Why antioxidant therapies have failed in clinical trials

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Abstract

In spite of considerable research, and many clinical trials involving thousands of patients, there is a conspicuous lack of antioxidant therapies available, Steinhubl (2008). In this short note we utilize highly reductive stochastic models, a stochastic model and a discrete Markov chain model, to analyze the generation of free radicals and their subsequent neutralization. An advantage of these models is that they incorporate values for the number of molecules present per unit volume, and with the Markov chain model, the relative dimensions of these molecules in contrast to ordinary differential equations. By means of thiese models we question the basis of antioxidant therapies based trapping or scavenging of reactive species. We demonstrate the extraordinary capacity of the enzymatic antioxidant defenses relative to non-enzymatic defenses. We conclude that, if the concentration of an non-enzymatic antioxidant is too low there is little chance of collision and interaction with a free radical species. If the rate of reaction between the free radical and the non-enzymatic antioxidant is below a necessary threshold then the effect of an antioxidant will be dwarfed by the free radical defense systems naturally present. As such we suggest that failure of most antioxidant therapies in clinical trials is to be expected.

Keywords: Antioxidant, Free Radical, Stochastic, Markov Model

1. Introduction

The free radical theory of ageing, Harman (2016). put forward the hypothesis that free radicals, usually reactive oxygen species, generated as a by product of respiration are a causal factor in the process of ageing. This is due to the fact that free radicals are highly reactive and are a cause of continual and cumulative damage to cellular proteins, lipid, and DNA (the soma). This damage, unless repaired, will lead to a progressive deterioration

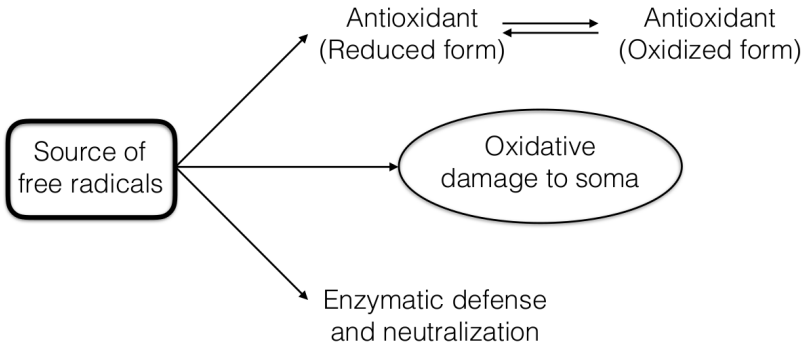

Figure 1: Generalized scheme for the interactions of free radicals. After generation free radical species can be neutralized by scavenging antioxidants, enzymatic defenses, or interact with and damage components of the cell.

of cellular function. Cumulative damage by free radicals has been associated with many diseases, notably age related neurodegenerative conditions such as Alzheimer's. This is in part because the brain has a high metabolic demand and a subsequent high oxygen demand, while being essentially post-mitotic, Cobley et al. (2018).

The process of oxidative damage and the mechanisms to avoid it can be reduced to a number of basic processes. The generation of free radicals and their subsequent neutralization by specific enzymatic means, neutralization by non-enzymatic antioxidants, or damage to the soma. This is shown schematically in Fig 1.

2. Stochastic Model

The initial modeling software used was Kinetiscope from Columbia Hill Technical Consulting[1]. Kinetiscope is based on stochastic algorithms, Gillespie (2016), instead of integration of differential equations.

The models were developed by imagining a highly simplified organelle, much like a mitochondrion. The parameters for the number and size of molecules in the cell were taken from "By the numbers" website[2] and associated publications, Milo and Phillips (2015). A typical human cell is

[1]http://www.hinsberg.net/kinetiscope/license.html
[2]http://bionumbers.hms.harvard.edu/

approximatly $4,000um^3$, and mitochondria comprise about one fifth of the volume of a cell or about $400um^3$. For input into the models we defined the contents of this organelle as 1×10^9 protein molecules, 1×10^{10} molecules of water, and 1×10^8 molecules of a non-enzymatic antioxidant (NEA). Ascorbic acid is present in millimolar concentration (cite begsten), though the exact intracellular concentration is not well defined. Of the protein present 3×10^7 molecules (3%) are an enzymatic antioxidant (EA). Superoxide dismutase comprises up to 3 percent of mamallian cellular protein (1-3% from PaxDb: Protein Abundance Database[3]) For interaction with the free radical the initial rate constant assumed for the non-enzymatic antioxidant was based on the reaction between glutathione and superoxide, $200M^{-1}s^{-1}$ Winterbourn (2016), or ascorbic acid and superoxide $3 \times 10^5 M^{-1}s^{-1}$ Buettner and Jurkiewicz (1996). The rate of neutralization of the free radical species by enzymatic means was based on the intearction of superoxide dismutase and superoxide and was defined as $1 \times 10^9 M^{-1}s^{-1}$. Superoxide dismutase is a special case and operates far faster than most enzymes, and may be as fast as it is theoratically possible Milgrom (2016). The rate for damage to the soma (all the components of the cell) was defined at $300M^{-1}s^{-1}$ based of the rate of superoxide interaction with methionine protein residues, Davies (2016). An arbitrary rate of free radical generation of $0.000001s^{-1}$ was selected to give a measurable rate of protein oxidation in a reasonable time span. The actual rate of generation of free radicals, specifically superoxide, by mitochondria has not been accurately determined Murphy (2009). This is in part due to the high catalytic rate of enzymes such as superoxide dismutase and catalase which maintain a low steady state level of superoxide and hydrogen peroxide.

In the absence of enzymatic antioxidants or non-enzymatic antioxidants the rate of damage to the protein was, as would be expected, linear and proportional to the rate of free raical generation (not shown). Enzymes involved in the defense against free radical damage such as superoxide dismutase and catalase have extremely high catalytic rates, and are some of the most abundant proteins present in the cell (1-3% from PaxDb: Protein Abundance Database[4]). If the concentration of the enzymatic antioxidant is set to 3% of the total protein and the kcat/Km is varied it can be seen (see Fig 2) that in order to reduce the rate of damage to the soma (protein in this model)

[3]https://pax-db.org
[4]https://pax-db.org

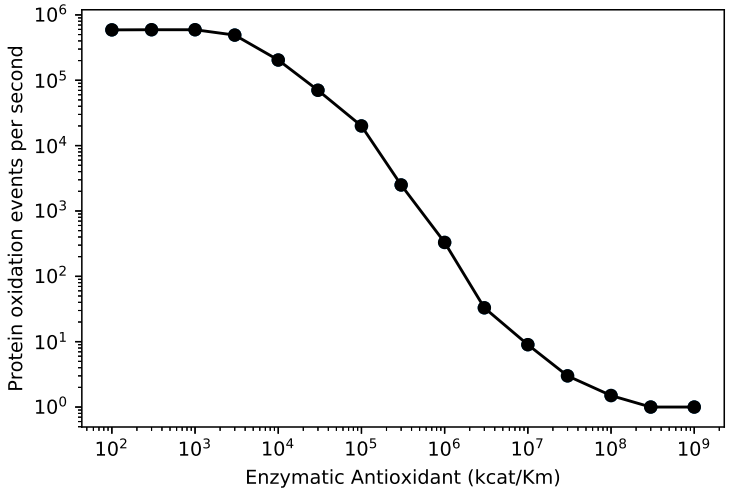

Figure 2: A. The abundance of the enzymatic antioxidant can be varied, as can be seen to be effective even with a kcat/Km of $1 \times 10^9 M^{-1}s^{-1}$ the number of molecules of the enzyme needs to be in excess of 1e8.

to a negligible level a kcat/Km of $3 \times 10^8 M^{-1}s^{-1}$ to $1 \times 10^9 M^{-1}s^{-1}$ is required. Coincidently this is close to the observed kcat/Km of enzymes such as catalase and superoxide dismutase, Milgrom (2016). Conversely if we define the kcat/Km of the enzymatic antioxidant as $1 \times 10^9 M^{-1}s^{-1}$ and vary the concentration of the enzymatic antioxidant, the plateau concentration at which there is negligible oxidative damage to the soma is at 0.3-1% of the total protein which is reasonably close to the concentration of enzymes such as catalase and superoxide dismutase at 1-3% (Fig 3).

As can be seen, in order to be effective as an antioxidant it is necessary to be present at a relatively high concentration and to have a high forward rate constant. If a non-enzymatic antioxidant is added to the system at a high concentration (1×10^8 molecules per cell) with a high kcat ($3 \times 10^5 M^{-1}s^{-1}$) in the absence of any enzymatic antioxidant it can be seen that it can function as an antioxidant, for a short while (see Fig 4). However, in the absence of a mechanism for the efficient regeneration of the reduced form from the oxidized form the antioxidant is rapidly depleted.

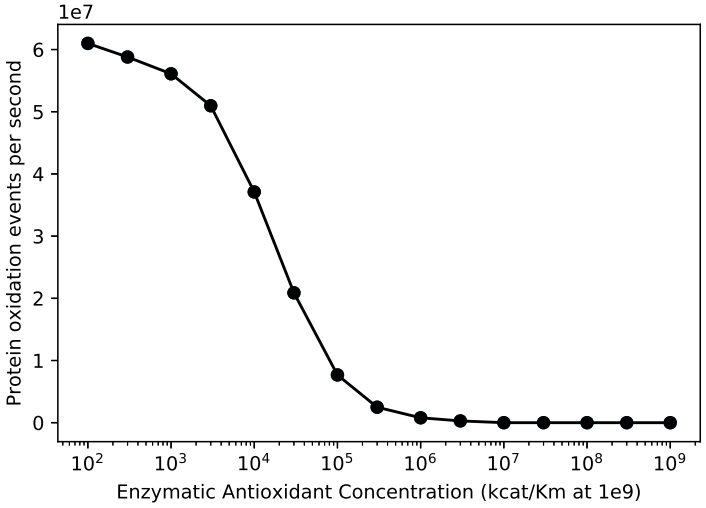

Figure 3: B. The assigned rate constant for the reaction of enzymatic antioxidant with the free radical species can be varied. If the number of enzyme molecules is defined as 1×10^8, then a kcat/Km of greater than $1 \times 10^6 M^{-1} s^{-1}$ is required to eliminate oxidative damage. For reference the kcat/Km of superoxide dismutase is in fact greater than $1 \times 10^8 M^{-1} s^{-1}$

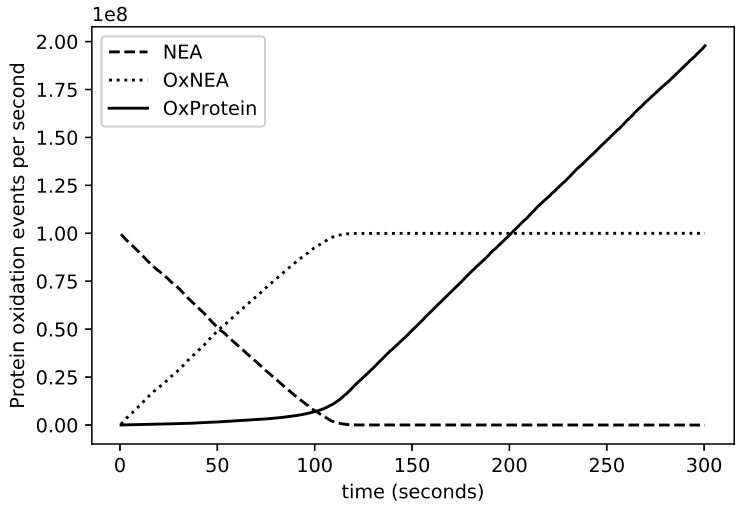

Figure 4: Rapid depletion of a non-regenerating non-enzymatic antioxidant, with accumulation of the oxidized form. When the store of the non-enzymatic antioxidant is exhausted there is rapid oxidation of the cellular proteins.

3. Markov Model

In this section we describe a discrete time Markov chain to model the trajectory of a free radical within a cell. The cell in this model is comprised of water molecules, protein molecules, enzymatic antioxidants, and non-enzymatic antioxidants. We further assume that there is a steady rate of free radical production. During each time interval a free radical encounters either a water molecule, a protein, an enzymatic antioxidant, or a non-enzymatic antioxidant. We consider the trajectory of a free radical within a cell and its likelihood of an encounter with each of the above components. These likelihoods are governed by the relative abundance of each component in the cell and their relative dimensions.

An encounter with a water molecule (which we call state w) has no effect on the free radical and it continues on its trajectory into the next time interval (and, consequently, the next state). The probability of an encounter with a water molecule $p(w)$ is derived from the ratio of water and the total amount of matter in the cell, thus:

$$p(w) = \frac{m_w v_w}{M_{total}} \tag{1}$$

We compute the total matter M_{total} in the cell as:

$$M_{total} = m_w v_w + m_{prot} v_{prot} + m_{ea} v_{ea} + m_{nea} v_{nea} \tag{2}$$

where m_w, m_{prot}, m_{ea} and m_{nea} are the number of molecules of water, protein, enzymatic antioxidant and non-enzymatic antioxidant respectively. Likewise, v_w, v_{prot}, v_{ea} and v_{nea} are the respective volumes for water, protein, enzymatic antioxidant, and non-enzymatic antioxidant.

Similarly, the probability of an encounter with a protein is derived from the ratio of the amount of protein and the total amount of matter in the cell (M_{total}). However, the effect of an encounter with a protein molecule can yield two different results. Either the free radical reacts with the protein or it does not. If the free radical reacts with the protein, then it is neutralised and its trajectory is terminated. We refer to this state as $prot_a$ and it is an *absorbing* state. If the outcome of the encounter is no reaction, then we have a *non-absorbing* state $prot_{na}$. For state $prot_{na}$, the free radical continues on its trajectory into the next time interval. Consequently, we have two probabilities for each of the states, namely, $p(prot_a)$ and $p(prot_{na})$. The probability $p(prot_a)$ is given by:

$$p(prot_a) = \left(1 - \frac{1}{k_{prot}}\right) \times \frac{m_{prot} v_{prot}}{M_{total}} \tag{3}$$

The probability $p(prot_{na})$ is given by:

$$p(prot_{na}) = \frac{1}{k_{prot}} \times \frac{m_{prot} v_{prot}}{M_{total}} \tag{4}$$

where k_{prot} is the kcat value for the protein (see Table 1).

As with protein molecules, we model an encounter with enzymatic antioxidant as two separate states to reflect the effect on the free radical, that is, the encounter either results in a reaction or no reaction with the enzymatic antioxidant. We use ea_a to represent the state where the free radical reacts with the enzymatic antioxidant. State ea_a is an absorbing state because the trajectory of the free radical is terminated during the current time interval. Conversely, ea_{na}, is a non-absorbing state whereby the free radical does not react with the enzymatic antioxidant and its trajectory continues on into the next time interval. Whether the free radical enters states ea_a or ea_{na} is governed by the relative transition probabilities. The relative transition

probabilities for ea_a and ea_{na} are derived from the kcat value of the enzymatic antioxidant as well as the relative quantity within the cell, thus, the probability $p(ea_a)$ is given by:

$$p(ea_a) = \left(1 - \frac{1}{k_{ea}}\right) \times \frac{m_{ea} v_{ea}}{M_{total}} \tag{5}$$

Similarly, the probability $p(ea_{na})$ is given by:

$$p(ea_{na}) = \frac{1}{k_{ea}} \times \frac{m_{ea_{na}} v_{ea_{na}}}{M_{total}} \tag{6}$$

where the kcat value for the enzymatic antioxidant is k_{ea} (Table 1).

As with an enzymatic antioxidant, a non-enzymatic antioxidant has a reaction state (nea_a) and a non-reaction state nea_{na} which are absorbing and non-absorbing, respectively. Functionally there is no difference between an enzymatic antioxidant or non-enzymatic antioxidant with rapid regeneration, the main factor is the processing time, which can be viewed as the equivalent of the forward rate constant. Based on the published rates of free radicals interaction with superoxide dismutase, catalase, glutathione, ascorbic acid, and protein it is evident that the rates are $k_{ea} \gg k_{nea} \gg k_{prot}$ where k_{nea} is the kcat value for non-enzymatic antioxidant (see Table 1). In each case, the rates of interaction and processing differ by orders of magnitude. The probability $p(nea_a)$ is given by:

$$p(nea_a) = \left(1 - \frac{1}{k_{nea}}\right) \times \frac{m_{nea_a} v_{nea_a}}{M_{total}} \tag{7}$$

The probability $p(nea_{na})$ is given by:

$$p(nea_{na}) = \frac{1}{k_{nea}} \times \frac{m_{nea_{na}} v_{nea_{na}}}{M_{total}} \tag{8}$$

Table 1 shows the values used to compute quantities of material in the cell along with their corresponding kcat values (where applicable). These values are used to derive transition probabilities for the transition matrix $\mathbf{T}$ of the Markov model:

	No. molecules	Vol. molecules nm^3	kcat $M^{-1}s^{-1}$
Water	1.3×10^{13}	0.01	NA
Protein	1.0×10^{9}	65.40	300
Enzymatic antioxidant	3.0×10^{7}	65.40	1×10^{9}
Non-enzymatic antioxidant	1.0×10^{8}	1.00	1×10^{5}

Table 1: Assigned values for the abundance, dimensions, and rate constants in the model

$$\mathbf{T} = \begin{bmatrix} p(w) & p(prot_a) & p(prot_{na}) & p(ea_a) & p(ea_{na}) & p(nea_a) & p(nea_{na}) \\ 0 & 1 & 0 & 0 & 0 & 0 & 0 \\ p(w) & p(prot_a) & p(prot_{na}) & p(ea_a) & p(ea_{na}) & p(nea_a) & p(nea_{na}) \\ 0 & 0 & 0 & 1 & 0 & 0 & 0 \\ p(w) & p(prot_a) & p(prot_{na}) & p(ea_a) & p(ea_{na}) & p(nea_a) & p(nea_{na}) \\ 0 & 0 & 0 & 0 & 0 & 1 & 0 \\ p(w) & p(prot_a) & p(prot_{na}) & p(ea_a) & p(ea_{na}) & p(nea_a) & p(nea_{na}) \end{bmatrix} \tag{9}$$

The probability of the system being in state i at time k is π_i^k.

$$\pi = \pi \mathbf{T} \tag{10}$$

where $\lim_{k\to\infty} \pi = \pi^{(k)}$. Without any loss of generality, we assume that the initial state of system is an encounter with water, thus, the initial state is:

$$\pi^0 = \begin{bmatrix} 1 & 0 & 0 & 0 & 0 & 0 & 0 \end{bmatrix} \tag{11}$$

While mechanistically different, the rate of neutralization of free radical species by enzymatic antioxidants is far greater than the rate of neutralization by non-enzymatic antioxidants, which in turn occurs at a much faster rate than damage to proteins by the free radical specise, that is, $k_{ea} \gg k_{nea} \gg k_{prot}$. Furthermore, these rates cover several orders of magnitude. However, for visualization purposes, these were set at lower ratios. Under these conditions it can be seen that the non-enzymatic antioxidant has little effect (Fig 5). If the forward rate constants assigned for the enzymatic antioxidant and non-enzymatic antioxidant are at more realistic values the effect of the non-enzymatic antioxidant is undetectable. Molecules with

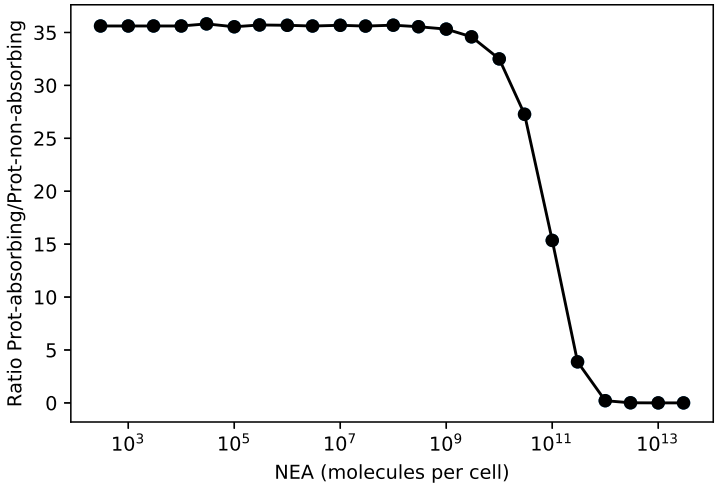

Figure 5: In the presence of an enzymatic antioxidant at 1×10^8 molecules per cell, with a kcat/Km of $1 \times 10^8 M^{-1}s^{-1}$ then to be an effective a non-enzymatic antioxidant with an assigned kcat of $3.0 \times 10^7 M^{-1}s^{-1}$ needs to be present at a high concentration, considerably in excess of a billion molecules per cell.

a size of the order of molecules such as glutathione and ascorbic acid are relatively small compared to cell components such as protein. To be effective physical interaction between the free radical and the putative antioxidant is necessary, this interaction is limited by physical size and numerical presence.

4. Conclusions

These models are highly simplified and reductive, however, they are not intended to mimic or replicate an actual biological system. The purpose was to explore the parameters that most influence the potential interaction and termination of a free radical species. The models used employ different approaches, stochastic and probabilistic, to investigate the interaction of free radicals and anti-oxidant defenses, they are however, are in qualitative agreement. They illustrate that enzymes involved in defense against oxidative damage, such as superoxide dismutase and catalase are already extremely effective. At the concentrations seen *in vivo* enzymatic antioxidants such as superoxide dismutase or catalase will essentially eliminate oxidative damage.

This is not surprising, to achieve the catalytic rates observed, enzymes such as superoxide dismutase and catalase must have been subject to strong selective pressure and as such are probably optimal in terms of catalytic rate and concentration. Non-enzymatic antioxidants such as glutathione or ascorbic acid have raltively modest rates of reaction with superoxide, in spite of the presumably identical selective pressures that acted on the enzymatic antioxidants. Based on these results we would infer that defence against free radicals is not a major role for these compounds. The results also permit a number of conclusions to be made concerning the role of therapeutic non-enzymatic antioxidants in the prevention of oxidative damage to the soma.

1. To be effective, a non-enzymatic antioxidant that operates by trapping or scavenging free radicals, needs to be present at a sufficiently high concentration to give a reasonable chance of interaction between the antioxidant, rather than with other components of the cell.
2. A potential free radical scavenging agent would also need to have a sufficiently high rate constant to effectively compete with the enzymatic defence system. Unless the rate of reaction is of the order of $1 \times 10^6 M^{-1} s^{-1}$ or greater, they will have little effect on the rate of protein oxidation.
3. There also needs to be an effective and efficient means of regenerating the reduced form of the antioxidant from the oxidized form. Without a mechanism to regenerate the reduced form of an antioxidant from the oxidized form an exogenous antioxidant would be rapidly depleted. For a synthetic or non- endogenous compound this is unlikely a mechansim for this will be present.

The models used are limited in the number of parameters considered and assume that the reactions take place in an aqueous milieu. Non-enzymatic antioxidants may have utility in a lipid membrane environment as damage by propogation by means of a chain reaction is possible. Nevertheless, within the scope of these models we suggest that there is limited therapeutic potential for non-enzymatic antioxidants that function by trapping or scavenging free radical species, and that the failure of many putative antioxidant compounds to demonstrate a therapeutic effect is to be expected.