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COMPARING THE EFFECT OF TOCOPHEROL AND INULIN ON FREE RADICALS PRODUCTION *IN VITRO*

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ABSTRACT

Free radicals such as superoxide and nitric oxide in moderate proportions are able to carry out vital functions such as necessitating cell renewal and cell proliferation, apoptosis and combating pathogens.Excess production could overwhelm the antioxidant mechanism used to maintain a stable redox state. This leads to pathological conditions such as inflammatory bowel diseases and colon cancer. The use of exogenous sources of antioxidant is being explored. α-tocopherol is a known antioxidant. However, the use of inulin (functional food) is an area of recent interest with little publications confirming its antioxidant ability. This project compares the antioxidant ability of α-tocopherol to inulin. Data was analysed using one-way ANOVA in the Minitab 17. Student two sample t.test was used for two-treatment groups treatment. Data observed from this project is not enough to confirm the antioxidant ability of inulin.

KEYWORD: Inulin, Free radicals, Antioxidant, α-tocopherol, Superoxide, Nitric oxide.

INTRODUCTION

The human biological system uses a variety of mechanisms to maintain a stable environment for cells to function normally, a general term known as homeostasis. A disruption in this balance will lead to deleterious effect on biological system as cells lose their capacity to function normally (Droge, 2002). One of these mechanisms is that involved in maintaining a stable redox state where free radicals and antioxidants are available in the right proportions to efficiently carry out their roles (Dröge, 2002). Studies have shown that free radicals (e.g superoxide and nitric oxide) carry out vital functions such as necessitating cell renewal and cell proliferation (cell signalling), apoptosis (gene regulation) and combating pathogens (immune surveillance) (Blaise *et al*., 2005; Davies, 2005). A situation where free radicals produced becomes more than the antioxidants (e.g superoxide dismutase and reduced glutathione), the free radicals can induce the cells adapting in response to proportions slightly above normal as seen in aging and in extreme pathological conditions stimulate uncontrolled cell proliferation, protein mutagenesis, evasion of apoptosis, uncontrolled inflammatory response to infective agents and cell death (Droge, 2002). Studies have shown that free radicals imbalance in the gastro intestinal tract has been linked to inflammatory bowel diseases such as Crohn's disease, ulcerative colitis and has also been implicated in the aetiology of colon cancer (Wasilewski *et al.*, 2015). However the mechanism by which they contribute to these diseases is yet to be fully understood.

In addition to the antioxidant mechanism which is the first line of maintaining redox homeostasis by which free radicals are evened out, the gastrointestinal tract also contributes to maintaining its homeostasis through the presence of microflora also referred to as beneficial bacteria or microbiota in the tract (Meyer, 2009). These bacteria's are able to contribute to the redox homeostasis through various ways which include adherence to the gastrointestinal tract (GIT) wall, hence, preventing the adherence of foreign bacteria's, and production of antimicrobial substances (Apajalahti, 2005).

Also, it has been frequently observed that several disorders associated with gastro intestinal tract motility disturbance and oxidative stress production has been attributed to lipopolysaccharide (LPS),an endotoxin present in the cell wall of gram negative bacteria (Fujihara *et al*., 2003 ; De Plaen *et al*., 2006). This LPS is a major virulent factor capable of mediating multisystem organ failure and has been reported to cause significant impairment of smooth muscle contractility in animal models (Niki, 2009).This impairment was caused by LPS activating macrophages capable of secreting various mediators such as hydrogen peroxide (H_2O_2), prostaglandins (PGs), cytokines and nitric oxide; most of these mediators are known to influence smooth muscle cells (SMCs) motility(Niki, 2009).Recently obtained results from an experimental model where human colonic mucosa was exposed to LPS showed that LPS is able to affect smooth muscle cell contractility due to its translocation throughout the mucosa and submucosa which subsequently suppresses muscle cell contractility through oxidative stress production (Niki, 2009).The progressive advancement in health awareness globally has propelled individuals as well as health professionals to adopt preventive approach to diseases rather than curative. This has led to intense studies of probiotics, prebiotics and symbiotics which aim to maintain or stimulate the growth of beneficial bacteria in the gut (Wasilewski *et al.,* 2015).

This project focuses on the use of inulin (a known example of prebiotic), but we will give a brief explanation of probiotics, prebiotics, and symbiotics.Probiotics are usually gram positive bacteria's of human origin (Wasilewski *et al.,* 2015) that are ingested to increase the population of beneficial bacteria in the gut and have been reported to have anti-inflammatory effects which has been employed in treating acute gastro intestinal infections (Wasilewski *et al.,*2015). Its modulation of the microflora population in turn prevents gastro intestinal diseases and has also been reported to alleviate symptoms of lactose intolerance in humans (Wasilewski*et al.,* 2015).Examples of bacteria ingested as probiotics include *Lactobacillus spp* and *Bifidobacterium spp* (Meyer, 2009*)*.Prebiotics on the other hand are usually non-digestible ingredients of plant origin that are resistant to digestive enzymes in the gastrointestinal tract (Wasilewski *et al.,* 2015)until it reaches the colon where it undergoes fermentation resulting in the alteration and promotion of the growth of beneficial microflora present here (Van den Ende *et al.,* 2011). Examples of prebiotics are galacto-oligosaccharides (lactulose), fructo-oligosaccharide (FOS) (oligofructose and inulin) and gluco and xylo-oligosaccharide (all of them being derived from oligosaccharides) (Wasilewski *et al.,* 2015). The most common example is fructo-oligosaccharides (FOS) (oligofructose and inulin). Symbiotics employs the resistance of prebiotic bacteria to digestive enzymes in the upper GIT in combination with a probiotic to give a more efficient result. This project explores free radical production and compares the effectiveness of α-tocopherol (a known antioxidant) to inulin (a prebiotic) in balancing out the free radicals produced. To give us a clearer insight into this project, it is imperative to have a general understanding of the physiological relevance of free radicals, antioxidants and inulin as a prebiotic to the biological system.

Aim

The main aim of this project is to investigate the ability of inulin in inhibiting free radical production and compare it to tocopherol a known antioxidant in vitro.

Objectives:

1. To stimulate the production of free radicals by bile acids using human keratinocyte (HK) cells
2. To investigate the inhibition of free radicals by the use of tocopherol and inulin
3. To study the tocopherol and inulin effects on free radical production on human epithelial cells in vitro

Hypothesis

Null hypothesis (H_0) states that inulin and tocopherol exhibit the same level of antioxidant effect on free radicals produced by human keratinocyte (HK) cells *invitro*. Alternative hypothesis (H_1) states that there is a significant difference between the antioxidant effect exhibited by inulin and tocopherol on free radicals produced by human keratinocyte (HK) cells *invitro*.

MATERIALS AND METHODOLOGY

Chemicals

Deoxycholic acid (CalBiochem, UK), α-Tocopherol (Sigma-Aldrich, UK), potassium hydroxide (Sigma-Aldrich, UK). Alcohol (ThermoFisher Scientific, UK), Benzo-a-pyrene (Sigma-Aldrich, UK). Inulin (Sigma-Aldrich, UK),dimethyl sulfoxide(DMSO)(ThermoFisher Scientific, UK, diphenyleneiodonium (DPI) (Sigma-Aldrich, UK) and phorbol myristate acetate (PMA) (Sigma-Aldrich, UK). Superoxide dismutase (SOD) (Sigma-Aldrich, UK).Metabolic test reagents was purchased from (Invitrogen, UK).. Griess reagent (Sigma-Aldrich UK)and Nitroblue tetrazolium test kit (Millipore, UK).

Cell culture

All tissue culture work was conducted under a class-2 laminar flow hood. Normal human keratinocytes (HK), neonatal (HEKn), catalog number: C-001-5C (Life technology, UK). Standard keratinocyte culture medium, keratinocyte-STF (1X) (GIBCO by life technologies ^{TM}). The cell culture flask containing cell lines were incubated in HK cell culture media treated with Penicillin- Streptomycin to prevent contamination of cells at 37C with 5% carbon dioxide (CO_2). Ethical was obtained from the Middlesex University London.

METHODOLOGY

Nitroblue tetrazolium (NBT)

Principles of Nitroblue tetrazolium (NBT) Assay

The conventional NBT assay is a quantitative test used to estimate the superoxide anion (O_2^-) production by phagocytic cells.This microscopic assay is conducted by counting the cells containing blue NBT formazan deposits, which are formed by reduction of the membrane permeable, water-soluble, yellow-colored, nitroblue tetrazolium (Y-NBT) by (O_2^-) (NLM, 2015). Cytokines are produced by normal human epithelial cells when the gut is exposed to pathogens. During this process, the phagocytes release superoxide among other free radicals which will be detected in the project using the modified NBT assay (Sim Choi *et al*., 2006). It has been shown that themodified NBT assay is able to override the limitations of the conventional assay by being more quantitative, more sensitive and easier to carry out (Sim Choi *et al.*, 2006).

Nitroblue tetrazolium test (NBT assay)Procedure

Cells were seeded at 1x 104/ well cells were seeded in total volume of 500µl in a 24well plate and allowed to adhere for 2-3 hours in the presence or absence of 30% inulin/tocopherol in the incubator at 37degree in 5% CO2. Thereafter, cells were treated with 50-150 µm deoxycholic acid and NBT reagent and further incubated for 20mins. After which, it was transferred to a 96 well plate and read at 620 Griess test.

Principles of Greiss Assay

The Griess reagent is most commonly used to measure the metabolites of nitric oxide more specifically nitrite (non-volatile and more stable) (Tsikas, 2007). This principle is based on the ability of nitrate to react with the amino group of sulphanilamide under acidic conditions to form the diazonium cation, which couples to N-(1-naphthyl)ethylenediamine in para-position to form the corresponding azo dye that is then measured(Tsikas, 2007).

Greiss Assay Procedure

Cells were seeded at 1 x104 / well in totalvolume of 500µl in 24 well plates in the appropriate media and allowed to adhere for 2-3 hours in the presence or absence of 10µM DPI, 30% inulin and/ tocopherol in the incubator at 370 C, 5%carbon dioxide CO2. Thereafter, cells were treated with 0.4 µM of BAP and 50 µM-150µM of deoxycholic acid and further incubated for 20-30 mins. Afterwards, 100 µl of supernatant of each designated well were pipetted in triplicate into a 96 well plate.This was followed by addition of 100 µl of Griess reagent then left to stand in the incubator for 10-15 minutes and read at 540nm (protected from light all through experiment).

Metabolic (MTT) assay

Principle of MTT assay

Cell proliferation and activity was measured using the metabolic test assay (MTT cell proliferation assay). The metabolic test assay is a widely accepted and highly reliable way of examining *invitro* cell proliferation, the yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide is reduced to a purple formazan in the presence of metabolic active cells.

MTT Assay Procedure

Cells were seeded at 5 x 10^4 / well in total volume of 200 µl in a 96 well plateand allowed to adhere for 24 and 48 hours in the presence or absence of 10µm DPI, 50-150 µm deoxycholic acid and 30% inulin / tocopherol in the incubator at 37degree in 5% CO_2.Afterwhich, 100 µl of supernatant was aspirated and MTT reagent added and further incubated for 4 hours and read at 570 nm.

Statistical analysis

Statistical analysis was performed using one way ANOVA for analysis of more than two treatment groups, based on 95% confidence interval and Turkey analysis. Data are average of three reproducible results, using the Minitab 17. Student two sample t.test was used for two-treatment groups treatment.

RESULTS

Inulin and Nitroblue Tetrazolium (NBT) Assay

Effect of Deoxycholic acid on Superoxide production by Normal Human Keratinocytes (HK) in the presence of Inulin.

The result in Fig 1 shows the superoxide production in normal human keratinocytes in the presence of deoxycholic acid (50-150 µm) and / absence of 30% inulin after 20mins. It was observed that superoxide production increased in cells treated with only 30% inulin by approximately 120% relative to the negative control (untreated cells). A progressive increase in superoxide production was observed in cells treated with only DC with increase in DC concentration and relative to the negative control (untreated cells), DC 50 µm showed about 72% increase in superoxide production, DC 100µm showed about 90% increase in superoxide production and DC 150µm approximately 92% increase in superoxide production. Superoxide production in the cells treated with DC + INU was seen to progressively decrease with increase in DC concentration with DC 50 µm + INU showing approximately 100% increase in nitric oxide production relative to the negative control (untreated cells), 85% increase in DC 100 µm + INU relative to negative control and about 75% increase in DC 150 µm + INU relative to negative control.

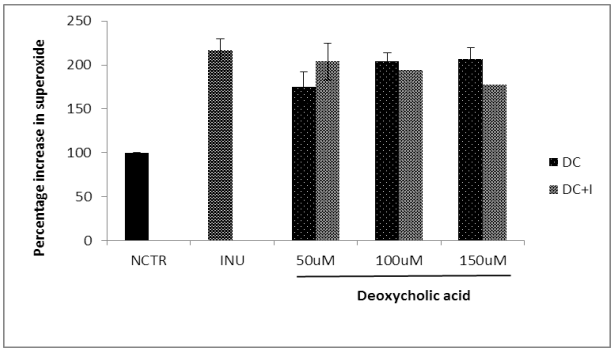

Figure 1: Effect of Deoxycholic acid on Superoxide production in Normal Human Keratinocytes (HK) in the presence of inulin. Cells were seeded at 1 x 10^4 in the presence of deoxycholic acid and/ 30% inulin for 20mins, Nitric oxide production was measured using NBT assay. Data are analysed as mean ± SE using One –Way ANOVA (Control vs treated groups).

Table 1: Showing p-values of different two sample t tests (P-value ≤ 0.05 is considered significant).

Two sample t- tests	p-value
(50µm) Dc – Dc+Inu	0.717
(100µm) Dc – Dc+Inu	0.999
(150µm) Dc – Dc+Inu	0.717

The null hypothesis (H_0) here states that there is no difference in superoxide production between the cells treated with only Dc (50-150 µm) and those treated with a combination of tocopherol and DC (50-150µm).

The alternative hypothesis (H_1) states that there is a difference between them.

The p-values obtained from the two sample t tests, 0.717, 0.999 and 0.717 are higher than 0.05 hence the difference in superoxide production between cells treated with only DC (50-150µm) and cells treated with a combination of DC (50-150µm) + INU are not considered statistically significant. We fail to reject the null hypothesis.

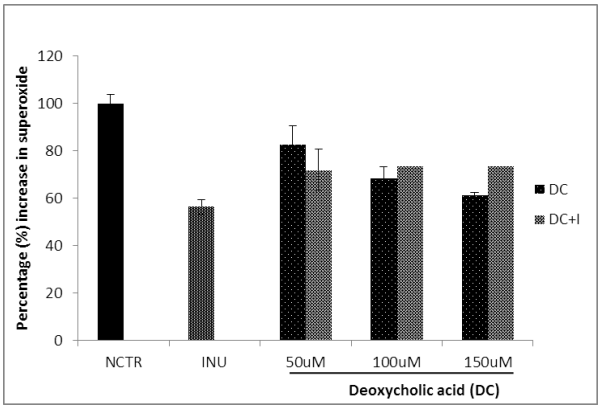

Figure 2: Effect of Deoxycholic acid on Superoxide production in Normal Human Keratinocytes (HK) in the presence of inulin. Cells were seeded at 1 x 10^4 in the presence of deoxycholic acid and/ 30% inulin for 30mins, Nitric oxide production was measured using NBT assay. Data are expressed as mean ± SE using One –Way ANOVA (Control vs treated groups).

The result in Fig 2 shows the superoxide production innormal human keratinocytes in the presence of deoxycholic acid (50-150 µm) and / absence of 30% inulin after 30mins. It was observed that superoxide production reduced in cells treated with only 30% inulin by approximately 42% relative to the negative control (untreated cells). A progressive decrease in superoxide production was observed in cells treated with only DC with increase in DC concentration and relative to the negative control (untreated cells), DC 50 µm showed about 16% decrease in superoxide production, DC 100µm showed about 30% decrease in superoxide production and DC 150µm approximately 40% decrease in superoxide production. Superoxide production in the cells treated with DC 50 µm + INU showed about 30% in reduction in superoxide production relative to the negative control (untreated cells) and 27% reduction for cells treated with DC 100 - 150µm + INU relative to negative control.

Table 1: Showing p-values of different two sample t tests (P-value ≤ 0.05 is considered significant).

Two sample t- tests	p-value
(50µm) Dc – Dc+Inu	0.858
(100µm) Dc – Dc+Inu	0.997
(150µm) Dc – Dc+Inu	0.760

The null hypothesis (H_0) here states that there is no difference in superoxide production between the cells treated with only Dc (50-150 µm) and those treated with acombination of tocopherol and DC (50-150µm).

The alternative hypothesis (H_1) states that there is a difference between them.

The p-values from the two sample t tests are not considered statistically significant. We fail to reject the null hypothesis.

Inulin and Griess Test

Effect of Deoxycholic acid on Nitric oxide production by Normal Human Keratinocytes (HK) in the presence of Inulin.

The result in Fig 3 shows the nitric oxide production innormal human keratinocytes in the presence of deoxycholic acid (50-150 µm) and / absence of 30% inulin after 20mins. It was observed that nitric oxide production increased in cells treated with only 30% inulin by approximately 16% relative to the negative control (untreated cells). A progressive increase in nitric oxide production was observed in cells treated with only DC with increase in DC concentration but relative to the negative control (untreated cells) DC 50 µm showed about 8% reduction in nitric oxide production while DC 100µm showed about 5% increase in nitric oxide production relative to negative control and DC 150µm approximately 10% increase in nitric oxide production

relative to the negative control. Nitric oxide production in the cells treated with DC + INU was seen to progressively increase with increase in DC concentration with DC 50 µm + INU showing approximately 19% increase in nitric oxide production relative to the negative control (untreated cells), 37% increase in DC 100 µm + INU relative to negative control and about 40% increase in DC 150 µm + INU relative to negative control.

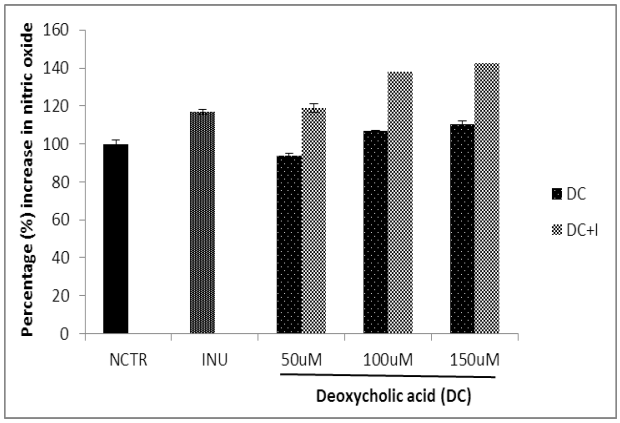

Figure 3: Effect of Deoxycholic acid on Nitric Oxide production in Normal Human Keratinocytes (HK) in the presence of inulin. Cells were seeded at 1 x 10^4 in the presence of deoxycholic acid and/ 30% inulin for 20mins, Nitric oxide production was measured using Griess assay. Data are analysed as mean ± SE using One –Way ANOVA (Control vs treated groups).

Table 3: Showing p-values of different two sample t tests (P-value ≤ 0.05 is considered significant).

Two samplet-tests	p-value
(50µm) Dc – Dc+Inu	0.000
(100µm) Dc – Dc+Inu	0.000
(150µm) Dc – Dc+Inu	0.000

The null hypothesis (H_0) here states that there is no difference in nitric oxide production between the cells treated with only Dc (50-150 µm) and those treated with a combination of inulin and DC (50-150µm).

The alternative hypothesis (H_1) states that there is a difference between them.

The P-values for the 3 paired tests are 0.000, a value lower than 0.05, hence we can say there is a significant difference between the cells treated with only DC (50-150 µm) and the cells treated with a combination of tocopherol and DC (50-150 µm). Therefore, we fail to accept the null hypothesis.

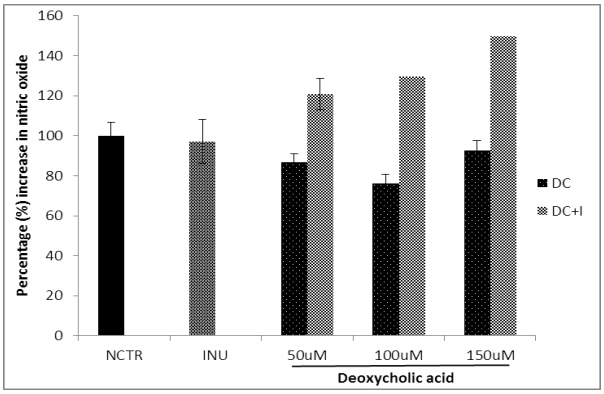

Figure 4: Effect of Deoxycholic acid on Nitric Oxide production in Normal Human Keratinocytes (HK) in the presence of inulin. Cells were seeded at 1 x 104 in the presence of deoxycholic acid and/ 30% inulin for 30mins, Nitric Oxide production was measured using Griess assay. Data are analysed as mean ± SE using One –Way ANOVA (Control vs treated groups).

The result in Fig 4 shows the nitric oxide production innormal human keratinocytes in the presence of deoxycholic acid (50-150 µm) and / absence of 30% inulin after 30mins. It was observed that nitric oxide production was slightly reduced in the presence of 30% inulin by approximately 2% relative to the negative control (untreated cell). Nitric oxide production in cells treated with only deoxycholic acid was observed to be further reduced relative to negative control with approximately 12% in DC 50 µm, 24% in DC 100 µm and 8% in DC 150 µm. In contrast, nitric oxide production was seen to increase progressively in cells treated with DC + INU showing about 20% increase in DC 50 µm + INU, 30% increase in DC 100 µm + INU and about 50% increase in DC 150 µm + INU.

Table 4: Showing p-values of different two sample ttests (P-value ≤ 0.05 is considered significant).

Two samplet-tests	p-value
(50µm) Dc – Dc+Inu	0.030
(100µm) Dc – Dc+Inu	0.001
(150µm) Dc – Dc+Inu	0.000

The null hypothesis (H_0) here states that there is no difference in nitric oxide production between the cells treated with only Dc (50-150 µm) and those treated with acombination of inulin and DC (50-150µm).
The alternative hypothesis (H_1) states that there is a difference between them.

The P-values of the paired tests, 0.030, 0.001 and 0.000 are lower than 0.05 hence statistically significant. In this case we fail to accept the null hypothesis.

Inulin and Metabolic test (MTT) assay
Effect of Deoxycholic acid on Metabolism of Normal Human Keratinocytes (HK) in the presence of Inulin
The result in Fig. 5 shows the metabolic activity of normal human keratinocytes (HK) in the presence

deoxycholic acid (50µM-150µM) and / absence of 30% inulin after 24 hrs. It was observed that metabolism of normal human keratinocytes was reduced relative to negative control (untreated cells) with approximately 42% in the presence of 30% inulin, 60% in the presence of 10µM diphenyleneiodonium chloride (DPI), 42% in the presence of 30% inulin + DPI (10µM), 30% in the presence 50µM-100µM deoxycholicacid (DC), and 42% in the presence of DC 150µM. Further reduction in metabolic activity of HK was observed in the presence of both 30% inulin and deoxycholic acid, and deoxycholic acid (DC) + DPI (relative to deoxycholic acid alone treated cells). DC 50 µM + inulin showed approximately 38% metabolic reduction, same with DC100 µM + inulin, while no reduction of metabolic activity was observed in the presence of both DC 150 µM and inulin. Metabolic activity in the presence of DPI +DC 50 µM - 100 µM were observed to be reduced further relative to deoxycholic acid alone or DC +INU.

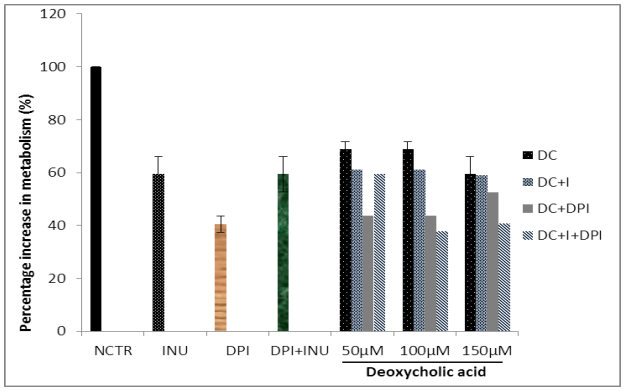

Figure 5: Effect of Deoxycholic acid on Metabolism of Normal Human Keratinocytes (HK) in the presence of Inulin. Cells were seeded at 5 x 10^4 in the presence of deoxycholic acid and/ 30% inulin for 24 hrs, cell metabolism was measured using MTT assay. Data are analysed as mean ± SE using One –Way ANOVA (Control vs treated groups).

Table 5: Showing p-values of different two sample t tests (P-value ≤ 0.05 is considered significant).

Two samplet-tests	p-value
(50µm) Dc – Dc+Inu	1.000
(100µm) Dc – Dc+Inu	1.000
(150µm) Dc – Dc+Inu	1.000

The null hypothesis (H_0) here states that there is no difference in the metabolism of cells treated with only DC(50-150µm) and the metabolism of cells treated with a combination of inulin and DC (50-150µm).

The alternative hypothesis (H_1) here states that there is a difference in the metabolism of the cells.

Despite the difference observed from the chart, the p-values which are 1.000 are considered to have no significant difference because the values are greater than 0.05. So we fail to reject the null hypothesis.

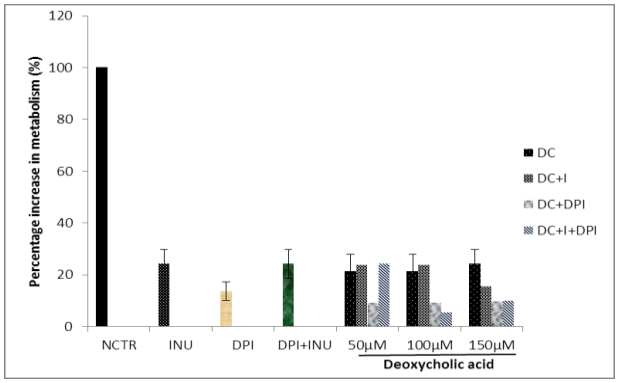

Figure 6: Effect of Deoxycholic acid on Metabolism of Normal Human Keratinocytes (HK) in the presence of Inulin. Cells were seeded at 5 x 10^4 in the presence of deoxycholic acid and/ 30% inulin for 48 hrs, cell metabolism was measured using MTT assay. Data are analysed as mean ± SE using One –Way ANOVA (Control vs treated groups).

The result in Fig. 6 shows the metabolic activity of normal human keratinocytes (HK) in the presence deoxycholic acid (50µM-150µM) and / absence of 30% inulin after 48 hrs. It was observed that metabolism of normal human keratinocytes was reduced relative to negative control (untreated cells) with approximately 74% in the presence of 30% inulin, 85% in the presence of 10µM diphenyleneiodonium chloride (DPI), 74% in the presence of 30% inulin + DPI (10µM), 78% in the presence 50µM-100µM deoxycholicacid (DC), and 77% in the presence of DC 150µM. Further reduction in metabolic activity of HK was observed in the presence of both 30% inulin and deoxycholic acid, and deoxycholic acid (DC) + DPI (relative to cells treated with only deoxycholic acid). DC 50 µM + inulin showed approximately 73% metabolic reduction, same with DC100 µM + inulin, with further reduction of metabolic activity observed in the presence of both DC 150 µM and inulin by approximately 85% relative to negative control. Metabolic activity in the presence of DPI +DC 50 µM - 100 µM were observed to be reduced further relative to deoxycholic acid alone or DC +INU.

Table 6: Showing p-values of different two sample t tests (P-value ≤ 0.05 is considered significant).

Two sample t-tests	p-value
(50µm) Dc – Dc+Inu	1.000
(100µm) Dc – Dc+Inu	1.000
(150µm) Dc – Dc+Inu	0.918

The null hypothesis (H_0) here states that there is no difference in the metabolism of cells treated with only DC (50-150µm) and the metabolism of cells treated with a combination of inulin and DC (50-150µm).
The alternative hypothesis (H_1) here states that there is a difference in the metabolism of the cells.
The p-values obtained which are 1.000, 1.000 and 0.918 are considered to have no significant difference because the values are greater than 0.05. So we fail to reject the null hypothesis. Hence there was no difference in the

effect of DC (50-150µm) and DC (50-150µm) +INU on the cell metabolism.

Tocopherol and Nitroblue Tetrazolium (NBT) Assay
Effect of Deoxycholic acid on Superoxide production by Normal Human Keratinocytes (HK) in the presence of Tocopherol.

The result in Fig 7 shows the superoxide production in normal human keratinocytes in the presence of deoxycholic acid (50-150 µm) and / absence of 30% tocopherol after 20mins. It was observed that superoxide production reduced in cells treated with only tocopherol by approximately 20% relative to the negative control (untreated cells). Further reduction in superoxide production was observed in cells treated with only DC (50-150 µm) relative to negative control where DC 50 µm showed about 26% reduction in superoxide production, DC 100µm about 22% increase in superoxide production and DC 150µm approximately 10% increase in superoxide production. Superoxide production in the cells treated with DC + TOC was seen to progressively decrease with increase in DC concentration with DC 50 µm + TOC showing approximately 22% decrease in nitric oxide production relative to the negative control (untreated cells), 30% increase in DC 100 µm + TOC relative to negative control and about 32% increase in DC 150 µm + TOC relative to negative control. Superoxide production was seen to be further reduced in cells treated with DC 150µm + DPI.

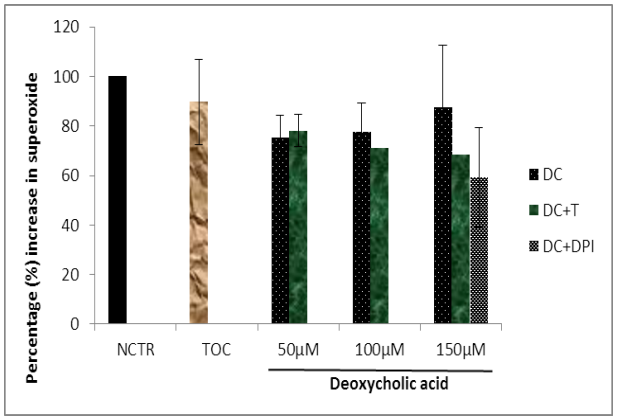

Figure 7: Effect of Deoxycholic acid on Superoxide production in Normal Human Keratinocytes (HK) in the presence of tocopherol. Cells were seeded at 1 x 104 in the presence of deoxycholic acid and/ tocopherol for 20mins, superoxide production was measured using NBT assay. Data are analysed as mean ± SE using One –Way ANOVA (Control vs treated groups).

Table 7: Showing p-values of different two sample ttests (P-value ≤ 0.05 is considered significant).

Two sample t-tests	p-value
(50µm) Dc – Dc+Toc	1.000
(100µm) Dc – Dc+Toc	1.000
(150µm) Dc – Dc+Toc	0.932

The null hypothesis (H_0) here states that there is no difference in superoxide production between the cells treated with only Dc (50-150 µm) and those treated with acombination of tocopherol and DC (50-150µm).

The alternative hypothesis (H_1) states that there is a difference between them.

It was observed that the p-values of the two sample t tests, 1.000, 1.000 and 0.932 are greater than 0.05 hence are not statistically significant. We therefore fail to reject the null hypothesis

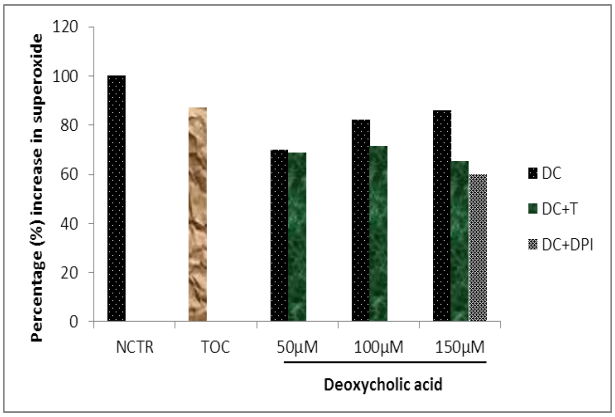

Figure 8: Effect of Deoxycholic acid on Superoxide production in Normal Human Keratinocytes (HK) in the presence of tocopherol. Cells were seeded at 1 x 10^4 in the presence of deoxycholic acid and/ tocopherol for 30mins, Superoxide production was measured using NBT assay. Data are analysed as mean ± SE using One –Way ANOVA (Control vs treated groups).

The result in Fig 8 shows the superoxide production in normal human keratinocytes in the presence of deoxycholic acid (50-150 µm) and / absence of tocopherol after 30 mins. It was observed that superoxide production reduced in cells treated with only 30% tocopherol by approximately 11% relative to the negative control (untreated cells). Further reduction in superoxide production was observed in cells treated with only DC (50-150 µm) relative to negative control where DC 50 µm showed about 30% reduction in superoxide production, DC 100µm about 18% increase in superoxide production and DC 150µm approximately 10% increase in superoxide production. Superoxide production in the cells treated with DC + TOC was also seen to decrease relative to negative control (untreated cells) with DC 50 µm + TOC showing approximately 32% decrease in superoxide production, DC 100 µm + TOC about 29% and DC 150 µm + TOC by approximately 34%. Superoxide production was seen to be further reduced in cells treated with DC 150µm + DPI.

Table 8: Showing p-values of different two sample t tests (P-value ≤ 0.05 is considered significant).

Two samplet-tests	p-value
(50µm) Dc – Dc+Toc	1.000
(100µm) Dc – Dc+Toc	0.998
(150µm) Dc – Dc+Toc	0.905

The null hypothesis (H_0) here states that there is no difference in superoxide production between the cells treated with only Dc (50-150 µm) and those treated with acombination of tocopherol and DC (50-150µm).

The alternative hypothesis (H_1) states that there is a difference between them.

The p-values of the two sample t tests obtained, 1.000, 0.998, and 0.905 are higher than 0.05 hence, are not statistically significant. We fail to reject the null hypothesis

Tocopherol and Griess Test

Effectof Deoxycholic acid on Nitric Oxide production by Normal Human Keratinocytes (HK) in the presence of Tocopherol.

The result in Fig 9 shows the nitric oxide production innormal human keratinocytes in the presence of deoxycholic acid (50-150 µm) and / absence of tocopherol after 20mins. It was observed that nitric oxide production was reduced in the presence of 30% tocopherol by approximately 40% relative to the negative control (untreated cell). Nitric oxide production in cells treated with only deoxycholic acid was observed to increase relative to negative control with approximately 210% in DC 50 µm, 280% in DC 100 µm and 350% in DC 150 µm. Nitric oxide production was also seen to increase progressively in cells treated with DC + TOC showing about 140% increase in DC 50 µm + TOC, 220% increase in DC 100 µm + TOC and about 300% increase in DC 150 µm + TOC relative to negative control. Nitric oxide production was seen to be reduced in BAP by about 30% relative to negative control (untreated cells). While nitric oxide production was increased in cells treated with DC 150 µm + DPI by approximately 310% relative to negative control.

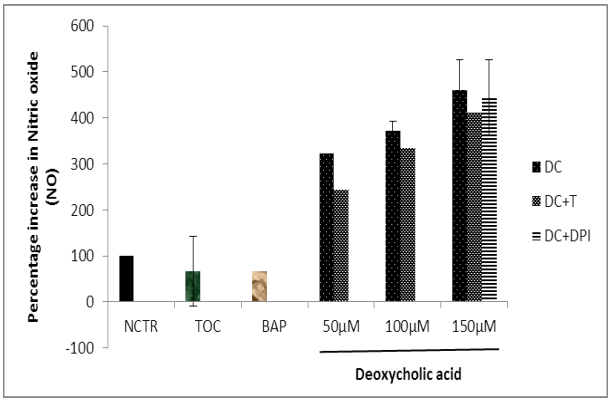

Figure 9: Effect of Deoxycholic acid on Nitric Oxide production in Normal Human Keratinocytes (HK) in the presence of tocopherol. Cells were seeded at 1 x 10^4 in the presence of deoxycholic acid and/ tocopherol for 20mins, Nitric Oxide production was measured using Griess assay. Data are analysed as mean ± SE using One –Way ANOVA (Control vs treated groups).

Table 9: Showing p-values of different two samplet tests (P-value ≤ 0.05 is considered significant).

Two samplet-tests	p-value
(50µm) Dc – Dc+Toc	0.986
(100µm) Dc – Dc+Toc	1.000
(150µm) Dc – Dc+Toc	0.999

The null hypothesis (H_0) here states that there is no difference in nitric oxide production between the cells treated with only Dc (50-150 µm) and those treated with a combination of tocopherol and DC (50-150µm).

The alternative hypothesis (H_1) states that there is a difference between them.

The p-values of the two sample t tests, 0.986, 1.000 and 0.999 are higher than 0.05, hence, not considered statistically significant. Therefore, we fail to reject the null hypothesis.

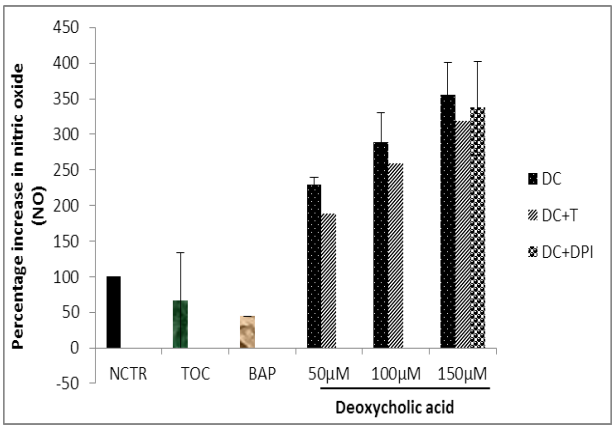

Figure 10; Effect of Deoxycholic acid on Nitric Oxide production in Normal Human Keratinocytes (HK) in the presence of tocopherol.Cells were seeded at 1 x 10^4 in the presence of deoxycholic acid and/ tocopherol for 30mins, Nitric Oxide production was measured using Griess assay. Data are analysed as mean ± SE using One –Way ANOVA (Control vs treated groups).

The result in Fig 10 shows the nitric oxide production innormal human keratinocytes in the presence of deoxycholic acid (50-150 µm) and / absence of tocopherol after 30mins. It was observed that nitric oxide production was reduced in the presence of 30% tocopherol by approximately 40% relative to the negative control (untreated cell). Nitric oxide production in cells treated with only deoxycholic acid was observed to progressively increase relative to negative control with approximately 130% in DC 50 µm, 190% in DC 100 µm

and 255% in DC 150 µm. Nitric oxide production was also seen to increase progressively in cells treated with DC + TOC showing about 80% increase in DC 50 µm + TOC, 160% increase in DC 100 µm + TOC and about 220% increase in DC 150 µm + TOC relative to negative control. Nitric oxide production was seen to be reduced in BAP by about 60% relative to negative control (untreated cells). While nitric oxide production was increasedin cells treated with DC 150 µm + DPI by approximately by approximately 235% relative to negative control.

Table 10: Showing p-values of different two sample t tests (P-value ≤ 0.05 is considered significant).

Two samplet-tests	p-value
(50µm) Dc – Dc+Toc	0.998
(100µm) Dc – Dc+Toc	1.000
(150µm) Dc – Dc+Toc	0.999

The null hypothesis (H_0) here states that there is no difference in nitric oxide production between the cells treated with only Dc (50-150 µm) and those treated with a combination of tocopherol and DC (50-150µm).

The alternative hypothesis (H_1) states that there is a difference between them.

The p-values of the two sample t tests, 0.998, 1.000 and 0.999 are higher than 0.05, hence, not considered statistically significant. Therefore, we fail to reject the null hypothesis.

Tocopherol and Metabolic Test (MTT) Assay

Effectof Deoxycholic acid on the metabolism of Normal Human Keratinocytes (HK) in the presence of Tocopherol (1mg/ml).

The result in Fig. 11 shows the metabolic activity of normal human keratinocytes (HK) in the presencedeoxycholic acid (50µM-150µM) and / absence of tocopherol after 24 hrs. It was observed that metabolism of normal human keratinocytes was reduced relative to negative control (untreated cells) with approximately 52% in the presence of tocopherol, 51% in the presence of 10µM diphenyleneiodonium chloride (DPI), 39% in the presence 50 µM deoxycholicacid (DC), 22% in the presence of 100 µM and 38% in the presence of DC 150µM. Further reduction in metabolic activity of HK was observed in the presence of both tocopherol and deoxycholic acid, and deoxycholic acid (DC) + DPI (relative to untreated cells).DC 50 µM + inulin showed approximately 73% metabolic reduction, same with DC100 µM + inulin, with further reduction of metabolic activity observed in the presence of both DC 150 µM and inulin by approximately 85% relative to negative control. Metabolic activity in the presence of DPI +DC 50 µM - 100 µM were observed to be reduced further relative to deoxycholic acid alone or DC +INU.

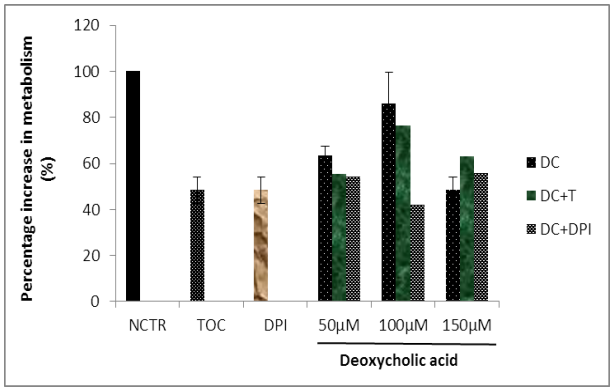

Figure 11: Effect of Deoxycholic acid on Metabolism of Normal Human Keratinocytes (HK) in the presence of tocopherol. Cells were seeded at 5 x 10^4 in the presence of deoxycholic acid and/ tocopherol for 24 hrs, cell metabolism was measured using MTT assay. Data are analysed as mean ± SE using One – Way ANOVA (Control vs treated groups).

Table 11: Showing p-values of different paired tests (P-value ≤ 0.05 is considered significant).

Two samplet-tests	p-value
(50µm) Dc – Dc+Toc	0.998
(100µm) Dc – Dc+Toc	0.993
(150µm) Dc – Dc+Toc	0.925

The null hypothesis (H_0) here states that there is no difference in the metabolism of cells treated with only DC (50-150µm) and the metabolism of cells treated with a combination of tocopherol and DC (50-150µm).

The alternative hypothesis (H_1) here states that there is a difference in the metabolism of the cells.

The p-values of the two sample t tests, 0.998, 0.993 and 0.925 are higher than 0.05 hence are not considered statistically significant. We fail to reject the null hypothesis.

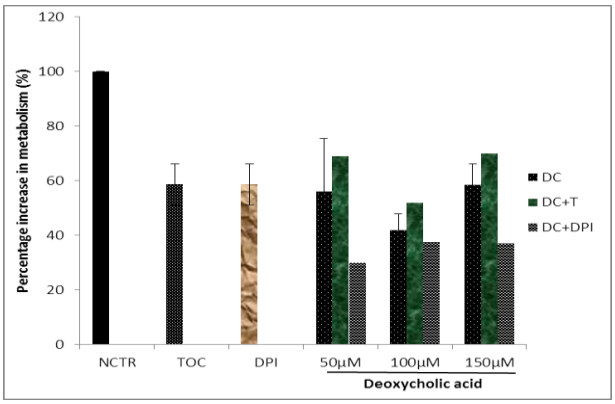

Figure 12: Effect of Deoxycholic acid on Metabolism of Normal Human Keratinocytes (HK) in the presence of tocopherol. Cells were seeded at 5 x 10^4 in the presence of deoxycholic acid and/ tocopherol for 48 hrs, cell metabolism was measured using MTT assay. Data are analysed as mean ± SE using One – Way ANOVA (Control vs treated groups).

The result in Fig. 12 shows the metabolic activity of normal human keratinocytes (HK) in the presencedeoxycholic acid (50µM-150µM) and / absence of tocopherol after 48 hrs. It was observed that metabolism of normal human keratinocytes was reduced relative to negative control (untreated cells) with approximately 42% in the presence of tocopherol, 42% in the presence of 10µM diphenyleneiodonium chloride (DPI), 45% in the presence 50 µM (DC), 48% in the presence of 100 µM and 42% in the presence of DC 150µM. Further reduction in metabolic activity of HK was observed in the presence of both 30% tocopherol and deoxycholic acid, and deoxycholic acid (DC) + DPI (relative to untreatedcells). DC 50 µM + tocopherol showed approximately 30% metabolic reduction, 50%in DC100 µM + tocopherol and about 29% in DC 150 µM and tocopherol relative to negative control. Metabolic activity in the presence of DPI +DC 50 µM - 100 µM was observed to be reduced further relative to deoxycholic acid alone or DC +TOC.

Table 12: showing p-values of different paired tests (P-value ≤ 0.05 is considered significant).

Two samplet-tests	p-value
(50µm) Dc – Dc+Toc	0.999
(100µm) Dc – Dc+Toc	1.000
(150µm) Dc – Dc+Toc	1.000

The null hypothesis (H_0) here states that there is no difference in the metabolism of cells treated with only DC (50-150µm) and the metabolism of cells treated with a combination of tocopherol and DC (50-150µm).

The alternative hypothesis (H_1) here states that there is a difference in the metabolism of the cells.

The p-values of the two sample t tests, 0.999, 1.000 and 1.000 are higher than 0.05 hence are not considered statistically significant. We fail to reject the null hypothesis.

DISCUSSION

Free radicals such as superoxide and nitric oxide which moderately produced by the cell are known to promote cell signalling that necessitate cell proliferation, cell renewal, induce apoptosis by gene regulation and enhance immune regulatory functions in response to invading pathogens (Victor *et al*., 2003). However, when produced in excess leads to harmful effects on cells rather than the intended protective function.This study focused on the impact of increased and sustained production of superoxide and nitric oxide; It also measured the cell metabolism which is reported to increase with a corresponding increase in free radical activity (Perez, 2009).

Nitric oxide (NO) is released by different types of cells which include denritic cells, natural killer cells (NK), phagocytes, mast cells, monocytes, macrophages, neutrophils, vascular smooth muscle cells, fibroblasts, Schwan cells, keratinocytes, chondrocytes, hepatocytes, eosinophils (Victor *et al.*, 2004). NO release is generated by specific nitric oxide synthases which have different isoforms, neuronal NOS (nNOS), Inducible NOS (iNOS) and endothelial NOS (eNOS). While nNOS and eNOS are constitutively expressed, iNOS is inducibly expressed in macrophages following stimulation by cytokines, LPS and other agents. NO carries out different physiologic functions such as blood pressure regulation, smooth muscle relaxation and inhibits the adhesion of platelets and leukocytes to the endothelium. NO also down-regulates endothelial adhesion of different adhesion molecule families (VCAM-1, ICAM-1, E-selectin), however, the extent to which it modulates them is variable. iNOS also regulates leukocyte recruitment during inflammatory responses. Induced NO production mechanism has been reported to be of great significance in macrophage cytotoxicity for tumour cells and bacterias (Victor *et al*., 2004).

It has been reported that reactive nitrogen species activates transcription nuclear factor κB (NF- κB) while antioxidants tends to reduce its expression. Also lipopolysaccharide (LPS) induced monocyte and macrophages activation lead to the expression of numerous mediators and inflammatory cytokines through transcription factors such as activator protein-1 (AP-1) and nuclear factor κB (NF- κB). (Fulihara *et al*.,2003). Inducable nitric oxide (iNOS) expression is one of the results of macrophage activation by LPS and subsequently increases the transformation of L-arginine to NO which is able to combine with superoxide ($O_2^{\cdot-}$) to form peroxynitrite ($ONOO^-$) (Cals-Grierson and Ormerod, 2004). Peroxynitrite is a cytotoxic oxidant, which NO toxicity is predominantly linked to and is capable of causing DNA fragmentation and lipid oxidation (Victor *et al*., 2004). Lipid peroxidation leads to oxidation of DNA and protein along the membrane leading to change in membrane permeability and modification of protein structure. Oxidative damage to mitochondrial membrane can also occur leading to membrane depolarization and uncoupling of oxidative phosphorylation with corresponding change in cellular respiration. This can result in the mitochondrial damage accompanied with the release of cytochrome *c*, the activation of caspases and eventual apoptosis (Victor *et al.*, 2004). NO dependent apoptosis has also been reported to be associated with decrease in cardiolipin concentration, reduced mitochondrial chain activity and release of mitochondrial cytochrome *c* into the cytosol. Increased resistance to NO-induced apoptosis has been reported to be associated high level of intracellular glutathione (Droge. 2002).

As earlier mentioned, antioxidants are pivotal in the maintenance of redox balance and function by either eliminating free radical precursors or by preventing catalysis, e.g., glutathione peroxidise or reacting with the reactive oxygen produced either to remove them or

inhibit them, e.g., vitamin E. This experiment employed the use of α-Tocopherol (fat soluble vitamin E), whose antioxidant ability has been reasonably established by different studies (Perez, 2009; Perse, 2013) in comparison to inulin whose antioxidant ability is currently widely investigated. Fat soluble vitamin E (α-tocopherol) is the most important antioxidant in the hydrophobic lipid interior of cell membranes as they help to prevent membrane polyunsaturated fatty acids from undergoing lipid peroxidation which leads to the loss of cell membrane integrity (Bostick, 2015 ; Victor *et al.*, 2004). Studies have shown that α-tocopherol is able to inhibit activation of NF- κB (associated with IBD) produced by LPS which could lead to subsequent decrease in TNFα. In the cause of neutrophil activation where reactive oxygen species are seen to be present in the extracellular matrix, the plasma and red cell components of the blood act as antioxidants; superoxide is inactivated by the copper-zinc SOD-dependent pathway present in the red blood cell.Apotransferrin, lactoferrin and ceruloplasmin are examples of metal-binding plasma proteins that function as important antioxidants in addition to their transport roles (Victor *et al.*,2004). Inulin is reported to have the ability to release short chain fatty acids (SCFAs) that in turn reduce the luminal pH thereby inhibiting the growth and adhesion of pathogen. Via this pathway, inulin is able to prevent the activation of the host immune response that would have led to the increase in protein expressions through the resulting adhesion molecules (Fujihara *et al*., 2003).

The p-values from Table 3.1.1 showed there was a significant difference in superoxide production between the cells treated with only DC 50 -150 µm and the cells treated with DC 50 -150 µm + INU after 20 mins incubation. This confirmed that deoxycholic acid which is a well established free radical stimulator (Perez, 2009) was able to stimulate superoxide production in the normal human keratinocytes used in amounts higher relative to the negative control. However the inhibitory activity of inulin in the cells treated with DC 50 µm + INU is questioned (Fig 3.1.1) as superoxide production is seen to be more in the cells treated with DC 50 µm + INU than the cells treated with DC 50 µm alone. It can be said that inulin inhibited superoxide production in cells treated with DC 100 – 150 µm (Fig 3.1.1). However, further analysis would have to be carried out to confirm this. In contrast, after 30 mins incubation, the superoxide production was seen to be lower relative to the negative control (untreated cells) (Fig 3.1.2) and the superoxide production was seen to reduce progressively in cells treated with only deoxycholic acid as the concentration increases. The p-values (Table 3.1.2) showed that there was a significant difference in superoxide production in cells treated with only DC 50 -150 µm and the cells treated with both DC 50 -150 µm + INU. The decrease in superoxide production in cells treated with only DC 50 -150 µm contradicts the result obtained in Fig 3.1.1. More so, because it is lower relative to the negative control it can be said that it was not a positive significance. Data from the results showed that there was a significant difference in nitric oxide production between the cells treated with only DC 100 – 150 µm and the cells treated with a combination of DC 100 – 150 µm + INU (Table 3.2.1 and Table 3.2.2). However the cells treated with a combination of DC 100 – 150 µm + INU were observed to have more nitric oxide production, hence, inferring reduced inhibitory activity of inulin. The data from this experiment also showed that there was no significant metabolic activity between the cells treated with only DC 100 – 150 µm and the cells treated with a combination of DC 100 – 150 µm + INU (Table 3.3.1 and Table 3.3.2).Again, this inconsistency is to be questioned because it has been reported that cell metabolism increases with increase in deoxycholic concentration but Fig 3.3.1 and Fig 3.3.1 depict otherwise (Monte, 2009 ; Ajouz and Shamseddine, 2014).

Data resulting from the experiments using tocopherol as the antioxidant showed no significant difference in superoxide production, nitric oxide production and cell metabolism between the cells treated with only DC 100 – 150 µm and the cells treated with a combination of DC 100 – 150 µm + TOC (Table 3.2.1 and Table 3.2.2). These results infers that there was an error during this experiment because a wide range of journals have established the antioxidant ability of α-tocopherol hence a significant difference was meant to be observed using α-tocopherol.

CONCLUSION

The data resulting from this experiment is not enough to compare the antioxidant activity of inulin and α-tocopherol. Various reasons could have resulted to the inconsistent data, such as the amount (3mg/ml) of inulin and tocopherol used (1mg/ml). Perhaps, a higher concentration could have resulted in more significant result. Also, there is a possibility that the timing of the incubation for the NBT and griess assays could have yielded better data if they were incubated longer. ELISA PLUS assay would have also helped to quantitatively detect the presence of any apoptotic cell death. In future, western blot can be used to analyse proteins that are upregulated during ROS activities such as E-cadherin (upregulated in colon cancer), since the expression of cell adhesion molecules have been correlated with cell growth, cell differentiation and wound repair and are said to be stimulated by tissue necrotic factor (TNF), LPS, ROS, and interleukin-1α. Also, ROS activity can further be determined by measuring the proteolytic degradation of NF-κB inhibitor IκB after ROS exposure. The LPS receptor complex plays a vital role in sensoring and mediating the response to LPS. Advance knowledge has been made in identifying the molecules of the LPS receptor complex which is composed of three proteins – CD14,Toll-like receptor 4 (TLR4), and myeloid differentiation protein-2.The need for establishment of biomarkers to measure oxidative stress invivo cannot be overlooked. This would immensely assist in early

discovery of disease, diagnose the progression of disease, and evaluate the effectiveness of treatment with antioxidants or functional foods. Lipid peroxidation products may serve as useful biomarkers, since the major reaction caused by oxidative stress is lipid peroxidation and is assumed to play a pivotal role in the pathogenesis and progression of many diseases despite the fact that they are non specific biomarkers.Furthermore, some lipid peroxidation products are chemically reactive and react with proteins, sugars and DNA to form stable adducts that are biologically specific to various protein.

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