

NUTRIENT PRELOADING OF CELLS

FIELD OF THE INVENTION

5 The invention pertains to cell culture media that improve stress resistance of cells, especially mammalian cells.

BACKGROUND OF THE INVENTION

10 Mammalian cells have become highly useful resources in healthcare, cosmetics and several related domains. They can be used directly in cell therapy which entails the injection or transplantation of viable cells to produce health and/or therapeutic benefits in recipients. Examples include transplantation of stem cells for tissue repair and regeneration, injection of stem cells for immunomodulatory effects and injection of immune cells to target and eliminate cancer cells in the body. Mammalian cells are also employed as “biofactories” in production of proteins, vesicles etc. for various applications downstream. In general, the value of mammalian cells across these applications is enormous and is often not easily replaced by

15 conventional pharmaceuticals.

Mammalian cells commonly face multiple forms of stress over the course of their application. The ability of mammalian cells to remain viable and functional against such stresses is therefore critical and is often the determinant of success in the applications the cells are employed in.

20 One instance is the transplantation of mammalian cells in cell therapy. A typical scenario entails thawing cryopreserved cells and soon after, injecting them into targeted locations in humans or animals via fine needles. Over this transplantation procedure alone, cells are already subjected to freeze-thaw stress and shear stress from fine needle injection. In addition, the sites, where the cells are introduced or where they are expected to effectuate their benefits, pose additional stress that severely reduce the viability of transplanted cells.

25 One key problem is the poor availability of nutrients such as energy substrates that are required by cells to fuel all the processes and pathways essential for survival and function. This problem is particularly apparent in instances where: 1) stem cells are transplanted into damaged/diseased tissues that are ischemic and poorly perfused or 2) immune cells are introduced into metabolically hyperactive tumor cells which can outcompete transplanted cells for nutrients or 3) cells are kept at hypothermic storage with a

30 limited volume of nutrient media or buffer.

Transplanted cells of adherent nature are further expected to attach as soon as possible to avoid detachment-induced cell death. Not surprisingly, the survival of transplanted stem cells in ischemic tissues had been documented to be as low as 0.44% at just 4 days after transplantation (*Human Mesenchymal Stem Cells Differentiate to a Cardiomyocyte Phenotype in the Adult Murine Heart*. Toma, Catalin, et al. 2002, *Circulation*, pp. 93 – 98).

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There are multiple strategies to improve the viability of cells in face of anticipated stresses during use e.g. poor nutrient availability after transplantation.

One widely explored strategy is the adjustment of cell culture conditions to precondition cells prior to their transplantation. Preconditioning arises from the recognition that: i) cells are typically cultivated in and

40 accustomed to culture media enriched with high concentration of nutrients to promote rapid cell growth

and; ii) there is a huge mismatch in nutrient availability between culture environments and transplantation sites.

In this strategy, cells are cultured in hypoxic environments and/or starved with low concentration of nutrients. The process of preconditioning can: A) select for cells that are inherently better adapted against anticipated stresses; B) induce metabolic adaptation such increased storage of intracellular glycogen and; C) enhance secretion of pro-survival and pro-growth cytokines.

Preconditioning with hypoxia and low nutrients can potentially be lengthy (e.g. 75 days starvation) and can lead to outcomes that are variable and not well-controlled. Moreover, the use of low nutrients to culture cells would likely affect cell proliferation and contradict the economic objectives of industrial cell cultivation to produce as many cells as within the shortest possible time.

Another strategy is the manipulation of gene expression in cells. Here, gene expression may be manipulated transiently via microRNAs and permanently with viral vectors. In targeting apoptosis inhibition or pro-survival genes e.g. heat shock protein 70, such genetic manipulation can enhance cell survival *in vitro* and *in vivo*.

A third strategy is the use of pharmacological compounds or biologicals to pre-treat the cells prior to transplantation. To date, a plethora of pharmacological compounds and biologicals such as sevoflurane, oxytocin, haemin, desferrioxamine, cyclic helix B peptide and lipopolysaccharide has been explored. Their effects are manifold and include: 1) induction of pro-survival and anti-apoptotic genes; 2) preservation of membrane stability and; 3) inhibition of mitochondria dysfunction during nutrient deprivation.

Lee et al. discloses the loading of bioactive-containing polymeric microparticles into MSCs to control their phenotype after transplantation. However, this prior art addresses the control of differentiation rather than nutritional needs of the transplanted MSCs in nutrient-poor environments. (*Cell Adhesion and Long-Term Survival of Transplanted Mesenchymal Stem Cells: A Prerequisite for Cell Therapy*. Lee, Seahyoung, et al. s.l. : Oxidative Medicine and Cellular Longevity, 2015).

Manipulation of gene expression produces significant alteration to the cells. The same can be said for treatment with pharmacological compounds and biologicals which, unlike nutrients, are potent triggers to countless cell signaling pathways even when present in small amounts. For gene manipulation and pharmacological compounds/biologicals, there will therefore be concerns over possible side effects to the overall behavior and functions of the cells until proven otherwise.

In other words, a population of cells with certain therapeutic functions cannot be automatically assumed to have exactly the same functions after genetic or pharmacological modification. In addition, there are safety and regulatory concerns regarding these strategies if the cells are already in the midst of their development and testing cycle. With the significant alterations mentioned above, regulatory agencies may recognize genetically or pharmacologically modified cells as completely new products that need a complete rework in terms of verification and validation.

Stress tolerance conferred by using fatty acids to induce lipid droplets is another option.

Khatchdourian et al. ("*Lipid droplets: Their role in nanoparticle-induced oxidative stress*" Molecular Pharmaceutics 2009) discloses that cells induced with oleic acid survive better under serum deprivation and nanoparticle induced oxidative stress.

This method has a limit because high fatty acid concentrations can eventually lead to lipotoxicity beyond

5 a certain concentration range. Smith et al ("Unsaturated fatty acids induce mesenchymal stem cells to increase secretion of angiogenic mediators" J Cell Physiol 2012, 227: 3225-3233) disclose excessive fatty acid concentrations to reduce viability of human mesenchymal stem cells.

Zhao et. al. ("Artificial Lipid Droplets" Nanomaterials 2022, 12, 672) discloses a method of formulating triglyceride particles which were coated with DOPC (1,2-dioleoyl-sn-glycero-3-phosphatidylcholine).

10 Although phosphatidylcholines would not have been expected to exert cell protective effects given known literature (US 7259273 and Yamagiwa et al), they facilitated the coating of triglyceride particles with perilipin which are proteins well-known for cell protective effects. When these particles assembled from triglycerides, phosphatidylcholines and perilipin were used to pretreat Hela cells. The cells developed better resistance against oxidative stress and lipotoxicity.

15 US2015175956 discloses the use of cholesterol, phosphatidylcholine and fatty acids as serum replacement for cell culture media. With a lipid profile that contained a low concentration range of fatty acids, the disclosed media demonstrated utility when used throughout regular cell culture to promote cell maintenance and growth in serum-free conditions.

Che et. al. ("Neuroprotective Effects of n-3 Polyunsaturated Fatty Acid-Enriched Phosphatidylserine Against Oxidative Damage in PC12 Cells" Cell Mol Neurobiol 2018, 38: 657-668) demonstrated that polyunsaturated phospholipids can be used to protect cells against oxidative stress. However, polyunsaturated phospholipids of defined compositions and high purity are significantly more expensive relative to saturated or monounsaturated counterparts. Furthermore, given the high number of double-bonds, polyunsaturated phospholipids are usually more susceptible to chemical instability over storage.

25 US7259273 discloses the use of lysophosphatidic acid (LPA) to inhibit cell apoptosis under serum-deprivation and tested the efficacy of LPA when presented in lipid membrane structures such as liposomes. Data in US7259273 show that empty phosphatidylcholine and phosphatidylglycerol liposomes had no protective effects as long as LPA was not included.

Similarly, Yamagiwa ("Phosphatidylcholine-plasmalogen-oleic acid has protective effects against arachidonic acid-induced cytotoxicity" Bio Pharm Bull 2022, 45:643-648) tested phospholipids for cytoprotective effects. The study demonstrated that only phospholipids containing plasmalogen as one of the two lipid tails showed cytoprotective effects. On the other hand, phospholipids with both oleic acid-like tails failed to produce any cytoprotective effects.

35 DESCRIPTION OF THE INVENTION

It is an object of the present invention to provide cell culture media that have a protective effect on the cells against a variety of stresses, particularly oxidative stress and other stresses like temperature stress or starvation stress in which the disadvantages of the prior art are at least reduced and which improve stress resistance and/or cell viability of the cells cultivated in said cell culture media.

This object is achieved by means of a cell culture medium comprising diacylglycerophospholipids and fatty acids in specific molar ratio of diacylglycerophospholipids: fatty acids.

According to any aspect of the present invention, there is provided a cell culture medium comprising diacylglycerophospholipids and fatty acids in specific molar ratio of diacylglycerophospholipids: fatty acids
5 ranging from 40:60 to 95:5 with the fatty acid concentration ranging from 10 μ M to 300 μ M and the diacylglycerophospholipid concentration ranging from 50 μ M to 2500 μ M.

Surprisingly, it could be shown that a cell culture medium according to any aspect of the present invention has a much higher protective effect on cells cultured in the said medium compared to a cell culture medium that comprises fatty acids alone. In particular, culturing of a cell in the cell culture medium

10 according to any aspect of the present invention leads to much higher cell viability of the cell after exposure of the cell to stress. While treatment with a fatty acid alone can lead to complete loss of cell viability due to cell toxicity of fatty acids at high concentrations, cells cultured in the cell culture medium according to any aspect of the present invention can resist the same stressors with little to no loss in cell viability. In particular, it is well known that the presence of fatty acids in cell culture medium has a

15 protective effect on the cells cultured in the medium resulting in the cells having a higher cell viability when exposed to stress. However, too high a concentration of fatty acid is also toxic to cells. It has however been surprisingly found here that the combination of fatty acids and diacylglycerophospholipids particularly in the ratio of the cell medium according to any aspect of the present invention removes, negates or reduces the toxic effect of high fatty acid concentration on the cells. This is even more

20 surprising as culturing cells in diacylglycerophospholipids alone do not impart any stress resistance on the cells. In particular, the molar ratio of diacylglycerophospholipids : fatty acids ranges from 50:50 to 95:5, more preferably a molar ratio of 60:40 to 95:5 and most preferably a molar ratio of diacylglycerophospholipids : fatty acids from 70:30 to 95:5. The fatty acid concentration preferably ranges from 5 μ M to 400 μ M, 10 μ M to 400 μ M, 15 μ M to 400 μ M, 20 μ M to 400 μ M, 25 μ M to 400 μ M, 30 μ M to
25 400 μ M, 35 μ M to 400 μ M, 40 μ M to 400 μ M, 45 μ M to 400 μ M, 50 μ M to 400 μ M, 5 μ M to 300 μ M, 10 μ M to 300 μ M, 15 μ M to 300 μ M, 20 μ M to 300 μ M, 25 μ M to 300 μ M, 30 μ M to 300 μ M, 35 μ M to 300 μ M, 40 μ M to 300 μ M, 45 μ M to 300 μ M, 50 μ M to 300 μ M, 5 μ M to 200 μ M, 10 μ M to 200 μ M, 15 μ M to 200 μ M, 20 μ M to 200 μ M, 25 μ M to 200 μ M, 30 μ M to 200 μ M, 35 μ M to 200 μ M, 40 μ M to 200 μ M, 45 μ M to 200 μ M, 50 μ M to 200 μ M, more preferably 20 μ M to 200 μ M and the diacylglycerophospholipid concentration
30 preferably ranges from 75 μ M to 2000 μ M. The fatty acid in the cell culture medium according to any aspect of the present invention is preferably a saturated, monounsaturated, diunsaturated or polyunsaturated C8 to C24 fatty acid. More preferably a saturated, monounsaturated, diunsaturated or polyunsaturated C12 to C22 fatty acid.

The term 'fatty acid' as used herein, refers also to a fatty acid salt wherein the cation is an organic cation
35 derived from at least lysine, arginine, ornithine or mixtures thereof. Using fatty acid salts may be considered more cost efficient and may work equally well as pure fatty acids.

Most preferably, the fatty acids in the cell culture medium according to any aspect of the present invention are selected from the group consisting of lauric acid, oleic acid, octanoic acid, decanoic acid, arachidonic acid, docosahexaenoic acid, eicosapentaenoic acid and mixtures thereof.

The cell culture medium according to any aspect of the present invention comprises diacylglycerophospholipids and fatty acids in a molar ratio of diacylglycerophospholipids : fatty acids ranging from 40:60 to 95:5. Preferably, the acyl groups of the diacylglycerophospholipids are saturated, monounsaturated or di-unsaturated C8 to C24 aliphatic chains. More preferably, the acyl groups of the diacylglycerophospholipids are saturated, monounsaturated or di-unsaturated C12 – C20 aliphatic chains and most preferably, saturated or monounsaturated or di-unsaturated C16 – C20 aliphatic chains. The acyl groups in the diacylglycerophospholipids can be the same or have different aliphatic chain length and saturation levels.

In one example, the acyl groups of the diacylglycerophospholipids in the culture medium according to any aspect of the present invention are saturated or monounsaturated C12 to C20 aliphatic chains, more preferably saturated or monounsaturated C16 to C20 aliphatic chains .

In particular, the diacylglycerophospholipids may bear a headgroup selected from the group consisting of choline, glycerol, serine, inositol and ethanolamine.

Preferably, the diacylglycerophospholipids in the cell culture medium according to any aspect of the present invention are selected from the group consisting of 1,2-dioleoyl-sn-glycero-3-phospholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphatidylglycerol (DOPG), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and mixtures thereof.

Fatty acids and diacylglycerophospholipids can be added to the cell culture media according to any aspect of the present invention by any means known in the art. In one example, the fatty acids and diacylglycerophospholipids are added into the culture medium in the form of fatty acids and diacylglycerophospholipids containing liposomes.

Fatty acids and diacylglycerophospholipids can be added to the cell culture medium separately or together through the use of carriers or delivery vehicles proteins e.g. albumin, cyclodextrin and solubilizing agents e.g. ethanol, dimethyl sulfoxide (DMSO). Other delivery vehicles and solubilizing agents can further include, but are not limited to, solid lipid nanoparticles as well as polymeric microparticles and nanoparticles.

Diacylglycerophospholipids can also be added as empty liposomes i.e. liposomes not containing fatty acids.

In one example, in the cell culture medium according to any aspect of the present invention, the diacylglycerophospholipids and fatty acids are present in the form of liposomes. The liposomes can be produced by any method known in the art, e.g. sonication, extrusion or homogenization.

The person skilled in the art can select the other nutrients in the cell culture medium according to the nutrient needs of the cells or alternatively select standard media formulations e.g. Dulbecco's modified eagle medium and supplement the cell culture medium with the diacylglycerophospholipids and fatty acids in the molar ratio and concentration according to any aspect of the present invention.

The diacylglycerophospholipids and/or fatty acids can be prepared as a concentrate with the concentrations reaching as high as 3000 times the intended final concentration in a basal cell culture media. The concentrate can then be diluted to the cell culture media according to any aspect of the present invention to achieve the desired final concentration and/or ratio of the diacylglycerophospholipids and/or fatty acids. Thus, a method of preparing a cell culture medium according to any aspect of the

present invention, forms part of the present invention in which the diacylglycerophospholipids and/or fatty acids are first prepared as concentrates and are subsequently diluted into cell culture medium to achieve the final desired concentration.

According to another aspect of the present invention, there is also provided a method of culturing cells comprising the steps of contacting the cells with the cell culture medium according to any aspect of the present invention and culturing the cells in said cell culture medium for 4 hours to 96 hours. In order to prepare the cells to withstand future stresses the cells are preferably cultured in the medium according to any aspect of the present invention for 24 hours to 96 hours, more preferably 24 hours to 72 hours and most preferably 36 hours to 72 hours.

Preferably, the step of contacting the cells with the cell culture medium according to any aspect of the present invention is carried out near or close to the end of cell expansion or cell exponential phase and prior to the transfer of the cells to downstream processes such as cryopreservation, hypothermic storage, cell transplantation and/or use in a pharmaceutical composition.

Preferably, the cells are cultured in the medium according to any aspect of the present invention prior to cryopreservation of the cells.

According to yet another aspect of the present invention, there is provided a use of the cell culture medium according to any aspect of the present invention for increasing cell viability of a cell post exposure to stress.

According to a further aspect of the present invention, there is provided a method of improving stress resistance of a cell, wherein the method comprises a step of:

- culturing the cell in the cell culture medium according to any aspect of the present invention for a suitable period prior to exposure to stress.

In particular, the suitable period may refer to 4 hours to 96 hours, more particularly, 24 hours to 96 hours, even more particularly 24 hours to 72 hours or 36 hours to 72 hours.

The stress factors that the cell may be exposed to, post culture in the cell culture medium according to any aspect of the present invention may include oxidative stress, cryopreservation, hypothermic storage, exposure of the cells to specific components that prepare the cell for use in cell transplantation and/ or in a pharmaceutical composition.

As used herein the term 'stress resistance' refers to the ability of the cell to survive various intrinsic and extrinsic stressors like but not limited to reactive oxygen species, mechanical forces and lack of nutrients. The stress resistance of the cell is evaluated through the ability of the cell to maintain its viability, morphology, and functionality during and after the presence of these stressors. In one example, stress resistance of a cell is determined by evaluation of cell viability. One example of an assay that measures cell viability may be ATP.

An example of cell function pivotal to successful transplantation of adherent cells is their adhesion to physical substrates (e.g. extracellular matrix and/or tissues at the transplantation site). Loss of cell adhesion often gives rise to poor retention of transplanted cells at the transplantation site and more critically, induce cell death through anoikis. The method of culturing cells in a cell culture medium according to any aspect of the present invention can also be applied to adherent cells prior to

transplantation. This step then improves the stress resistance of the adherent cells enabling the cells to have higher chance of survival and higher success rate of the transplant.

In another example, the method of culturing cells in the cell culture medium according to any aspect of the present invention is applied prior to producing a pharmaceutical composition containing the cells.

- 5 According to another aspect of the present invention there is provided a pharmaceutical composition comprising cells cultured in a cell culture medium comprising diacylglycerophospholipids and fatty acids in a molar ratio of diacylglycerophospholipids : fatty acids ranging from 40:60 to 95:5 with the fatty acid concentration ranging from 10 μM to 300 μM and the diacylglycerophospholipid concentration ranging from 50 μM to 2500 μM . In particular, the pharmaceutical composition according to any aspect of the
- 10 present invention comprises cells cultured in a cell culture medium comprising diacylglycerophospholipids and fatty acids in a molar ratio of diacylglycerophospholipids : fatty acids ranging from 50:50 to 95:5, more preferably a molar ratio of 60:40 to 95:5 and most preferably a molar ratio of diacylglycerophospholipids : fatty acids ranging from 70:30 to 95:5.

15 BRIEF DESCRIPTION OF FIGURES

Figure 1 is a graph showing viability of human mesenchymal stem cells (hMSCs) after 48 hours of pretreatment with varying concentrations of oleic acid.

- Figure 2 is a graph showing showing cellular lipid levels of hMSCs with no pretreatment (cont), pretreatment for 48 h with 150 μM of oleic acid alone (OA) or pretreatment with various types of
- 20 liposomes containing oleic acid and diacylglycerophospholipids (L1-L4).

Figure 3 is a graph showing showing cell viability of hMSCs at day 0 of starvation and after 48 hours of pretreatment with 150 μM of oleic acid alone (OA) or pretreatment with various types liposomes containing oleic acid and diacylglycerophospholipids (L1-L4).

Figure 4 is a graph showing cell viability of hMSCs after 5 days of starvation.

- 25 Figure 5 is a graph showing cell viability of hMSCs after six days of starvation.

Figure 6 is a graph showing cellular lipid levels of hMSCs with no pretreatment (Control), pretreatment for 48 h with 150 μM of oleic acid alone (OA), pretreatment with 150 μM oleic acid and a separate dose of 350 μM diacylglycerophospholipids (OA + GPL-1) and pretreatment with 150 μM oleic acid with a separate dose of 1993 μM diacylglycerophospholipids (OA + GPL-2).

- 30 Figure 7 is a graph showing cell viability of hMSCs at day 0 of starvation and after 48 hours of pretreatment with 150 μM of oleic acid alone (OA), pretreatment with 150 μM oleic acid and a separate dose of 350 μM diacylglycerophospholipids (OA + GPL-1) and pretreatment with 150 μM oleic acid with a separate dose of 1993 μM diacylglycerophospholipids (OA + GPL-2).

Figure 8 is a graph showing cell viability of hMSCs after 5 days of starvation.

- 35 Figure 9 is a graph showing cell viability of hMSCs after 7 days of starvation.

Figure 10 is a graph showing cellular lipid levels of hMSCs with no pretreatment (cont), pretreatment with oleic acid-containing liposomes that yielded a final concentration of 150 μ M of oleic acid and 1993 μ M diacylglycerophospholipids (L4) as well as pretreatment with 125 μ M, 150 μ M or 175 μ M oleic acid alone (OA-1 – OA-3).

- 5 Figure 11 is a graph showing cell viability of hMSCs at day 0 of starvation and after a 48 hour pretreatment with oleic acid-containing liposomes (L4) as well as pretreatment with 125 μ M, 150 μ M or 175 μ M oleic acid alone (OA-1 – OA-3).

Figure 12 is a graph showing cell viability of hMSCs after 4 days of starvation.

Figure 13 is a graph showing cell viability of hMSCs after 6 days of starvation.

- 10 Figure 14 is a graph showing cell viability of hMSCs on day 0 of starvation and after 48 hours of pretreatment with 150 μ M of oleic acid alone (OA), pretreatment with 350 μ M diacylglycerophospholipids alone (GPL 1), pretreatment with 2000 μ M diacylglycerophospholipids alone (GPL 4), pretreatment with 150 μ M oleic acid and 350 μ M diacylglycerophospholipids (L1) and pretreatment with 150 μ M oleic acid and 1993 μ M diacylglycerophospholipids (L4).

- 15 Figure 15 is a graph showing cell viability of hMSCs after 4 days of starvation.

Figure 16 is a graph showing cell viability of hMSCs after 5 days of starvation.

Figure 17 is a graph showing cell viability of hMSCs after 24 hours exposure to oleic acid-containing liposomes composed of C18:3 PC and DOPC.

- 20 Figure 18 is a graph showing cell viability of hMSCs on day 0 of starvation and after 48 hours of pretreatment with 150 μ M of oleic acid alone (OA), pretreatment with 150 μ M oleic acid + 350 μ M DSPC (L (DSPC)), pretreatment with 150 μ M oleic acid + 350 μ M DOPC (L (DOPC)) and pretreatment with 17 μ M oleic acid + 20 μ M C18:3 PC + 20 μ M DOPC (L (C18:3 PC)). "NA" denotes non applicable.

Figure 19 is a graph showing cell viability of hMSCs after 4 days of starvation. "NA" denotes non applicable.

- 25 Figure 20 is a graph showing cell viability of hMSCs after 6 days of starvation. "NA" denotes non applicable.

Figure 21 is a graph showing cell viability of hMSCs on day 0 of starvation and after 48 hours of pretreatment with 150 μ M of oleic acid alone (OA) and with liposomes containing 150 μ M oleic acid, 175 μ M DSPC and 175 μ M DOPC (L(DSPC + DOPC)).

- 30 Figure 22 is a graph showing cell viability of hMSCs after 4 days of starvation.

Figure 23 is a graph showing cell viability of hMSCs after 6 days of starvation.

Figure 24 is a graph showing cell viability of hMSCs at day 0 of starvation and after a 48 hour pretreatment with lauric acid and diacylglycerophospholipids containing liposomes (LA-1 + GPL) as well as pretreatment with 300 μ M, 350 μ M or 400 μ M lauric acid alone (LA-1 – LA-3).

Figure 25 is a graph showing cell viability of hMSCs after 6 days of starvation and initial pretreatment for 48 hours with 300 μ M (LA-1), 350 μ M (LA_2) and 400 μ M (LA-3) lauric acid as well as combined pretreatment with 300 μ M lauric acid and 350 μ M diacylglycerophospholipids (LA-1 + GPL).

5 Figure 26 is a graph showing viability of hMSCs that were pretreated with 150 μ M oleic acid (OA) and pretreated with 150 μ M oleic acid and 350 μ M diacylglycerophospholipids (L1), underwent cell processing steps including enzymatic dissociation, multiple centrifugation steps, shear forces during dispersal of cells into gel materials and storage on ice and then held in regular culture medium for 4 days.

Figure 27 is a graph showing viability of hMSCs after 7 days of culture that underwent cell processing steps followed by starvation and poor adherence.

10 Figure 28 is a graph showing viability of hMSCs after four (4) days of culture that underwent cell processing steps followed by starvation and oxidative stress.

Figure 29 is a graph showing VEGF secretion of untreated and pretreated hMSCs over seven days of culture in the presence of starvation and oxidative stress.

15 Figure 30 is a graph showing cell viability of HDFs at day 0 of starvation and after 48 hours of pretreatment with liposomes containing oleic acid and diacylglycerophospholipids (L1).

Figure 31 is a graph showing cell viability of HDFs after 3 days of starvation.

Figure 32 is a graph showing cell viability of THP-1 monocytes at day 0 of starvation and after 48 hours of pretreatment with liposomes containing oleic acid and diacylglycerophospholipids (L1).

Figure 33 is a graph showing cell viability of THP-1 monocytes after 6 days of starvation.

20 Figure 34 is a graph showing cellular lipid levels of hMSCs with no pretreatment (cont), pretreatment for 48 h with 70 μ M and 80 μ M of docosahexaenoic acid (DHA-1 and DHA-2) as well as pretreatment with liposomes containing DHA and diacylglycerophospholipids (DHA-1 + GPL and DHA-2 + GPL).

25 Figure 35 is a graph showing cell viability of hMSCs at day 0 of starvation and after a 48 hour pretreatment with docosahexaenoic acid and diacylglycerophospholipids containing liposomes (DHA-1 or 2 + GPL) as well as pretreatment with 70 μ M and 80 μ M docosahexaenoic alone (DHA 1 and 2).

30 Figure 36 is a graph showing cell viability of hMSCs after 6 days of starvation and initial pretreatment for 48 hours with 70 μ M (DHA-1) and 80 μ M (DHA-2) as well as combined pretreatment with 70 μ M and 80 μ M docosahexaenoic acid and diacylglycerophospholipids (DHA-1 + GPL, DHA-2 + GPL) in the form of liposomes.

EXAMPLES

Example 1

35 *General procedure to prepare liposomes from fatty acids and diacylglycerophospholipids.*

The liposomes were prepared via sonication. As diacylglycerophospholipids 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphatidylglycerol (DOPG) were used. Oleic acid was used as fatty acid. Diacylglycerophospholipids and fatty acid were first dissolved in
5 chloroform at a total lipid mass concentration of 40mg/ml. The molar ratio of DOPC to DOPG was 92.5 : 7.5.

The molar ratio of diacylglycerophospholipids to fatty acids is indicated in the examples below and ranged from 70:30 to 95:5. The solution was blown-dry with nitrogen gas to form a thin film.

DOPG was added in addition to DOPC so as to impart negative surface charges to the resultant
10 liposomes which helps to maintain colloidal stability of the liposomal formulation.

To form liposomes containing oleic acid and diacylglycerophospholipids, degassed phosphate buffered saline was added to hydrate and resuspend the thin lipid film to a final lipid mass concentration of 20 mg/ml. The suspension was vortexed to form a milky suspension and was further sonicated in an ultrasonic water bath for approximately 15 min until the suspension turned opalescent. For
15 characterization, liposomal preparations were diluted in deionized water to final mass concentration of 1 mg/ml.

The liposomal preparations were filtered through 0.45 µm membrane as means of sterilization before use in cell culture. Liposomal preparations were then added to cell culture media to desired concentrations prior to incubation with cells.

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Example 2

General procedure of preloading human mesenchymal stem cells (hMSCs) with fatty acids and diacylglycerophospholipids

25 The hMSCs were obtained from a commercial source (STEMCELL Technologies). The hMSCs were first seeded onto suitable culture ware e.g. well plates or tissue culture flasks. In some cases, the cell culture ware was first coated with cell attachment factors (e.g. fibronectin) before cells were seeded. After hMSCs attached overnight, they were preloaded by replacing existing cell culture media with cell culture media containing oleic acid and diacylglycerophospholipids. Cells were cultured at 37°C and 5% CO₂ in
30 the presence of oleic acid and diacylglycerophospholipids for preloading to occur. Duration of the culture is indicated in the examples below.

Effects of preloading on intracellular lipids were evaluated by Nile red staining and downstream quantitative fluorescence measurements. Cells were washed 1 time with phosphate buffered saline, fixed with 4% paraformaldehyde, stained with 5 µg/ml Nile Red solution and measured for fluorescence at
35 475/570 nm and 515/590 nm. Cell nuclear or DNA staining (e.g. 4',6-diamidino-2-phenylindole DAPI) followed by quantitative fluorescence measurements was carried out to evaluate the impact of preloading on growth in cell number over the duration of preloading. Cells were washed 1 time with phosphate buffered saline, fixed with 4% paraformaldehyde, stained with 1 µg/ml DAPI and measured for fluorescence at 358/461 nm.

Quantitative measurement of cellular adenosine triphosphate (ATP) levels was carried out to evaluate the impact of preloading on cell metabolic viability. A bioluminescent assay (Promega) using a luciferase for quantification of ATP was used. The cells were lysed and incubated with the luciferase reagent and subsequently measured for luminescence.

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Example 3

Test of Cell Viability of hMSCs at different concentrations of fatty acid, oleic acid

Human mesenchymal stem cells (hMSCs) were pretreated for 48 h with cell culture media (Dulbecco's modified eagle medium containing 10% v/v fetal bovine serum) to which oleic acid stock solution (100mM in ethanol) had been added to varying targeted final concentrations 150µM, 200µM and 250µM. Results are shown in Figure 1. Pretreatment of hMSCs with oleic acid induces lipid droplet formation which in turn imparts cells with tolerance against cell stress. However, the concentration of oleic acid used in pretreatment cannot be increased indefinitely in the attempt to increase the stress tolerance effects. High concentration of oleic acid induces toxicity on cells beyond a certain concentration. Analysis of Variance (ANOVA) was carried out and statistical significance was determined by Tukey post-hoc test and applied to pairwise comparisons. A p-value below 0.05 denotes statistical significance.

As shown in Figure 1 oleic acid reduces cell viability at a concentration of 200 µM or above ($p < 0.001$ for 200 µM and 250 µM compared with Control). Fatty acids can induce toxicity at high concentrations and it is not possible to keep increasing fatty acid concentrations to increase the stress tolerance of the cells.

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Example 4

Test of Cell Viability of hMSCs in presence of liposomes of Example 1

Human mesenchymal stem cells (hMSCs) were cultured in Dulbecco's modified eagle medium containing 10% v/v fetal bovine serum, 25mM glucose, 1mM sodium pyruvate and 2mM L-alanyl-L-glutamine dipeptide. The hMSCs were pretreated with different types of liposomes for 48 h. The liposomes were produced via sonication as described in Example 1. The diacylglycerophospholipids content comprised of 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphatidylglycerol (DOPG) in the molar ratio of 92.5:7.5. Oleic acid was used as fatty acid.

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Across all liposome types L1 to L4, the liposomes were added to the culture medium to a final oleic acid concentration of 150 µM while the final concentration of diacylglycerophospholipids ranged from 350 to 1993 µM. For comparison, hMSCs were also pretreated with 150 µM oleic acid for 48 h by adding oleic acid stock solution (100 mM in ethanol) directly to the culture medium.

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The various pretreatments performed on hMSCs are listed as follows:

Cont: Control (no addition of oleic acid and no addition of diacylglycerophospholipids)

OA: 150µM oleic acid

L1: 150µM oleic acid + 350µM diacylglycerophospholipids

L2: 150µM oleic acid + 850µM diacylglycerophospholipids

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L3: 150µM oleic acid + 1350µM diacylglycerophospholipids

L4: 150µM oleic acid + 1993µM diacylglycerophospholipids

For samples L1 to L4 oleic acid and diacylglycerophospholipids were added in the form of liposomes.

Figure 2 shows the cellular lipid levels measured by Nile red staining after 48 hours of pretreatment with oleic acid and various types of liposomes. Cellular lipid levels were normalized to that of untreated

5 hMSCs (Cont – control). Figure 2 shows that hMSCs pretreated with 150 µM oleic acid alone or various types of liposomes containing oleic acid and diacylglycerophospholipids increased their cellular lipid levels relative to untreated control (Cont) ($p < 0.001$). The increases in lipid levels among the various pretreatments were statistically similar.

After the 48 h pretreatment, existing culture media was removed and hMSCs were washed once with
10 phosphate-buffered saline to ensure removal of culture media. After washing, hMSCs were starved for 6 days in Dulbecco's modified eagle medium that was serum-free and had the concentrations of glucose, sodium pyruvate and L-alanyl-L-glutamine dipeptide reduced significantly to 0.25 mM, 0.01 mM and 0.02 mM respectively. The viability of the starved hMSCs was then evaluated at various timepoints by measuring cellular adenosine triphosphate (ATP) levels as described in Example 2.

15 a) Cell viability at Day 0

Quantitative measurement of cellular adenosine triphosphate (ATP) levels was carried out as described in Example 2. Figure 3 shows that viability of hMSCs right before the start of starvation was similar across all types of pretreatment.

b) Cell viability at Day 5

20 Quantitative measurement of ATP levels after 5 days of starvation showed that viability of untreated hMSCs decreased significantly (Figure 4). The viability of hMSCs pretreated with 150 µM oleic acid (OA) alone was higher than that of untreated hMSCs (Cont) ($p < 0.001$). The viability of hMSCs pretreated with liposomes (L1 – L4) was even higher ($p < 0.001$ referenced to untreated hMSCs (Cont)) at levels similar to those observed at day 0 before the start of starvation.

25 c) Cell viability at Day 6

Figure 5 shows the measurement of ATP levels after six days of starvation. The viability of untreated hMSCs (Cont) and hMSCs treated with oleic acid alone (OA) had dropped to nearly zero after six days. In contrast, the viabilities of hMSCs treated with oleic acid and diacylglycerophospholipids containing liposomes (L1-L4) remained substantially higher than untreated hMSCs (Cont) ($p < 0.001$). The viabilities
30 of hMSCs treated with oleic acid and diacylglycerophospholipids containing liposomes (L1-L4) also remained substantially higher than hMSCs treated with oleic acid alone (OA) ($p < 0.001$). Among them, it was also observed that cultures L3 and L4 which are hMSCs pretreated with liposomes that contained higher ratios of diacylglycerophospholipids exhibited even higher viabilities.

The use of diacylglycerophospholipids together with fatty acids increases starvation tolerance beyond that
35 achieved by oleic acid alone. In addition to that the high amounts of added diacylglycerophospholipids surprisingly did not induce toxicity.

Example 5

40 *Test of Cell Viability of hMSCs in presence of fatty acids and diacylglycerophospholipids were separately dosed into the culture medium*

Example 5 was carried out similar to Example 4 with the difference that fatty acids and diacylglycerophospholipids were separately dosed into the culture medium. Human mesenchymal stem cells (hMSCs) were cultured in Dulbecco's modified eagle medium containing 10% v/v fetal bovine serum and 25mM glucose, 1mM sodium pyruvate and 2mM L-alanyl-L-glutamine dipeptide. hMSCs were pretreated with oleic acid and separate doses of diacylglycerophospholipids in the form of fatty acid-free liposomes for 48 h. For these pretreatments, oleic acid stock solution (100mM in ethanol) was directly diluted into the culture medium to achieve a final oleic acid concentration of 150 μ M, followed by the addition of fatty acid-free liposomes to achieve a final diacylglycerophospholipid concentration of 350 μ M or 1993 μ M.

All fatty acid-free liposomes were produced via sonication and the diacylglycerophospholipid content comprised of 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphatidylglycerol (DOPG) in the molar ratio of 92.5:7.5. For comparison, hMSCs were also pretreated with 150 μ M oleic acid alone by diluting oleic acid stock solution (100mM in ethanol) into the culture media.

The various pretreatments are listed as follows:

Control (no addition of fatty acid and no addition of diacylglycerophospholipids)

OA: 150 μ M oleic acid

OA + GPL-1: 150 μ M oleic acid + 350 μ M diacylglycerophospholipids

OA + GPL-2: 150 μ M oleic acid + 1993 μ M diacylglycerophospholipids

Figure 6 shows the cellular lipid levels of hMSCs after pretreatment for 48 hours. The cellular lipid levels were determined via Nile red staining. Cellular lipid levels are shown normalized to that of untreated hMSCs (Control). As can be seen in Figure 6, hMSCs pretreated with 150 μ M oleic acid (OA) alone or combinations of 150 μ M oleic acid and diacylglycerophospholipids in the form of fatty acid-free liposomes (OA + GPL-1 & OA + GPL-2) increased their cellular lipid levels substantially relative to untreated control ($p < 0.001$, Tukey test). While the addition of oleic acid and monounsaturated diacylglycerophospholipids as separate doses appeared generally similar to oleic acid-containing liposomes, 150 μ M oleic acid and a separate high dose of 1993 μ M diacylglycerophospholipids (OA+GPL-2) was slightly less potent in terms of increasing cellular lipid accumulation relative to oleic acid alone (OA) ($p < 0.05$, Tukey).

After the 48 h pretreatment, existing culture media was removed and hMSCs were washed once with phosphate-buffered saline to ensure removal of culture media. After washing, hMSCs were starved in Dulbecco's modified eagle medium that was serum-free and had the concentrations of glucose, sodium pyruvate and L-alanyl-L-glutamine dipeptide reduced significantly to 0.25 mM, 0.01 mM and 0.02 mM respectively. The viability of the starved hMSCs was then evaluated at various timepoints by measuring cellular adenosine triphosphate (ATP) levels as described in Example 2.

a) Cell viability at Day 0

Quantitative measurement of cellular adenosine triphosphate (ATP) levels was carried out as described in Example 2. Figure 7 shows that viability of hMSCs right before the start of starvation was similar across all types of pretreatment. Similar to Example 4, the separate addition of diacylglycerophospholipids did not induce toxicity in the cells.

b) Cell viability at Day 5

Quantitative measurement of cellular adenosine triphosphate (ATP) levels was carried out as described in Example 2. Quantitative measurement of ATP levels after 5 days of starvation showed that viability of untreated hMSCs (Cont) decreased significantly (Figure 8). The viability of hMSCs pretreated with 150 μ M oleic acid alone as well as hMSCs pretreated with 150 μ M oleic acid and a separate dose of 350 μ M diacylglycerophospholipids (OA + GPL-1) and pretreated with 150 μ M oleic acid with a separate dose of 1993 μ M GPL (OA + GPL-2) were higher than that of untreated hMSCs ($p < 0.001$, Tukey test).

c) Cell viability at Day 7

Quantitative measurement of cellular adenosine triphosphate (ATP) levels was carried out as described in Example 2. Figure 9 shows the quantitative measurement of ATP levels after seven days of starvation. The viability of untreated hMSCs and hMSCs treated with oleic acid alone had dropped to nearly zero. In contrast, hMSCs treated with a combination of oleic acid and diacylglycerophospholipids (cultures "OA + GPL-1" and "OA + GPL-2") remained substantially higher than untreated control (Cont) ($p < 0.01$, Tukey test) and substantially higher than hMSCs treated with oleic acid alone (OA) ($p < 0.01$, Tukey test). The separate addition of diacylglycerophospholipids and oleic acid (i.e. not formulated together as liposomes) also produced enhanced starvation tolerance compared to oleic acid alone. This observation was surprising for "OA + GPL-2" given that "OA + GPL-2" resulted in a slightly lower lipid accumulation than OA alone.

20 Example 6*Optimisation of concentration of Oleic acid alone with hMSCs*

The purpose of Example 6 was to investigate whether optimizing the concentration of oleic acid and using it alone could replicate the enhanced stress resistance observed with the combination of oleic acid and diacylglycerophospholipids (GPL). As shown in Example 3 fatty acids reduce cell viability at a concentration of 200 μ M or above. Thus, concentrations of 125 μ M, 150 μ M and 175 μ M were applied. Human mesenchymal stem cells (hMSCs) were cultured in Dulbecco's modified eagle medium containing 10% v/v fetal bovine serum and 25mM glucose, 1mM sodium pyruvate and 2mM L-alanyl-L-glutamine dipeptide. For 48 h, hMSCs were pretreated with varying concentrations of oleic acid alone, namely 125 μ M, 150 μ M and 175 μ M, for 48 h by diluting oleic acid stock solution (100mM in ethanol) into the culture media. The hMSCs were also pretreated for 48 h with oleic acid and diacylglycerophospholipids containing liposomes. The liposomes were produced via sonication and the diacylglycerophospholipids content comprised of 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphatidylglycerol (DOPG) in the molar ratio of 92.5:7.5. The liposomes yielded a final concentration of 150 μ M oleic acid and 1993 μ M diacylglycerophospholipids.

The various pretreatments are listed as follows:

Cont: Control

L4: 150 μ M oleic acid + 1993 μ M diacylglycerophospholipids (in the form of liposomes)

OA-1: 125 μ M oleic acid

40 *OA-2: 150 μ M oleic acid*

OA-3: 175µM oleic acid

After the 48-h pretreatment, existing culture media was removed and hMSCs were washed once with phosphate-buffered saline to ensure complete removal of culture media. After washing, hMSCs were starved in Dulbecco's modified eagle medium that was serum-free and had the concentrations of glucose, sodium pyruvate and L-alanyl-L-glutamine dipeptide reduced significantly to 0.25 mM, 0.01 mM and 0.02 mM respectively. The viability of the starved hMSCs was then evaluated at various timepoints by measuring cellular adenosine triphosphate (ATP) levels as described in Example 2.

Figure 10 shows the cellular lipid levels after 48 hours of various pretreatments. Cellular lipid levels were normalized to that of untreated hMSCs (Cont – control). After 48 h pretreatment hMSCs were measured for cellular lipid levels via Nile red staining. After 48 h, all pretreated hMSCs (i.e. L4, OA-1, OA-2 & OA-3) increased their cellular lipid levels relative to untreated hMSCs (control) ($p < 0.001$, Tukey test). The hMSCs pretreated with 150 µM oleic acid alone (OA-2) exhibited lipid levels similar to hMSCs pretreated with liposomes containing oleic acid and diacylglycerophospholipids (L4). On the other hand, hMSCs pretreated with 125 µM (OA-1) and 175 µM (OA-3) oleic acid exhibited lipid levels lower and higher than hMSCs pretreated with oleic acid-containing liposomes (L4) respectively ($p < 0.05$, Tukey test).

a) Cell viability at Day 0

Quantitative measurement of cellular adenosine triphosphate (ATP) levels was carried as described in Example 2. Figure 11 shows that viability of hMSCs right before the start of starvation was similar across all types of pretreatment.

b) Cell viability at Day 4

Quantitative measurement of cellular adenosine triphosphate (ATP) levels was carried out as described in Example 2. Quantitative measurement of ATP levels after 4 days of starvation showed that viability of untreated hMSCs decreased significantly (Figure 12). On the other hand, the viabilities of pretreated hMSCs remained substantially higher relative to untreated control ($p < 0.001$, Tukey test), particularly those pretreated with oleic acid and diacylglycerophospholipids containing liposomes (L4) and those with high concentrations of oleic acid (OA-3).

c) Cell viability at Day 6

Quantitative measurement of cellular adenosine triphosphate (ATP) levels was carried out as described in Example 2. Figure 13 shows the quantitative measurement of ATP levels after 6 days of starvation. The viability of untreated hMSCs (Cont) and hMSCs treated with oleic acid alone (OA-1, OA-2, OA-3) had dropped to nearly zero after six days. Only the viability of hMSCs pretreated with oleic acid and diacylglycerophospholipids containing liposomes (L4) remained unchanged since day 4 and substantially higher than the rest ($p < 0.001$, Tukey test).

The combination of oleic acids with diacylglycerophospholipids (GPL) outperforms oleic acid alone at the various concentrations selected based on lipotoxicity limits. This outperformance is surprising particularly with respect to OA-3 which could induce the highest level of cellular lipid accumulation across all pretreatment groups. Thus, the protective effect of oleic acid combined with diacylglycerophospholipids cannot be replicated with oleic acid alone through optimizing concentrations.

Example 7

Cell Viability test using hMSCs in presence of liposomes of Example 1, oleic acid alone and diacylglycerophospholipids alone

Example 7 was carried out similar to Example 4 with the difference that combinations of oleic acid and diacylglycerophospholipids were compared directly against oleic acid alone and diacylglycerophospholipids alone.

Human mesenchymal stem cells (hMSCs) were cultured in Dulbecco's modified eagle medium containing 10% v/v fetal bovine serum and 25mM glucose, 1mM sodium pyruvate and 2mM L-alanyl-L-glutamine dipeptide. The hMSCs were pretreated with 150µM oleic acid alone for 48 h by diluting oleic acid stock solution (100mM in ethanol) into the culture media. The hMSCs were also pretreated with 350µM diacylglycerophospholipids alone and 2000µM diacylglycerophospholipids alone for 48 h by adding fatty acid-free liposomes to the culture media. Finally, hMSCs were pretreated with combinations of oleic acid and diacylglycerophospholipids by adding oleic acid and diacylglycerophospholipids containing liposomes that yielded a final concentration of 150 µM oleic acid + 350 µM diacylglycerophospholipids and by adding oleic acid- and diacylglycerophospholipids containing liposomes at a final concentration of 150 µM oleic acid + 1993 µM diacylglycerophospholipids.

Both, fatty acid-free liposomes and oleic acid/diacylglycerophospholipids containing liposomes were produced via sonication. Across all pretreatments that involved diacylglycerophospholipids, the diacylglycerophospholipids content comprised of 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphatidylglycerol (DOPG) in the molar ratio of 92.5:7.5.

The various pretreatments were as follows:

Cont: Control (no fatty acid, no diacylglycerophospholipids)

OA: 150µM oleic acid

GPL1: 350µM diacylglycerophospholipids (in the form of fatty acid-free liposomes)

GPL4: 2000µM diacylglycerophospholipids (in the form of fatty acid-free liposomes)

L1: 150µM oleic acid + 350µM diacylglycerophospholipids (in the form of oleic acid and diacylglycerophospholipids containing liposomes)

L4: 150µM oleic acid + 1993µM diacylglycerophospholipids (in the form of oleic acid and diacylglycerophospholipids containing liposomes)

After the 48 h pretreatment, existing culture media was removed and hMSCs were washed once with phosphate-buffered saline to ensure removal of culture media. After washing, hMSCs were starved in Dulbecco's modified eagle medium that was serum-free and had the concentrations of glucose, sodium pyruvate and L-alanyl-L-glutamine dipeptide reduced significantly to 0.25 mM, 0.01 mM and 0.02 mM respectively. The viability of the starved hMSCs was then evaluated at various timepoints by quantitative measurement of cellular adenosine triphosphate (ATP) levels carried out as described in Example 2.

a) Cell viability at Day 0

Quantitative measurement of cellular adenosine triphosphate (ATP) levels was carried out as described in Example 2. Figure 14 shows that viability of hMSCs right before the start of starvation was similar across all types of pretreatment.

b) Cell viability at Day 4

Figure 15 shows the quantitative measurement of ATP levels after four days of starvation. The viability of untreated hMSCs (Cont) decreased to nearly zero. The viability of hMSCs pretreated with oleic acid alone (OA) or diacylglycerophospholipids alone (GPL 1, GPL 2) were higher than untreated hMSCs, but they had also dropped significantly. In contrast, the viabilities of hMSCs pretreated with liposomes containing oleic acid and diacylglycerophospholipids were higher than untreated hMSCs (Cont) and hMSCs pretreated with oleic acid (OA) or diacylglycerophospholipids alone (GPL-1, GPL-2) ($p < 0.001$, Tukey test). The enhancement was synergistic i.e. significantly better than what could be expected from combining individual effects of oleic acid and diacylglycerophospholipids. Surprisingly, the combination of monounsaturated diacylglycerophospholipids and oleic acid imparts significantly higher starvation tolerance than each component alone and the expected sum of both components.

c) Cell viability at Day 5

Figure 16 shows the quantitative measurement of ATP levels after five days of starvation. The viability of untreated hMSCs (Cont) as well as hMSCs pretreated with oleic acid alone (OA) or GPL alone (GPL 1, GPL 4) had decreased to nearly zero. In contrast, the viability of hMSCs pretreated with liposomes containing oleic acid and diacylglycerophospholipids (L1, L4) remained much higher than all other samples ($p < 0.01$, Tukey test). Similar to Example 4, the liposomes having a higher diacylglycerophospholipids content (L4) imparted better starvation resistance to the pretreated hMSCs. ($p < 0.001$, Tukey test).

Again, the combination of monounsaturated diacylglycerophospholipids and oleic acid surprisingly imparts higher starvation tolerance than each component alone and the expected sum of both components.

Example 8

Cell Viability test using hMSCs with different diacylglycerophospholipids in the liposomes

Example 8 was carried out similar to Example 4 with the difference that as diacylglycerophospholipids 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 1,2-dilinolenoyl-sn-glycero-3-phosphocholine (18:3 PC) were used alongside 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) to produce three types of oleic acid-containing liposomes. 18:3 denotes alkyl chain length of C18 comprising 3 double bonds. Specifically, the diacylglycerophospholipids composition in each type of liposomes by molar % was

- 1.) 100% DSPC,
- 2.) 100% DOPC
- 3.) 50% 18:3 PC + 50% DOPC.

Across all three types of diacylglycerophospholipids, the acyl groups are 18-carbon long but contain different number of double bonds. DSPC contains 0 double bonds, DOPC contains 1 double bond and C18:3 PC contains 3 double bonds on each of the two acyl groups respectively. Human mesenchymal stem cells (hMSCs) were cultured in Dulbecco's modified eagle medium containing 10% v/v fetal bovine serum and 25mM glucose, 1mM sodium pyruvate and 2mM L-alanyl-L-glutamine dipeptide.

Figure 17 shows the result for testing cytocompatible concentrations of diacylglycerophospholipids composed of 50% 18:3 PC + 50% DOPC.

The final concentrations were:

0 μM – no oleic acid, no C18:3 PC and no DOPC

25 μM – 4 μM of oleic acid + 25 μM C18:3 PC + 25 μM DOPC

87.5 μM – 13 μM of oleic acid + 87.5 μM C18:3 PC + 87.5 μM DOPC

5 200 μM – 25 μM of oleic acid + 200 μM C18:3 PC + 200 μM DOPC.

Viability is normalized to control hMSCs and expressed as %. Because toxic effects have been reported for polyunsaturated glycerophospholipids such as C18:3 PC ("Interference of phosphatidylcholines with in-vitro cell proliferation – no flock without black sheep" *Biochimica et Biophysica Acta* 1848 (2015) 1599 – 1608), oleic acid-containing liposomes composed of 50% C18:3 PC + 50% DOPC were first tested on

10 hMSCs to determine cytocompatible concentrations. Figure 17 shows that when hMSCs were cultured for 24 h with oleic acid-containing liposomes prepared with C18:3 PC, a final C18:3 PC concentration of 25 μM was cytocompatible while a final C18:3 PC concentration of 87.5 μM or higher lowered viability of hMSCs. Subsequently, hMSCs were pretreated with the three types of liposomes for 48 h by diluting each liposome stock into the culture media. All liposomes are produced via sonication and did not contain

15 DOPG. For comparison, hMSCs were also pretreated with 150 μM oleic acid alone by diluting oleic acid stock solution (100mM in ethanol) into the culture media.

The pretreatments were as follows:

Cont: Control

OA: 150 μM oleic acid

20 *L (DSPC): 150 μM oleic acid + 350 μM DSPC*

(in the form of oleic acid and diacylglycerophospholipids containing liposomes)

L (DOPC): 150 μM oleic acid + 350 μM DOPC

(in the form of oleic acid and diacylglycerophospholipids containing liposomes)

L (C18:3): 17 μM oleic acid + 20 μM C18:3 PC + 20 μM DOPC

25 *(in the form of oleic acid and diacylglycerophospholipids containing liposomes)*

After the 48 h pretreatment, existing culture media was removed and hMSCs were washed once with phosphate-buffered saline to ensure complete removal of culture media. After washing, hMSCs were starved in Dulbecco's modified eagle medium that was serum-free and had the concentrations of glucose, sodium pyruvate and L-alanyl-L-glutamine dipeptide reduced significantly to 0.25 mM, 0.01 mM and 0.02

30 mM respectively. The viability of the starved hMSCs was then evaluated at various timepoints by quantitative measurement of cellular adenosine triphosphate (ATP) levels carried out as described in Example 2.

a) Cell viability at Day 0

Figure 18 shows that viability of hMSCs right before the start of starvation was similar across all types of

35 pretreatment.

b) Cell viability at Day 4

Figure 19 shows the quantitative measurement of ATP levels after four days of starvation. The viability of untreated hMSCs decreased to nearly zero. The average viability of hMSCs pretreated with oleic acid alone (OA) was statistically similar to untreated hMSCs. The viability of hMSCs pretreated with L (DSPC) and L (DOPC) were substantially higher than the viability of untreated hMSCs ($p < 0.001$, Tukey test) and

40

hMSCs pretreated with oleic acid alone (OA) ($p < 0.001$, Tukey test). The viability of hMSCs pretreated with L (C18:3 PC), however, also decreased to nearly zero. This may be due to low concentration of oleic acid and diacylglycerophospholipids.

c) Cell viability at Day 6

- 5 Measurement of ATP levels after 6 days of starvation showed that viabilities of untreated hMSCs as well as hMSCs pretreated with oleic acid alone had decreased to nearly zero. Similar to day 4, hMSCs pretreated with L (C18:3) did not exhibit any enhanced starvation tolerance relative to those pretreated with oleic acid alone. In contrast, the viabilities of hMSCs pretreated with L(DSPC) and L(DOPC) remained significantly higher than untreated hMSCs and hMSCs pretreated with oleic acid alone ($p < 0.05$ and $p < 0.01$ respectively, Tukey test).
- 10 Similar to DOPC, addition of fully saturated diacylglycerophospholipids (DSPC) enhances starvation tolerance beyond oleic acid alone.

Example 9

- 15 *Cell Viability test using hMSCs with different diacylglycerophospholipids in the liposomes*

Example 9 was carried out similar to Example 8 with the difference that 1,2-dilinolenoyl-sn-glycero-3-phosphocholine (DSPC) were used together with 1,2-dioleoyl-sn-glycero-3-phospholine (DOPC) as a mixture of diacylglycerophospholipids to produce oleic acid-containing liposomes L (DSPC + DOPC). The molar ratio of DSPC to DOPC is 1:1. The content of diacylglycerophospholipids and oleic acid was fixed at a ratio of 70:30. Human mesenchymal stem cells (hMSCs) were cultured in D L-alanyl-L-glutamine dipeptide Dulbecco's modified eagle medium containing 10% v/v fetal bovine serum, 25mM glucose, 1 mM sodium pyruvate and 2mM L-alanyl-L-glutamine dipeptide.

- For 48 h, hMSCs were pretreated with L (DSPC + DOPC) at a concentration that yielded a final concentration of 150 μ M oleic acid and 350 μ M GPL. hMSCs were also pretreated with 150 μ M oleic acid alone for comparison.

The pretreatments were carried out as follows:

Cont: Control

OA: 150 μ M oleic acid

- 30 *L (DSPC + DOPC): 150 μ M oleic acid + 175 μ M DSPC + 175 μ M DOPC (in the form of oleic acid and diacylglycerophospholipids containing liposomes)*

After the 48 h pretreatment, existing culture media was removed and hMSCs were washed once with phosphate-buffered saline to ensure complete removal of culture media. After washing, hMSCs were starved in Dulbecco's modified eagle medium that was serum-free and had the concentrations of glucose, sodium pyruvate and L-alanyl-L-glutamine dipeptide reduced significantly to 0.25 mM, 0.01 mM and 0.02 mM respectively. The viability of the starved hMSCs was then evaluated at various timepoints by quantitative measurement of cellular adenosine triphosphate (ATP) levels carried out as described in Example 2.

a) Cell viability at Day 0

Quantitative measurement of cellular adenosine triphosphate (ATP) levels was carried out as described in Example 2. Figure 21 shows that viability of hMSCs right before the start of starvation was similar across all types of pretreatment.

b) Cell viability at Day 4

- 5 Figure 22 shows the quantitative measurement of ATP levels after four days of starvation. The viability of untreated hMSCs (Cont) decreased to nearly zero. The viability of hMSCs pretreated with oleic acid alone also dropped significantly despite exhibiting improvement over untreated hMSCs. The viability of hMSCs pretreated with L (DSPC + DOPC) remained substantially higher than untreated hMSCs ($p < 0.001$, Tukey test) and hMSCs pretreated with oleic acid alone ($p < 0.001$, Tukey test), demonstrating the
- 10 effectiveness of the mixture of saturated and monounsaturated diacylglycerophospholipids in enhancing starvation tolerance.

c) Cell viability at Day 6

- Figure 23 shows the quantitative measurement of ATP levels after six days of starvation. The viability of untreated hMSCs (Cont) as well as hMSCs pretreated with oleic acid alone (OA) had decreased to nearly
- 15 zero. Again, the viability of hMSCs pretreated with oleic acid and diacylglycerophospholipids containing liposomes composed from a 50:50 mixture of DSPC and DOPC (L (DSPC + DOPC)) remained significantly higher than untreated hMSCs ($p < 0.001$, Tukey test) and hMSCs pretreated with oleic acid alone ($p < 0.001$, tukey test).

20 **Example 10**

Cell Viability test using hMSCs with different types of fatty acids (i.e. Lauric acid) in combination with diacylglycerophospholipids

- In example 10 it was investigated whether the enhanced protective effect imparted by the combination of
- 25 diacylglycerophospholipids and oleic acid also applies to other types of fatty acids. Lauric acid was investigated as another fatty acid example since with the difference being that oleic acid is a 18-carbon long fatty acid with one carbon-carbon double bond while lauric acid is a 12-carbon long fatty acid without any carbon-carbon double bond. Human mesenchymal stem cells (hMSCs) were cultured in Dulbecco's modified eagle medium containing 10% v/v fetal bovine serum, 25mM glucose, 1mM sodium pyruvate
- 30 and 2mM L-alanyl-L-glutamine dipeptide.

- The hMSCs were pretreated with lauric acid and a separate dose of diacylglycerophospholipids in the form of fatty acid-free liposomes for 48 h. For these pretreatments, lauric acid stock solution (100mM in ethanol) was directly diluted into the culture medium to achieve a final lauric acid concentration of 300 μ M, followed by the addition of fatty acid-free liposomes to achieve a final diacylglycerophospholipid
- 35 concentration of 350 μ M. All fatty acid-free liposomes were produced via sonication and the diacylglycerophospholipids content comprised of 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphatidylglycerol (DOPG) in a molar ratio of 92.5:7.5. For comparison, hMSCs were also pretreated with 300 μ M, 350 μ M or 400 μ M lauric acid alone by diluting lauric acid stock solution (100mM in ethanol) into the culture media. After the 48 h pretreatment, existing culture media
- 40 was removed and hMSCs were washed once with phosphate-buffered saline to ensure complete removal

of culture media. After washing, hMSCs were starved in Dulbecco's modified eagle medium that was serum-free and had the concentrations of glucose, sodium pyruvate and L-alanyl-L-glutamine dipeptide reduced significantly to 0.25 mM, 0.01 mM and 0.02 mM respectively. The viability of the starved hMSCs was then evaluated at various timepoints by quantitative measurement of cellular adenosine triphosphate

5 (ATP) levels carried out as described in Example 2.

Fatty acids and diacylglycerophospholipids (GPL) were added as follows:

Cont: Control without any added fatty acids or diacylglycerophospholipids

LA-1 + GPL: 300µM lauric acid + 350µM diacylglycerophospholipids

LA-1: 300µM lauric acid

10 *LA-2: 350µM lauric acid*

LA-3: 400µM lauric acid

a) Cell viability at day 0

Quantitative measurement of cellular adenosine triphosphate (ATP) levels was carried out as described in Example 2. Figure 24 shows that viability of hMSCs right before the start of starvation was similar across all types of pretreatment. In line with previous studies reporting cytotoxicity of saturated fatty acids such as lauric acid, all the pretreated hMSCs exhibited slightly lower viability than untreated hMSCs (Cont) ($p < 0.001$, Tukey test).

15 all types of pretreatment. In line with previous studies reporting cytotoxicity of saturated fatty acids such as lauric acid, all the pretreated hMSCs exhibited slightly lower viability than untreated hMSCs (Cont) ($p < 0.001$, Tukey test).

b) Cell viability after 6 days

In Figure 25, measurement of ATP levels after 6 days of starvation showed that viabilities of untreated hMSCs as well as hMSCs pretreated with lauric acid alone had decreased substantially.

20 In contrast, the viability of hMSCs pretreated with 300 µM lauric acid and 350 µM GPL remained significantly higher than untreated hMSCs (Cont) and hMSCs treated with lauric acid alone (LA-1, LA-2 and LA-3) ($p < 0.001$, Tukey test). Therefore, diacylglycerophospholipids can also be used together with other fatty acids such as saturated fatty acids to increase starvation tolerance beyond that achieved by fatty acid alone.

Example 11

Exposure of hMSCs to different stresses to test tolerance of cells post treatment with fatty acids and diacylglycerophospholipids

30 The above examples observed enhanced starvation tolerance in hMSCs pretreated with the combination of diacylglycerophospholipids and fatty acid in comparison to hMSCs pretreated with fatty acid alone. Example 11 investigated whether this enhanced tolerance would apply to other types of stresses such as shear stress, poor adherence and oxidative stress.

35 a) Exposure to stresses during typical processing steps in cell therapy

Human mesenchymal stem cells (hMSCs) were cultured in Dulbecco's modified eagle medium containing 10% v/v fetal bovine serum and 25 mM glucose, 1 mM sodium pyruvate and 2 mM L-alanyl-L-glutamine dipeptide. The hMSCs were pretreated with oleic acid and diacylglycerophospholipids containing liposomes for 48 h. All liposomes were produced via sonication. The diacylglycerophospholipids content comprised of 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-

40 comprised of 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-

phosphatidylglycerol (DOPG) in the molar ratio of 92.5:7.5. The oleic acid and diacylglycerophospholipids containing liposomes were added to the culture medium to a final oleic acid concentration of 150 μ M and a final diacylglycerophospholipids concentration of 350 μ M. For comparison, hMSCs were also pretreated with 150 μ M oleic acid for 48 h by adding oleic acid stock solution (100mM in ethanol) directly to the culture medium. To mimic stresses afflicted upon cells during typical processing steps in cell therapy workflow, the pretreated hMSCs were dissociated from culture ware using trypsin and ethylenediaminetetraacetic acid (EDTA). The harvested hMSCs were then centrifuged for 5 min at 300 g, resuspended at 1 million per ml in Dulbecco's modified eagle medium containing 10% v/v fetal bovine serum and stored on ice for 3 hrs. After this, the hMSCs were centrifuged for 5 min at 300g glucose, washed once with phosphate-buffered saline and resuspended at a high density of 10 million cells per ml into 24 wt% pluronic acid gel. To ensure thorough dispersion of the cell pellet in the pluronic acid and to mimic shearing forces during such cell processing steps, resuspension of hMSCs into pluronic gel was performed by pipetting 25 times through a 200 μ l pipette tip. 5 μ l of the hMSC-laden gel suspension was then deposited into tissue culture plate and incubated at 37°C for 10 min for gelation to occur. The hMSCs were then cultured in full nutrient culture media (Dulbecco's modified eagle medium containing 10% v/v fetal bovine serum) so that there was no nutrient starvation and changes to cell viability could be attributed to the stresses that occurred during the cell processing step. The ATP levels of the hMSCs was then evaluated after four days by quantitative measurement of cellular adenosine triphosphate (ATP) levels carried out as described in Example 2. The quantitative measurement of ATP levels after 4 days of culture is expressed as percentage of day 0 ATP levels.

The various pretreatments were as follows:

Cont: Control (no pretreatment)

OA: 150 μ M oleic acid

L1: 150 μ M oleic acid and 350 μ M diacylglycerophospholipids (in the form of liposomes)

Figure 26 shows that for untreated hMSCs, the cell processing steps had a significant impact such that the ATP level of hMSCs only recovered to 31 % of Day 0 after 4 days of growth in regular culture medium. Pretreatment with oleic acid alone reduced the impact of cell processing steps on hMSCs such that the ATP level of hMSCs returned to 97 % of Day 0 after 4 days of growth in regular culture medium. Pretreatment with liposomes containing oleic acid and GPL fared the best in minimizing the impact of cell processing steps on hMSCs such that the ATP level of hMSCs after 4 days of growth in regular culture medium reached 134 %, which was significantly higher than untreated hMSCs (Cont) ($p < 0.001$, Tukey test) and hMSCs pretreated with oleic acid alone ($p < 0.001$, Tukey test).

b) Exposure to stresses typical after cell transplantation

Human mesenchymal stem cells (hMSCs) were cultured in Dulbecco's modified eagle medium containing 10% v/v fetal bovine serum and 25 mM glucose, 1 mM sodium pyruvate and 2 mM L-alanyl-L-glutamine dipeptide. The hMSCs were pretreated with oleic acid-containing liposomes for 48 h. All liposomes were produced via sonication. The diacylglycerophospholipids content comprised of 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphatidylglycerol (DOPG) in the molar ratio of 92.5:7.5. The oleic acid-liposomes were added to the culture medium to a final oleic acid

concentration of 150 μM and a final diacylglycerophospholipids concentration of 350 μM . For comparison, hMSCs were also pretreated with 150 μM oleic acid for 48 h by adding oleic acid stock solution (100mM in ethanol) directly to the culture medium. The hMSCs were then processed via the same steps as above into 24 wt% pluronic acid and plated onto tissue culture plate. To mimic stresses such as starvation and poor adherence that were commonly encountered during cell transplantation, the tissue culture plate was not coated with any attachment factors and hMSCs were starved in Dulbecco's modified eagle medium that was serum-free and had the concentrations of glucose, sodium pyruvate and L-alanyl-L-glutamine dipeptide reduced significantly to 0.25 mM, 0.01 mM and 0.02 mM respectively. The viability of the stressed hMSCs was then evaluated at day seven (7) by quantitative measurement of cellular adenosine triphosphate (ATP) levels as described in Example 2. Figure 27 shows the quantitative measurement of ATP levels after 7 days of culture and expressed as a percentage of ATP level at day 0 right before culture as an indicator of cell viability.

The various pretreatments were as follows:

Cont: Control (no pretreatment)

15 *OA: pretreatment with 150 μM oleic acid*

L1: pretreatment with 150 μM oleic acid and 350 μM diacylglycerophospholipids (in the form of liposomes)

The measurement of cell viability after 7 days of starvation and poor adherence showed a significant drop in cell viability to 4.5 % for untreated hMSCs (Cont). Pretreatment with oleic acid (OA) helped to improve the viability of hMSCs to 11.4%. Pretreatment with liposomes containing oleic acid and diacylglycerophospholipids (L1) further enhanced the improvement against starvation and poor adherence conditions beyond that seen with oleic acid alone. In this case, cell viability remained at 17.9 % which was significantly higher than untreated hMSCs (Cont) and hMSCs pretreated with oleic acid alone (OA) ($p < 0.01$, Tukey test).

25 c) Exposure to oxidative stress

Human mesenchymal stem cells (hMSCs) were cultured in Dulbecco's modified eagle medium containing 10% v/v fetal bovine serum and 25mM glucose, 1mM sodium pyruvate and 2mM L-alanyl-L-glutamine dipeptide. The hMSCs were pretreated with oleic acid-containing liposomes for 48 h. All liposomes were produced via sonication. The diacylglycerophospholipids content comprised of 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphatidylglycerol (DOPG) in the molar ratio of 92.5:7.5. The oleic acid-liposomes were added to the culture medium to a final oleic acid concentration of 150 μM and a final diacylglycerophospholipids concentration of 350 μM . For comparison, hMSCs were also pretreated with 150 μM oleic acid for 48 h by adding oleic acid stock solution (100mM in ethanol) directly to the culture medium. The hMSCs were then processed via the same steps as above into 24 wt% pluronic acid and plated onto tissue culture plate coated with fibronectin. To mimic stresses such as starvation and oxidative stress that were commonly encountered during cell transplantation, hMSCs were starved in Dulbecco's modified eagle medium that was serum-free and had the concentrations of glucose, sodium pyruvate and L-alanyl-L-glutamine dipeptide reduced significantly to 0.25 mM, 0.01 mM and 0.02 mM respectively. In addition, the medium contained 80 μM H_2O_2 to induce oxidative stress. The viability of the stressed hMSCs were then evaluated at day 4 by quantitative

measurement of cellular adenosine triphosphate (ATP) levels as described in Example 2. Figure 28 shows the quantitative measurement of ATP levels after 4 days of culture and expressed as a percentage of ATP level at day 0 right before culture as an indicator of cell viability.

The various pretreatments were as follows:

5 *Cont: Control (no pretreatment)*

OA: pretreatment with 150 μ M oleic acid

L1: pretreatment with 150 μ M oleic acid and 350 μ M diacylglycerophospholipids (in the form of liposomes)

The measurement of cell viability after 4 days of starvation and oxidative stress

showed a significant drop in cell viability to 3.8 % for untreated hMSCs (Cont). Pretreatment with oleic acid helped to improve average viability of hMSCs to 9.9 % but the increase was not statistically significant from untreated hMSCs (Cont).

On the other hand, pretreatment with liposomes containing oleic acid and diacylglycerophospholipid (L1) enhanced the viability of hMSCs to 19.5%, which was significantly higher than untreated hMSCs (Cont) ($p < 0.01$, Tukey test) and hMSCs pretreated with oleic acid alone (OA) ($p < 0.05$, Tukey test).

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Example 12

Production of vascular endothelial growth factor (VEGF) in hMSCs during stress after treatment of diacylglycerophospholipids and oleic acid

20 Example 12 differed from example 11 in that it investigated whether pretreatment of hMSCs with the combination of diacylglycerophospholipids and oleic acid could result in enhanced production of vascular endothelial growth factor (VEGF) by hMSCs in the presence of starvation and oxidative stress.

Human mesenchymal stem cells (hMSCs) were cultured in Dulbecco's modified eagle medium containing 10% v/v fetal bovine serum and 25 mM glucose, 1 mM sodium pyruvate and 2 mM L-alanyl-L-glutamine

25 dipeptide. The hMSCs were pretreated with oleic acid and diacylglycerophospholipids containing liposomes for 48 h. All liposomes were produced via sonication. The diacylglycerophospholipids content comprised of 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphatidylglycerol (DOPG) in the molar ratio of 92.5:7.5. The oleic acid-liposomes were added to the culture medium to a final oleic acid concentration of 150 μ M and a final diacylglycerophospholipids

30 concentration of 350 μ M. For comparison, hMSCs were also pretreated with 150 μ M oleic acid for 48 h by adding oleic acid stock solution (100mM in ethanol) directly to the culture medium. The hMSCs were then processed via the same steps as in Example 12 c) into 24 wt% pluronic acid gel and plated onto tissue culture plate coated with fibronectin. To induce starvation stress and oxidative stress, hMSCs were starved in Dulbecco's modified eagle medium that was serum-free and had the concentrations of glucose, sodium pyruvate and L-alanyl-L-glutamine dipeptide reduced significantly to 0.25 mM, 0.01 mM and 0.02 mM respectively. In addition, the medium contained 80 μ M H₂O₂ to induce oxidative stress. Culture media was collected at day 7 for the quantification of vascular endothelial growth factor (VEGF). Secretion of cytokines such as VEGF is a critical aspect behind the therapeutic value of cells such as hMSCs. VEGF was measured via enzyme-linked immunosorbent assay (ELISA) and normalized to the DNA content.

40 DNA content was measured by fluorescence based assay. Figure 29 shows the quantitative

measurement of VEGF normalized to the DNA content of the sample after seven days of culture under starvation and oxidative stress.

The various pretreatments were as follows:

Cont: Control (no pretreatment)

5 *OA: pretreatment with 150 μ M oleic acid*

L1: pretreatment with 150 μ M oleic acid and 350 μ M diacylglycerophospholipids (in the form of liposomes)

Measurements of VEGF showed that untreated hMSCs (Cont) secreted 0.62 pg VEGF per ng DNA under starvation and oxidative stress. Average VEGF secretion for hMSCs pretreated with oleic acid alone (OA) rose to 1.62 pg VEGF per ng DNA but was not statistically significant relative to untreated hMSCs (Cont).

10 Pretreatment of hMSCs with liposomes containing oleic acid and diacylglycerophospholipids (L1) resulted in the largest enhancement in VEGF secretion to 3.24 pg VEGF per ng DNA, which was significantly higher than untreated hMSCs (Cont) ($p < 0.01$, Tukey test) and hMSCs pretreated with oleic acid (OA) ($p < 0.05$, Tukey test). This was because of the improved resistance against stresses as demonstrated in Example 11.

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Example 13

Test of Cell Viability of Human dermal fibroblasts (HDFs) in presence of liposomes of Example 1

Human dermal fibroblasts (HDFs) were cultured in Dulbecco's modified eagle medium containing 10% v/v fetal bovine serum, 25mM glucose, 1mM sodium pyruvate and 2mM L-alanyl-L-glutamine dipeptide.

20 HDFs were pretreated with oleic acid and diacylglycerophospholipids containing liposomes for 48 h. All liposomes were produced via sonication. The diacylglycerophospholipids content comprised solely of 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC). The oleic acid-containing liposomes were added to the culture medium to a final oleic acid concentration of 150 μ M and a final diacylglycerophospholipids concentration of 350 μ M.

25

The various pretreatments performed on HDFs are listed as follows:

Cont: Control (no addition of oleic acid and no addition of diacylglycerophospholipids)

L1: 150 μ M oleic acid + 350 μ M diacylglycerophospholipids (in the form of oleic acid and diacylglycerophospholipids containing liposomes)

30 After the 48 h pretreatment, existing culture media was removed and HDFs were washed once with phosphate-buffered saline to ensure removal of culture media. After washing, HDFs were starved in Dulbecco's modified eagle medium that was serum-free and had the concentrations of glucose, sodium pyruvate and L-alanyl-L-glutamine dipeptide reduced significantly to 0.25 mM, 0.01 mM and 0.02 mM respectively. The viability of the starved HDFs was then evaluated at various timepoints by measuring cellular adenosine triphosphate (ATP) levels as described in Example 2.

35

a) Cell viability at Day 0

Quantitative measurement of cellular adenosine triphosphate (ATP) levels was carried out as described in Example 2. Figure 30 shows that viability right before the start of starvation was similar between untreated HDFs (Cont) and HDFs pretreated with oleic acid-containing liposomes (L1).

40

b) Cell viability at Day 3

Quantitative measurement of ATP levels after 3 days of starvation showed that viability of untreated HDFs decreased to almost zero (Figure 31). The viability of HDFs pretreated with oleic acid and diacylglycerophospholipids containing liposomes (L1) remained at a much higher level ($p < 0.001$ referenced to untreated HDFs (Cont)), showing the increased stress resistance imparted by the combination of diacylglycerophospholipids and fatty acids.

Example 14

Test of Cell Viability of Human monocytic cell line (THP-1) in presence of liposomes of Example 1

Human monocytic cell line (THP-1) were cultured as suspension cells in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% v/v fetal bovine serum and 0.05 mM 2-mercaptoethanol. THP-1 monocytes were seeded at 300 000 cells per ml and pretreated with oleic acid and diacylglycerophospholipid containing liposomes for 48 h. All liposomes were produced via sonication. The diacylglycerophospholipids content comprised solely of 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC). The oleic acid and diacylglycerophospholipids containing liposomes were added to the culture medium to a final oleic acid concentration of 150 μ M and a final diacylglycerophospholipids concentration of 350 μ M.

The various pretreatments performed on THP-1 monocytes are listed as follows:

Cont: Control (no addition of oleic acid and no addition of diacylglycerophospholipids)

L1: 150 μ M oleic acid + 350 μ M diacylglycerophospholipids (in the form of liposomes)

After the 48 h pretreatment, THP-1 monocytes were centrifuged at 200 g for 5 min to remove existing culture media. The monocytes were washed by being resuspended in phosphate-buffered saline and centrifuged again at 200 g for 5 min. Afterward, the monocytes were resuspended to a cell density of 3 million cells per ml in serum-free Dulbecco's modified eagle medium containing 0.5 mM glucose, 0.02 mM sodium pyruvate and 0.04 mM L-alanyl-L-glutamine dipeptide. The viability of the starved THP-1 monocytes was then evaluated at various timepoints by measuring cellular adenosine triphosphate (ATP) levels as described in Example 2.

a) Cell viability at Day 0

Quantitative measurement of cellular adenosine triphosphate (ATP) levels was carried out as described in Example 2. Figure 32 shows that viability right before the start of starvation was similar between untreated monocytes (Cont) and monocytes pretreated with oleic acid and diacylglycerophospholipids containing liposomes (L1).

b) Cell viability at Day 6

Quantitative measurement of ATP levels after 6 days of starvation showed that viability of untreated THP-1 monocytes decreased significantly (Figure 33). However, the viability of THP-1 monocytes pretreated with oleic acid and diacylglycerophospholipids containing liposomes (L1) remained much higher ($p < 0.001$ referenced to untreated THP-1 monocytes (Cont)), showing the increased stress resistance imparted by the combination of diacylglycerophospholipids and fatty acids.

Example 15

Test of Cell Viability of hMSCs in presence of liposomes comprising Docosahexaenoic acid

Docosahexaenoic acid was investigated as another fatty acid example with the difference being that oleic acid is an 18-carbon long fatty acid with one carbon-carbon double bond while docosahexaenoic acid is a 22-carbon long fatty acid with 6 carbon-carbon double bonds. Human mesenchymal stem cells (hMSCs) were cultured in Stempro MSC SFM XenoFree media (Gibco). The hMSCs were pretreated with docosahexaenoic acid alone or a liposomal formulation containing diacylglycerophospholipids and docosahexaenoic acid for 48 h. For these pretreatments, docosahexaenoic acid stock solution (152mM in ethanol) was used to form liposomes via sonication as described in example 1. The molar ratio of diacylglycerophospholipids to docosahexaenoic acid used was 70:30. The diacylglycerophospholipids content comprised of 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphatidylglycerol (DOPG) in the molar ratio of 92.5:7.5. For comparison, hMSCs were also pretreated with 70 μ M or 80 μ M docosahexaenoic acid alone by diluting docosahexaenoic acid stock solution (152mM in ethanol) into the culture media.

Figure 34 shows the cellular lipid levels of hMSCs after pretreatment for 48 hours. The cellular lipid levels were determined via Nile red staining. Cellular lipid levels are shown normalized to that of untreated hMSCs (Control). As can be seen in Figure 34, hMSCs pretreated with docosahexaenoic acid alone (DHA-1 & DHA-2) or combinations of docosahexaenoic acid and diacylglycerophospholipids in the form of fatty acid-free liposomes (DHA-1 + GPL & DHA-2 + GPL) increased their cellular lipid levels relative to untreated control. The cellular lipid level was also seen to increase with the increase in concentration of docosahexaenoic acid for all conditions. After the 48 h pretreatment, existing culture media was removed and hMSCs were washed once with phosphate-buffered saline to ensure complete removal of culture media. After washing, hMSCs were starved in Dulbecco's modified eagle medium that was serum-free and had the concentrations of glucose, sodium pyruvate and L-alanyl-L-glutamine dipeptide reduced significantly to 0.25 mM, 0.01 mM and 0.02 mM respectively. The viability of the starved hMSCs was then evaluated at various timepoints by quantitative measurement of cellular adenosine triphosphate (ATP) levels carried out as described in Example 2

Fatty acids and diacylglycerophospholipids (GPL) were added as follows:

Cont: Control without any added fatty acids or diacylglycerophospholipids

DHA-1 + GPL: 70 μ M docosahexaenoic acid + 163 μ M diacylglycerophospholipids

DHA-2 + GPL: 80 μ M docosahexaenoic acid + 186 μ M diacylglycerophospholipids

DHA-1: 70 μ M docosahexaenoic acid

DHA-2: 80 μ M docosahexaenoic acid

a) Cell viability at day 0

Quantitative measurement of cellular adenosine triphosphate (ATP) levels was carried out as described in Example 2. Figure 35 shows that viability of hMSCs right before the start of starvation was similar across all types of pretreatments.

b) Cell viability after 6 days

In Figure 36, measurement of ATP levels after 6 days of starvation showed that viabilities of untreated hMSCs as well as hMSCs pretreated with docosahexaenoic acid alone had decreased substantially. In contrast, the viability of hMSCs pretreated with 80 μ M docosahexaenoic acid and 186 μ M GPL remained significantly higher than untreated hMSCs (Cont) and hMSCs treated with docosahexaenoic acid alone (DHA-1 and DHA-2). Therefore, diacylglycerophospholipids can also be used together with other fatty acids such as polyunsaturated fatty acids to increase starvation tolerance beyond that achieved by fatty acid alone.

CLAIMS:

1. Cell culture medium comprising diacylglycerophospholipids and fatty acids in a molar ratio of diacylglycerophospholipids : fatty acids ranging from 40:60 to 95:5 with the fatty acid concentration
5 ranging from 10 μM to 300 μM and the diacylglycerophospholipid concentration ranging from 50 μM to 2500 μM .
2. Cell culture medium according to claim 1, characterized in that the molar ratio of diacylglycerophospholipids : fatty acids ranges from 40:60 to 95:5 with the fatty acid concentration
10 ranging from 20 μM to 200 μM and the diacylglycerophospholipid concentration ranging from 75 μM to 2000 μM .
3. Cell culture medium according to claim 1 or 2, characterized in that the fatty acid is a saturated, monounsaturated, diunsaturated or polyunsaturated C8 to C24 fatty acid.
- 15 4. Cell culture medium according to claim 3, characterized in that the fatty acids are selected from the group consisting of lauric acid, oleic acid, octanoic acid, decanoic acid, arachidonic acid, docosahexaenoic acid and mixtures thereof.
- 20 5. Cell culture medium according to any one of claim 1 to 4, characterized in that the acyl groups of the diacylglycerophospholipid are saturated, monounsaturated or diunsaturated C8 to C24 aliphatic chains.
6. Cell culture medium according to claim 5, characterized in that the acyl groups of the
25 diacylglycerophospholipid are saturated or monounsaturated C12 to C20 aliphatic chains.
7. Cell culture medium according to claim 5 or 6, characterized in that the diacylglycerophospholipids are selected from the group consisting of 1,2-dioleoyl-sn-glycero-3-phospholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphatidylglycerol (DOPG), 1,2-dilinolenoyl-sn-glycero-3-phosphocholine (DSPC) and mixtures thereof.
30
8. Cell culture medium according to any one of claim 1 to 7, characterized in that the diacylglycerophospholipids and fatty acids are present in the medium in the form of liposomes.
- 35 9. Method of preparing a cell culture medium according to any one of claims 1 to 7, characterized in that the diacylglycerophospholipids and/or fatty acids are first prepared as concentrates and are subsequently diluted into cell culture medium to achieve the final concentration.

10. Method of culturing cells comprising the steps of contacting the cells with the cell culture medium according to claim 1 and culturing the cells in said cell culture medium for 4 to 96 hours.
- 5 11. Method of culturing cells according to claim 10, characterized in that the step of contacting the cells with the cell culture medium according to claim 1 is carried out near the end of cell expansion and prior to the transfer of cells to downstream processes.
12. Method of culturing cells according to claim 10, characterized in that the method is applied prior to
10 cryopreservation of the cells.
13. Method of culturing cells according to claim 10, characterized in that the method is applied prior to producing a pharmaceutical composition containing the cells.
- 15 14. Pharmaceutical composition comprising cells cultured according to the method of culturing cells of claim 10.