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RESEARCH ARTICLE

The Redox Stress Test: A novel technique reveals oxidative stress in Parkinson's disease

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ABSTRACT

A novel Redox Stress Test has been developed to identify symptoms of diseases associated with oxidative stress by observing symptom changes induced by short-term activation of the transcription factor Nrf2, to restore redox homeostasis. The Nrf2 pathway is triggered by a herbal preparation, Broccoli Seed Tea, developed to deliver a therapeutic dose of highly bioavailable sulforaphane, a potent activator of Nrf2. We discuss the rationale behind the tea and describe the methods used to optimise the bioavailability of sulforaphane to match the pharmacodynamics of Nrf2 activation. When consumed by people with Parkinson's disease, the Redox Stress Test induced powerful and concurrent attenuation of a diverse group of Parkinson's non-motor symptoms, including fatigue, constipation and urinary urgency. Motor symptoms were strictly unaffected. This observation indicates that oxidative stress may be a common factor contributing to non-motor symptoms involving sites in the CNS and peripheral organs. We tentatively interpret the results in terms of a hypothetical model for Parkinson's Syndrome which we describe as a multisystem redox disorder with reservoirs of the disease in peripheral organs as well as in the brain. Eliminating the disease in peripheral organs is therefore a prerequisite to stopping disease progression in the brain. According to this model, the redox disorder in the brain provokes progressive neurological damage, which is not recognised as such in the early years. More specific neurological symptoms only come to light many years later, when damage to dopaminergic neurons creates a dopamine deficiency which eventually exceeds the threshold required for normal motor control, generating a new coherent group of neurological symptoms which define movement disorder. Given the apparent ease with which oxidative stress can be quenched in several locations simultaneously, we briefly discuss possible implications for public health, medical research, patients and patient advocacy groups. We note that the Redox Stress Test may have the potential to explore symptoms of other diseases where oxidative stress is believed to play a major role, although this remains subject to validation by further research.

Introduction

We report on the development and first results of a novel technique, the Redox Stress Test (RST), designed to identify symptoms associated with oxidative stress. The test is based on a special Broccoli Seed Tea (BST), containing a highly bioavailable form of sulforaphane, a potent activator of the transcription factor Nrf2 (Nuclear factor (erythroid-derived 2)-like 2). We expose the rationale behind the test and describe the methods used to optimise the sulforaphane content to facilitate consistent activation of Nrf2. A critical factor regulating Nrf2 activation by sulforaphane is its bioavailability at the point of ingestion. Many aspects of the preparation and delivery of the RST are determined by this factor.

The Redox Stress Test induced a powerful and concurrent attenuation of a diverse and seemingly unrelated array of non-motor symptoms in people suffering from Parkinson's disease. In contrast, motor symptoms were strictly unaffected. These results indicate that oxidative stress may be a common factor contributing to this symptom group, but not to motor symptoms. There is considerable evidence that oxidative stress plays a major role in Parkinson's disease,[1–4] but how this translates into symptoms is not well understood. The RST distinguished between symptoms of Parkinson's disease caused by oxidative stress from those caused by dopamine depletion. Among the symptoms attenuated, two are located in peripheral organs, indicating that oxidative stress occurring outside of the brain may contribute to Parkinson's disease.

These results imply that our current understanding of Parkinson's disease may be in question. We tentatively interpret them in terms of a hypothetical model of multisystem redox disorder affecting peripheral organs as well as the brain, that we call "Parkinson's Syndrome". According to this model, redox disorder in the brain provokes slow, but progressive neurological damage which goes largely unrecognized for years. Neurological symptoms only come to light when the scale of neurodegeneration exceeds a threshold for normal brain function. Potential implications for further research and public health, focusing on eliminating oxidative stress as the common cause of non-motor symptoms are also briefly discussed.

The origin of the technique

The idea for the RST emerged from a pilot study which indicated that several non-motor symptoms associated with Parkinson's disease were attenuated after drinking BST containing sulforaphane, whereas motor symptoms were unaffected. [5]Sulforaphane is a potent activator of the transcription factor Nrf2 (Nuclear factor (erythroid-derived 2)-like 2) and can be synthesized from its precursor, glucoraphanin, present in the seeds of broccoli (*Brassica oleracea var. Italica*). We hypothesized that if the symptoms attenuated by BST were the manifestation of oxidative stress, then it might be possible to develop a technique to distinguish symptoms associated with Redox disorder from symptoms due to other causes. The existing version of BST was not optimized for this purpose, so a program of standardization, optimization and control was initiated to match the pharmacokinetics of delivery of sulforaphane to the pharmacodynamics of the target mechanism. The most important information to emerge from this study was that all potential Nrf2 activators (sulforaphane and other isothiocyanates) should be present in a state which ensures their highest degree of bioavailability at the moment of ingestion. Consumption of the precursors, glucoraphanin and myrosinase enzyme, which deliver sulforaphane much more slowly and with lower bioavailability, is incompatible with the objective to consistently and reliably activate Nrf2.

Oxidative stress

Although the term oxidative stress is widely used, it remains a vague concept, considered to be harmful, yet poorly understood by medical professionals and patients. In current language, stress refers to passive tension induced by a threatening situation. Oxidative stress, on the other hand, is caused by extremely aggressive chemicals which attack and damage cells and organelles within them. Oxidative stress is the biological equivalent of corrosion, where the corrosive agents are generated inside the cells. It occurs due to an imbalance between the production and elimination of aggressive oxidising free radicals, grouped under the names: Reactive Oxygen Species, (ROS) and Reactive Nitrogen Species (RNS).[6–8] If not neutralised rapidly (<1microsecond) by deoxidizing enzymes, ROS and RNS oxidize cellular and mitochondrial DNA, cellular organelles and membranes, causing widespread damage, leading eventually to cell death.[9,10] Oxidative stress may be caused by exposure to toxic substances, pesticides[11–13] and radiation,[14] but its major source is endogenous, due to electron leakage from the Electron Transport Chain (ETC)[15–18] during energy production by oxidative phosphorylation in mitochondria. Cells with high energy consumption such as those in the CNS,[9,10,15,19] the cardiovascular system,[20–23] the gastrointestinal system,[24–31] the urinary system[32–37] and the skin[14,38,39] are most vulnerable to oxidative stress. Mitochondria are both a source and a target

of oxidative stress.[10,40–45] When redox homeostasis is not fully restored inside mitochondria (see next section), damage to mitochondrial DNA, lipid membranes and elements of the ETC[15,46–48] creates a vicious circle and an increasingly oxidazing environment that ultimately leads to their failure, energy shortages and cell death.

The NRF2 Pathway

a) Nrf2, the master regulator of redox homeostasis

The Nrf2/Keap1/ARE pathway is a mechanism which has evolved to protect cells from the most damaging aspects of oxidative stress. It is a chain reaction involving 3 major players (described below), working together to detect and neutralise the free radicals causing oxidative stress.[49–53] With increasing age, the activity of the Nrf2 pathway declines allowing oxidative stress to develop with greater frequency or intensity.[50]

Nrf2 (Nuclear factor (erythroid-derived 2)-like 2) are signalling proteins (transcription factors) whose ultimate function is to promote the transcription (synthesis) of antioxidants, antioxidant enzymes and anti-inflammatory cytokines which act to stop or prevent damage caused by oxidative stress. The Nrf2 protein is constantly synthesized, degraded and recycled in the cytosol of cells.[8,45,52] However, the transcription of cytoprotective enzymes and cytokines only occurs when high levels of ROS are detected (Fig.1).

Keap1 (Kelch-like ECH-associated protein 1) are proteins which form dimers to create a barrier which regulates the life-cycle of Nrf2:

(i) In the absence of high levels of ROS or electrophiles, the Keap1 dimers sequester almost all Nrf2 protein and program its degradation and recycling.[54–56]

(ii) Keap1 also monitors the redox state in the cytosol via 27 cysteine sensors that bind oxidants and toxins.[57–59]

(iii) Keap1 regulates the flow of Nrf2 to the nucleus according to the state of oxidation of its cysteine sensors and is the ultimate controlling element in the Nrf2 pathway.

(iv) The most important cysteine sensor is C151.[59] Oxidants, toxins and electrophiles, including sulforaphane[56] bind to C151 which is the target for the Redox Stress Test.

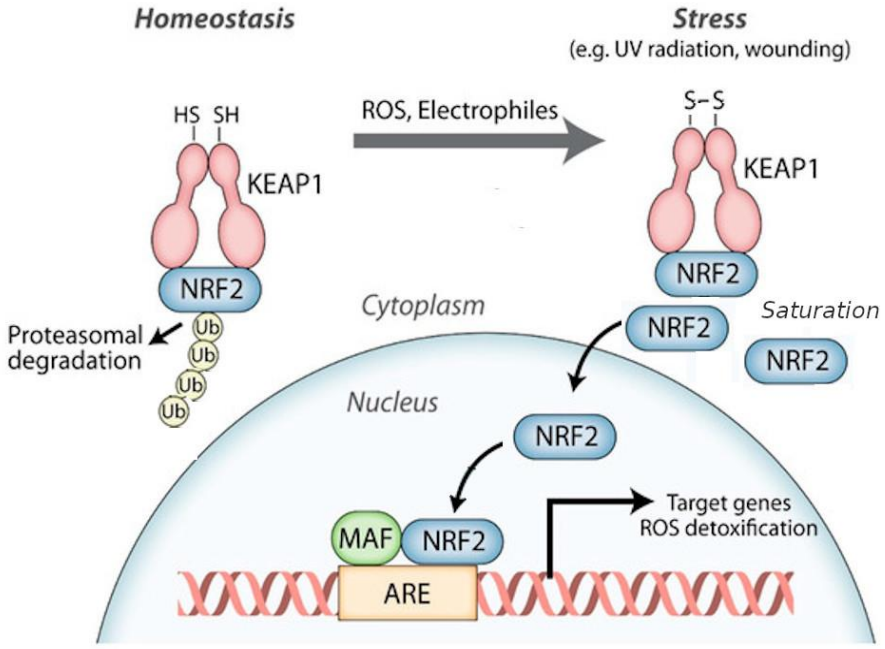

Fig. 1, The Nrf2 pathway. *When oxidative stress is low (homeostasis), Keap1 protein dimers act as gatekeepers, sequestering free Nrf2 protein and degrading it, thus preventing transcription (left). When ROS levels rise, they bind to cysteine sensors of Keap1 which then undergo a conformational change that deactivates the degradation of Nrf2, whist keeping it bound to Keap1. Newly synthesized Nrf2 is then free to migrate to the nucleus where it forms a hetero-dimer with MAF (small musculoaponeurotic fibrosarcoma) proteins60 and binds to ARE gene promoter sequences. This promotes the transcription of cytoprotective genes which express antioxidant molecules and enzymes and anti-inflammatory cytokines that protect cells from oxidative stress and inflammation. Sulforaphane acts as an electrophile (reversible oxidant), temporarily inactivating Keap1. At high concentrations, sulforaphane saturates Keap1, which provokes mass migration of Nrf2 to the nucleus and transcription of cytoprotective genes. (Illustration from P. Hiebert and S Werner.)*

ARE (Antioxidant Response Elements) are sequences in promoter regions of DNA.[40] Nrf2 forms a heterodimer with sMAF (small musculoaponeurotic fibrosarcoma)[60] which then binds to ARE and activates the transcription of up to 300 genes which express antioxidant and detoxifying enzymes, anti-inflammatory cytokines and simultaneously suppresses inflammatory cytokines. Cytokines are small messenger proteins that generate or suppress inflammation. Many of the antioxidant enzymes expressed by this process have relatively long half-lives and provide protection against ROS over periods lasting from many hours to a few days.[61]

(b) Nrf2 activators: So-called Nrf2 activators are in fact Keap1 inhibitors, most of which bind to the C151 sensor of Keap1.[55,57] Sulforaphane binds to C151 by reversible covalent bonding of the reactive cysteine sulfhydryl group to the central carbon of the isothiocyanate group, effectively oxidizing the sensor[62] (Fig. 2). This change of electrical charge induces a conformational change in Keap1 that deactivates the degradation of Nrf2[52,53,56,58].

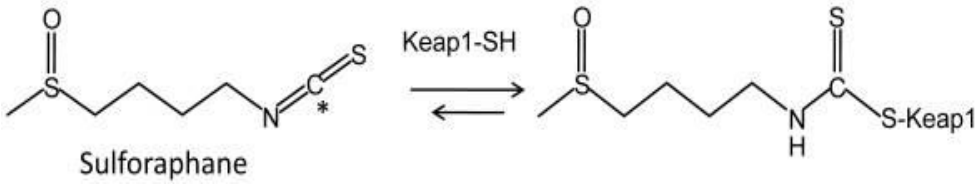

Fig. 2: Sulforaphane binding to Keap1 sensors. *The electrophilic carbon of the isothiocyanate group of sulforaphane binds covalently to the reactive sulfhydryl group in a cysteine sensor of Keap1. Ref. Hu et al. 2011.*

All isothiocyanates present in the broccoli tea including those generated by the white mustard seed powder, are electrophiles capable of binding to C151 and contributing to the overall activity of the tea. The sulfhydryl group (R-S-H) of C151 is located in a shallow trough surrounded by 5 basic amino acids, creating a polarised docking site with a high affinity for electrophiles.[57,59]

c) Nrf2 activity is regulated by a simple floodgate mechanism

When the sensors of Keap1 detect high levels of ROS, toxins or electrophiles, the capacity of the Keap1 barrier to capture Nrf2 is reduced as the concentration of oxidants and electrophiles increases. However, the Keap1 barrier does not micro-manage the release of Nrf2. On the contrary, it operates via a simple floodgate mechanism[54,63–66] whereby newly synthesized Nrf2 overflows the Keap1 barrier when the latter is diminished by the action of ROS. The overflow point corresponds to a threshold where the overall capacity of active Keap1 to bind and degrade Nrf2 matches the inward flow of newly synthesised Nrf2. This mechanism is of critical importance when considering how to control oxidative stress by activating Nrf2. Inactivation of Keap1 beyond the critical threshold requires a corresponding activator concentration in blood vessels supplying the affected cells. It implies that activation of Nrf2 will be systematically triggered only when the serum sulforaphane concentration exceeds the threshold value to saturate the Keap1 barrier independently of the dose ingested over a longer period. In practice, this means that all isothiocyanates, including sulforaphane must be present in their free, fully bioavailable form at the point of ingestion in order to generate a high serum peak.

Design of the Redox Stress Test

Two sources of error are commonly present when using herbal products to deliver a bioactive substance:

(i) the variability of the source molecule or its precursors per unit of dry weight of the raw material,[67–69]

(ii) the bioavailability and pharmacokinetics of absorption of the active molecule after ingestion.[69–72]

Both of these are important in this case where the bioactive substance is sulforaphane and the source materials are its precursors. Sulforaphane slowly degrades in contact with humidity. It is therefore not present in significant amounts in any broccoli plant tissues, seeds or seedlings. The precursor molecule, glucoraphanin (GR) and the enzyme myrosinase are stored in separate vacuoles in the plant tissue. When the plant tissue is crushed or chewed, free glucoraphanin comes into contact with myrosinase enzyme which then cleaves the glucose molecule from its sinolate chain. The resulting unstable intermediate then rearranges to form sulforaphane and/or other molecules.

The bioavailability of sulforaphane when ingested as glucoraphanin, with and without myrosinase, has been measured at 35-40% and 5% respectively, with considerable person to person variation,[71,72] whereas when ingested as the free molecule it exceeds 70 %.[70] In addition, the serum concentration of free sulforaphane peaks at about

one hour after ingestion and has a half-life of about 1.8 h, whereas for equivalent doses of the precursors, the serum concentration profile is spread out over 24 hours such that the concentration at any one time remains low. The precursor method of delivery of sulforaphane is therefore incompatible with the objective of obtaining a high peak serum concentration of sulforaphane.

Given the lack of information published on the kinetics of hydrolysis of glucoraphanin to sulforaphane,[73] we initiated a program to collect the kinetic data required to define good conditions to make fully hydrolysed broccoli seed tea.

Materials and Methods

STANDARDISATION AND OPTIMISATION OF THE RST

The preparation and delivery method was optimised and standardized as follows:

- A unique seed batch with a measured GR content of 78 µmol/g, generously donated by Brassica Protection Products, Baltimore, was used to make all preparations. It has recently been independently measured at 84 µmol/g.[74]
- The parameters used to optimize and standardize the RST were derived from data obtained via indirect quantitative analysis of the enzymic hydrolysis of the glucosinolates and from studies of the hydrolysis reaction kinetics using manual pH-stat titration combined with ionic conductivity measurements.
- These measurements were used to establish a preparation protocol designed to ensure at least 95% hydrolysis of glucosinolates in the seed extract to produce fully bioavailable isothiocyanates immediately prior to ingestion.

CONTROL OF THE CONVERSION OF GLUCOSINOLATES TO ISOTHIOCYANATES

The reaction equation for the conversion of glucoraphanin to sulforaphane is shown in Fig. 3. In the final step of this reaction (the Lössen rearrangement), a bivalent acidic sulfate ion is liberated for every monovalent O-SO^{3-} group in the original GR molecule. This release of sulfuric acid enables the process to be followed quantitatively and kinetically by pH-stat titration. The upper branch of the final reaction produces an inactive nitrile. It requires the presence of Fe^{2+} and an epithiospecifier protein, ESP, also present in broccoli seeds.[75] To block this reaction and achieve maximum sulforaphane yield, ESP was thermally deactivated by extracting the glucoraphanin into boiling, demineralised water.[75]

As a visual control, the upper branch produces a bright yellow suspension (possibly due to iron sulphide), whereas the products of the lower branch are colourless. Boiling water extraction also denatures the original myrosinase enzyme in the broccoli seeds. To compensate for this loss, a small quantity of white mustard (*Synapis alba*) seed powder (~10 – 15 % w/w compared to the broccoli powder), was added after extraction to replace the original enzyme.

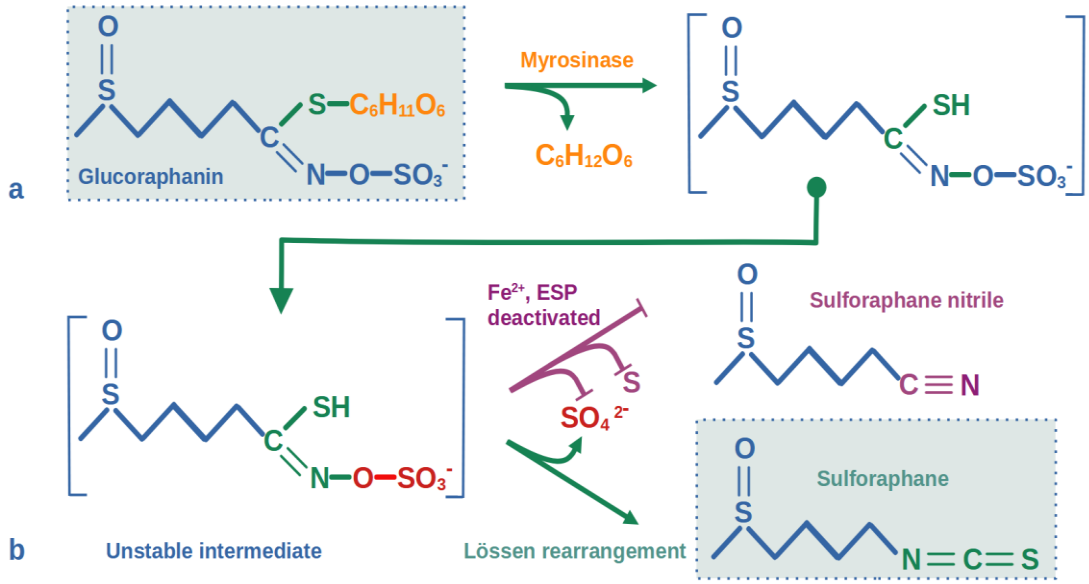

Fig. 3: Reaction equation for the enzymatic conversion of glucoraphanin to sulforaphane. *(a) cleavage of the glucose molecule by the myrosinase enzyme to form an unstable intermediate. (b) Lössen rearrangement of the unstable intermediate to form either sulforaphane nitrile (upper branch, shown blocked in the absence of Fe^{2+} and ESP), or sulforaphane (lower branch,).*

Quantitative acid/base titrations, set to pH = 7.0, were used to calculate the total quantity of glucosinolates in the seed powder. To obtain kinetic data, real-time pH-stat titrations were recorded by time-lapsed video (to avoid operator errors) and used to calibrate conductivity data collected in parallel. Time-recorded conductivity measurements were then used to follow the reaction kinetics. Under conditions where the quantity of enzyme is abundant compared to quantity of glucoraphanin, the reactions follow first order kinetics (Fig. 4, 60°C). With lower enzyme availability, they revert to much slower, zero order kinetics. These reactions are dependent on stirring, dilution and temperature, with an upper temperature limit defined by thermal degradation of the enzyme. At body temperature the reaction time for full conversion exceeds 2 hours. The temperature range for processing was chosen to be marginally below the onset of thermal degradation of myrosinase, where the reaction is both rapid and least sensitive to minor temperature variations.

These studies were used to determine a rapid, practical and reproducible way to ensure maximum enzymatic hydrolysis of isothiocyanates for the seed stock concerned. A protocol was established to enable participants to reliably achieve total (>95 %) conversion of the glucosinolates from 1 g of crushed broccoli seeds, for the preparation of 100 ml of finished BST, with a comfortable margin. The dose consumed was determined as a proportion of the 100 ml prepared in a range from 30 to 50 ml, and the rest was discarded. This standardized version of BST was used in all experiments reported in this article.

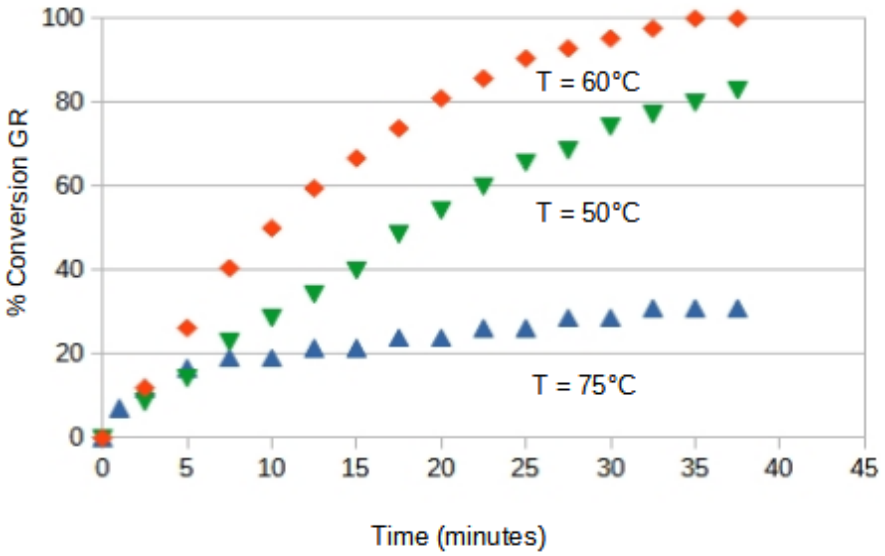

Fig. 4: Kinetics of the hydrolysis of glucosinolates. *% of total hydrolysis calculated from electrical conductivity data calibrated against quantitative pH-stat titration under actual preparation conditions. Note the breakdown of the reaction after a few minutes at 75°C due to thermal degradation of the enzyme*

EXPERIMENTAL DESIGN, SELF-EXPERIMENTATION AND DATA COLLECTION

The study described here was a multiple (n=1) self-experiment carried out by people with Parkinson's with the aim of slowing down their own disease progression. While keeping other medications unchanged, each participant prepared and consumed freshly-brewed BST, initially once per week, with the option of increasing the frequency to twice per week for a period of at least 4 weeks. Participants were requested to record their symptom changes at weekly intervals on a randomised symptom record spreadsheet, adding comments and modifying symptoms to match their own situation. Symptoms were graded on a scale of 0 - 3 as indicated in the table. Data records were anonymised and then selected to include cases with at least 2 well-established non-motor symptoms and 2 well-established motor symptoms of Parkinson's disease at baseline. Individual records were sorted into motor and non-motor symptom groups and the summed to observe the total symptom group changes for a given individual. Table 1 shows a sorted symptom record for a well-documented case.

These results are only relevant to this particular profile of Parkinson's patients and should not be interpreted as applying to a wider Parkinson's population.

ID 415	Baseline	WK1	WK2	WK3	WK4	WK5	WK6
Non-Motor Symptoms							
cramps	2	0	0	0	0	0	0
pain	3	1	1	1	1	1	1
smell	0	0	0	0	0	0	0
urinary urgency	2	0	0	0	0	0	0
nocturia	1	0	0	0	0	0	0
constipation	1	1	1	0	0	0	0
fatigue	0	1	1	0	0	0	0
sleep	0	2	1	1	0	0	0
speech	0	0	0	0	0	0	0
apathy	0	0	0	0	0	0	0
memory	0	0	0	0	0	0	0
brain fog	0	0	0	0	0	0	0
Total non- motor	**9**	**5**	**4**	**2**	**1**	**1**	**1**
Motor Symptoms							
tremors	2	1	1	1	1	1	1
foot drag	1	1	1	1	1	1	1
arm swing	1	1	1	1	1	1	1
freezing	0	1	1	1	1	1	1
stiffness/rigidity	2	2	2	2	2	2	2
bradykinesia	2	1	1	1	1	1	1
dyskinesia	2	2	2	2	2	2	2
soft voice	0	0	1	1	0	0	0
handwriting	1	1	1	1	1	1	1
dystonia	1	2	2	2	2	2	2
Total motor	**12**	**12**	**13**	**13**	**12**	**12**	**12**
Symptom scale	**0 = barely noticeable/absent**				**1 = manageable**		
	2 = severe				**3 = handicapping**		

Table 1. Self-assessed symptom record sheet: Individual record sheet, sorted into motor and non-motor symptoms scores for RST test over 6 weeks. Dosing was 50 ml of standard BST once per week.

All participants were informed of the experimental nature of the broccoli tea and the risks involved (some known, many unknown), within the limits of the collective knowledge of the whole group, none of whom were medical professionals. They each signed a declaration of informed consent and were also asked to discuss the experiment with their physician before taking part. The equipment necessary to prepare the RST was purchased by participants at their own expense. They were then coached in the technique of tea brewing. Several participants provided feedback which enabled the protocol to be improved and simplified. All participants received broccoli seed from the standard stock once they felt comfortable about making the tea. The final responsibility for correct brewing and appropriate dosing remained with the participants themselves. Datasets from participants who acknowledged low compliance with the preparation protocol or irregular dosing, were excluded.

DOSE RANGE AND FREQUENCY

Dozens of clinical trials using sulforaphane in the form of broccoli sprouts, sprouts or seed extract have been dosed in the range 100 – 200 µmol/day and have reported few adverse effects.[69,70] However, the amount of highly bioavailable sulforaphane delivered at the time of ingestion in these trials could have been less than the numbers quoted and may not be comparable to the present study. Toxicity tests showed no indication of thyroidal toxicity or changes in liver or kidney function at 40 µmol sulforaphane daily for 84 days.[70]

The first tests using optimised RST indicated a positive symptom response in a dose range corresponding to 30 to 50 ml of the standard preparation of 100 ml of broccoli seed tea, at a frequency of once per week. This is equivalent to the glucoraphanin content of 0.3 to 0.5 g of the

seeds used or 25 – 40 µmol of sulforaphane per week, very much lower than for comparable studies where broccoli sprout extracts or unconverted glucoraphanin and myrosinase had been used. Some participants took up the option of increasing the dosing frequency to twice per week after the first week while keeping to the same individual dose. For some participants, exceeding this dose range resulted in a temporary increase in the severity of Parkinson's disease symptoms, in particular urinary urgency, whereas good results were obtained at lower doses.

Results

SYMPTOM SPECIFICITY OF THE REDOX STRESS TEST

Figure 5 shows the weekly evolution of the total motor and non-motor symptom group scores for the data record n° 415 given in Table 1. The progressive decline in non-motor scores over the first 4 weeks, in contrast to the stability of the motor scores, is well established and replicates earlier observations.[55]

To determine the selectivity of the RST, the difference in symptom scores at week 4 compared to baseline was extracted from the records from 17 participants and summed over all the recorded cases of each symptom. The results are summarised in Table 2. Symptoms that responded most strongly to the RST were a triad made up of fatigue, urinary urgency and constipation. These are some of the most frequently observed non-motor symptoms of Parkinson's disease and have a major impact on the quality of life of patients.[76,77] Several participants reported total resolution in just 2 – 3 weeks after years of severe constipation. For participants with several non-motor symptoms, their attenuation was observed to be concurrent rather than sequential. In contrast, participants expressed their disappointment with regard to the lack of improvement of motor symptoms. In a few cases, tremor was reported to increase slightly but returned to previous levels after a few days.

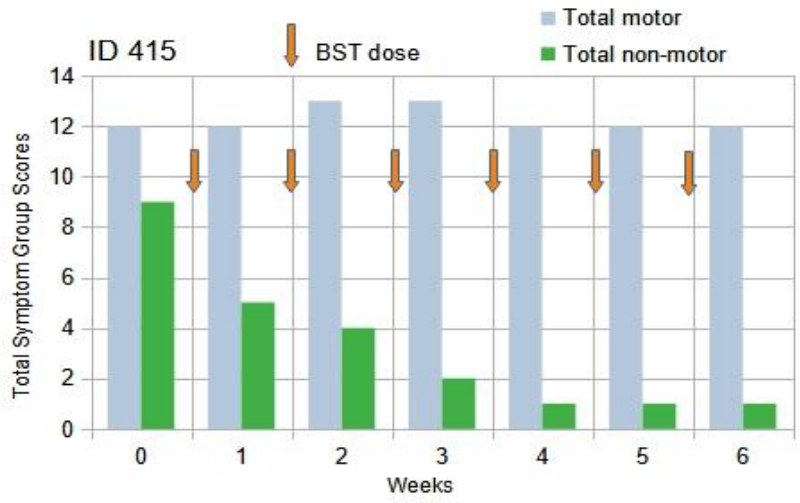

Fig. 5: Total motor and non-motor symptom response: *Individual response of grouped motor and non-motor symptom scores to RST over 6 weeks. Dosing was 50 ml standard BST once per week.*

The data is reported in the first instance in terms of a qualitative (binary) response to the RST compared to the total number of cases of each symptom. The results show a highly-selective response that categorically distinguishes between two symptom types. Symptoms which showed a strong positive response to RST all belong to the non-motor group, whereas those which were unchanged all belong to the motor group. This result is the exact opposite of the response of the same symptoms to dopamine replacement therapy (DRT).[76,77] Taken together, they indicate that the two symptom groups are independent of each other. In Table 2, symptoms that respond to RST were assigned the label Redox Stress-positive (RS+) and those that did not respond, were assigned the label Redox Stress-negative (RS-).

Secondly, we quantified the magnitude of symptom attenuation as high, partial or none. The leading redox symptoms also showed consistently high quantitative attenuation, reaching almost total resolution of the symptoms for some participants at 4 weeks. In all cases the symptoms returned to their previous levels 2 – 4 weeks after stopping the treatment.

Symptom type	N° of symptom cases	Attenuation by BST	Magnitude of attenuation	RS Label
Non-motor Symptoms				
Urinary urgency	14	13	High	RS+
Constipation	11	9	High	RS+
Fatigue	16	16	High	RS+
Brain fog/confusion	8	6	Partial	RS+
Apathy, mood	10	8	Partial	RS+
Sleep impairment	8	6	Partial	RS+
Total non-motor	67	58		RS+
Motor symptoms				
Bradykinesia	13	0	–	RS-
Rigidity	13	0	–	RS-
Tremor	10	1	–	RS-
Total motor	36	1		RS-

Abbreviations: RS+: Redox-Stress-positive; RS-: Redox-Stress-negative

Table 2. Self-assessed symptom response: Extracted from the records of 17 volunteers following self-administered consumption of freshly-brewed BST for at least 4 weeks.

ANECDOTAL OBSERVATIONS, DATA RELIABILITY AND TIMESCALES

In this pilot study, all the information collected was subjective, based on self-assessment of the symptom changes by the participants. This logically raises the question of data reliability, especially when the magnitude of changes are small or occur over a long period. However, this was not an issue given the clear, positive response to the Redox Stress Test regarding the key non-motor symptoms, and the total lack of response for motor symptoms. Furthermore, participants did not feel the need for caution when describing the degree of change for these symptoms, whereas they were more nuanced when describing symptoms which were only partially attenuated, such as sleep disorders, quoting general improvements in their quality of life, more energy and appetite for life. They also expressed disappointment at the lack of improvement in motor symptoms and held out hope that this might change over time. However, the pattern of symptom attenuation observed in this study is in line with that observed over the previous 3 years using earlier versions of the BST. This pattern consistently shows the greatest improvements in fatigue and daytime energy, constipation and daytime urinary urgency, variable degrees of improvement in brain fog, motivation, mood and sleep quality, and a total absence of any improvement in motor symptoms.

Discussion and interpretation

QUALIFYING THE REDOX STRESS TEST

In this first study, the RST successfully distinguished between two symptom groups of Parkinson's disease which we tentatively relabel RS+ and RS-. There was no overlap in the response of these two groups to the RST indicating their total independence from one another. That result was an essential, albeit insufficient step to qualify the RST as a reliable technique to identify symptoms related to oxidative stress. To fully achieve that qualification, the actual redox state of the target organs or tissues would need to be controlled clinically before and after the application of the RST, not only for Parkinson's disease but for a range of medical conditions where oxidative stress is involved. Numerous analytical techniques are available to measure biomarkers of oxidative stress, lipid peroxidation and DNA damage in clinical samples.[87] We currently do not have the means to make such measurements but we strongly encourage research groups to replicate our work with the additional clinical controls necessary to validate the Redox Stress Test. Until then, the present results should only be considered as potential indicators of the redox state of the tissues underlying the symptoms studied.

PARKINSON'S SYMPTOM GROUPS ARE ASSOCIATED WITH DIFFERENT REDOX STATES

Whilst keeping in mind the provisional nature of the test results, we explore potential implications. The discussions and hypotheses that follow should be understood as subject to the RST results being validated by future measurements.

The Redox Stress Test applied to the symptoms of Parkinson's disease shows two notable features:

(i) Parkinson's symptoms may be divided into two well-characterized groups:

- RS+ symptoms, previously designated as non-motor symptoms.
- RS- symptoms, previously designated as motor symptoms.

(ii) A positive response to RST was typically observed over a timescale of 1 – 4 weeks and was concurrent for all RS+ symptoms for a given participant.

The division into two groups, with non-motor symptoms labelled as RS+ and motor symptoms labelled as RS-, supports the hypothesis that oxidative stress and mitochondrial dysfunction are the driving forces of Parkinson's progression throughout the prodromal phase, but also continuing for the whole duration of the disease. For motor symptoms, which respond to dopamine replacement therapy (DRT), but not to RST, this result is an indication that motor symptoms are not directly affected by oxidative stress, even though OS is considered to be the cause of the loss of dopaminergic neurons.

The timescale for RS+ symptom attenuation (1 – 4 weeks) is consistent with that required for recycling and replacing dysfunctional mitochondria. This differentiates RS+ symptoms from RS- motor symptoms which respond more rapidly (~30 minutes) to DRT, since DRT simply compensates for the shortage dopamine in the brain.

SYMPTOMS OF PARKINSON'S ARE LOCATED IN DIFFERENT SYSTEMS

Among the frequent symptoms that responded most positively to the Redox Stress Test are fatigue, constipation and urinary urgency, three symptoms located in three distinct systems, the CNS, the gastrointestinal (GI) tract and the lower urinary tract (LUT). This indication of three active sites of the disease deserves careful consideration. Ostensibly, it suggests that the GI tract, the LUT and the CNS are active reservoirs of oxidative stress and mitochondrial dysfunction and implies that a system of "communication" among the sites exists[26,79–81]. Yet it provides no information about the original site of the disease nor about the order of progression of the disease.

The nature of the tissues which host the reservoirs of oxidative stress has not been established. We have been accustomed to thinking about Parkinson's as a brain disease affecting neurons, but faced with the possibility of reservoirs of the disease in peripheral organs, we may have to enlarge our conception of Parkinson's to allow for disease activity in non-neuronal tissues. The current brain centred approach to Parkinson's accommodates the occurrence of constipation and urinary dysfunction through neuronal damage in the CNS also affecting or being transmitted to peripheral nervous systems such as enteric nervous system, the autonomic nervous system and those controlling bladder function. There is little doubt that all of these nervous systems are damaged by the oxidative stress of Parkinson's disease[28,29,82–84], but without proof to the contrary, we cannot rule out the possibility of reservoirs of oxidative stress being located in non-neural tissues such as the epithelial cells lining the intestines and the bladder. Indeed, oxidative stress in the GI tract and the LUT of Parkinson's patients is well documented.

The Redox Stress Test indicates the presence of oxidative stress in peripheral organs by activating the Nrf2 pathway to neutralise it. The NRf2 pathway is however suppressed across all neurons in the human CNS.[85] The task of neutralizing oxidative stress in neurons of the CNS is carried out by astrocytes that host an active Nrf2 pathway and can deliver the benefits of Nrf2 activation to neurons via distributed connections to synapses.[86] However astrocytes may also host reservoirs of oxidative stress and mitochondrial dysfunction, in which case their capacity to protect neurons from oxidative stress and to recycle mitochondria is impaired and even reversed. Indeed, oxidative stress-damaged astrocytes may then contaminate neurons with dysfunctional mitochondria and accelerate the process of neurodegeneration rather than controlling it.[87,88]

PARKINSON'S SYNDROME IS PRIMARILY A MULTISYSTEM REDOX DISORDER (HYPOTHESIS)

The overall pattern of oxidative stress and mitochondrial dysfunction in several sites, supports our hypothesis that Parkinson's is a Syndrome, which we describe in terms of a multisystem redox disorder. This provokes progressive neurological damage for the total duration of the disease. However, in the early years, the symptoms generated by the neurological damage are relatively mild and any relationship between peripheral and neurological symptoms is not recognised. When left untreated for a long period, the accumulated damage to dopaminergic neurons induces a progressive dopamine deficiency which exceeds the threshold for normal motor control, creating a new group of symptoms of neurological origin which define the condition as a movement disorder. The neurological component of Parkinson's Syndrome might therefore be considered as the sequela to a relatively benign and potentially treatable chronic multisystem redox disorder.

Whilst there is convincing evidence that oxidative stress plays a major role in Parkinson's disease[2–4,6,8,10] and several authors have described Parkinson's in terms of a multisystem disorder,[89–92] to our knowledge, the idea that oxidative stress might be the common factor driving the disease in several systems simultaneously has not been proposed previously or supported by experimental evidence. The present work, if confirmed by further research, would be a powerful indicator that redox disorder in multiple systems drives the progression of Parkinson's syndrome and may be the root cause of neurodegeneration.

LOOKING FORWARDS

It will require robust research to substantiate and build upon our findings. However, we are acutely aware of the possible implications of the work presented here. On the assumption that the hypothesis that Parkinson's is a Syndrome rooted in distributed redox disorder is valid, one might expect to see a trend towards targeting the recognition of early redox disorders and developing preventative therapies. Indeed, it is quite possible that today's neurological component of Parkinson's might eventually come to be considered as the preventable sequela of a relatively benign multisystem redox disorder.

In the long term, the gain in quality of life for Parkinson's patients and the reduction of the burden for patients, carers and public health authorities could be considerable. There are however many hurdles to be overcome before that goal can be achieved.

The first step would be to confirm the redox states of the locus of the RS+ symptoms through complementary clinical analyses. That might best be achieved through a shared effort with research groups having established competence in the field. Secondly, short clinical trials to establish "proof of concept" to eliminate reservoirs of oxidative stress in peripheral organs as a prerequisite to slowing disease progression could likely be accomplished relatively rapidly and at modest cost.

Beyond that, the barriers to progress are likely to be much higher. For public health authorities, the move from the present symptomatic treatment of movement disorder to disease control and prevention could represent considerable savings, but would require significant upfront expenditure to pass clinical trials before new therapies could be made available.

At the time of writing, the BST method is not compatible with the business model for commercial drug development since it is not available in a form adapted to widespread application and the active agent, sulforaphane, is a natural product that cannot be patented.

Finally, the overall pace of change is likely to be heavily influenced by the determination of patient advocacy groups to influence public health authorities to take direct action. Given the apparent ease with which the redox disorder can be quenched in many sites simultaneously, the demand from Parkinson's patients and advocacy groups for an effective and safe therapy to eliminate oxidative stress might become irresistible.

Conclusions

A Redox Stress Test (RST) in the form of a herbal tea containing fully bioavailable sulforaphane has been developed to identify symptoms caused by oxidative stress in Parkinson's disease, by observing symptom changes induced by short-term activation of the Nrf2 pathway to restore redox homeostasis. To ensure high selectivity, the composition of the tea was optimised to match the pharmacodynamics of the mechanism of Nrf2 activation. When consumed by people with Parkinson's disease, the RST induced powerful and concurrent attenuation of a diverse array of symptoms, known as non-motor symptoms and, for the first time, distinguished them from a second group whose cause (dopamine depletion) was previously known. Being able to assign a common cause to these symptoms might also help patients understand and manage their symptoms better.

Whilst being fully aware of the need to validate these findings by further research, we tentatively interpret the results in terms of a hypothetical model for Parkinson's Syndrome which we describe as a multisystem redox disorder with reservoirs of the disease in peripheral organs as well as in the brain. According to this model, the redox disorder in the brain provokes a slow but progressive neurological impairment which goes largely unrecognized for years. Neurological symptoms only come to light many years later, when the damage to dopaminergic neurons exceeds a threshold for normal brain function.

If validated, potential applications might offer significant advantages reaching beyond Parkinson's disease: the RST could also be used to reveal new information about other diseases in which oxidative stress may play a role such as Amyotrophic Lateral Sclerosis (ALS), Alzheimer's disease, cardiovascular diseases, irritable bowel disease, chronic obstructive pulmonary disease and long Covid. We stand ready to explore opportunities to help replicate our

experiments under clinically controlled conditions that might confirm and further enrich our understanding of how redox disorder relates to Parkinson's disease and beyond.

LIMITATIONS OF THIS WORK

In this paper, we have clearly stated that the work presented here represents the provisional results of an ongoing patient-led research program designed and carried out by people with Parkinson's disease at their own expense, with the aim of slowing the progression of their own disease and sharing their findings with other Parkinson's patients. As such we are acutely aware of the need for further robust research to substantiate and build upon our findings. We strongly encourage research groups to replicate our work with the additional clinical controls necessary to verify the findings of the Redox Stress Test.

Conflict of interest statement

The author was diagnosed with Parkinson's disease in 2018. He declares no other conflict of interest.

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