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Report for:

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Report 174-001. Foundational properties at the cellular level.

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Report 174-001. Foundational properties at the cellular level.

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1 Executive Summary

NNB Nutrition is in the process of launching Ergothioneine in the US market. A strategic research plan is mapped out, and this report represents the results of the initial project.

Ergothioneine produced by NNB's proprietary method was tested in a basic panel of bioassays for antioxidant and anti-inflammatory properties.

Results:

Ergothioneine showed antioxidant properties and cellular antioxidant protection of red blood cells, indicative of cellular bioavailability and demonstrating that Ergothioneine produced by NNB's method accumulates in red blood cells with intact antioxidant properties.

Ergothioneine showed protection of cell viability, and enhanced mitochondrial function under conditions of oxidative stress.

[redacted]

Conclusion:

Ergothioneine from NNB showed cellular support and protection in a variety of bioassays using human cells from healthy donors.

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2 Purpose

To initiate the first step of a systematic, strategic research plan, where this first protocol focused on foundational antioxidant, cell-protection, and anti-inflammatory effects of Ergothioneine.

3 Background

Ergothioneine is a potent antioxidant and cellular regulating natural compound that is known to have its own transport system for mammalian uptake via food, and to selectively accumulate in specific cells including red blood cells and immune cells (macrophages).

Ergothioneine is naturally present in food, including beans and oat bran, and especially in some edible mushrooms such as oyster mushrooms. Purifying Ergothioneine from food sources is a tedious process leading to an expensive dietary supplement. NNB has a method based on fermentation that has allowed NNB to significantly reduce the cost of production.

NNB wants to document efficacy on its own product produced by their methodology. A strategic research plan has been discussed and is in ongoing development to support a comprehensive marketing direction and explore multiple niches (ingestible, topical).

The project reported here was the first in a series of phases to help document the biological effects of NNB's Ergothioneine. The comprehensive inclusion of all key marketing claims must be protected at all phases of testing. These **focus areas** include:

1. Antioxidant effects
2. Anti-inflammatory effects
3. Immune modulation
4. Anti-ageing effects (This is a broad area and will be approached in multiple different ways as the strategic research plan unfolds.)

4 Work Performed

4.1 Test Products

Table 1. Test product for this project.

Name	Source	Handling	Solubility in water
Ergothioneine	NNB USA	Aqueous	50 g/L

The different tests have different levels of sensitivity, and effects are detectable at different dose ranges. Below is a table that summarizes the dose ranges tested.

Table 2. Dose range in lab tests.

Test name	Dose range tested (g/L)*
Total Antioxidant Capacity	0.005 – 10
Red blood cell integrity**	0.002 – 3.3
Protection of PMN cells**	0.005 – 3.3
Protection of PBMC	0.05 – 3.3
Cellular antioxidant protection**	0.002 – 3.3
Effects on ROS formation, PMN cells**	7.5×10^{-9} – 5

**All tests included the 0.6 g/L dose estimated in human and mammalian tissue as a natural antioxidant.[1]*

***This includes the dose range of 0.009 g/L (40,000 nM in whole blood), reported in blood 2 weeks after ingesting a daily dose of 0.025 g (25 mg) Ergothioneine for 7 days.[2]*

4.2 Product Handling

Ergothioneine has some solubility in aqueous media needed for cell culture, so the product was added directly to physiological saline and allowed to hydrate under gentle agitation. The solution was sterile-filtered and serial dilutions were prepared in physiological saline for cell culture testing.

4.3 Tests Performed

The product was tested in a selected panel of lab assays to expand on our knowledge of its fundamental biological activities.

The tests included:

- Total Antioxidant Capacity
- Cell-based Antioxidant Protection of erythrocytes (CAP-e)
- Cellular viability and support of mitochondrial metabolic activity
- Effects of free radical formation by human inflammatory immune cells

The results from these assays serve as a hub for further decision-making on which directions may be most productive for the test product. See also the section below "Future Directions".

4.4 Statistical analysis

Data analysis involved calculation of statistical significance when comparing the data from cell cultures treated with Ergothioneine to control cultures, using the two-tailed unpaired t-test. Statistical trends are indicated if $P<0.1$, statistical significance is indicated if $P<0.05$, and a high level of significance is indicated if $P<0.01$.

Statistical significance is indicated for each dose of Ergothioneine in tables immediately below graphs (above the figure legends), where asterisks for each dose in the table below the graph, where $P<0.10$: (*), $P<0.05$: * and $P<0.01$: **.

5 Results

5.1 Cellular Viability – Preparation for Further Bioassay Work

For the particular purpose of testing the activating effects of the test products on immune cell activation and cytokine production, a cell viability assay was needed as a preparatory step when starting work on the biological effects of complex natural products. The data generated from this testing helped identify the ***optimal dose range*** for the immune cell testing.

The testing involved 3 different cell types: red blood cells, polymorphonuclear (PMN) cells, and peripheral blood mononuclear cells (PBMC).

5.1.1 Red Blood Cells

We use a red blood cell model to test effects of a new test product on the integrity of the cell membrane. The red blood cell is a convenient model, because of its simplicity; it does not contain a nucleus or mitochondria, and therefore cannot undergo apoptosis or display effects pertaining to mitochondrial distress. The red blood cells contain hemoglobin, and the red color of hemoglobin leaking from stressed cells with compromised cell membranes is a convenient measure of cell lysis. The red color is measured by spectrophotometry, and the results are displayed below as optical density at 576 nanometers (nm) where hemoglobin absorbs light.

Synopsis:

- Ergothioneine did not cause stress to the cell membrane at any dose tested.
- Ergothioneine at 0.1 – 1 g/L showed higher optical density than untreated cells:
    - This may reflect support of the membrane integrity, for example by protecting the red blood cells from lysing during the assay (pipetting and centrifugation).
    - Alternatively, the product may have interacted with hemoglobin to alter the absorbance properties.

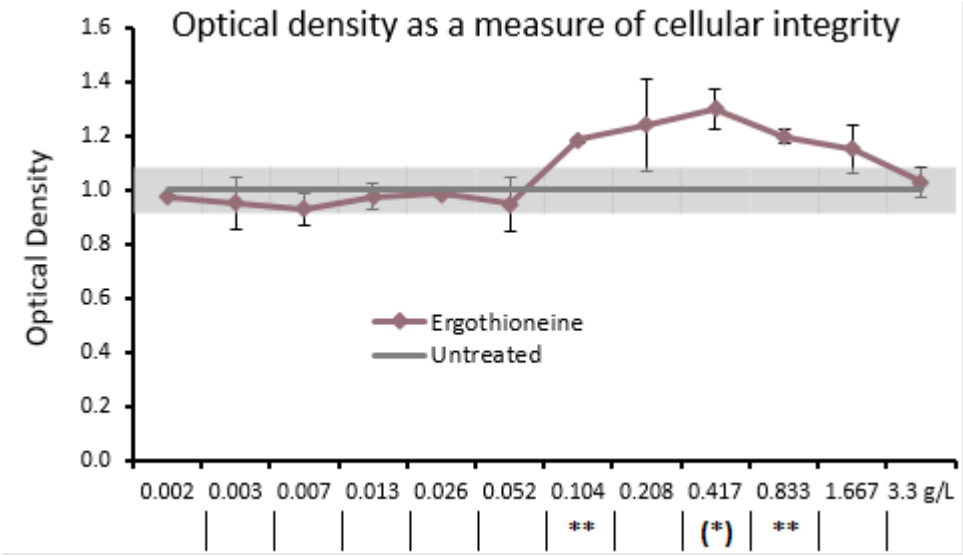

Figure 1. *Optical density as a measure of cellular integrity. The measurements of optical density at 576 nm (where hemoglobin shows light absorbance) are shown as a relative measure for the number of red blood cells per culture. The results are shown as the average ± standard deviation of duplicate wells. The grey line shows the average of untreated cells (average of 12 cultures), and the lighter grey shade shows the standard deviation for untreated cells.*

5.1.2 White Blood Cells – Polymorphonuclear Cells

Since Ergothioneine did not stress the cell membrane at the 3.3 g/L dose, this was used as the highest dose in the next 2 cell viability assay as well. PMN cells are highly inflammatory, and the cytoplasm filled with granules with various functions involved in oxidative stress, phagocytosis, and secretion of free radicals (oxidative burst). When the cells are stressed or triggered to produce free radicals, they degranulate. If the stress is very intense, the cells progress to apoptosis after degranulation. The protection of a high level of intracellular granularity (i.e. absence of degranulation and apoptosis) is measured by the forward and side scatter properties by flow cytometry.

Synopsis:

- When PMN cells were treated with Ergothioneine there were more PMN cells with a high level of intracellular granularity, suggesting less inflammatory degranulation, and demonstrating an anti-inflammatory effect.

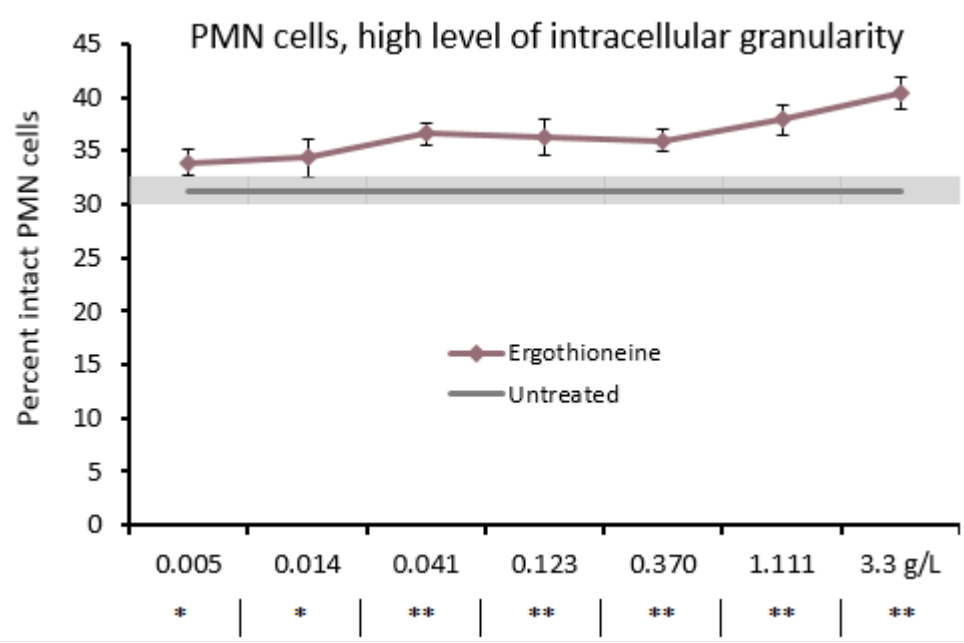

Figure 2. *PMN cells with a high level of intracellular granularity. Flow cytometry analysis of the light scatter properties of PMN cells was performed. The average of 12 untreated PMN cell cultures is shown as a grey line, and the lighter grey shade shows the standard deviation for untreated cells. In comparison, the increased protection of intact PMN cell granularity is shown and reached a high level of statistical significance at the 5 highest doses.*

5.1.3 White Blood Cells – Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells were tested for cell viability using the MTT assay. This assay utilizes a dye that changes color dependent on mitochondrial function. Freshly harvested cells were cultured to allow the color formation to take place in proportion to mitochondrial function. In the MTT bioassay, chemical reactions trigger a specific color development based on cellular functions:

- When a reduction in color is measured, this is linked to a reduced cellular viability, either as a result of direct killing, or inhibition of mitochondrial function.
- When an increase in color is measured, this has several possible explanations: 1) increased cell numbers (growth); 2) increased mitochondrial mass, and 3) increased mitochondrial function (energy production).

This is a cost-effective screening of potential effects of a test product on ***mitochondrial metabolic activity,*** i.e. cellular energy production.

Synopsis:

- The peripheral blood mononuclear cells tolerated Ergothioneine well at all doses tested and no cellular stress was observed. This helps planning further immune-based bioassays in future test protocols.
- Under normal, un-stressed culture conditions, the lowest dose of Ergothioneine showed a mild support of mitochondrial function. This was statistically significant when comparing to untreated control cultures ($P<0.05$).
- Under conditions of oxidative stress, Ergothioneine supported a higher level of mitochondrial function. This was highly significant ($P<0.01$) at the 3 lowest doses tested.

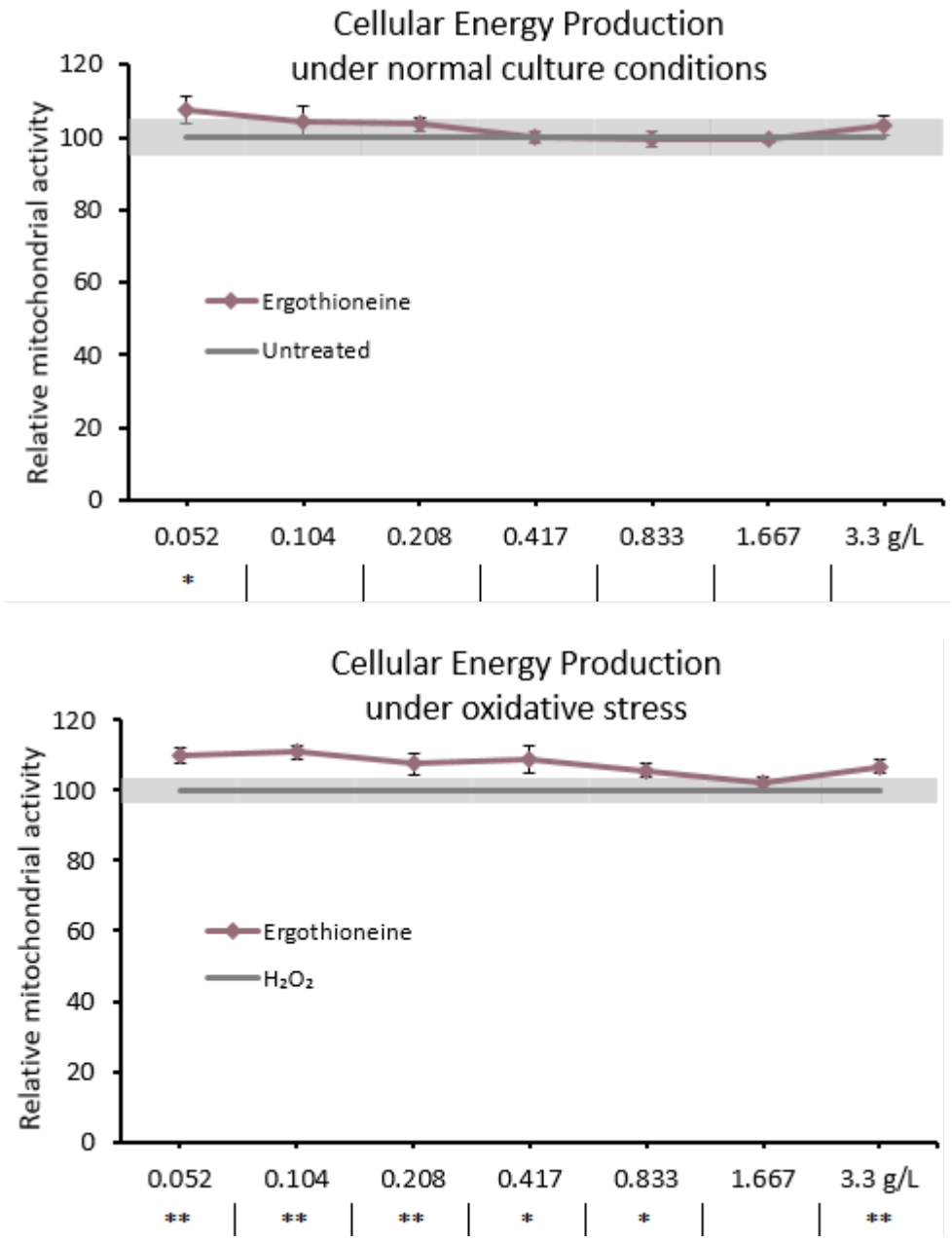

Figure 3. *Cellular energy production as a measure of relative mitochondrial metabolic activity in PBMC cultures under normal culture conditions (top) and under oxidative stress (bottom). PBMC were exposed to serial dilutions of products for 24 hours after which time the cultures were processed in the colorimetric MTT assay. Results reflect the sum of the metabolic activity of cells in each culture. The solid grey line shows the average of untreated cells, and the lighter grey shade shows the standard deviation for untreated cells. The colorimetric readings were used to calculate the % change from untreated control cultures (top, grey line at 100%) and the % change from H_2O_2 control cultures (bottom, grey line at 100%).*

5.2 Total Antioxidant Capacity

The product was tested in the Folin-Ciocalteu assay (also known as the total phenolics assay).[3] This assay makes use of the Folin-Ciocalteu reagent to measure antioxidants. The assay was performed by adding the Folin-Ciocalteu's phenol reagent to serial dilutions of extract, thoroughly mixing, and incubating for 5 minutes. Sodium carbonate is added, starting a chemical reaction producing a color. The reaction was allowed to continue for 30 minutes at 37°C. Optical absorbance was measured at 765nm in a colorimetric plate reader. Gallic acid was used as a reference standard,[4] and the data are reported in Gallic Acid Equivalents per gram product.

Synopsis:

- Ergothioneine showed strong antioxidant capacity.
- The Total Antioxidant Capacity value was 343 Gallic Acid Equivalents.
- To put this value into perspective, antioxidant-rich juices and juice concentrates rarely exceeds values of 50. In contrast, many types of extracts from teas, berry seeds and berry skins can exceed this value (on a per-weight basis).

Table 3: Total antioxidant capacity - mg GAE per gram product

Ergothioneine	343

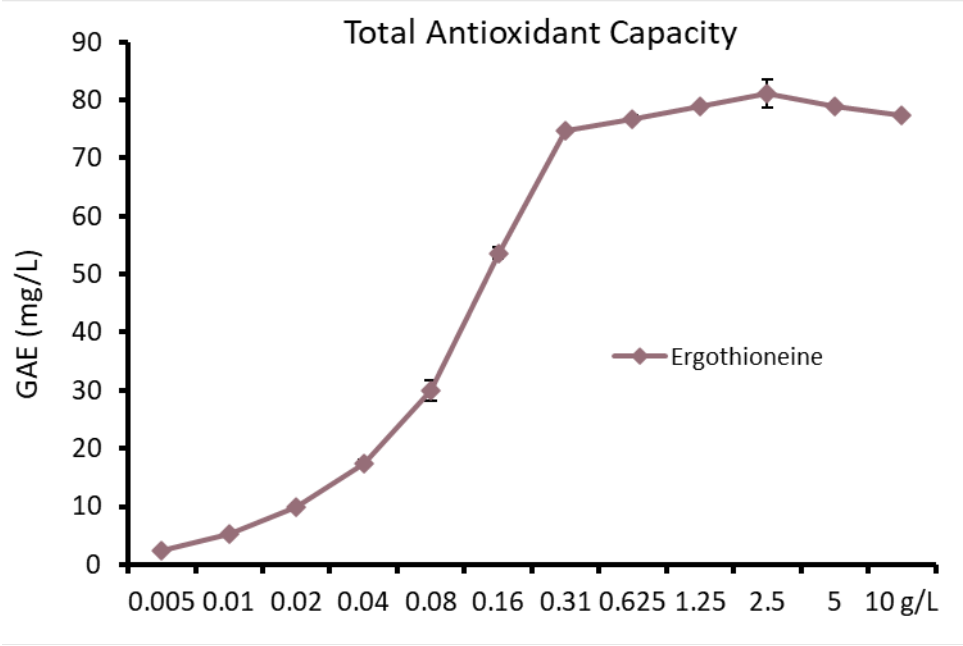

Figure 4. *Total antioxidant capacity of Ergothioneine is shown in Gallic Acid Equivalents (mg/L). The colorimetric readings are used to plot the results onto the gallic acid standard curve and calculate the Gallic Acid Equivalents (GAE). The data is shown as the average ± standard deviation of duplicate data points for each dose of Ergothioneine.*

5.3 Cell-based Antioxidant Protection Assay

The rationale[5] behind the method that we use is important: It allows assessment of antioxidant potential in a method that is comparable to the 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.

We developed the CAP-e bioassay specifically to work with natural products and ingredients.[6] The method has been used on multiple types of natural products and ingredients, published in the peer-reviewed scientific literature.[7] [8] [9] [10] [11] [12]

As a model cell type, we use the red blood cell (RBC). This is an inert cell type, in contrast to other cell types such as PMN cells (often used for subsequent testing of anti-inflammatory effects of natural product and extracts) (see section 7.4 below). We developed the red blood cell-based assay particularly to be able to assess antioxidants from complex natural products in a cell-based system and help interpret subsequent data from more complex cellular models.

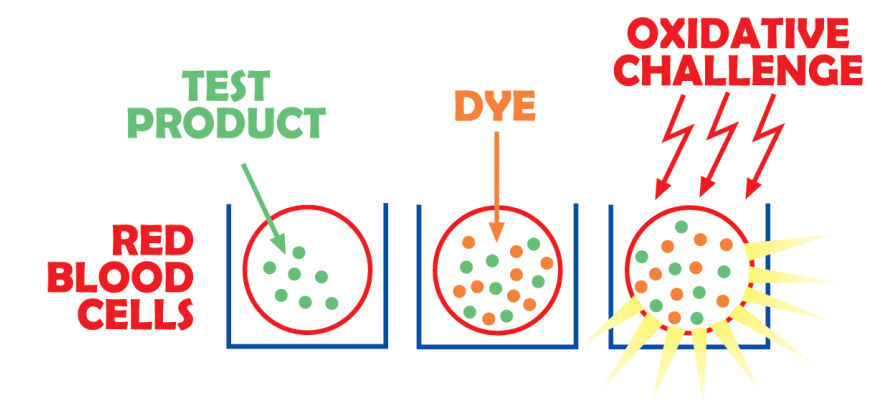

Figure 5. *Cellular antioxidant protection (CAP-e) assay principles: Living cells are loaded with a test product, allowing any compounds that are able to enter into the cell, reflecting bioavailability at the cellular level. Un-absorbed test product is removed. Cells are loaded with a precursor that becomes fluorescent upon oxidative stress damage. If a test product triggers reduced fluorescence, this directly translates to cellular protection from free radical stress.*

Human RBC were washed repeatedly in physiological saline, and then exposed to the test products. During the incubation with a test product, any antioxidant compounds able to cross the cell membrane can enter the interior of the RBC. Then the RBC are washed to remove compounds that were not absorbed by the cells, and loaded with the DCF-DA dye, which turns fluorescent upon exposure to reactive oxygen species. Oxidation is triggered by addition of the peroxyl free radical generator AAPH. The fluorescence intensity is evaluated. The low

fluorescence intensity of untreated control cells serves as a baseline, and RBC treated with AAPH alone serve as a positive control for maximum oxidative damage.

If we observe a reduced fluorescence intensity of RBC exposed to a test product and subsequently exposed to AAPH, this indicates that the test product contains antioxidants available to penetrate into the cells and protect these from oxidative damage.

Based on the low fluorescence of the untreated control wells, and the high fluorescence of the cell cultures exposed to oxidative damage, the fluorescence intensity in cell cultures treated with test products prior to exposure to oxidative stress is used to calculate the percent inhibition of cellular oxidative stress.

Synopsis:

- Ergothioneine was able to enter into and protect living cells from free radical stress.
- This is a measure of bioavailability at the cellular level.
- To put this value into perspective, antioxidant-rich foods and juices rarely exceeds 50, whereas highly pure extracts of teas may reach levels 10 times higher (on a per-weight basis).

Table 4: Cellular antioxidant protection - µM GA/gram

Ergothioneine	130

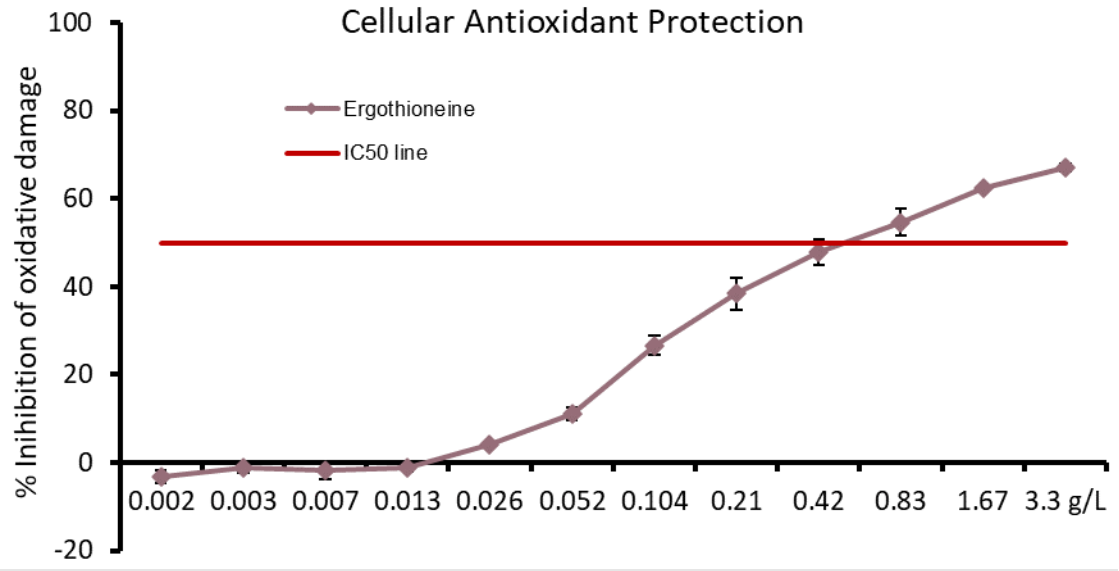

Figure 6. *Cellular antioxidant protection provided by Ergothioneine. The percent inhibition of cellular oxidative damage was calculated based on the fluorescence readings for cells treated with the test product, compared to negative control cultures (untreated cells) and positive control cultures (treated with the oxidizer AAPH in the absence of antioxidants). The percent inhibition is shown as the average ± standard deviation of duplicate data points for each dose.*

5.4 Effect on Free Radical Formation by Polymorphonuclear Phagocytes

Many natural products with antioxidant capacity also reduce the Reactive Oxygen Species (ROS) formation in inflammatory immune cells.[13] [14] Other products may increase the ROS formation, despite antioxidant capacity, and this may indicate an interesting cooperation between support of antimicrobial defence mechanisms and antioxidant capacity.[5] Thus, natural products may affect PMN cell ROS formation by three different mechanisms (see figure below):

1. Neutralizing ROS by direct antioxidant affect;
2. Triggering an anti-inflammatory cellular signal, leading to reduced ROS formation;
3. Triggering an immune reaction, leading to enhanced ROS formation.

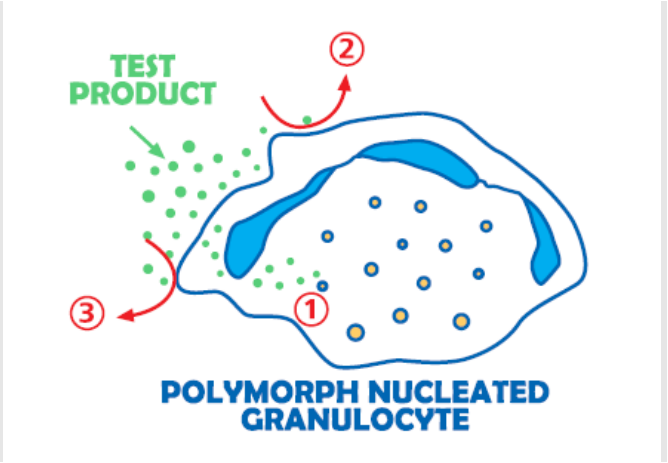

Figure 7. *Diagram of human inflammatory immune cell. The polymorphonuclear cell may react to a test product in several different ways: 1) Antioxidants may penetrate into the cells and help neutralize pre-made free radicals inside the cell; 2) Immunogenic compounds may engage with cell surface receptors (such as Toll-Like Receptors) and trigger immune activation and a pro-inflammatory behavior, or 3) anti-inflammatory compounds may engage with different cell surface receptors and trigger a re-programming of the cell to a less inflammatory behavior. Results from the CAP-e assay (above) helps interpret the complex data.*

Human polymorphonuclear (PMN) cells in whole blood samples were used for testing effects of a product on ROS formation. This cell type constitutes approximately 70% of the white blood cells in humans. PMN cells produce high amounts of ROS upon certain immune-activating challenges, including bacterial invaders, as part of the cells' normal phagocytic immune defense activities.

The testing was performed on cells from a healthy blood donor. Minute samples of blood were exposed to the test product. During the incubation with a test product, antioxidant compounds able to cross the cell membrane can enter the interior of the PMN cells, and compounds that trigger a signaling event can do so. Then the cells were loaded with the dihydrorhodamine dye,

which turns fluorescent upon exposure to ROS. Formation of ROS was triggered by addition of the bacterial peptide f-Met-Leu-Phe (fMLP). The samples were treated with a lysing buffer to eliminate most of the red blood cells in the samples, after which the samples were fixed in formalin. The fluorescence intensity of the PMN cells was evaluated by flow cytometry. The low fluorescence intensity of untreated control cells served as a baseline and PMN cells treated with fMLP alone serve as a positive control.

If the fluorescence intensity of PMN cells exposed to an extract, and subsequently exposed to fMLP, is reduced compared to fMLP alone, this indicates that a test product has anti-inflammatory effects.

In contrast, if the fluorescence intensity of PMN cells exposed to a test product is increased compared to fMLP alone, this indicates that a test product has pro-inflammatory effects by enhancing this aspect of anti-microbial immune defense mechanisms.

Synopsis:

- The effect of Ergothioneine on Reactive Oxygen Species formation was tested twice:
  - **Test 1** used an extremely broad dose range, covering doses reported in blood plasma and in erythrocytes after consuming 25 mg Ergothioneine.[2]
    - [redacted]
  - **Test 2** focused on the higher end of the dose range.
    - [redacted]

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6 Conclusions

Ergothioneine showed antioxidant properties and cellular antioxidant protection, indicative of cellular bioavailability.

Ergothioneine showed protection of cell viability and enhanced mitochondrial function under conditions of oxidative stress.

Ergothioneine protected the high level of granularity of polymorphonuclear phagocytes, reflecting their readiness to release immune protective Reactive Oxygen Species and other defense bioactives, which demonstrated protection of the cells. This protection was statistically significant ($p<0.05$) at the low dose of 0.005 g/L, which is lower than the dose of 0.009 g/L measured in whole blood after consuming 25 mg Ergothioneine.[2]

[redacted]

7 Further Work

Further steps in the **Strategic Research Plan** include several market applications and directions:

- **Energy**
  - Relevant for anti-ageing, sports nutrition, and long-term fatigue.
  - Documentation may include more in-depth testing of mitochondrial effects.
- **Immune modulation:**
  - Relevant for **preventive health protection** as a part of **longevity**.
  - **Management of inflammation** to allow acute inflammation involved in initiation of a protective immune response to pathogens, while also assisting a return to homeostasis to avoid development of chronic inflammatory conditions.
- **Skin health:**
  - The skin is our third brain and a very important organ for protection against environmental stress, UV light, pollution, and microbial pathogens.

- The **anti-ageing** effects of skin involves all 4 focus areas that NNB USA uses to market the product. Testing using human skin cells will address all 4 focus areas.
  - A first focus area will include processes involved in **wrinkle reduction.**
  - A second focus area will involve effects on skin cell **senescence.**

- **Clinical testing**
  - Clinical testing will be planned around documentation of acute effects on **antioxidant status**, **immunity** and **inflammation management**, to support claims for a product designed to work fast.
  - **Stem cell**-related effects are of course also highly relevant for regulation of inflammation and for direct reparative functions in anti-ageing.

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