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DetoxiTide®, a Natural Nanopeptide with Systemic Beneficial Effects *in vivo* Exerts Signal Transduction Profiles of Anti-Inflammatory and Anti-Toxicity Properties *in vitro*

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Short Abstract: The DetoxiTide is a natural nanopeptide extracted by animal organs that, registered as a food supplement, are widely used in vivo to control and cure several pathological conditions in patients. Its application leads toward several direction has been associated in patient treatments to standard drugs or medic attenuating toxicity after chemiotherapy cycles and in physiopathology affecting several organs.

Abstract

We have used the DetoxiTide, an active natural compound enriched for natural micropeptides and essential amino acids that has been extensively used in vivo as a potent antioxidant. We extracted the content of package tablets, solubilized in Dimethyl Sulfoxide (DMSO) for treating in vitro cultured cell, namely THP-1, a human cell line derived from a monocytic leukemia whose phenotype can be reverted into macrophages by adding

PMA, a chemical inducer of cellular differentiation. As widely described, the differentiated THP-1 macrophages treated with the bacterial lipopolysaccharide (LPS) can activate the primary steps of inflammation increasing the expression of the IL-6 and TNF pro-inflammatory cytokines. In this report we demonstrate that co-incubating the THP-1 cells with the LPS and detoxiTide, a marked decrease in IL-6 release measured by ELISA assays was observed. Moreover, in order to test a non-immunogenic cell background, we used the Hacat, a keratinocyte cell line that grows in vitro as an adherent mono layer in order to induce a pro-oxidant background by challenging the cell cultures with hydrogen peroxide (H_2O_2). As a matter of fact, several reports document that the promotion of prooxidative state levels activates signal transduction mechanisms that are specific to the inflammatory process. Therefore, applying such in vitro cell model, we have demonstrated that detoxiTide treatments on keratinocytes also attenuated the inflammation profile by decreasing the expression of the IL-6 proinflammatory cytokine, induced by LPS. Furthermore, on the keratinocytes hyper oxidized by H_2O_2 not only the amount of IL-6 decreased after co-incubation with the detoxiTide, but at the same time it was observed a marked increase on the synthesis of the Nrf-2 nuclear factor which has been shown to be a crucial key element that allows the detoxification of cells under prooxidative stress. In particular, being a nuclear trans-acting factor, the increase of the Nrf-2 protein was detected by using western blot assays on extracts isolated from H_2O_2 treated or untreated cells. These data give a demonstration that the detoxiTide acts in vitro as a beneficial factor that induce anti-inflammatory mechanisms in human and potentiates the antioxidant signal transduction pathways involved in the cell detoxification process.

Keywords: Nanopeptides; Inflammatory pathways; Anti-oxidants

Introduction

Nanopeptides identified a heterogeneous group of biological molecules spanning few amino acids in the backbone structure. Conventionally polypeptides are assumed to contain up to 100 amino acid residues whereas oligonucleotides only contain up to 10 **[1-5]**. Since the last 50 years extensive work has been carried out on the biological characterization of many different peptides whose ability to modulate the most prominent biological function in the human body has represented the basis for the complex homeostatic control of the human and animal physiology. In particular enough scientific emphasis has been linked to the isolation and characterization of nano and micropeptides with few (2 up to 5) amino acid residues in their structure **[3-5]**. Initially these micro molecules were isolated and chemically characterized from animal organs acting in a specific way on the physiology and functionality of the same organs and with the property to modulate important biological functions due to their antibacterial, antimicrobial, anti-inflammatory, anti-tumoral antioxidant and immunoregulatory characteristics **[6-8]**. Such properties were first reported by Khavinson giving a directed demonstration of the capacity of Nanopeptides to control gene expression. Further experimental studies confirmed in the last years the extensive biological activity of Nanopeptides with short residues (**2-4** AA) in immunomodulatory, anticarcinogenic and geroprotective functions and in most of biological activity of human physiology. Several studies have investigated the molecular and biochemical mechanisms that control these functions. In particular many reports confirm that principally the micropeptides modulate gene expression by binding to DNA and to regulatory regions of target genes **[6-22]**. In particular peptides as ED, AGED, DEAL KEG can bind to histone protein unfolding the chromatin structure and increasing the transcription activity of target genes. The expression of such genes can be activated by direct or indirect binding of specific

Nanopeptides. In a recent study the AEDG and the KE peptides were found to control the expression of 98 and 36 transcripts, respectively. It was revealed that the AEDG and AEDP tetra peptides activate the differentiation of pluripotent cells towards epidermis, mesenchymal, and nervous tissue. Peptides KE, AED, KED, AEDG, and AAAAEKAAAAEKAAAAEK are activators of neuronal differentiation. The AEDL peptide stimulates the lung cells' differentiation, while the KEDW peptide stimulates the maturation of various types of human pancreatic cells. Beside all these different functions some interests have risen from the observation that most of the micropeptides control the modulation of inflammation, proliferation and antioxidant status of the cells. We and others **[23]** have recently demonstrated that du, tri e tetraptide can decrease the expression of pro-inflammatory cytokines by co incubating in vitro differentiated macrophages with the bacterial Lipopolysaccharide (LPS) and Nanopeptides derived from different animal organs or chemically synthesized. A direct effect on proliferation of monocytes was also seen in vitro by measuring the rate of cell division and the amount of exosome release that represents a mark of induced mitogenic activity in eukaryotic cells **[23]**. Considering the fact that a latent inflammatory status exists in organisms that move toward an irreversible state of cellular aging, some Nanopeptides may counteract this effect. It has been shown that senescence mechanisms that slow down the doubling capacity of mesenchymal stem cells in vitro can be attenuated by treating MSCc isolated by dental ligament with tetra and dipeptide **[24-27]**. This property of peptides is of the utmost importance as it indicates the significant role of the peptide regulation of gene expression in such biologically important processes as cell differentiation, functional activity, senescence, apoptosis, immunogenesis, and neurogenesis **[28-31]**. Furthermore, some reports also indicate that Nanopeptides may have distinct antioxidant properties by binding to free radicals or to antioxidant enzymes suggesting a detoxification mechanism in animal cell models that seems to be quite conserved through evolution. However, there is no scientific evidence of the molecular and biochemistry pathways that can mediate these phenomena neither a direct control of transcription on target genes. The detoxiTide is a recent product licensed as a food integrator which contains a composite mix of amino acids and a natural tripeptide derived from animal organs whose sequence and chemistry properties has been blinded for industrial secret as it is not covered by any patent. According to a wide documentation on the physiological properties of similar tripeptides, we have studied the effect *in vitro* of the detoxiTide in term of anti-inflammatory and antioxidant properties. As a primary consideration, we have to report that the administration of the compound *in vivo* to persons affected by degenerative disorders that compromise important physiological functions, confers a beneficial effect on patients suffering from drug-induced hepatotoxicity, as experienced in several administrations to patients with various pathologies (Jose Alberto Casonato, Paolo Bordin, personal communication and unpublished data). According to these observations, using two different established cell lines, one derived from a human monocytic leukemia (THP-1) and the other derived from normal immortalized skin cells (HACAT) both co incubated in vitro with the detoxiTide and the pro-inflammatory inducer Lipopolysaccharide (LPS), we demonstrated that the micro peptide exerts an anti-inflammatory effect that was manifested by the decrease in the pro-inflammatory cytokine (IL-6). Furthermore, on the HACAT cells, when we induced toxicity by challenging the cells with increasing concentration of hydrogen peroxide (H_2O_2), the nfr2 transcription factor that is activated and increased during the physiological pat5hway of detoxification is enhanced upon treatment with the detoxiTide as documented by western blot assay. As illustrated in published reports by us and others, mixture of free amino acids is not able to induce ant inflammatory pathways neither involved in regulation of gene expression. Therefore, we can suggest that the

detoxiTide bearing the DetoxiTide tripeptide has properties of an anti-inflammatory and detoxification agent whose actions on human cells and human physiology could be better investigated for putative administration on well-defined therapeutic protocols.

Materials and Methods

Cell Culture

Human leukemia monocytic cell line (THP-1) was purchased from ATCC. Cells were grown under 5% CO_2 incubator at 37 °C degrees in RPMI 1640 (Sigma–Aldrich; Merck Millipore, Darmstadt, Germany) supplemented with 10% FBS, 2 mM L-glutamine and 1% Pen–Strep (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Series of experiments were performed when the cells reached approximately 80% confluence. THP-1 cells (~2 × 105/mL) were incubated with 100 ng/mL PMA (Sigma–Aldrich, Saint Louis, MO, USA) for 3 days at 37 °C, in order to induce monocyte–macrophage differentiation. The PMA-containing media was removed, and cells were incubated for a further four days in conventional medium, before treating THP-1-derived macrophages with 100 ng/ml of detoxiTide alone solved in Dimethyl sulfoxide (stocked at 1 mg/ml) alone or supplemented by 100 ng/ml Lipopolysaccharide (LPS). LPS derived from E. coli was purchased from Sigma–Aldrich, Saint Louis, MO, USA. THP-1-derived macrophages were detached incubating into Trypsin-EDTA 1X (Euro Clone) for 10 min at 37 °C. Macrophages were counted using 0.4% of Trypan–blue solution (Sigma–Aldrich, Saint Louis, MO, USA) in a Burker chamber and tested for viability.

Cell Viability

The cell viability was measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-dipenyl tetrazolium bromide (MTT) (Sigma–Aldrich, Saint Louis, MO, USA) assay according to standard procedures for adherent cells. Absorbance (optical density) was measured at 530 nm using a micro-plate reader (Infinite F50, Tecan, Männedorf, Switzerland). The experiment was performed in triplicate.

Cell extracts and western blot

Protein Extraction and Western Blot Total proteins were extracted from monocytes and macrophages in 50 mM Tris-HCl pH 7.8, 1% Triton X100, 0.1% SDS, 250 mM NaCl, 5 mM EDTA lysis buffer in the presence of the mini protease inhibitor cocktail at 150 µL/ml (Roche Diagnostics, Mannheim, Germany) as described **[32]**. Cell lysates were separated on 12% or 15% SDS-PAGE depending on molecular weight of target proteins and transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). Membranes were incubated overnight at 4 °C with specific primary Abs (diluted 1/100, Santa Cruz Biotechnology, Dallas, TX, USA). Antibodies directed against the Nfr2 factor were purchased from Cell Signaling Technologies (Danvers, MA, USA). Membranes were incubated overnight at 4 ◦C on a shaker with specific primary antibodies to Nfr2 factor, and β-actin as a control. Specific secondary goat anti-rabbit IgG-HRP conjugated antibodies (1:2000, Santa Cruz) (dilution according to Cell Signaling Technology specific protocol) were used for detection in all experiments, incubating membranes for one hour at RT with gentle shaking. Immunocomplexes were visualized using the ECL detection system (Amersham Pharmacia Biotech, Little Chalfont, UK). Western Blot densitometry band quantification was by ImageJ software (Rasband, 1997–2014).

ELISA assay

ELISA assay samples for detecting the IL-6 release were prepared from liquid media. A validated kit assay for IL-6 detection was purchased from Thermo Fisher (Thermo Fisher Scientific, USA). Briefly a standard curve

was prepared with recombinant IL-6 protein starting from 40 ng/ml up to 8 scalar dilutions preparation (40 ng/ml, 20 ng/ml, 10 ng/ml, 5 ng/ml, 1.5 ng/ml, 1.25 ng/ml, 0,635 ng/ml, 0,312 ng/ml). Standards and samples were added to an IL-6 antibody pre-coated 96 wells plate in order to allow the IL-6 to adhere to the bottom of plates. Incubation was at RT for 60 minutes. Reading was at 480 nm using a conventional automated ELISA reader.

Results

Modulation of IL-6 pro-inflammatory cytokine by detoxiTide in THP-1 cells

In order to activate pro-inflammatory signaling on the THP-1 macrophages, we used the Lipopolysaccharide as an inducer factor. LPS derives from bacterial membranes and is able to interact in vivo and in vitro with the Cd II complex receptors that induces the transcription activation of pro-inflammatory genes in particular IL-6 and TNF-alfa. The release of the IL-6 was detected from the supernatants of treated cells using an ELISA assay where antibodies raised against the IL-6 were used and the increase in IL-6 expression was evident in a time course **(Figure 1)**. Interestingly enough when macrophages were co-incubated with LPS plus 10 ng/ml of DetoxiTide a marked decrease on the IL-6 expression was found and such relevant condition was verified repeating the experiment at least three times. The exact quantification in the diminished expression of the cytokine was normalized against the densitometry analysis of the expression of beta-actin as a control **Table 1**.

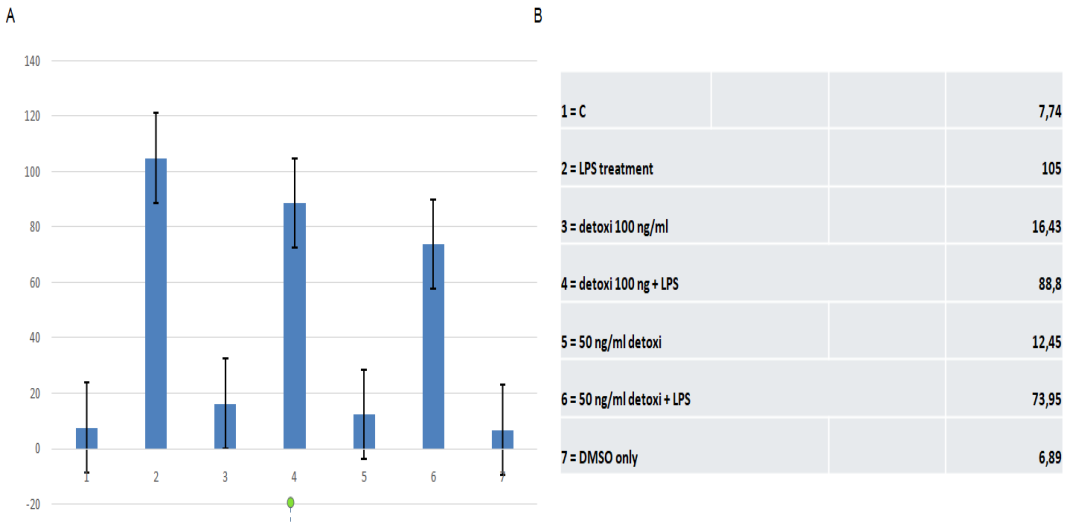


Figure 1: 48 hrs treatment of THP-1 with detoxi: evaluation of IL-6. A. Numbers are as indicated in Fig.1B. ELISA assay was performed as indicated. $ and 6 shows the decrease in IL-6 release after co-incubation with 100 ng/nl and 50 ng/ml of detoxiTide. Numbers show fold of induction and are the mean of three experiments. The 50 ng/ml detoxiTide concentration shows a better anti-inflammatory activity. The 3 and 5 detoxi treatment do not gice any substantial modulation of IL-6 release comparable to the control.

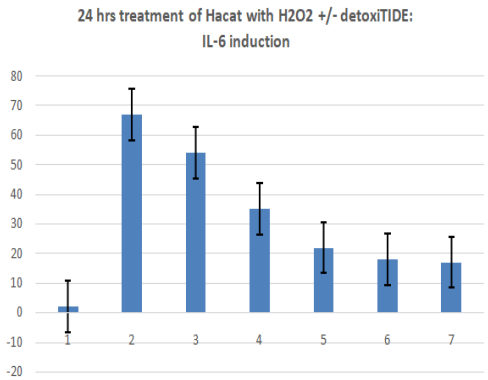
Table 1: The TedoxiTide tablet composition. The tripeptide NJ sequence is covered as an industrial secret. Not displayed. Exact amount of the other components are indicated. Tablet contents were dissolved in 100% Dymethylsulfoxide (DMSO). All the controls were adding the DMSO to cell cultures. Viability was always assayed using MTT.

Ingredients	
*Tripeptide complex NJ	100 mg
Vitamin C	80 mg
Vitamin B6	1,4 mg
Tiamin	1,4 mg
L-leucin, L-isoleucin	60 mg
L-valin	40 mg
DL-methionin	50 mg
Chlorella vulgaris	50 mg

Modulation of IL-6 pro-inflammatory cytokine by detoxiTide in HACAT keratinocytes

Furthermore, we used another cell line that has no immunogenic function but derived from immortalized keratinocytes excised by normal skin biopsies. The immortalized cell line, namely HACAT, has been extensively used for testing pro-oxidative pathways that can be easily activated using UV light exposure or hydrogen peroxide (H_2O_2). The induction of pro-oxidation usually is parallel by an increase of the pro-inflammatory status of the cells. As a matter of fact, keratinocytes treated with H_2O_2 can display an increase of IL-6 or TNF-alfa expression since the oxidization of membrane phospholipids promote the release of cytoplasmic phospholipase A2 (cPLA2) with accumulation of Arachidonic acid that ultimately activates the inflammatory pathways of prostaglandin and the consequent induction of target pro inflammatory genes. Also, in our sets of experiments the H_2O_2 was able to activate inflammatory pathways with an increase on IL-6 release in the supernatants of treated keratinocytes **(Figure 2)**. Moreover, the co-incubation of the detoxiTide decrease the release of IL-6 giving a strong support that the detoxiTide interferes with the activation of the pro-inflammatory steady state in the cells and that at least a complex network of cellular transducers is player in this experimental phenomenon.

A



B

1 = C	2,75
2 = H_2O_2 treatment 500 uM	67
3 = H_2O_2 treatment 1 mM	54
4 = H_2O_2 treatment 5 mM	35
5 = H_2O_2 treatment 500 uM + detoxi	21,5
6 = H_2O_2 treatment 1 mM + detoxi	18,6
7 = H_2O_2 treatment 5 mM + detoxi	17,6

Figure 2: 24 hrs treatment of Hacat with H2O2 +/- detoxiTIDE: IL-6 induction. A. Data from ELISA experiment was depicted as fold of induction. 2,3 and 4 are reuslts after treating HACAT cells with increasing concentrations of H2O2. 5,6 and 7 were co-incubation of the 500 um H2O2 with 50 ng/ml of detoxiTide.

Induction of Nrf-2 expression on HACAT cell extract after detoxiTide treatments

Finally, we focused our attention in investigating the primary elements that could be activated in the co-treatment of keratinocytes with detoxiTide. In particular we tried to understand if the canonical trans-acting factor that acts at nuclear level after extensive exposure of cells to pro-oxidants could be involved and could be modulated upon the detoxiTide exposure. We treated cells with the hydrogen peroxidase and first we tested the capacity of H_2O_2 to induce the nuclear expression of Nrf-2 using cellular total extracts that were fractionated in an electrophoresis system for a western immunoblot assay. As expected, the Nfr2 was activated upon H_2O_2 exposure. This s usually due to an activation of an anti-oxidant process that involve many different intermediates of redox reactions including the balancing of the NADP+/NADPH redox molecules. As shown in the **Figure 3** the coincubation of the H_2O_2 with the detoxiTide gave evidence to an increase in the expression of the Nrf-2 factor. Such observation may lead to the hypothesis that a direct link is associated to the entire network of cytoplasmic signaling involved in the regulation of detoxification inside eukaryotic cells.

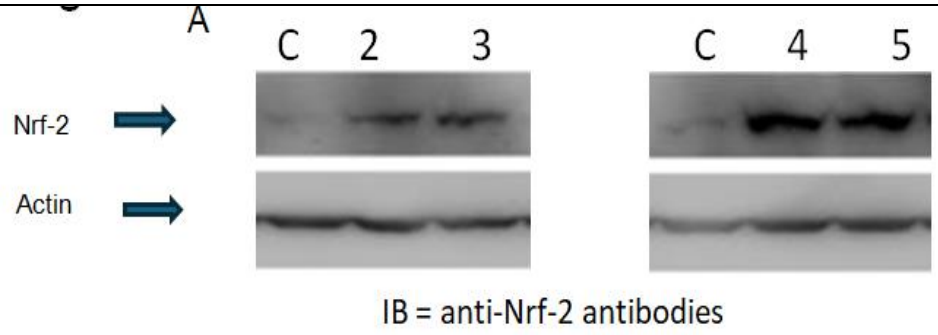


Figure 3: WESTERN BLOT ANALYSIS USING Nrf-2 ANTIBODY ON HACAT CELLULAR EXTRACTS AFTER OXIDATIVE CHALLENGE WITH H_2O_2. A. Extracts from HACAT cells treated with H_2O_2 or a combination of H2O2 + DETX were used for western blot assay as described in materials and methods.

Discussion

In this study, we have investigated the anti-inflammatory activity of the detoxiTide®, a compound containing a natural tripeptide using terminal differentiated macrophages derived from THP-1 cell line, a human monocytic leukemia cell line. Using THP-1 cells as an immune cellular model, both mechanisms of inflammatory induction and modulation of the inflammatory response were evaluated related to the detoxiTide influence. Since it has been shown that such nano peptides may act as anti-senescence factors able to prolong the life span of mesenchymal stem cells in culture, macrophages were treated with a standard dose of detoxiTide tripeptide to investigate some cellular functions. Initially we extracted the detoxiTide compound from packaged tablets dissolved in Dimethyl Sulfoxide (DMSO). Macrophages were plated and treated with bacterial Lipopolysaccharide (LPS) to induce pro-inflammatory cytokines. Therefore, we found an increase in IL-6 expression as expected being a marker of the early phase of inflammation. Surprisingly enough, the co-

incubation with 50 ng/ml of detoxiTide decreased the amount of IL-6 detectable by an ELISA assay, suggesting a direct role of detoxiTide in modulating the process of inflammation. The more plausible hypothesis is that the tripeptide may influence gene transcription since it has been widely documented that most micropeptides can pass the cytoplasmic nuclear barrier with no energy requirement. Alternatively, the interaction of peptides may even act at receptor level and thus influencing the signaling cascade inside the cells. These observations suggest that, under cell duplication conditions, peptides influence the duplication rate without causing dysfunction in the cell cycle phases. To better understand if this anti-inflammatory activity is also present in non-immunogenic cell, we used an immortalized cell line derives from normal keratinocytes (HACAT). Normal immortalized keratinocytes have been used in vitro to test prooxidative stress and consequent immunity response based on hypothesis that epidermis may act as an immunological barrier against infective agents, preserving the biological activity of natural pro-oxidant particles that impact the human body from the environment. Moreover, since the modulation of anti-oxidant activity involve nuclear factors that activate genes involved in enzymatic pathways controlling the oxidoreductive level of different molecular substrates, we investigated the role of the Nrf-2 trans-acting nuclear protein. The nuclear factor erythroid 2 related a factor (Nrf-2) is a transcription polypeptide that is encoded by the NFE2L2 gene. It is a basic leucine zipper protein that regulates the expression of antioxidant complexes that protect against oxidative damage triggered by injury and inflammation. So, we performed western blot assays to specifically study the role of Nrf-2, steady state level and mechanisms of activation. We found as shown on **Figure 3** that the incubation of HACAT cells with 50 ng/ml of the detoxiTide increase the steady state level of the protein when we run total cellular extracts from treated cells immunoblotted to a specific anti-Nrf-2 antibody. This discovery underlines the role of the antioxidant activity of detoxiTide acting at molecular level in a pathway mostly involved in cellular detoxification. In addition, this enforces the concept that the detoxiTide could be used in therapeutic protocols in patients following cycles of intense chemotherapy treatments and in all cases where the hepatic activity is compromised.

Conclusions

In this study we found that Nfr-2, a transacting factor that regulates the transcription of genes involved in the detoxification processes, can be modulated upon the detoxiTide treatment of HACAT cells. This is a quite interesting observation. As a matter of fact, as already discussed challenging cells with substances as H_2O_2 leads to marked oxidation of the cellular proteins. This determines an increase in all the processes that bring to a pro inflammatory response, mostly the transcription activation of IL-6 and TNF-alfa. However, very little is known about the capacity of a detoxifying agent to determine an up regulation of the Nrf-2 that is an involved in a multitask process that combines all the biochemical and molecular mechanisms of detoxification. It is plausible that other molecular mechanisms could be investigated since we theoretically know but not completely understand that the micro peptide can pass through the nuclear barrier and directly bind the regulatory regions of target genes. We do want also to emphasize that many clinicians that administer the detoxiTide to their patients principally for hepatic detoxification exert beneficial effects and experience the healing from most of the hepatic dysfunctions (Jose Casonato, personal communication). More additional experiments assaying the transcription regulation of these genes need further scientific evaluation.

Patents

No patent was released to the DetoxiTide because formulation was from natural products. The NJ tripeptide formula is covered by industrial secrecy.

Author Contributions

The following statements should be used "Conceptualization, S:M: and E.T..; methodology, E.J.E.; software, R.P.; validation, R.M., G.U.; formal analysis, S.M.; investigation, S.M.; resources, O.D.; data curation, R.P; writing—original draft preparation, E.T and S.M.; writing—review and editing, S.M.; visualization, E.T..; supervision, S.M. and O.D.; project administration, S.M.; funding acquisition, G.U. All authors have read and agreed to the published version of the manuscript.". Authorship must be limited to those who have contributed substantially to the work reported.

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Conflicts of Interest

The authors declare no conflict of interest.

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