

Inflamyar™ Protects from Oxidative Stress

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Abbreviations: OS: Oxidative Stress; PMN: Polymorphonuclear Cells; PBMC: Peripheral Blood Mononuclear Cells; ROS: Reactive Oxygen Species

ABSTRACT

Introduction: Oxidative stress (OS) is associated with significant damage to cell structures leading to multiple disease patterns. Dietary supplementation or topical administrations of non-enzymatic antioxidants are described to prevent OS-related disease rates. Many of the most powerful and most discussed natural non-enzymatic antioxidants can be found in the plant kingdom. In this study, the antioxidant effect of a commercially available plant extract remedy was evaluated.

Methods: Primary human cells (peripheral blood mononuclear cells [PBMC], erythrocytes and polymorphonuclear cells [PMN]) were pre-incubated with Inflamyar™ (PEKANA Naturheilmittel GmbH, Germany). Cell viability (MTT-assay), cellular antioxidant protection (CAP-e assay) and reactive oxygen species (ROS)-formation assays were performed.

Results: The MTT test showed a predominantly consistent to slightly increased cellular viability. A mild protection of oxidative damage in human erythrocytes at higher doses of the test substance was seen in the CAP-e assay. In the ROS assay, slightly reduced levels of ROS formation for all tested concentrations of the test substance were documented in PMN cells from two of three donors.

Conclusion: This study documented stable vitality and protective capacities against cellular oxidative stress of cells treated with the test substance.

Introduction

Oxygen is one of the most important elements for life, but in certain circumstances oxygen can also harm the organism. Influences of the outside world such as redox chemicals, ionizing radiation, UV light, heavy metal ions, ozone exposure and cigarette smoke can readily generate reactive oxygen species (ROS), which have an oxidizing effect on other substances [1,2]. A large part of these reactive species are represented by free radicals, for example hydroxyl radicals, superoxide radicals, nitric oxide radicals and the lipid peroxy radical, and have an excess of free electrons. These substances are unstable and have a highly reactive potential on other substances [2]. In addition to the environmental formation of ROS, these can also be found as by-products in the oxygen

metabolism of aerobic organisms. ROS are generated in connection with the electron transport in mitochondrial energy generation, immune reactions against microorganisms and viruses [3,4] as well as processes of the cytochrome P-450 system [5,6].

The human antioxidant defense system consists out of two components, an enzyme component on the one hand, comprising enzymes such as glutathione peroxidase, catalase and superoxide dismutase and the small molecule antioxidant component on the other, which comprises vitamin A and E, ascorbate, glutathione and thioredoxin. These substances are the natural protection against endogenous ROS and other free radicals. Oxidative stress occurs when the production of ROS exceeds the natural antioxidant

defense mechanisms of the body and damages biomolecules such as lipids, proteins and nucleic acids [1].

Oxidative stress has been thought to contribute to the general decline in cellular functions that are associated with many human diseases including amyotrophic lateral sclerosis [7,8], Alzheimer's disease [9-11], Parkinson's disease [12,13], ischemia/reperfusion neuronal injuries, degenerative disease of the human temporomandibular-joint [14], atherosclerosis [15], macular degeneration [7,16], degenerative retinal damage [17], cataract formation [18], multiple sclerosis [19], muscular dystrophy [20,21], rheumatoid arthritis [22], human cancers [23,24] the aging process itself [25,26] as well as inflammatory disorders and tissue injury [27-29].

Several studies associated an additional supply of antioxidants with lower disease rates and preventive protection by dietary supplementation or topical administration [30-32]. Thus, the supplementation of non-enzymatic antioxidants could be a feasible way of restoring redox homeostasis and reduce ROS-associated diseases. Many of the most powerful and most discussed natural non-enzymatic antioxidants, for example the polyphenols, can be found in the plant kingdom. Polyphenols are naturally occurring compounds found in multiple plants, exhibiting largest antioxidant potential of secondary plant metabolites [33]. Five major groups of phytochemicals are classified, flavonoids, phenolic acids, stilbenes, lignans and tannins [34-36]. A special antioxidative activity has been described for substances from the flavonoid and phenolic acid classes both *in vitro* and *in vivo*. They also show a synergistic enhancement when interacting with other antioxidants such as tocopherol and ascorbate [33,37].

In this study, the antioxidant effect of a commercially available plant extract, consisting of extracts from *Arnica montana*, *Bryonia cretica*, *Guajacum*, *Toxicodendron quercifolium*, *Bellis perennis*, *Ledum palustre*, *Ruta graveolens* and *Viscum album* was evaluated.

Material and Methods

All experiments were conducted by NIS Labs, Klamath Falls, USA.

Test Substance

The test substance, Flamyar™, is a natural plant extract remedy manufactured according to the German homeopathic pharmacopoeia by PEKANA Naturheilmittel GmbH (Kießlegg, Germany) and distributed in the USA under the name Inflammatory™. The test substance was developed for the treatment of sports injuries, sprains, joint problems, bruises, and muscle strains. Active ingredients are *Arnica montana* spag. Peka Dil. D12, *Bryonia cretica* spag. Peka Dil. D4, *Guajacum* Dil. D4, *Toxicodendron quercifolium* Dil. D12, *Bellis perennis* spag. Peka Dil. D8, *Ledum palustre* Dil. D4, *Ruta graveolens* spag. Peka Dil. D6, *Viscum album* spag. Peka Dil. D4.

Isolation of Peripheral Blood Mononuclear and Polymorphonuclear Cells and Erythrocytes

Upon written informed consent from healthy human donors, and as approved by the Sky Lakes Medical Center Institutional Review Board (Federalwide Assurance 2603) for ethical standards, peripheral blood was drawn into heparinized vacutainer tubes. Blood was layered onto Lympholyte-Poly (Thermo Fisher Scientific, Waltham, MA, USA) and centrifuged at 1800 rpm for 35 minutes. Peripheral blood mononuclear cells (PBMC), polymorphonuclear (PMN) cells, and erythrocytes were harvested separately and each cell fraction washed in PBS (Sigma-Aldrich, Saint Louis, MO, USA) by centrifugation at 2400 rpm for 10 minutes. PBMC and PMN cells were washed twice and erythrocytes four times prior to use in assays.

MTT Assay

Freshly purified PBMCs cells were plated in flat-bottom 96-well microtiter plates [NUNC, Roskilde, Denmark] at 2×10^6 cells/ml in 90 μ l. 10 μ l PBS was added to untreated control wells (n=6), and 10 μ l of serial dilutions of the test substance in PBS added to assay wells (n=3 per group). Cells were incubated at 37°C, 5% CO₂ for 24 hours. Then 10 μ l of Component A from Vybrant® MTT Cell Proliferation Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) was added, and allowed to incubate for 4 hours. Component B (SDS-HCl) was added, and the plate returned to the incubator for an additional 18 hours. At the end of the incubation, the optical density was measured as the absorbance at 570 nm, using a colorimetric plate reader (BioTek PowerWave, Winooski, VT, USA).

Cellular Antioxidant Protection (CAP-e) Assay

For the cellular CAP-e assay (antioxidant protection of erythrocytes assay), inert human erythrocytes (RBC) were transferred in V-bottom 96-well microtiter plates (NUNC, Roskilde, Denmark), and incubated in a serial dilution of the test substance for 20 minutes (n=2 per group) [38]. During this period, antioxidant compounds could cross the cell membrane and get inside the cells. In order to remove any compounds that were not absorbed by the cells, the erythrocytes were then washed twice with PBS and subsequently loaded with 0.4 μ g/ml of the indicator dye 2', 7'-dichlorofluorescein diacetate (DCF-DA, Thermo Fisher Scientific, Waltham, MA, USA) and 1mg/ml of α, α' -Azodiisobutyramidine dihydrochloride (AAPH, Wako Chemicals, Richmond, VA, USA). DCF-DA becomes fluorescent when oxidized and AAPH is a free radical generator which induces intracellular oxidative stress. The fluorescence was quantified via fluorescence plate reader at 488nm (Tecan Spectrafluor, Tecan, Durham, NC, USA). The fluorescence intensity of untreated cells was defined as baseline, only AAPH-treated cells served as positive control (n=6). A lower fluorescence intensity in cells that were previously exposed to the test product indicates the availability of bioavailable antioxidants in the test substance.

Reactive Oxygen Species Assay

Since they are known to produce high levels of ROS, freshly purified PMN cells were used for the testing of effects of the test substance on reactive oxygen species (ROS) formation [39]. PMN cells (2×10^5 cells in 200 μ l per well) were exposed to serial dilutions of the test substance during an incubation period of 20 minutes. The PMN cells were then washed and loaded with the indicator dye DCF-DA (0.5 μ g/ml), which turns fluorescent upon exposure to ROS. The ROS formation was triggered by addition of 2mM H_2O_2 (Sigma-Aldrich, Saint Louis, MO, USA) for 45 minutes. The PMN cells were washed, transferred to cold RPMI-1640 medium (Sigma-Aldrich, Saint Louis, MO, USA), and stored in the dark on ice. Fluorescence intensity was evaluated by flow cytometry using an Attune[®] acoustic focusing cytometer (Thermo Fisher Scientific, Waltham, MA, USA). The low fluorescence intensity of untreated control cells served as a baseline and PMN cells treated with H_2O_2 alone served as a positive control. The testing was performed on PMN cells from three different healthy donors, where each dose of the test substance was tested in triplicate.

Statistical Analysis

Averages and standard deviations for each data set were calculated using Microsoft Excel. Statistical analysis was performed using the two-tailed, dependent t-test. Statistical significance was designated as $p < 0.05$, and a high level of significance was designated as $p < 0.01$.

Results

In order to exclude any cytotoxic or cytostatic effects of the test substance, the impact of serial dilutions of the test substance on cell viability of PBMCs were evaluated using MTT Cell-Viability Assay. The data obtained showed no negative effects on cellular viability or mitochondrial function at any of the doses of the test substance; only at the highest concentration of 100ml/l a slightly, but not significantly reduced cell viability was seen ($93.95 \pm 3.42\%$, $p = 0.0756$). Highest cell viability was observed after incubation with 12.5ml/l ($105.58 \pm 2.90\%$, $p = 0.1903$). All other samples possessed viability data between $99.31 \pm 0.86\%$ (0.05ml/l) and $103.18 \pm 2.39\%$ (1.56ml/l) compared to untreated controls.

For investigation of the ability for cellular oxidative damage, a Cellular Antioxidant Protection (CAP-e) assay was performed on human red blood cells (RBCs) after incubation with serial dilutions of the test substance. The treatment of cells with the test substance led to an inhibition of oxidative damage at the 41.67ml/l ($8.88 \pm 2.42\%$) and 83.33ml/l ($20.22 \pm 2.39\%$) doses. Concentrations of 5.21ml/l to 20.83ml/l showed a comparable moderate protection from $2.53 \pm 0.59\%$ (20.83ml/l) to $4.38 \pm 1.91\%$ (5.21ml/l), and the highest concentration of the test substance used in this experiment (166.67ml/l) led to an inhibition of $4.64 \pm 2.36\%$.

PMN cells from donor 1 showed a reduction of ROS formation at lower concentrations of 0.1ml/l ($4.94 \pm 10.38\%$; $p = 0.542$) and

0.39ml/l ($0.92 \pm 10.29\%$; $p = 0.907$). Treatment of the cells with test substance concentrations of 1.56 ml/l to 100ml/l led to a ROS formation range between $0.62 \pm 10.22\%$ (1.56ml/l; $p = 0.5480$) and $3.15 \pm 3.61\%$ (6.25ml/l; $p = 0.9366$). In contrast, reduced levels of ROS formation for all tested concentrations of the test substance were assessed in PMN cells from donor 2 and 3. Cells from donor 2 showed a range of $-3.33 \pm 9.40\%$ (0.1ml/l; $p = 0.6950$) to $-10.38 \pm 10.01\%$ (6.25ml/l; $p = 0.2608$). Data from donor 3 were evaluated from $-1.30 \pm 9.95\%$ (0.39ml/l; $p = 0.8578$) to $-6.00 \pm 2.45\%$ (100ml/l; $p = 0.1811$).

Discussion

The application of plant extract remedies is widespread among the people. However, the effect of these products is controversially discussed in the scientific community. In the past years, plant extract agents have come more and more into the focus of research and a rising number of studies, showing a positive effect of these remedies can be found in the databases. In contrast to this, different studies conclude that these preparations are not significantly different from placebo treatment [40]. In this study, a plant extract remedy from *A. montana*, *B. cretica*, *Guajacum*, *T. quercifolium*, *B. perennis*, *L. palustre*, *R. graveolens* and *V. album* was evaluated *in vitro* regarding its anti-oxidative capacity on human cells. At the beginning of this study, a MTT cell viability assay was performed on PBMCs in order to get an insight in effects of the test substance on cellular mitochondrial activity, also serving as a baseline for the concentrations used in further experiments. Given the dilution (D4 - D12) of the ingredients, we expected to see little or no cellular stress, even at higher doses, as we typically do with many traditional botanical extracts [41-44]. As expected, the test substance showed no significant reduction of cell viability or mitochondrial function (Figure 1).

In the next step, the antioxidant potential of the test product was assessed by a Cellular Antioxidant Protection (CAP-e) assay [45,46]. This test allows assessment of antioxidant potential in a method that is comparable to the Oxygen Radical Absorbance Capacity (ORAC) test, but only allows measurement of antioxidants that are able to cross the lipid bilayer cell membrane, enter the cells, and provide biologically meaningful antioxidant protection under conditions of oxidative stress. A mild protection of up to 20% inhibition of oxidative damage in human RBCs at doses of 41.6 to 83.3 ml/l reflects the antioxidant capacity of this product at higher concentrations (Figure 2). Combining data from CAP-e assay with results from ROS assay, some interesting conclusions for the test substance could be made. A large number of antioxidative natural substances are also able to reduce the ROS release in immune cells [27,33]. In contrast, ROS formation is increased in part despite the initial antioxidative effect of a substance through the interaction with the inflammatory signaling pathways of cells. The following three effects of natural substances on the ROS formation of PMN cells are therefore possible:

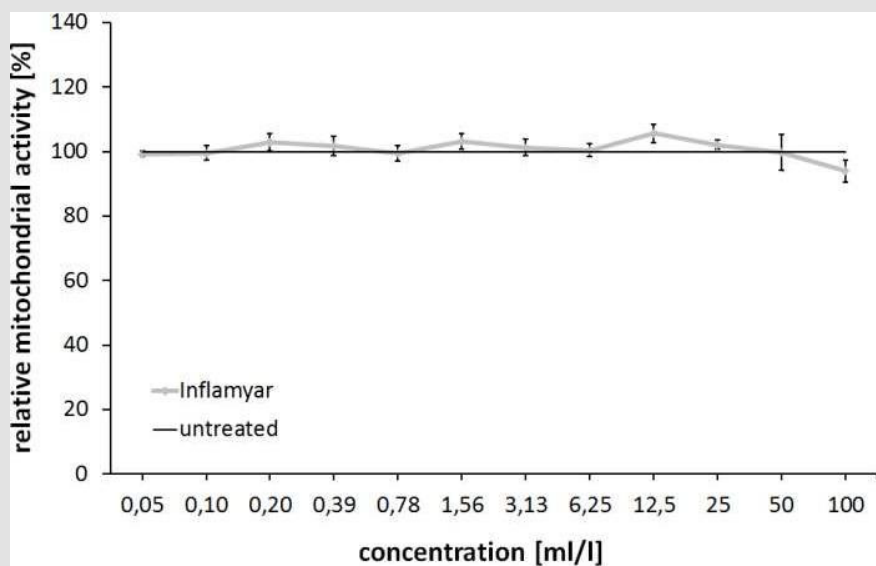


Figure 1: MTT Cell-Viability Assay. Viability (relative mitochondrial activity) of PBMCs after 24 hours incubation in serial dilutions of the test substance compared to untreated control cells.

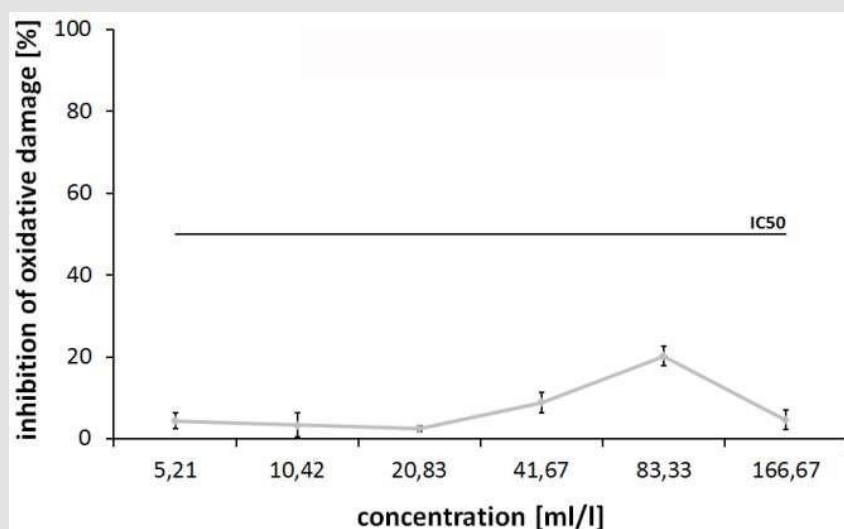


Figure 2: Cellular Antioxidant Protection (CAP-e) Assay. Inhibition [%] of oxidative damage for the test substance in human RBC after addition of α,α' -Azodiisobutyramidine dihydrochloride (AAPH) to induce intracellular oxidative stress (IC_{50} : half maximal inhibitory concentration).

- (1) Direct inhibitory effect on the ROS formation;
- (2) Indirect inhibitory effect through activation of an anti-inflammatory signaling pathway or
- (3) Activation of an inflammatory cascade, which results in an increased release of ROS [27,47].

In this study, three patients were analysed, since due to the current health status of the patients, experiments on immune cells (PMN cells in the ROS assay) of a single patient are not very meaningful. Interestingly, two out of three donors showed a slight but non-significant decrease in reactive oxygen species formation by PMN cells under oxidative stress conditions after

pretreatment of cells with all doses of the test substance (Figure 3). This, in combination with the results from CAP-e assay, indicates a triggering of an anti-inflammatory mechanism leading to a reduced ROS-formation at low concentrations of the test substance, whereas the antioxidant capacities at these doses are low. This might be an interesting issue for further analysis of the test substance. In comparison to previous published data, only a few literatures on applications of the active substances contained in the test substance are available. Many of the published data show the effect of undiluted or not in potencies diluted extracts from the herbs. In this context, Gaspar, et al. described no cytotoxicity for isolates from *A. montana* at low concentrations [42].

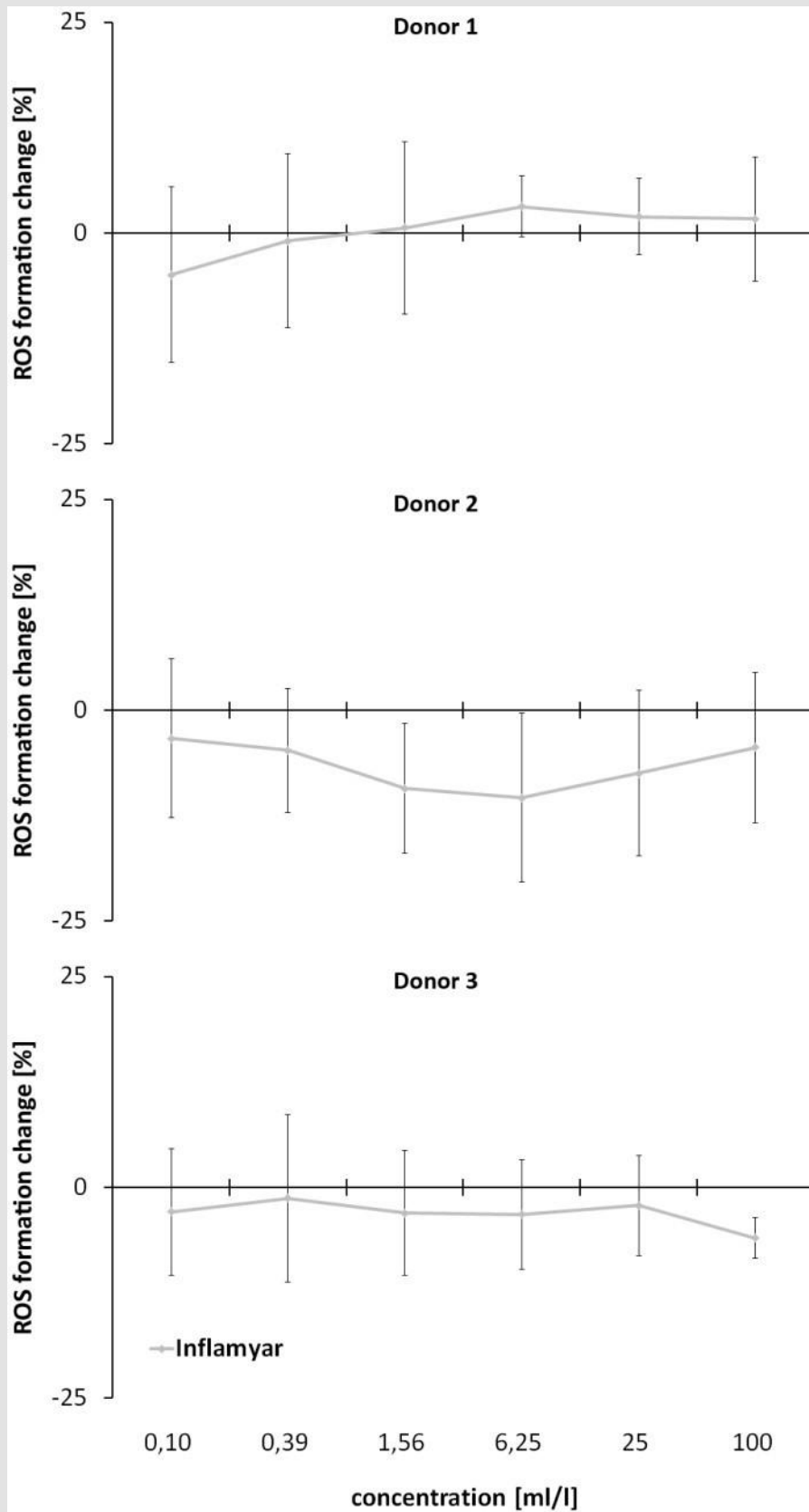


Figure 3: Reactive Oxygen Species (ROS) Assay. Evaluation of effects of the test substance under oxidative stress. PMN cells were incubated with serial dilutions of the test substance before adding H₂O₂ to induce ROS formation.

Other studies also described no or only low cytotoxicity for Arnica-species [43,44]. For other plant species enclosed in the test substance, the information of cytotoxicity is very poor. No data regarding mitochondrial activity was found. Interestingly, an anti-oxidant capacity was described for each plant enclosed in the test substance: *A. montana* [43,48], *B. cretica* [49], *Guajacum* [50,51], *T. quercifolium* [52], *B. perennis* [53,54], *L. palustre* [55], *R. graveolens* [56,57] and *V. album* [58,59]. Moreover, *A. montana*, members of *Bryonia* species, *Guajacum* and *R. graveolens* were also described to have anti-inflammatory [56,60-63] and immunomodulatory [52,63] capacities. This might be an interesting issue for further analysis, since data from this study also suggest an anti-inflammatory effect of the test substance (ROS-inhibition together with low anti-oxidative capacity at low doses). Interestingly, some of the ingredients possess oppositional actions on human cells. For example, *T. quercifolium* exhibits genotoxic capabilities at higher concentrations, whereas lower concentrations of the same isolate showed anti-genotoxic capacities [64], suggesting a beneficial impact of the dilutions used for the production of the test substance.

Conclusion

This study documented stable vitality and protective capacities against cellular oxidative stress of cells treated with the test substance.

Acknowledgements

Not applicable.

Conflict of Interest

The authors declare that they have no competing interests.

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Ethics approval and consent to participate.

All the procedures performed on human cells are approved by the Sky Lakes Medical Center Institutional Review Board (Federalwide Assurance 2603) for ethical standards.

Author's Contributions

All experiments were conducted by NIS Labs, Klamath Falls, USA. MS, VS, MH and LH have performed data analysis and interpretation, MS prepared the manuscript. VS, MH, UK and LH critically reviewed the manuscript and contributed intellectual content. All authors read and approved the final version of the manuscript.

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