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Report 185-001. Antioxidant Capacity and Cellular Uptake – Foundational Testing in Preparation for Further Preclinical Research.

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1 Executive Summary

Chondroitin sulfate Is a structural component of cartilage and the vascular system, composed of long strands of specific sugars, providing tensile strength and resilience from compression.

Purpose:

This project involved testing of two different forms of chondroitin-4-sulfate for antioxidant capacity and cellular bioavailability.

Results and conclusions:

Chondroitin-4-sulfate (powder) from EternaPure has antioxidant capacity, and this was also easily documented in the injectable product Equine Max.

Both the source material (powder) and the injectable product Equine Max, showed a very mild level of cellular antioxidant protection in the CAP-e bioassay, suggesting that a small amount of the chondroitin-4-sufate chains were sufficiently small to penetrate into living cells and protect the cells from the inside-out.

The emulsified product pureC4S designed for oral consumption was produced from the same source material. Interpretation of the antioxidant properties was clouded by the antioxidant properties of the emulsifier itself, and the stress caused to cell membranes by higher doses of the emulsifier and the emulsified chondroitin-4-sulfate.

Further work:

Additional testing is warranted and should continue to compare the powder, the injectable and the emulsified products. For the emulsified product we need to explore doses below 0.01 g/L (chondroitin sulfate) and evaluate cellular models that will provide outcomes for this dose. This will then allow a comparison between the injectable and the emulsified versions to examine whether the emulsified product is delivered to cells more efficiently.

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2 Purpose

Testing of different delivery systems for chondroitin-4-sulfate for antioxidant capacity and cellular bioavailability in preclinical assays. Two fundamental questions were asked:

1. Does the specific source of chondroitin-4-sulfate used by EternaPure possess antioxidant properties?
2. If so, are at least some of the molecules of a size and composition that are capable of entering into and protecting living cells from oxidative stress?

3 Background

Chondroitin sulfate (CS) is a linear heteropolysaccharide consisting of repeating disaccharide units of glucuronic acid and galactosamine, which is commonly sulfated at C-4 and/or C-6 of galactosamine. Chondroitin-4-sulfate (C4S) is a glycosaminoglycan that is sulfated at C4. It is isolated from pure bovine trachea and the moiety size can vary greatly.

The novel consumable emulsified product available for testing in this research project may be present in smaller units with a relatively lower molecular weight, and the average molecular weight may be around 15 kDa.

There is minimal data on cellular delivery and penetration. Some biological and immunological activity may be through C4S biding to cell surface receptors, triggering immune activation and modulating cytokine production.

There is a need to initiate research to establish basic understanding of the product and move forward with a stepwise plan to establish where the specific source and delivery system for EternaPure's C4S may show superiority. This project was the first step in this process.

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4 Work Performed

4.1 Test Products

Table 1. Test product compared in this project.

Name	Source	Description	Handling
Chondroitin-4-sulfate*	Eternapure	Powder	Aqueous
Equine Max	Summit JP	Injectable solution	Aqueous
PureC4S	Eternapure	Emulsified	Aqueous
Summit Large Canine Control	Cellg8	Emulsifier control	Aqueous

**This powder is the source from which the other 2 test products are produced.*

4.2 Product handling

All 4 test products were used for generating a suspension in aqueous media necessary for cell cultures. Serial dilutions were prepared in physiological saline at neutral pH, in preparation for the lab testing.

4.3 Tests performed

The 4 compounds were tested and compared in an initial set of lab assays to start generating a foundational understanding and help plan further work. The focus for the initial testing was to document basic antioxidant properties and bioavailability at the cellular level.

The testing included:

- Ensuring that all compounds are tested at doses that do not cause direct damage to cells;
- Testing the levels of antioxidant properties, to help plan the CAP-e cellular antioxidant protection assay;
- Testing cellular uptake in the CAP-e bioassay: Bioavailability and bio-delivery at the cellular level.

Results from these assays will serve as a hub for further decision-making on which direction(s) may be most productive for documenting superiority of the emulsified test product.

5 Results

5.1 Total Antioxidant Capacity

The products were tested in the Folin-Ciocalteu assay (also known as the total phenolics assay). This assay makes use of the Folin-Ciocalteu reagent to measure antioxidants. The assay is 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 is allowed to continue for 30 minutes at 37°C. Optical absorbance is measured at 765 nanometer in a colorimetric plate reader. Gallic acid is used as a reference standard, and the data reported in Gallic Acid Equivalents (GAE) per gram product.

Synopsis:

- Chondroitin-4-sulfate from EternaPure showed a clear dose-dependent antioxidant capacity.
- The injectable of the product, Equine Max, showed antioxidant properties within a similar range although slightly less potent.
- The novel emulsified oral consumable product pureC4S appeared to possess antioxidant properties; the exact magnitude of the effect was masked by 2 factors:
    - The inherent antioxidant properties of the emulsifier
    - The opaque properties of the emulsifier.

All 4 products were tested at maximum doses, and at 5 serial 2-fold lower doses. Due to the dilute properties of the product pureC4S, it was not possible to test this product at higher doses. Further extrapolation must be made from the starting material, the powder, which clearly possessed antioxidant capacity.

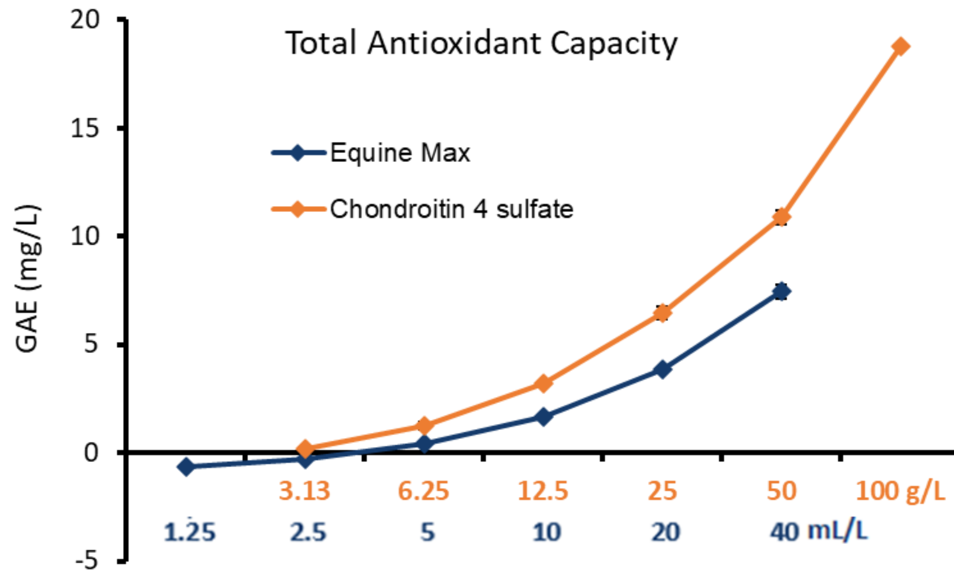


Figure 1. Total antioxidant capacity of Equine Max and Chondroitin-4-sulfate. The colorimetric readings are used to plot the results onto the gallic acid standard curve and calculate the Gallic Acid Equivalents (GAE). Each dose represents the amount of chondroitin sulfate at that dose for that product. The data is shown as the average ± standard deviation of duplicate data points for each dose.

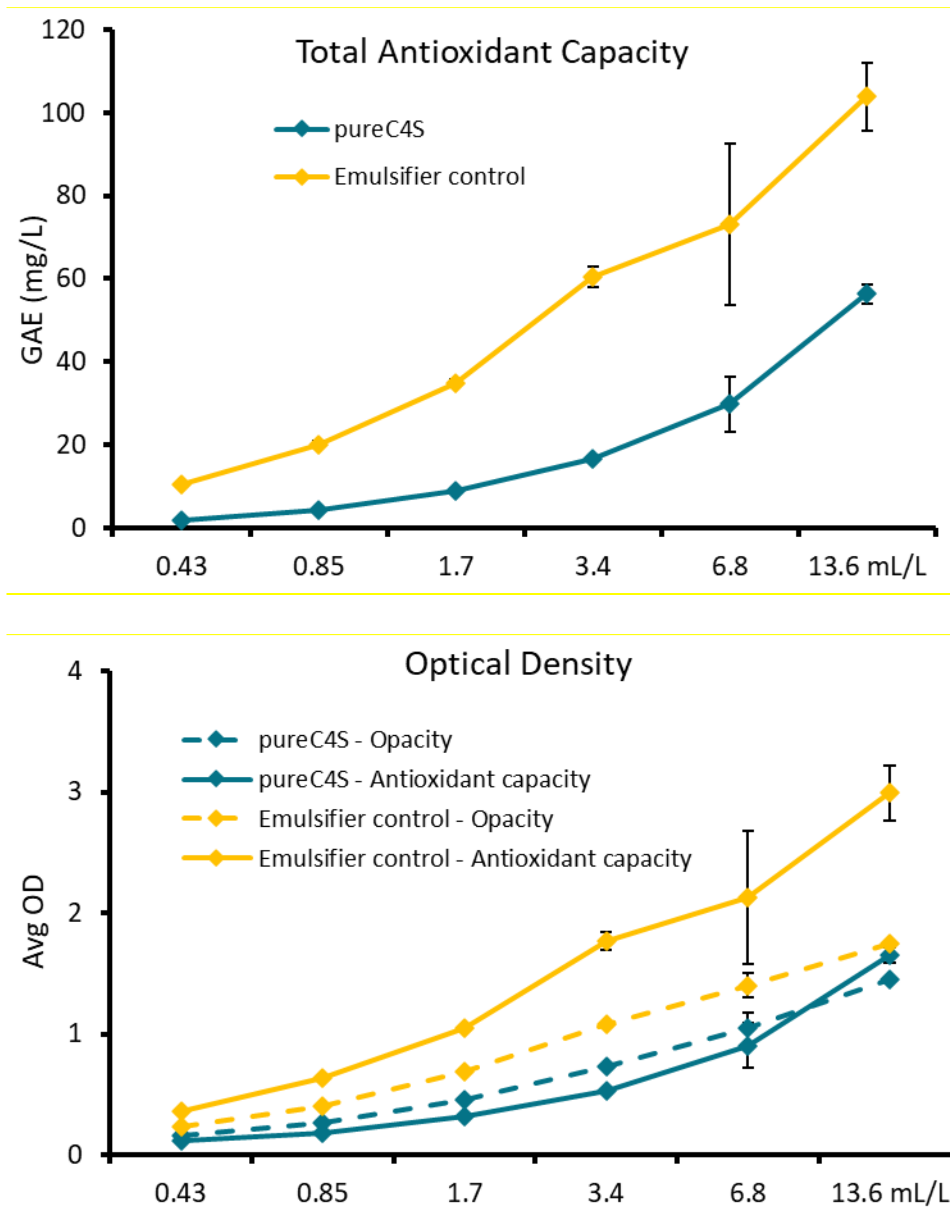


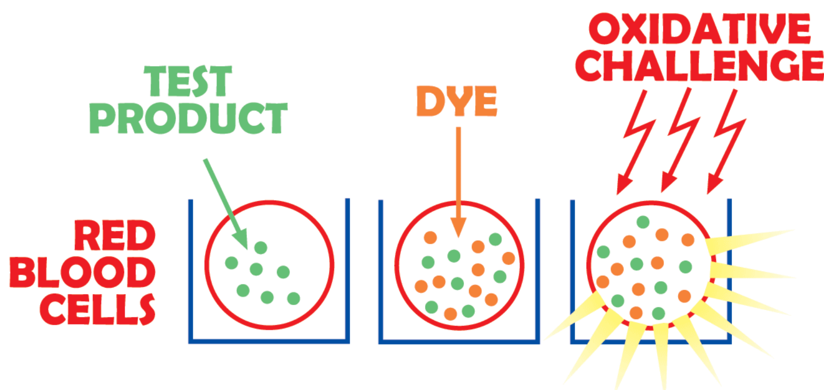
Figure 2. Total antioxidant capacity of pureC4S and Emulsifier control. ***Top:*** *The colorimetric readings are used to plot the results onto the gallic acid standard curve and calculate the Gallic Acid Equivalents (GAE).* ***Bottom:*** *The colorimetric readings for each test product are shown for the colorimetric development in the Total Antioxidant Capacity assay and for the inherent opacity of the emulsifier and the emulsified pureC4S (dashed lines).* ***Both graphs:*** *The data is shown as the average ± standard deviation of duplicate data points for each dose.*

5.2 Cell-based Antioxidant Protection assay.

The rationale[i] behind the method that we use is important: It allows assessment of anti-oxidant 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.[ii] The method has been used on multiple types of natural products and ingredients, published in the peer-reviewed scientific literature.[iii iv v vi vii viii]

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). We developed the red blood cell-based assay particularly to be able to assess antioxidants from complex natural products in a cell-based system, as well as help interpret subsequent data from more complex cellular models.



Human RBC are 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:

- The 2 products chondroitin-4-sulfate and Equine Max were tested at full strength in the CAP-e assay, after first documenting that these doses did not cause stress to the cellular integrity in the assay.
    - At the highest dose tested, there was a very mild protection of the cells from oxidative stress, suggesting that some of the chondroitin-4-sulfate is present in a simple and small enough form to enter into the cells and protect the cells from oxidative damage from the inside-out.
- The emulsified pureC4S and the emulsifier control were tested at slightly reduced doses after an initial test that documented the higher doses of the emulsifier caused stress to the cell membranes, an expected occurrence since cell membranes are composed of lipids. At the same time it was important for this initial test to not dilute the products so much that no effect would be seen – therefore we did expect some cellular stress at higher doses.
    - At higher doses the emulsified product and the emulsifier control triggered cellular stress and lysis.
    - At the lower doses the products were too dilute and potential cellular antioxidant protection was below levels of detection.

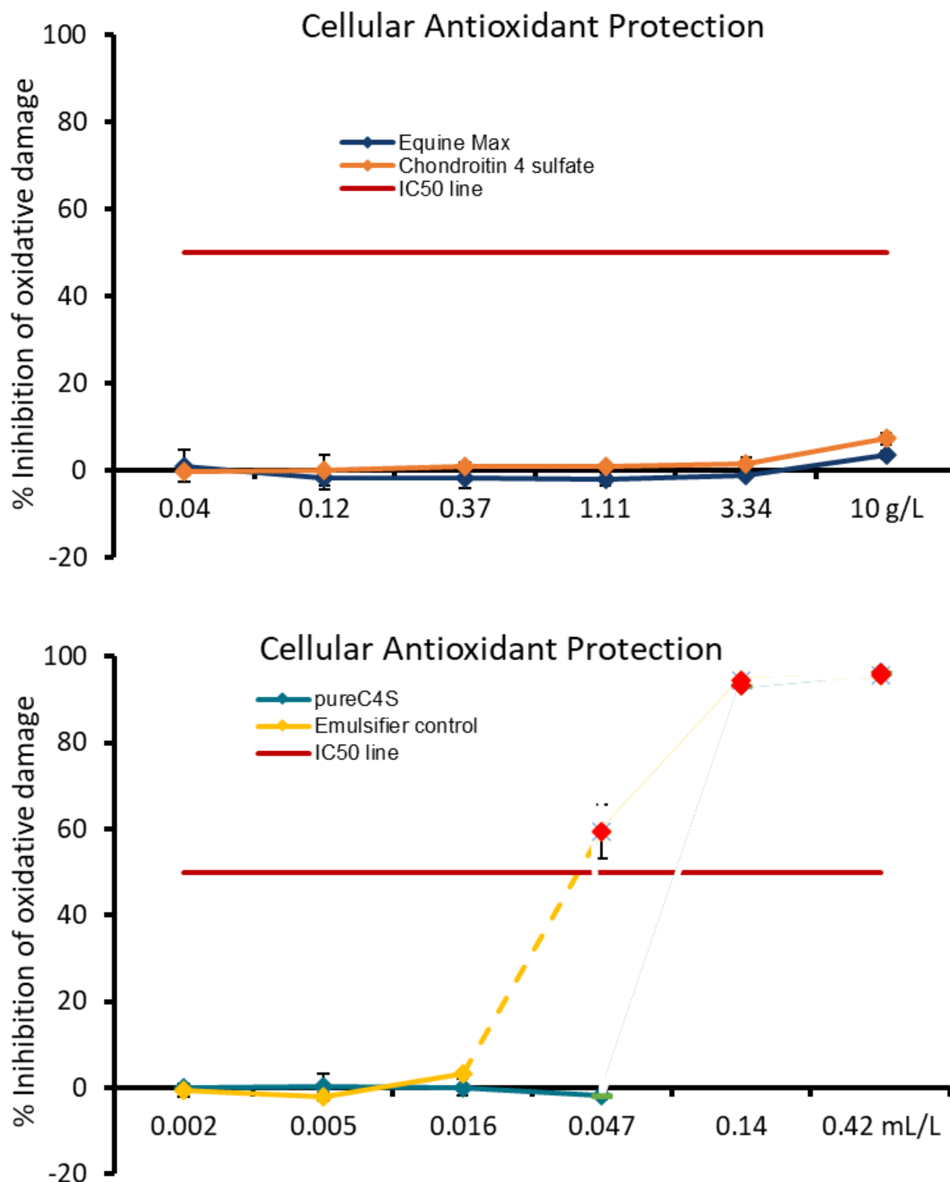


Figure 3. Cellular antioxidant protection of Equine Max and Chondroitin-4-sulfate (top graph), and pureC4S and Emulsifier control (bottom graph). The percent inhibition of cellular oxidative damage was calculated based on the fluorescence readings for cells treated with the test product, compared to 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. Red data points indicate cell lysing. Cell lysing can happen at higher doses of test products that for various reasons are not well tolerated by the live cells. Lysing can be caused by unfavorable pH, salt concentration and other factors.

6 Further work

The results reported here are of foundational value and is typically part of the ***Strategic Research Plan*** when we initiate work on novel compounds and natural extracts. Other parts of the foundational testing in bioassays include:

- Effects on cellular viability and metabolic health;
- Effects on free radical formation in human inflammatory cells;
- Immune activation, natural killer and T cell activation, and production of cytokines and growth factors involved in immune activation, anti-inflammatory effects, and tissue regeneration and reparative functions.

Further preclinical testing may include effects on immune defense mechanisms and inflammation regulation.

Additional work may be conducted in fibroblast cell cultures under oxidative stress and inflammatory conditions.

7 References

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These statements have not been evaluated by the Food and Drug Administration. These products are not intended to diagnose, treat, cure or prevent any disease.