucleus or DNA and thus to display live, apoptotic, and/or dead cells. The first dot plot also includes two other markers - one to separate the live cells from the dead cells and the other, to separate dead from apoptotic cells. The second dot plot includes a marker which discriminates cells from debris that might have been associated with DNA. In these experiments, cells were removed from flasks and thoroughly suspended with a range of albumin concentrations. At the specified time points, the cells were stained, by mixing with Guava ViaCount Reagent in a sample tube at room temperature. The cells were allowed to stain for at least 5 min before samples were read in the FACS machine, according to the specified protocol. Accurate cell counting on the Guava system occurs at a concentration range of 1×10^4-5×10^5 cells/ml. Each evaluation was conducted in triplicates.

Analysis of cell adherence

Cell adherence was tested after a 24-hour incubation of trypsinized cells in cold injection solution (4°C) in the presence or absence of 2.5% albumin. After 24 hours in cold suspension, cells were centrifuged and seeded in growth medium at a concentration of 1×105 cells, on gelatin-coated, 12-well plates. The medium was changed after two hours in order to wash away nonadherent cells. The cells were then cultured in fresh growth medium for an additional 24 hours and visualized under a light microscope, at 200x magnification. Ten random fields of the seeded wells were imaged in a blinded.

Statistical analysis

The multiple comparisons (two-way ANOVA) tests examined quantitative parameters of the albumin concentrations and of the three time points (0 h, 24 h and 48 h). The non-parametric sign rank test

was applied for testing differences between 0 h and 24 h assessments for quantitative parameters. All tests applied were two-tailed, and a p value of ≤5% was considered statistically significant. The data were analyzed using the SAS® version 9.1 for Windows (SAS Institute, Cary North Carolina).

Results

Effect of albumin on viability of cells suspended in cold injection solution

Endothelial and smooth muscle cells were trypsinized and then suspended, for up to 48 hours, in cold injection solution with increasing concentrations of albumin. Albumin clearly maintained a higher degree of cell viability ($P<0.01$), even at time zero, namely, a simple washing step with albumin helped maintain high viability (Figure 1). Higher cell viability ($P<0.001$) was obtained when ECs were incubated in any of the albumin-supplemented injection solutions (Figure 1A and 1B), as compared to a 24-hour incubation in the control albumin-free solution (Figure 1). No differences in viability were found between the three albumin concentrations at any of the tested incubation times (0, 24 and 48 h). Similar results were found with SMCs following incubation with increasing concentrations of albumin in cold injection solution (Figure 1C and 1D). At times zero and 24 h, all SMC samples incubated with albumin contained a significantly greater number of viable cells than the control groups ($P<0.01$), however no difference was found between the three tested albumin concentrations.

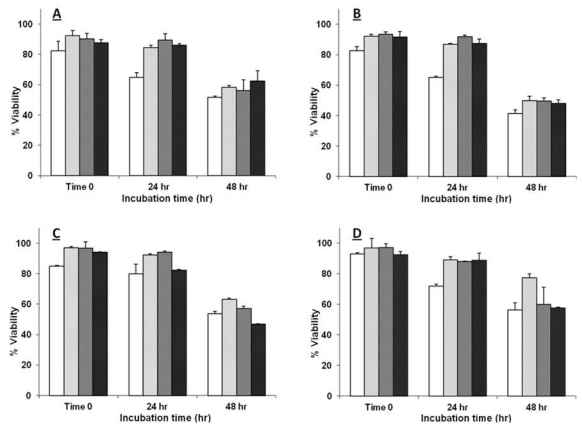

Figure 1: Viability of Endothelial and Smooth Muscle cells. Cell viability was determined by Trypan blue exclusion assay following different durations of incubation in injection solution with various concentrations of albumin. All histograms from white to black bars indicate 0%, 1%, 2.5%, and 5% albumin, respectively. Panel A depicts endothelial cells from human donor 1, where panel B depicts endothelial cells from human donor 2. Panel C depicts human smooth muscle cells from donor 1, where panel D depicts smooth muscle cells from human donor 2. The statistical analysis utilized two-way ANOVA. Error bars indicate ± SD of three consecutive experiments.

Viability of cells as determined by the ViaCount Assay

In order to verify the viability of SMCs, an alternative cell viability assay was performed. SMCs were suspended in injection solution supplemented with 1, 2.5, or 5% albumin. Cells at a density of 1×106/ml were assayed for viability in duplicates, according to the protocol described above. Suspensions in injection solution containing any of the three concentrations of albumin contained more viable cells, when compared to those lacking albumin. This high viability was sustained over the first 24 hours of incubation and was reduced in the 48 hour incubation period. In contrast, SMCs suspended in the control solution lacking albumin, showed a high reduction in cell viability within 24 hours; by 48 hours postincubation, approximately 85% of cells were not viable, as compared to the 44% and 38% viability observed in suspensions containing 1% and 2.5% albumin, respectively. These results stand in line with the Trypan blue staining method that showed that addition of albumin maintains cell viability for more than 24 hours (Table 1).

Effect of albumin on cell adherence

In order to evaluate the physiology of ECs and SMCs cultured in the presence of albumin, we compared adherence of cells incubated and stored (24 h) in injection solution supplemented with 2.5% albumin, to cells stored without albumin. Cells were then plated and incubated overnight in their regular growth media. The two cell types pretreated with albumin adhered better and proliferated faster, when compared to those that were not treated with albumin (Figure 2). Although more ECs were found in a few plates seeded with albumin-treated cells, no significant differences were found compared to ECs grown without albumin pretreatment. SMCs displayed fairly increased numbers of adherent cells at the lower concentration of albumin (1% albumin) after 24 h ($P<0.01$), whereas cell adherence in all other groups did not significantly differ from that of the control groups (Figure 2).

Guava #	Albumin 0%	Albumin 1%	Albumin 2.5%	Albumin 5%
1	91.4	95.6	96.7	96.7
2	92.4	97.6	97.9	97
Average	91.9	96.6	97.3	96.9
1	73.1	93.7	93	74.1
2	65.3	91.6	91.4	88.1
Average	69.2	92.6	92.2	81.1
1	16.5	48.4	42.9	5.4
2	14.1	38.9	33.7	15.1
Average	15.3	43.6	38.3	10.3

Table 1: Percentage of viable SMCs (Guava assay)

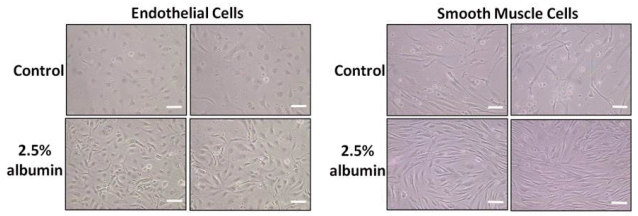

Figure 2: Adherence of endothelial and smooth muscle cells following albumin pretreatment. Cells pre-incubated (24 h) in suspension in cold injection solution with or without 2.5% albumin were tested for their adherence. ECs, from donor 1, and SMCs, from donor 2, were seeded and incubated overnight before being visualized by the microscope. Representative Fields were imaged at 200x Magnification. Scale bar=25 μm.

These results indicate that albumin supplementation preserves cell viabilities to a great extent. The viability of both cell suspensions was preserved within the first 24 hours of incubation, which is far beyond the preset acceptance criterion (>70%) and, as such, these cell suspensions can still be injected for treatment. Thus, albumin supplementation provides significant support to previous results on cell viability, although the reference point of the albumin effect is not the injection solution free of albumin, rather, the time zero of injection solution containing albumin.

Rabbit #	Time 0h (%)		Time 24h (%)	
	EC	SMC	EC	SMC
2593	96	100	83	98
2424	96.5	97	98	98
2435	99.1	100	91	92.5
3257	97.8	98.5	90.5	94.2
3431	100	98.8	90.9	92.5
Average	97.9	98.9	90.7	95
STDEV	1.7	1.2	5.3	2.8

Table 2: Percent viability of EC and SMC incubated in 2.5% albumin injection solution

Discussion

Short-term (hours to days) hypothermic storage (4°C) of primary human cells in solution containing up to 2.5% albumin, proved to be efficient and safe. Tissue culture media are designed to provide optimal conditions for cell preservation in their viable and functional state. The injection solution used in this study consisted of Lactated Ringer's solution, which is widely used in intravenous injection. While this solution is physiological, due to its isotonicity with the blood, it lacks some important nutrients and is therefore considered a poor storage medium for cells. In this study, the solution was supplemented with glucose, heparin, sodium bi-carbonate and serum albumin. Such additives maintained higher cell viability for more than 24 hours in hypothermic conditions. Various studies have indicated that sustained exposure of cells to a certain solutions can cause histological changes and intracellular injuries due to the generation of free radicals [6,7]. The damaging effect of free radicals results mainly from the lipid peroxidation of the cell membrane, and thus, cell viability in suspension may be maintained by specific scavenging antioxidants [26,27].

Albumin is clinically approved for intravenous injection, and is considered a non-toxic additive acting as a serum peroxidase [12,14], and therefore, may provide protection from free radicals. It has been found to preserve some organs during transplantation processes [28]. In mammals, albumin is a key contributor to the colloid osmotic pressure of the blood plasma that tends to pull water into the circulatory system [29]. Moreover, albumin binds various ligands, such as inorganic ions, drugs, amino acids, fatty acids and others, facilitating their transport throughout the circulation [30,31]. Physiological concentrations of albumin appear to increase viability of various cell types in vitro. It has been found to inhibit human EC apoptosis, most likely through scavenging free radicals [32], due to its sulfhydryl group at Cys34 that protects several cell types from oxidative damages [14,33]. It directly scavenges reactive oxygen species (ROS), such as superoxide anion ($O^{2\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$) and nitric oxide ($NO^{\bullet}$) [34,35]. Within the context of cell apoptosis, albumin binding to cell surface receptors, like megalin, may prevent apoptosis through activation of intracellular signaling activating PI3K [36-39] and p44/p42 MAPK [40], as seen in several cell types. Due to its essential role, albumin is also abundant in the extravascular compartment [41]. These works suggest that the protective effect of albumin may be mediated by reducing ROS formation, inhibiting caspase-3 activation, and suppressing MAPK signaling. As a result, albumin may have exerted cellular protective effects via both antioxidant and anti-apoptosis mechanisms. Albumin has been shown to protect against apoptosis of chronic lymphocytic leukemia cells [42] and endothelial cells [36], to decrease reactive oxidant-induced cellular apoptosis, to promote neuronal survival [43], and to increase energy metabolism in primary astrocytes [44]. These studies strongly indicate that albumin exerts its cellular protective effects through anti-oxidative and anti-apoptotic mechanisms. In other studies, it has been shown that intravenous administration of albumin reduces brain injury [45-47].

In the current study, we evaluated the effect of serum albumin on the viability of ECs and SMCs suspended in cold injection solution. Albumin significantly improved cell viability and to a certain extent, cell adherence as well, when compared to the control, albumin-free injection solution. The increased cell viability, or in other words, the reduction in the damaging effect that is possibly due to free radical generation, may be attributed to the protective effect of albumin. The enhanced viability due to addition of albumin, as shown with the Trypan blue staining assay, was further confirmed with the ViaCount assay and adherence tests. In both assays, cell viability in most of the albumin-containing solutions was higher than that of the controls. The viability of ECs and SMCs was maintained at higher levels for more than 24 hours in the injection solution containing 1-5% albumin, suggesting that most cells were in a stationary or stable state. As they constitute the blood vessels, ECs and SMCs are constantly in contact with the blood albumin which ranges between 3-5 gr/100 ml. The amount of albumin that is in contact with or needed by these cells within the body is most probably at its lower range (i.e. 3 gr/100 ml or less) due to the other functions that albumin performs in vivo. This would explain that the 2.5% albumin-treated group has shown better result than the 5% albumin-treated group (Figure 1, Tables 1 and 2). The integrity of the cobblestone-like morphology of ECs and hills and

valleys morphology of SMCs stored in the albumin-containing solution, were maintained after seeding, as seen using a phase-contrast microscope (Figure 2). Such preservation of the cellular integrity and adhesion capacity seemingly stems from the inhibition of processes involved in cell apoptosis [48]. Interestingly, the protective effect of albumin was apparent as early as time zero (i.e., within 30 min of preparation of the cell suspension). Such protection of cells from free radicals and/or from onset of apoptosis strongly indicates the immediate protective effect of albumin (Figure 1). Albumin in its native and soluble form can quickly protect cells, whereas denatured albumin induces cell apoptosis [49]. The decreased cell viability observed in albumin-containing solutions during the 24-48 hours postincubation, is likely due to albumin denaturation. Addition of fresh albumin to cell solutions may provide further protection to cells in suspension.

The increased viability of cells in the albumin-containing solution is important for extending the shelf life of cell-based products. Hypothermic solution containing albumin seems to provide the optimal conditions to preserve cell integrity, viability and functionality. Assessing ECs and SMCs in similar preservative solutions has not been previously studied. ECs seem to be more susceptible to cell death during preservation, whereas SMCs seem to be more tolerant.

The crucial challenge in the field of cell therapy is presented by the short life span of therapeutic cells. Therefore, it is essential to maintain high cell viability in order to allow treatment efficiency and to provide a sufficient time interval until administration of the therapeutic cells. This work demonstrates that ECs and SMCs significantly retain their viabilities upon exposure to supportive environments supplemented with albumin. The ability to prolong cell viability in-vitro may extend shelf life of cellular-based therapies aimed to replace diseased and damaged tissues in the body.

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