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Featured Topic: Anti-aging

A Crucial Antioxidant Mechanism by Nutraceuticals: Glutathione Antioxidant Response/ Glutathione Redox Cycling

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Molecular Links between Caloric Restriction and Sir2/SIRT 1 Activation

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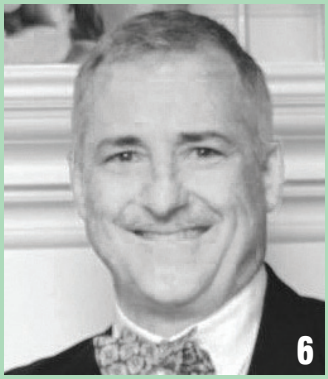

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A Word from the Editor in Chief

Prof. Jack Wong,
Editor in Chief
Director, Regulatory Affairs, Asia Pacific,
Terumo BCT (Asia Pacific) Ltd.
(Singapore Branch)
Email: speedxquality@yahoo.com

Dear Readers,

Thank you for reading Asia Health Care Journal Issue April 2016. There are various healthcare articles provided by professionals and academic experts. We have the featured topic of "Anti-aging" which is one of the most exciting topics in today's medical world. I hope this journal can facilitate different healthcare professionals sharing their knowledge and opinions, giving the most latest development of healthcare industry information to the public.

Some key updates in 2016:

1. We are now working on the 2nd edition of "Handbook of Medical Device Regulatory Affairs in Asia". It is our honor to have many government officers from US FDA, UK and Japan as well as many Asia regulatory experts to contribute the articles. This new book has a new section which will cover Latin America regulatory system. Look forward to getting the latest edition around mid of this year.
2. For training, we just finished a regulatory and medical device research training in National Taiwan University last month. We will complete the training in Nanyang Technological University, Singapore by April and we are planning a new programme in Tohoku University around the end of 2016.

Finally, I would like to thank everyone who contributed to this publication, hope you enjoy reading it.

Prof. Jack Wong
Asia Regulatory Professional Association

ARPA
Asia Regulatory Professional Association

The Asia Regulatory Professional Association (ARPA) is an organization of healthcare regulatory affairs professionals in Asia. ARPA aims to raise the standard and social recognition of regulatory professionals as part of healthcare team.

Details of ARPA can be found in
http://www.healthcare.org.hk

Life Science Entrepreneurship in Hong Kong: What Works, What Doesn't and How to Improve It?

A Town and Gown Lecture hosted by The Innovation Forum Hong Kong

by Oliver Cheung and Cecilia Tsui

The Innovation Forum Hong Kong ("IFHK") is a branch of The Innovation Forum, a global NGO connecting academia with industries and policy makers in the innovative sectors. We aim to unleash the transformative potential of scientific entrepreneurship, and to act as an accumulator, accelerator and broker of ideas and expertise (for more details, please see endnotes).

The IFHK hosted its very first "Town and Gown" lecture on 27 February, 2016 at the Charles K. Kao Auditorium, Hong Kong Science Park. The lecture's theme was on life sciences entrepreneurship in Asia, conducted by heavy-weighted speakers as follows:

- **The Hon Mrs. Fanny Law**, GBS, JP, Chairperson, Hong Kong Science & Technology Parks Corporation ("HKSTP")
  Professor Ronald Li, Director, Stem Cell & Regenerative Medicine Consortium, The University of Hong Kong, Li Dak-Sum Research Centre for Regenerative Medicine, The University of Hong Kong, Ming-Wai Lau Centre for Regenerative Medicine, Karolinska Institutet
- **Professor Albert Yu**, Chairperson, Hong Kong Biotechnology Organisation (Vice Director, Neuroscience Research Institute, Peking University, and Chairman and CSO, Hai Kang Life Corporation Limited)
- **Dr. Victor Ng**, Senior Manager, Productivity Training Institute, Hong Kong Productivity Council ("HKPC")
- **Mr. Bernard Chan**, Head of Investments, China Regenerative Medicine International Limited (CRMI); and
- **Dr. Dominic Chan**, Founder and Chief Consultant, Decisive Consulting Limited, Co-Founder and Partner, Dark Horse Investment Limited, (Hon Director, Empowering Young Entrepreneurs Programme (a joint initiative of the Google Inc and The Chinese University of Hong Kong)

After the welcome speech given by Dr. Cecilia Tsui, Founding President of IFHK, Mrs. Law kick-started the event with a presentation on the work of Hong Kong Science and Technology Parks Corporation (HKSTP), the opportunities in Asia and Hong Kong's competitiveness as a start-up hub for life science entrepreneurs.

With an aging population in many Asian cities, Mrs. Law observed that this is an exciting time to be involved in the development of biotech innovations, as the public health challenges in the next decade imply that there are many opportunities for young innovators in the field to explore. Mrs. Law listed three focal points of the HKSTP's work, that is to Connect, Collaborate and Catalyse on Hong Kong's potential. As well, she called for rebranding the city as one that not only is a financial centre but also one that is supportive of technological innovations.

Mrs. Law concluded her presentation by emphasizing on the need for Hong Kong to incentivize STEM (science, technology, engineering and mathematics) education as a means to reinforce and to diversify the existing talent pool. At the same time, the academia should migrate from "publish or perish" to a "deploy or die" mentality; that is not just focusing on scientific discoveries, but also emphasising on translational research, turning scientific discoveries into useful products.

The lecture continued with a presentation given by Professor Ronald Li, who shared his views on life science entrepreneurships in Asia, from his experience as a founder of "Novo Heart", a global stem cell biotechnology company dedicated to develop and engineer bio-artificial human heart prototypes, drastically improving drug discovery and development of heart therapeutics, and to further develop their bio-engineered human heart constructs into transplantable heart grafts for cell based regenerative therapies.

From his work, Professor Li observed that though Hong Kong is an ex-

cellent platform for biotech start-ups to thrive, the general public still has a slightly conservative attitude towards the idea of young entrepreneurs introducing new innovation, instead of taking up careers that are considered to be more stable. Though he is positive that this will change in the future, Professor Li said that there is still much to be done to attract more talents in Hong Kong to pursue a career in STEM fields.

Professor Li also pointed out on the need for young entrepreneurs to make the choice by passion and not by elimination, as the road to success will not be an easy one.

The last presentation was given by Professor Yu, who discussed about his work as a professor in neuroscience and as an entrepreneur with Hai Kang Life Corporation Limited (HKLife), a company specialized in the R&D and commercialization of clinical and veterinary molecular diagnostics.

Professor Yu echoed the views of Professor Li, and focused on the one aspect that many young entrepreneurs failed to realize – as a life science entrepreneur, one has to wear many hats at the same time – he can be a salesman, consultant, accountant, and a negotiator. The skills required to succeed in life sciences entrepreneurship is drastically different from those as an academic researcher. The entrepreneur has to develop a sense in identifying which of the many ideas in his head is commercially sustainable, otherwise he will not be able to retain his investor's interests.

Professor Yu concluded his presentation with one important advice: young entrepreneurs must be able to attract talent and delegate work effectively in order to advance a project to the next level, as Professor Yu said in his own words, "you can never do it alone".

After the presentations, the three speakers are joined by Mr. Bernard Chan, Dr. Dominic Chan and Dr. Victor Ng for a panel discussion. Dr. Chan was the moderator of the panel.

When discussing about investors' attitudes towards biotechnology, Mr. Chan observed that many of the investors in Asia are still relatively uninformed about the potential and technicalities of biotech innovations. As a result, highlighting the importance for young entrepreneurs in this field called for good communication skills, so their ideas may come across in a way that is persuasive and easily understood.

Mr. Chan also advised that young entrepreneurs should avoid the pitfall of working on a proposal for a "perfect product". Instead, they should think about whether the product is commercially viable. That way, future iterations of the product can be funded, as well as making the venture more sustainable, and hence attractive to investors.

On the other hand, Dr. Dominic Chan and Dr. Victor Ng commented that many of the shortcomings observed in start-ups can be sourced from miscommunication within the team, where the partner with a business background fails to understand the work of the technical expert, and vice versa. Dr. Ng added that the SME ONE programmes offered by the Hong Kong Productivity Council aims to address this issue, and provides a platform that young entrepreneurs may not have otherwise.

In terms of markets, Professor Li commented that young entrepreneurs in Hong Kong should not just aim for the Hong Kong market, but should also utilize the city's advantages and reputation in promoting their products to other parts of the world.

The panel discussion ended with a laugh from the audience when Professor Yu, Dr. Ng and Dr. Chan concluded their comments with a pitch to the audience to join their respective organizations/programmes that they are managing.

The event ended with two idea pitches where Dr. Tsui, Professor Li and Professor Yu acted as judges. The first pitch was made by HSK GeneTech, a start-up with a focus on health management products and services based on gut microbiome analysis. The second pitch was made by Health View Bioanalytic Limited, a start-up that put state-of-the-art research into reality by accurately assessing the risk of stroke through a fundus photo in the fastest and convenience manner.

About The Innovation Forum

Innovation FORUM

Founded in a laboratory at the University of Cambridge, UK in 2012, nowadays, The Innovation Forum (IF) is operating 16 branches in Europe, USA and Asia, including Barcelona, Cambridge, Copenhagen, Edinburgh, Hong Kong, KraKow, Lausanne, London (KCL), London (LBS), London (UCL), London (Imperial), Manchester, New York, Oxford, Tokyo, and Munich. It is a global platform encouraging cross-disciplinary and cross-generation collaborative efforts among policy leaders, investors, industry-academia and industry-startups with open access. The Innovation Forum is an annual conference that gathers more than 400 high profile delegates and a non-profit network of over 10,000 young innovators who work together to promote scientific entrepreneurship locally, nationally and globally.

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Mr. Tom French

With over 11 years experience of managing role, Mr. Tom French has a clear vision of delivering Strategy for Market Leadership, Business Growth and Shareholder Value.

Mr. French is now the President CEO of Zoono USA since September 2015. He has successfully revitalized and build a functional organization to launch innovative antimicrobial technology into the $1.2B US Disinfectant marketplace. Prior to his current role at Zoono USA, Mr. French was the Chief Marketing Officer and Managing Director at French & Company LLC from November 2009 to August 2015. He has proven his professional record by profitably managed a strategy consultancy dedicated to creating disruptive opportunities to precipitate rapid growth.

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Trial of Zoono Microbe Shield Surface Sanitizer within a New Jersey hospital Intensive Care Unit

Abstract

A trial of the Zoono antimicrobial was conducted within the Intensive Care Unit at a Saint Barnabas Health, a New Jersey, USA hospital showing statistically significant reduction in pathogens over an extended period of time as compared to standard hospital cleaning protocols alone.

The initial Laboratory Test validated existing Test Result data supplied by the manufacturer relating to the efficacy and durability of the Zoono product as there was a statistically significant reduction in pathogens over the 8-hour test period, compared to increases in the non-treated areas / rooms. The delta between the two trend lines, was significant. There was a statistically significant reduction in pathogens over the 7-day period, compared to increases in the non-treated areas / rooms. The delta between the two trend lines, was significant.

This reduction in pathogenic presence will benefit both the hospital and patients alike. The use of QACs will provide an extra layer of protection for patients and reduce the risk of nosocomial infection (HAI's) and this will lead to a corresponding reduction in costs.

Introduction

The following is an initial summary of results from the environmental portion of a clinical evaluation of the Zoono Z-71 Surface Sanitizer. A medical white paper of the complete study including patient outcomes is in development.

Background

In March 2015, the US White House released a comprehensive National Action Plan identifying critical actions to be taken to combat the rise of antibiotic-resistant bacteria. The No.1 goal of this National Plan, is to "Slow the Emergence of Resistant Bacteria and Prevent the Spread of Resistant Bacteria."

This Presidential National Action Plan [Executive Order 13676: Combating Antibiotic-Resistant Bacteria] was a catalyst for this trial.

Technology

Quaternary Ammonium Compounds (also referred to as QACs or "quats") have been recognized as having bactericidal and antimicrobial properties since 1916. QACs have been used within the hospital environments globally for several decades and several are approved by the US EPA as antimicrobial substances in the United States.

The Zoono Product

Zoono represents the 9th generation of the original technology. Zoono Microbe Shield is a proven, long lasting water based surface sanitizer currently sold in 40+ countries / territories globally. It is distributed in the USA by Shrewsbury (NJ) based, Zoono USA LLC.

The Zoono Difference

When manufactured, the Silane part of the Zoono molecule starts as trimethoxysilane (or silyl), but once placed in water an equilibrium is formed between the trimethoxy and trihydroxysilane. This equilibrium keeps the molecule as a monomer and inhibits the molecule from bonding or growing as a polymer until it dries. Once drying occurs, the first of the three available hydroxyl groups forms a covalent bond immediately, the second bond occurs several days later and the third a similar period again. The initial association to the substrate is made through the attraction of the positively charged cation to the surfaces that exhibit a negative character in the aqueous media. Once the first bond occurs, the molecule is permanently attached to the surfaces during the time frame for the second and third covalent bond, sterochemical-rotation occurs and the molecule may covalently bond to the surface or to itself forming a network polymer that encapsulates the surface being treated. This rotation allows for a uniform layer of antimicrobial protection. The silanol functionality which enables this molecule to homopolymerize helps account for the durability of this technology.

The mechanism works the same on bacterial or viral cells. On direct contact with microorganisms the technology works by disrupting (or rupturing) the cell membrane. This interrupts the normal life processes and destroys the cell. Two forces cause this interruption: the quanternised Nitrogen acts as an 'electrocuting' charge and the carbon chain acts as a sword or puncturing agent.

The structure is ideal for taking advantage of the anionic nature and the liproprotein composition of the microbial membranes. Since the antimicrobial acts only on the membrane and does not lose strength over time, it does not create the conditions which allow for a sublethal dose - thus allowing microorganisms to develop an increased resistance to the given microorganism. Like bacteria which have soft and hard shell species, viruses are classified as envelope or non-envelope microorganisms.

The Objective

The Trial objective was to confirm that the introduction of a QAC antimicrobial (in addition to standard protocols) would result in an effective, statistically significant and long lasting reduction in total bacteria count within the intensive care units (ICU). Ultimately, these reductions will contribute to a decrease in Healthcare Acquired Infections (HAIs) within the Hospital.

Key Issue

Cleaning and disinfecting surfaces using traditional products (poisons or high alcohol based dehydrating agents) can remove / kill pathogens on surfaces, but for short term efficacy only - usually surfaces are re-contaminated within minutes. In addition, these same traditional products promote mutation (resistance) and the evolution of superbugs. By comparison, Zoono Microbe Shield has both proven efficacy plus durability over long periods and pathogens cannot develop immunity against the QAC based formulation.

The emergence of resistant bacteria (and other pathogens) is not new. According to the World Health Organization (WHO), Health Care associated Infections (or infections acquired in healthcare settings) are the most frequent adverse event in health-care delivery worldwide. Hundreds of millions of patients are affected by HAI's each year, leading to significant mortality and financial losses for health systems.

HAI's (also referenced as "nosocomial infections") include infections acquired by patients in the hospital or facility but appearing after discharge, and occupational infections among staff. HAI's are not confined to 'developing countries' - in high-income countries, approximately 30% of patients in ICU are affected by at least one health care-associated infection.

Infections are common in patients in contemporary ICUs, and risk of infection increases with duration of stay. Infection and related sepsis are the leading cause of death in non-cardiac ICUs, with mortality rates that reach 60% and account for approximately 40% of total ICU expenditures. Importantly, the incidence of sepsis is increasing, as is the number of consequent infection-related deaths.

In a United States Centres for Disease Control and Prevention (CDC) Report (March 2015), it was estimated HAI's in US hospitals alone result in up to $33 billion in excess medical costs every year. The CDC 'HAI Prevalence Survey' (October 2015) concludes that 1 in 25 US hospital patients has at least one healthcare-associated infection. There were an estimated 722,000 HAIs in U.S acute care hospitals in 2011 and 75,000 hospital patients with HAIs died during their hospitalizations. Whilst the nosocomial infection rate has remained relatively stable (around 5-6 HAI's per 100 admissions), due to the progressively shorter inpatient stays over the last 20 years, the rate of HAI's per 1,000 patient days has actually increased 36%, from 7.2 in 1975, to 9.8 in 1995.

New forms of antibiotic resistance can cross international boundaries and spread between continents with alarming ease. Each year in the United States, at least 2 million people acquire serious infections with bacteria that are resistant to one or more of the antibiotics designed to treat those infections. At least 23,000 people die each year as a direct result of these antibiotic-resistant infections. Many more die from other conditions that were complicated by an antibiotic resistant infection.

Antibiotics have revolutionised medicine in many respects and countless lives have been saved; their discovery was a turning point in human history. Regrettably, the use of these wonder drugs has been accompanied by the rapid appearance of resistant strains. Medical pundits are now warning of a return to the pre-antibiotic era; a recent database lists the existence of more than 20,000 potential resistance genes (r genes) of nearly 400 different types, predicted in the main from available bacterial genome sequences.

Location

The trial was conducted over a ten-week period during May, June and July 2015, within the (non-surgical) Intensive Care Unit (ICU) of a major New Jersey Hospital.

Test Areas

There were 12 rooms in the non-surgical section of the ICU. Of the 12 rooms, 3 were designated control rooms and 9 used for product evaluation purposes. In order to avoid any area receiving special attention from Housekeeping staff, they were all kept blind to both test locations and designated control room.

Within all rooms, 4 identical locations were tested. In addition, three hand scanners on patient Electronic Medical Records (EMR) computers were used for testing purposes.

To minimise patient (and staff) disruption, all swab testing was undertaken outside regular hours - between 5.00am-7.00am.

Benchmarking

All areas identified as testing locations for the trial, were subjected to full benchmark tests. This provided base measurements.

Protocol

In order the Trial did not affect existing protocols, the application of the Zoono antimicrobial within the ICU was completed as an additional feature rather than as an alternative to existing practices. This ensured any significant change in CFU counts would only be a result of using the Zoono product.

Prior to the commencement of the study, the manufacturer provided the Hospital Internal Review Board (IRB) with copies of several Test Results relating to their product against bacteria, fungi, viruses, mould and yeast. As a result, it was agreed this trial would be based on CFU Total Plate Count rather than being pathogen specific.

In parallel to CFU testing, ATP tests were completed in the immediate vicinity of each test area. The ATP testing was designed to provide a trend analysis only - to compare and contrast with the CFU results.

Zoono Microbe Shield was applied both topically (once every 7 days) and via a standard, cold fogging unit (once per month). In order to maintain total separation from any possible housekeeping influence, treatments were conducted by non-hospital (non-housekeeping) personnel.

The non-control test locations were treated and re-evaluated at 8-hour / 7 day intervals across a total of 10 weeks. Samples were cultured for total bacteria (Total Plate Counts) and compared with non-treated, control locations.

Chain Of Custody

In order to maintain total integrity of the swabbing / testing regime, the CFU swabbing and ATP swabbing procedures were completed independently. The only person permitted to handle CFU swabs, was the independent Microbiologist. He was solely responsible for all swabbing, the packing of completed swabs into the specialty containers, the sealing of cartons, signing of seals etc. This entire process was photographed with digital copies sent by email to the Testing Laboratory the same day. Upon receipt, the Laboratory was able to compare the photos of the carton sent, with the carton that arrived.

Sample Enumeration And Analysis

All sample identities were masked with a numerical code; samples were identified only after results delivered. The neutralization broth was 10m1 of 1:10 dilute D/E neutralization broth in 15m1 conicals. Samples were sufficiently vortexed for 30±10 seconds. Suspensions were enumerated using standard dilution and plating techniques to determine the number of surviving microorganisms. Tryptic soy agar (TSA) enumeration plates were incubated for 120±6 hours at ambient temperature.

Results

Zoono Microbe Shield Efficacy Test

An initial Efficacy Test on the Zoono antimicrobial resulted in a 4.78 log reduction (99.998%) against Staphylococcus. aureus (ATCC 6538) after 24 hours. This result validated earlier Laboratory testing for this batch completed as part of the manufacturer's normal QA process.

8-Hour Test (CFU)

For the CFU 8-Hour Test (8 hours between application of Microbe Shield and testing), the average CFU count reduction in pathogens across the test areas, was 59.82%. By comparison, the average for the test areas within the control rooms over the same period, increased by 45.30%.

This delta is statistically significant.

7-Day Test (CFU)

For the CFU Duration test (7 days between application of the antimicrobial and testing), the average reduction in pathogens across the test areas, was still 31.66% after 7 days. By comparison, the average for the test areas within the control rooms over the same period, increased by 593.95%.

Again, this delta is statistically significant.

Was The Trial Objective Achieved?

The objective of this trial was to confirm whether the incorporation of a new generation antimicrobial product into existing Housekeeping protocols, would result in an effective, statistically significant and long lasting reduction in total bacteria count on nominated surfaces within a high risk ICU environment.

As these objectives were satisfied, these reductions will contribute to a decrease in Healthcare Acquired Infections (HAls) and an improved ROI to the Hospital.

The objective of reducing the total bacteria count on nominated surfaces, was clearly achieved.

Conclusions

The trial of the Zoono QAC antimicrobial within the Intensive Care Unit at this hospital, was successful resulting in a statistically significant reduction in pathogens over an extended period of time as compared to standard hospital cleaning protocols alone.

- The initial Laboratory Test validated existing Test Result data supplied by the manufacturer relating to the efficacy and durability of the Zoono product.
- There was a statistically significant reduction in pathogens over the 8-hour test period, compared to increases in the non-treated areas / rooms. The delta between the two trend lines, is significant.
- There was a statistically significant reduction in pathogens over the 7-day period, compared to increases in the non-treated areas / rooms. The delta between the two trend lines, is significant.
- This reduction in pathogenic presence will benefit both the hospital and patients alike.
- The use of QACs will provide an extra layer of protection for patients and reduce the risk of nosocomial infection (HAI's) and this will lead to a corresponding reduction in costs.

Dr. George Tzortzis

Chief Scientific Officer at Clasado Research Services Ltd., a manufacturer of novel prebiotic galactooligosaccharides (GOS). Dr. George Tzortzis is a Food Biotechnologist with research experience at the University of Reading (UK) in the area of colonic functional food ingredients. He is the author of numerous papers and patents regarding prebiotics and synbiotics preparations involving galactooligosaccharides.

The other authors: D.B.A. Silk, A. Davis, J.Vulevic & G.R.Gibson

Clinical trial: the effects of a trans-galactooligosaccharide prebiotic on faecal microbiota and symptoms in irritable bowel syndrome

Abstract

Background

Gut microflora-mucosal interactions may be involved in the pathogenesis of irritable bowel syndrome (IBS).

Aim

To investigate the efficacy of a novel prebiotic trans-galactooligosaccharide in changing the colonic microflora and improve the symptoms in IBS sufferers.

Methods

In all, 44 patients with Rome II positive IBS completed a 12-week single centre parallel crossover controlled clinical trial. Patients were randomized to receive either 3.5 g/d prebiotic, 7 g/d prebiotic or 7 g/d placebo. IBS symptoms were monitored weekly and scored according to a 7- point Likert scale. Changes in faecal microflora, stool frequency and form (Bristol stool scale) subjective global assessment (SGA), anxiety and depression and QOL scores were also monitored.

Results

The prebiotic significantly enhanced faecal bifidobacteria (3.5 g/d $P < 0.005$; 7 g/d $P < 0.001$). Placebo was without effect on the clinical parameters monitored, while the prebiotic at 3.5 g/d significantly changed stool consistency ($P < 0.05$), improved flatulence ($P < 0.05$) bloating ($P < 0.05$), composite score of symptoms ($P < 0.05$) and SGA ($P < 0.05$). The prebiotic at 7 g/d significantly improved SGA ($P < 0.05$) and anxiety scores ($P < 0.05$).

Conclusion

The galactooligosaccharide acted as a prebiotic in specifically stimulating gut bifidobacteria in IBS patients and is effective in alleviating symptoms. These findings suggest that the prebiotic has potential as a therapeutic agent in IBS.

Introduction

Irritable bowel syndrome (IBS) is the most common functional gastrointestinal disorder. Depending on the criteria used to define IBS, its prevalence in the general adult population is up to 20%.[1,2] In many sufferers, it is a chronic disorder[3] and patients experience significant impairment in health related quality of life.[4] The contribution of IBS to annual healthcare costs is high[5] and patients also lose significant amounts of time off work.[3,6] The diagnosis of IBS has importance therefore in both human and economic terms.[7] IBS continues to represent a significant therapeutic challenge and based on what is known of its pathophysiology, multidisciplinary approaches have been proposed.[7,8] Current interest has focused on the role of gut microflora-mucosa interactions linked to inflammatory and immune processes that are likely to contribute towards enteric neuromuscular dysfunction in these patients.[9,10] The development of IBS following bacteriologically proven gastroenteritis,[11-15] the presence of low grade mucosal inflammation,[15,16] endoendocrine cell hyperplasia,[15,16] abnormal fermentation[17,18] and evidence of small bowel bacterial overgrowth in IBS[19] are some of the observations that have pointed to a pivotal role for indigenous microbiota in the pathogenesis of IBS. Probiotics are defined as live microbes that confer a significant benefit to the host.[20] The results of early studies of probiotic therapy in IBS produced conflicting results.[7] Methodological differences and variability of probiotic strains were factors that contributed to the initial scepticism of this therapeutic approach. In a recent and important pilot study, treatment with Bifidobacterium infantis 35624 but not Lactobacillus salivarius UC4331 resulted in symptom improvement and correction of a pro-inflammatory, Th-1 IL-10/IL-12 ratio.[21] This study was followed by a large scale trial that confirmed the benefits of therapy with Bifidobacterium infantis 35624 in primary care female IBS patients.[22] Subsequently, the beneficial effects of another Bifidobacterium probiotic strain (Bifidobacterium animalis DN-173010) have been shown in IBS adult subjects with predominant constipation.[23]

Probiotics suffer the disadvantage of degradation during transit through the gastrointestinal tract and the need for stringent quality controls during and after bacteria already resident in the colon.[24] As such, they manufacture. In contrast, prebiotics are nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity ofexert positive changes in the resident gut microbiota.

In response to the findings pointing to the benefits of Bifidobacterium probiotic strains, a novel prebiotic, composed of a trans-galactooligosaccharide mixture B-GOS has been synthesized (Clasado, Milton Keynes, UK). Its capacity to act as a bifidogenic prebiotic was recently confirmed in healthy humans.[25] Using this compound, we report here the results of

a pilot study undertaken to investigate the efficacy of a prebiotic as therapy in patients with IBS.

Patients And Methods

Study population

A total of 42 subjects were required for an 80% probability that the study would detect, at a two sided 5% significance level, an effect on the colonic bifidobacterial population if the true change in the bifidobacterial population was 0.495 times the standard deviation. Patients were recruited from gastroenterology clinics at Central Middlesex Hospital (CMH) and by direct advertisement in the Irritable Bowel Syndrome Network publication. Individuals aged between 18 and 80 years who satisfied Rome II criteria for diagnosis of IBS[26] and in whom organic gastrointestinal disease, including inflammatory bowel disease, had been excluded were considered for inclusion in the study. Patients were excluded if they had a functional disorder of the upper gastrointestinal tract for which treatment had not been stable for the preceding three months. All patients were excluded if they had abnormal hematological and biochemical indices and excluded, if they had abnormal findings on barium enema or colonoscopy undertaken within the previous 5 years.

Patients were also excluded, if they had been ingesting products containing prebiotics or probiotics in the 2 weeks preceding entry into the trial. Ethics Committee approval was received for the study which has been registered with ISRCTN (ISRCTN54052375).

Trial protocol

This was a single centre, parallel, patient blinded, randomized cross over controlled trial. Each potentially eligible patient was invited to attend CMH to discuss the study in detail (Visit 1). All patients were given an information sheet and allowed 72-hr to decide whether or not to take part (Table 1). Prior to enrolment in the study, patients were required to read, sign and date a consent form. General practitioners of patients recruited from the IBS Network were contacted to confirm suitability. All patients were evaluated by a full review of clinical history (Visit 2) and based on clinical symptoms, the patients were categorized to one of the following groups (Table 1); diarrhoea predominant (IBS-D), constipation predominant (IBS-C) or alternating (IBS-A) the latter patients having symptoms of both diarrhoea and constipation. Eligible subjects then entered a 2-week baseline period (Visit 3). At this visit, the investigator recorded symptoms and completed a subjective global assessment (SGA) on the Case Report Form (CRF). At the end of the baseline period, bowel symptoms were re-assessed to confirm that IBS symptoms remained unchanged. During this time and throughout the rest of the study, subjects were instructed not to take any IBS treatment. Patients fulfilling any one of the exclusion criteria were excluded from the study at this stage. At the end of the baseline period, subjects were randomized to one of three treatment groups. Randomization was performed by stratified block randomization within each IBS symptom subgroup. Trial design consisted of a 2-week "baseline" period followed by 2 treatment periods of 4 weeks each, separated by a 2-week "wash out" phase.

During the first treatment period, patients were asked to ingest once daily, before breakfast, a drink containing either 3.5 g (Group I) or 7.0 g (Groups II and III) placebo (chocolate or banana flavoured). After the 2 week wash out period, patients were then asked to drink once daily, before breakfast, either 3.5 g (Group I), 7.0 g (Group II) B-GOS or 7.0 g placebo (Group III) (chocolate or banana flavoured). At 7 day intervals during the 3 months study period, patients were contacted over the telephone and symptoms and SGA of relief were recorded. Compliance was assessed by direct questioning every week over the telephone.

Prebiotic preparation

The trans-galactooligosaccharide mixture used in this study was produced from the activity of galactosyltransferases from Bifidobacterium bifidum NCIMB 41171 on lactose.[25] The final product was in dry powder form consisting of (wt:wt) 48% galactooligosaccharides with a degree of polymerization between 2–5, 22% lactose 18% glucose and 12% galactose. Based on previous studies, the bifidogenic properties of B-GOS have been established for daily intake levels of 3.5 and 7.0 g[25] and as the aim of the intervention was to increase the bifidobacterial numbers in the colonic microfora, those intake levels were chosen for this study as well. Placebo (maltodextrins DE20) and B-GOS samples were packaged in a double blind fashion and placed in boxes, A (3.5 g chocolate or banana flavoured placebo), B (7.0 g chocfolate or banana flavoured placebo), C (3.5 g chocolate or banana flavoured B-GOS), D (7.0 g chocolate or banana B-GOS) and delivered to the study centre. Subjects were asked to reconstitute sachets immediately before consumption by mixing the powder with water.

Table 1. Summary of trial procedures

	Trial Procedures
Visit 1	Study discussion, provision of information sheet
Visit 2	Categorisation of patients to IBS-D, IBS-C or IBS-A. Assessment of symptoms and subjects global assessment (SGA) of relief 14 day baseline period (week 1–2)
Visit 3	Reassessment of symptoms and SGA of relief, study entry, randomisation, assessment of quality of life, assessment of dietary fibre intake, assessment of anxiety and depression scores, provide faecal sample, provide with test sample 28 day study period 1 (week 3–6)
Visit 4	Reassessment of symptoms, quality of life, dietary fibre intake, anxiety and depression scores. Provide faecal sample 14 day washout period (week 7–8)
Visit 5	Reassessment of symptoms, quality of life, dietary fibre intake, anxiety and depression scores. Provide faecal sample. Provide with test samples 28 day study period 2 (week 9–12)
Visit 6	Reassessment of symptoms, quality of life, dietary fibre intake, anxiety and depression scores. Provide faecal sample

Study population

Throughout the entire study, subjects were contacted on a weekly basis and symptoms recorded on the CRF. Bowel movement frequency was recorded as number per day and consistency was scored on a 7-point scale and evaluated using the Bristol stool scale.[27] The following 3 cardinal IBS symptom clusters were assessed:

(i) abdominal pain or discomfort;
(ii) bloating or distension;
(iii) bowel movement difficulty.

The latter could reflect difficulty with evacuation (e.g. straining or sense of incomplete evacuation) or urgency. Each symptom was evaluated using an ordinal scale (Likert scale: maximum score 7).[28] A composite score that comprised the sums of the three cardinal symptoms (pain / discomfort, bloating / distension and the bowel movement difficulty score) was also calculated for each patient (maximum score 21). The subjective global assessment (SGA) of relief was recorded at weekly intervals during the course of the study. SGA were scored from 1–5 (1 = completely relieved, 2 = considerably relieved, 3 = somewhat relieved, 4 = unchanged and 5 = worse).

Stool samples for faecal flora analysis were obtained at baseline, at the end of both treatments and during the washout period. Patients collected samples in a vessel provided. Each faecal sample was transferred to a vial and 1:1 (weight:weight) glycerol was added. Samples were stored at 20°C until required for analysis.

Bacterial enumeration

Faecal homogenates were subjected to fluorescent in situ hybridization using synthetic oligonucleotide probes, targeting specific regions of the 16S rRNA molecule and labelled with fluorescent dye Cy3 as described by Rycroft et al.[29] Briefly, samples were fixed overnight (4°C) in 4% (w / v) paraformaldehyde. Fixed cells were re-suspended in 150 lL PBS and stored in ethanol (1:1 v / v) at 20°C. Following overnight hybridization with each probe, the fixed cells were washed and vacuum filtered (2 lm polycarbonate isopore membrane filter, Millipore UK Ltd., Watford, UK). They were then mounted onto a glass slide of 20 lm of slowfade (Molecular Probes, Leiden, The Netherlands) and enumerated using the Fluor 100 lens (Eclipse 400 Epifluorescent Microscope, Nikon, Kingston-upon-Thames, UK). The probes used were Bif164 for Bifidobacterium genus.[30] His150 for Clostridium perfringens–histolyticum subgroup,[31] Bac303 for Bacteriodes-Prevotella[32] Lab158 for Lactobacillus–Enterococcus spp.,[33] Erec482 for Eubacterium rectale / C.coccoides group,[31] Ec1531 for E. coli[34] and Srb687 for Desulfovibrio spp.[35] 4,6-diamidino-2-phenylindole (DAPI) was used for the enumeration of total cell counts. All probes were provided by MWG-Biotech (London, UK).

Quality of life and psychological assessments

The impact of IBS symptoms on quality of life was assessed by administration of an IBS specific questionnaire developed and validated by Drossman et al.[36] at each study phase. The IBS-36 questionnaire scores symptoms on a 7-point Likert scale where 0 = never and 6 = always. The highest possible score is 216 and the lowest is 0. Anxiety and depression were also measured using the Hospital Anxiety and Depression (HAD) scale at each study phase. This scale detects states of anxiety and depression. The authors provided score ranges for "non-case", "doubtful" cases, and "definite" case of anxiety and depression.[37] With either of the HAD subscales, a score above 11 indicated definite clinically significant anxiety or depression.

Dietary intake was assessed and recorded for 5 days at each trial phase throughout the study. Each patient was instructed not to alter his / her diet during the treatment. Fibre intake including soluble and insoluble fibre (measured in grams) was calculated using a diecentrifuged at 1500 g for 5 mins and washed twice in 1 mL filtered PBS.

Safety assessments

Safety assessment consisted of monitoring and recording all adverse events. This information was reported by the patient or discovered by investigator questioning. Each adverse event was described by:

(i) duration (start and end dates);
(ii) severity grade (mild, moderate, severe);
(iii) relationship to the study treatment (suspected, not suspected);
(iv) action(s) taken.

Statistical methods

All statistical tests were performed using SPSS version 15.0 (SPSS Inc., Chicago, IL, USA) and a value of $P < 0.05$ was taken to indicate statistical significance. All data were analysed by an analysis of variance (ANOVA) model of repeated measurements taking into account the crossover design. In the ANOVA model, volunteer, treatment period (Baseline week 2, end of Placebo week 6 and end of treatment week 12) and treatment were introduced as fixed effects and subject measurements as random effect. Significant differences between treatments were determined by Tukey's Honestly Significant Difference test.

Role of the funding source

The funding source, Clasado Limited, had no role in study design, collection, analysis and interpretation of the data or in the writing of the report. The decisions to submit the paper for publication was made by the authors.

Results

Subjects

A total of 153 subjects were invited to participate in the study. A total of 60 subjects consented and were enrolled into the study. Sixteen of the enrolled patients did not complete the study. Figure 1 summarizes the reasons for patient dropouts. Forty-four subjects completed all phases of the study.

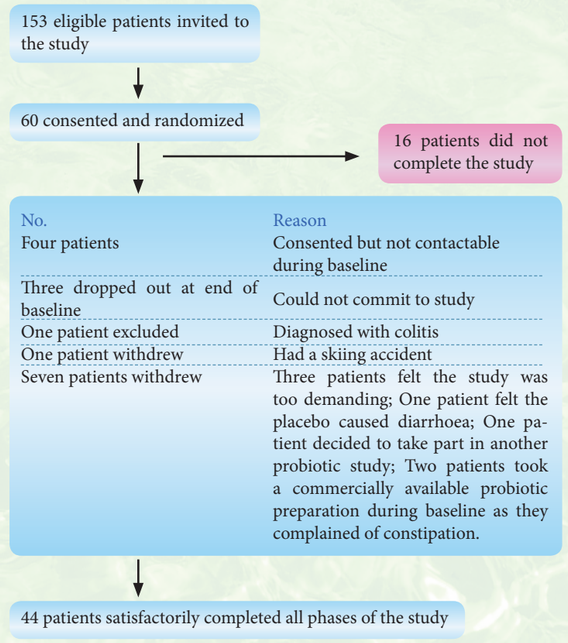

Figure 1. Flow diagram of participants

Baseline characteristics

Among the 44 subjects, 28 were female and 16 were male and as shown in Table 1, there was a female predominance in each treatment group. Subjects averaged 54 years in age (range, 20-79 years).

Categorization of subjects at baseline indicated that 23 (52%) had IBS-D, 9 (21%) IBS-C and 12 (27%) IBS-A. The gender and age of the patients are shown in Table 2.

Table 2. Gender and age of IBS patients recruited into the study

Gender and age	IBS-D (n = 23)		IBS-C (n = 9)		IBS-C (n = 9)	
Female (F) Male (M)	9F	14M	8F	1M	11F	1M
Age mean	44	47	42	61	56	61
(Standard Deviation)	(13.8)	(12.6)	(9.8)		(12.0)	

Sixteen of twenty three patients with IBS-D were randomized to receive prebiotic following placebo (3.5 g n = 8; 7.0 g n = 8). Seven patients were randomized to receive placebo for both treatment periods. Seven of nine patients in the IBS-C group received prebiotic (3.5 g n = 4; 7.0 g n = 3). Two patients were randomized to the placebo group. Seven of twelve patients in the IBS-A group received prebiotic (3.5 g n = 4; 7.0 g n = 3) and five were randomized to the placebo group.

Table 3. Log10 numbers of the monitored bacterial groups in the faecal microflora of the volunteers at the beginning of each treatment

	Group I (n = 16)		Group II (n = 14)		Group III (n = 14)	
	Placebo/Prebiotic 3.5 g		Placebo/Prebiotic 7.0 g		Placebo/Placebo	
Bacterial group	Placebo (week 6)	Prebiotic (week 12)	Placebo (week 6)	Prebiotic (week 12)	Placebo (week 6)	Placebo (week 12)
Total	10.51 0.34	10.59 0.24	10.50 0.25	10.56 0.26	10.51 0.31	10.46 0.35
Bifidobacterium spp.	9.00 0.32	9.10 0.33	8.96 0.31	9.03 0.42	8.97 0.33	8.94 0.31
Lactobacillus-Enterococcus spp.	8.46 0.25	8.57 0.23	8.36 0.22	8.48 0.22	8.52 0.19	8.44 0.28
Clostridium perfringens-hystolyticum subgroup	8.52 0.29	8.60 0.29	8.56 0.28	8.58 0.32	8.62 0.36	8.56 0.31
Eubacterium rectale/C.coccoides	9.54 0.31	9.59 0.24	9.51 0.29	9.51 0.36	9.57 0.33	9.47 0.29
Bacteroides-Prevotella spp.	9.44 0.32	9.52 0.25	9.41 0.21	9.51 0.23	9.45 0.29	9.41 0.32
E.coli	7.62 0.29	7.73 0.28	7.5 0.29	7.63 0.22	7.58 0.32	7.63 0.27
Desulfovibrio spp.	7.84 0.30	7.93 0.21	7.78 0.31	7.82 0.23	7.86 0.30	7.88 0.25

Table 4. Bacterial proportion of the monitored faecal microflorazomponents during the treatments used for IBS patients

		Group I (n = 16)		Group II (n = 14)		Group III (n = 14)	
		Placebo/Prebiotic 3.5 g		Placebo/Prebiotic 7.0 g		Placebo/Placebo	
Bacterial group		Placebo	Prebiotic	Placebo	Prebiotic	Placebo	Placebo
Bifidobacterium spp.	b	3.08 0.31	3.25 0.51	2.90 0.42	3.01 0.38	2.85 0.54	2.99 0.45
	e	3.42 0.29	5.51 0.43^{**,b}	2.94 0.38^{a}	7.48 0.59^{***,c}	2.90 0.67	2.95 0.27
Lactobacillus-Enterococcus spp.	b	0.89 0.12	0.95 0.17	0.74 0.57	0.85 0.75	1.00 0.15	0.95 0.44
	e	0.99 0.11	1.19 0.10	0.81 0.77	1.16 0.67	0.96 0.23	1.04 0.33
Clostridium perfringens-hystolyticum subgroup	b	1.02 0.10	1.03 0.08	1.15 0.13	1.07 0.18	1.26 0.16	1.24 0.29
	e	0.95 0.28^{a,b}	0.71 0.15^{b}	1.19 0.28^{a}	0.76 0.09^{*,b}	1.01 0.17^{a}	1.38 0.35^{a}
Eubacterium rectale/C.coccoides	b	10.79 1.21^{a,b}	9.95 0.76^{a,b}	10.19 1.42^{a,b}	9.07 0.74^{a}	11.47 1.32^{b}	10.07 1.03^{a,b}
	e	10.32 0.99^{a}	13.12 1.21^{**,b}	9.32 1.01^{a}	10.16 0.96^{a}	10.94 0.89^{a}	11.91 0.78^{a}
Bacteroides-Prevotella spp.	b	8.57 0.95	8.56 0.06	8.16 0.63	8.93 0.93	8.51 0.64	8.95 0.72
	e	8.59 0.69^{a}	8.03 0.73^{a}	8.67 0.59^{a}	5.75 0.75^{**,b}	8.01 0.59^{a}	9.50 0.67^{a}
E.coli	b	0.13 0.03	0.14 0.02	0.10 0.01	0.12 0.02	0.12 0.02	0.15 0.02
	e	0.16 0.02^{a}	0.14 0.01^{a}	0.11 0.02^{a,b}	0.09 0.02^{b}	0.13 0.01^{a}	0.15 0.02^{a}
Desulfovibrio spp.	b	0.21 0.03	0.22 0.03	0.19 0.02	0.18 0.01	0.22 0.02	0.26 0.01
	e	0.24 0.03^{a}	0.24 0.04^{a}	0.18 0.01^{b}	0.16 0.01^{b}	0.24 0.02^{a}	0.27 0.02^{a}

b, baseline; e, end of 4-week treatment.
Values are means s.d.
* Indicates difference from beginning of treatment ($P < 0.05$).
** Indicates difference from beginning of treatment ($P < 0.005$).
*** Indicates difference from the beginning of the treatment ($P < 0.001$). (paired t-tests).
Mean values (s.d.) with unlike superscript letters within each row were significantly different ($P < 0.05$).
The monitored bacterial group ratios were analysed by ANOVA using three fixed effect (treatment, period of treatment, sequence of treatment).

Bacteriology

Total bacterial counts and individual bacterial groups were determined by FISH. As shown in Table 3, there were no differences in the numbers of bacteria in any of the bacterial groups at the beginning of the placebo and prebiotic treatment periods. The relative proportions of each bacterial group assessed at baseline and at the end of each 4-week treatment period taking into account changes in total bacterial numbers are shown in Table 4. With one exception, group proportions at the beginning of the placebo treatment periods were similar to the group proportions at the beginning of the prebiotic treatment periods. In group 3 patients (placebo/placebo), E. rectale/C. cocciodes proportions were lower ($P < 0.05$) at the start of the second placebo treatment period compared with the first.

Four weeks treatment with placebo did not change proportions of bacteria in any of the groups assessed. In contrast, prebiotic treatment resulted in significantly higher proportions of bifidobacterium spp. ($P < 0.05$ and $P < 0.001$, for 3.5 and 7.0 g/d doses respectively). There were significantly lower proportions of C.perfringenssubgroup histolyticum ($P < 0.05$) and Bacteroides/Prevotella spp. ($P < 0.005$) after administration of 7.0 g/d prebiotic and higher proportions of E.rectale/C.coccoides after administration of 3.5 g/d ($P < 0.005$).

Compared to the effects of 4 weeks treatment with placebo, the prebiotic treatment had a clear and significant effect on the relative proportions of Bifidobacterium spp. after administration of both 3.5 ($P < 0.005$) and 7 g ($P < 0.001$) and it was also significantly ($P < 0.05$) higher with the higher dose of a pre-biotic. The higher dose of the prebiotic also resulted in the lower proportion of C.perfringens subgroup histolyticum and Bacteroides-Prevotella spp. ($P < 0.05$ in both cases) when compared with the placebo in the same treatment group and a higher proportion of E.rectale/C.coccoides spp. was achieved with the lower prebiotic dose ($P < 0.05$).

Table 5. Mean scores for efficacy variables during the treatments used for the IBS patients

		Group I (n = 16)		Group II (n = 14)		Group III (n = 14)	
		Placebo/Prebiotic 3.5 g		Placebo/Prebiotic 7.0 g		Placebo/Placebo	
		Placebo	Prebiotic	Placebo	Prebiotic	Placebo	Placebo
Defecation No/d	b	2.7 1.3	2.1 1.1	2.4 1.3	1.6 1.1	2.0 1.1	2.5 0.9
	e	2.1 1.3	1.8 1.1	1.9 1.1	1.8 1.4	2.1 0.9	2.0 1.1
Stool consistency	b	3.5 1.4	4.4 1.4	4.0 1.1	4.6 1.1	4.6 1.1	4.3 0.8
	e	4.1 1.4^{*,b}	3.8 1.0^{*,b,a}	3.4 1.3^{a}	4.3 1.1^{b}	4.3 1.1^{b}	4.1 1.1^{b}
Flatulence	b	1.8 0.8^{b}	2.0 0.6	2.1 0.8^{b}	1.6 0.9^{a}	2.0 0.6^{b}	1.9 0.7^{a}
	e	1.8 0.7^{b}	1.3 0.6^{*a}	1.5 0.9^{*a,b}	1.5 1.0^{a,b}	1.7 0.7b	1.7 0.7^{*b}
Abdominal Pain	b	4.2 2.0	3.5 2.2	3.6 2.6	2.9 2.5	3.0 2.3	3.5 2.1
	e	2.4 2.1^{*a,b}	1.9 1.7^{a}	3.1 2.5^{a,b}	2.6 2.1^{a}	3.6 1.5^{a,b}	3.4 1.9^{a,b}
Bloating	b	4.4 15^{b}	4.1 1.3^{b}	3.9 1.3^{a,b}	3.4 1.5^{a}	3.9 1.4^{a,b}	2.2 2.3^{a,b}
	e	3.9 1.1^{b}	2.8 0.9^{*a}	3.8 1.3^{b}	4.1 1.2^{b*}	3.8 1.4^{b}	3.5 1.6^{b}
Incomplete evacuation/urgency/straining	b	2.7 2.0	2.4 2.5	3.2 2.8	3.6 2.2	2.7 2.5	2.7 2.3
	e	2.0 1.9	1.8 1.7	3.6 2.3	2.6 2.2	2.9 2.6	2.6 2.2
Likert scale	b	11.1 5.3^{b}	9.9 6.2^{a}	10.2 6.3^{b}	8.2 5.7^{a}	10.4 4.6^{b}	9.4 4.2^{a}
	e	7.9 4.2^{*b,c}	6.2 4.3^{*a}	9.4 5.6^{c}	8.1 4.6^{ba}	10.1 4.4^{c}	7.7 5.4^{*c}
SGA	b	4.3 0.5	4.2 0.5	4.2 0.6	4.1 0.8	4.1 0.6	4.0 0.7
	e	4.0 0.7^{b}	3.1 0.8^{*a}	4.1 0.8^{b}	3.6 0.9^{*a,b}	4.1 0.7^{b}	3.5 0.5^{b}
HAD (A)	b	7.8 4.0	7.9 4.2	10.6 4.3	9.7 4.3	8.1 4.3	9.0 5.0
	e	7.5 4.4^{b}	8.1 3.8^{b}	9.7 4.3^{a}	7.8 4.6^{*b}	9.1 5.5^{b}	8.5 5.3^{b}
HAD (D)	b	5.8 3.9	5.3 2.7	7.9 3.3	6.7 5.8	6.9 4.1	6.9 4.8
	e	5.4 3.6^{a}	5.1 2.8^{a}	7.4 3.6^{b,c}	5.8 3.4^{a,b}	6.0 4.2^{b}	6.6 4.1^{b,c}
QOL	b	92.8 44.2^{b}	81.4 40.9^{a,b}	97.4 31.4^{b}	77.1 39.8^{a}	93.4 48.1^{b}	97.1 46.4^{b}
	e	89.4 41.4^{b,c}	79.8 40.4^{a,b}	91.1 34.8^{b,c}	74.3 41.6^{a}	98.5 48.1^{c}	97.0 49.2^{c}

b, baseline; e, end of 4-week treatment.
Values are means s.d.
* Indicates difference from beginning of treatment ($P < 0.05$). (Matched pairs t-tests).
Mean values (s.d.) with unlike superscript letters within each row were significantly different ($P < 0.05$).
The recorded scores were analysed by ANOVA using three fixed effect (volunteer, treatment, period of treatment).

Response to treatment (Table 5)

The clinical and psychological parameters were similar at the beginning of the placebo and prebiotic treatment periods. In group I patients, treatment with placebo resulted in a significant change in stool consistency ($P < 0.05$) and an improvement in abdominal pain ($P < 0.05$). In group II patients, treatment with placebo resulted in a significant improvement in flatulence ($P < 0.05$). Treatment with placebo (3.5 g/d, group I; 7.0 g/d group II; 7.0 g/d group II) was without effect on any of the other clinical parameters monitored and on none of the psychological parameters. Treatment of group I patients with 3.5 g/d prebiotic resulted in a significant change in stool consistency ($P < 0.05$), flatulence ($P < 0.05$) bloating ($P < 0.05$), Composite Likert scale ($P < 0.05$) and SGA ($P < 0.05$). Treatment of group II patients with 7.0 g/d prebiotic resulted in a significant improvement in SGA ($P < 0.05$) and anxiety (HAD[A]) scores ($P < 0.05$).

Compared with the effects of 4 weeks treatment with placebo, prebiotic treatment (3.5 g/d) had a significantly greater effect on flatulence ($P < 0.05$), bloating ($P < 0.05$), Composite Likert Scale ($P < 0.05$) and SGA ($P < 0.05$). Compared with the effects of a 4-week treatment with placebo, prebiotic treatment (7.0 g/d) had a significantly greater effect on composite Likert Scale ($P < 0.05$), anxiety scores ($P < 0.05$) and quality of life ($P < 0.05$).

Fibre intake

Mean daily fibre intakes during the treatment periods are shown in Table 6. During the course of all treatment periods in the three groups of patients, fibre intake remained unchanged. Neither placebo nor prebiotic treatment was associated with any significant change in fibre intake.

Adverse events

Three patients reported adverse events. One (IBS-D) reported an increase in severity of diarrhea while taking 3.5 g placebo. Symptoms were described as moderate and the patient withdrew from the study. Two patients reported nausea (1 patient 7.0 g placebo;1 patient 3.5 g prebiotic), described as mild events; the nausea lasted a mean of two days.

Table 6. Mean daily fibre intakes during the treatments used for the IBS patients (g/day)

		Group I (n = 16)				Group II (n = 14)				Group III (n = 14)			
		Placebo/Prebiotic 3.5 g				Placebo/Prebiotic 7.0 g				Placebo/Placebo			
		Placebo		Prebiotic		Placebo		Prebiotic		Placebo		Placebo	
Fibre	b	13.1	4.06	13.8	6.64	9.4	3.46	10.5	4.73	10.9	5.04	10.5	4.66
	e	13.7	5.6	14.3	6.74	10.6	4.89	9.9	3.75	8.6	3.29	9.11	3.82
Soluble Fibre	b	4.6	1.44	4.5	1.32	3.2	1.38	3.5	1.61	3.9	2.10	3.6	2.15
	e	4.3	1.23	4.3	1.68	3.6	1.78	3.4	1.56	2.9	1.27	3.4	2.02
Flatulence	b	3.0	1.59	2.6	1.16	1.9	1.01	2.2	1.51	2.3	1.78	2.3	1.63
	e	2.9	1.26	2.7	1.66	2.3	1.26	2.0	0.93	2.3	1.71	2.0	1.29

b, baseline; e, end of 4-week treatment.
Values are means s.d.

Discussion

The present study is the first to have been undertaken to investigate the potential efficacy of a prebiotic in the management of patients with IBS. The prebiotic consisting of a galactooligosaccharide mixture has been confirmed as being selective towards the beneficial genus effects of probiotic bifidobacteria in the management of IBS.[21-23] The test strains were shown not only to improve abdominal symptoms[21-23] but also provided an insight into possible mechanisms of action in that one study demonstrated a pro-inflammatory THI-like cytokine IL-10/IL-12 ratio in the peripheral blood of the IBS patients that was normalized by Bifidobacterium infantis treatment.[21] Importantly, in a more recent study, treatment with fermented milk containing Bifidobacterium animals resulted in a significant improvement in health related quality of life.[23]

Orally applied prebiotics have also been associated with immune-modulatory effects on parameters of the innate and specific immunity, mostly in animal experiments or in vitro. As prebiotics are fermented to various degree by the intestinal beneficial bacteria (e.g. bifidobacteria, lactobacilli), the immune-modulatory properties have mainly been attributed to a microflora dependent effect. However, specific prebiotics have also been reported to bind to specific receptors on cells of the immune system suggesting that a direct interaction between prebiotics and the host is in place.[40] One of the important findings of our study is that the prebiotic was shown to have a bifidobacterial enhancing effect in the IBS patients at both doses administered, with levels of bifidobacteria becoming similar to those of healthy humans.[25] In addition, at the 3.5 g daily dose, the numbers of Eubacterium rectale/C.coccoides were increased and at the 7.0 g daily dose, those of Clostridium perfringens subgroup histolyticum and Bacteroides-Prevotella Spp. were reduced. Whether IBS is accompanied by quantitative or qualitative changes in the bacterial flora of the large intestine remains a contentious issue. Using older microbiological culture techniques, a qualitative alteration in the faecal flora was found[41-43] as was increased colonic fermentation in some patients.[17-23] Recently, it has been reported that there are higher numbers of bacteroides and lower numbers of bifidobacteria in the mucosa associated microflora of IBS sufferers.[44] Further studies using the more advanced analytical technique such as used here are clearly required to characterize in more detail possible quantitative or qualitative changes in colonic bacterial flora of different subgroups of IBS patients.

Treatment with the prebiotic, which induced qualitative changes in the faecal flora, was associated with significant changes in stool consistency, flatulence, composite scores (abdominal pain/discomfort, bloating/distension and bowel movement difficulty) as well as subjective global assessment values in our patients. Our results are thus reminiscent of the beneficial clinical effects obtained when treating IBS patients with Bifidobacterium spp. containing probiotics.[21-23] The lower flatulence production seen in the prebiotic treatment arm may have been due to the increased bifidobacterial load, as these are not a gas producing genus of gut bacteria.[45] The IBD-D patients were the only subgroup to have clinically significant anxiety (HAD (A)>11). In these, anxiety scores were significantly reduced during treatment with 7.0 g/d prebiotic and in these patients, quality of life also significantly improved.

One of the objectives of this study was to try and obtain some information about what might constitute an optimum dose of prebiotic. Towards this end, the bacteriological data suggest that the 7.0 g rather than3.5 g daily dose had the best prebiotic effect on the faecal microflora. On the other hand, from the clinical standpoint, the 3.5 g/d dose was overall more effective than the 7.0 g/d dose, although both doses showed improvements. There is no clear explanation yet to explain those differences. It is of some interest that in the dose comparison of the probiotic Bifidobacterium infantis 35624 in IBS patients, a dose of 1 x $10^{[8]}$ CFU provided the greatest benefit, whereas the higher 1-$10^{[10]}$ CFU dose was without effect as compared to placebo.[22] This lack of effect was ascribed to the difficulties in "packaging" the higher dose of probiotic.[22,46]

The possibility might exist that in both that probiotic study[22] and in the present prebiotic study, the lack of effects of the higher doses were real ones.

Both doses were well tolerated and no adverse side effects were reported by any of the patients. The study provides no information, however, as to how long treatment might have to be continued nor information as to how long the alterations in bacteriological milieu might last after discontinuation of treatment. It was because of the known carry over effect of prebiotic treatments on the faecal bacteriology in cross over studies, that our patients always received the placebo treatment before the probiotic.

IBS is a very common disorder[1,2] with many patients suffering chronic abdominal symptoms[3] and poor quality of life.[4] Treatment remains problematic for many patients, particularly since the recent withdrawal of clinically proven receptor active drugs.[15]

Although the results of this study point to the need for multicentre, randomized, controlled, clinical trials of large numbers of patients to assess more formally the efficacy of prebiotic interventions in IBS patients, our data do support the concept that dietary intervention directed towards gut microbiota modulation represents a significant step forward in therapy.

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A crucial antioxidant mechanism by nutraceuticals: Glutathione antioxidant response/glutathione redox cycling†

Abstract

The "Mitochondrial Free Radical Theory of Aging" hypothesizes that reactive oxygen species (ROS) arising from aged and/or defective mitochondria are associated with the pathogenesis of various age-related diseases. The glutathione antioxidant response, in particular glutathione redox cycling, is a critical mechanism for protection against ROS-induced cell death. Over the past few decades, a number of phytochemicals [such as curcumin, epigallocatechin gallate (EGCG), resveratrol and schisandrin B (Sch B)], which all possess the ability to elicit a nuclear factor (erythroid-derived 2)-like 2 (Nrf2)-mediated antioxidant response, have been identified. Despite the fact that these phytochemicals can produce cyto/tissue protection against oxidant-induced injury in various types of cultured cells/rodent tissues, the underlying protective mechanism can vary. While curcumin, EGCG and resveratrol likely confer cytoprotection via the activation of glutathione S-transferase and glutathione peroxidase, Sch B is thought to produce its protective effect via the induction of glutathione redox cycling, which is of primary importance in preventing cell death. The differences in structures of phytochemicals could produce different degrees of Nrf2 activation and antioxidant gene expression. In the hope of developing safe and effective interventions for protection against oxidant-induced injuries, further studies are required to define the protective mechanism(s), particularly the array of antioxidant enzyme expressions, induced by the various phytochemicals.

Oxidative stress and oxidative stress-related diseases

Mitochondria, the powerhouse of the cell, are involved in metabolic processes of energy production in cells under aerobic condition. At the same time, reactive oxygen species (ROS) are inevitably generated as a by-product. The "Mitochondrial Free Radical Theory of Aging" (MFRTA) hypothesizes that the gradual and continuous production of ROS in mitochondria can cause oxidative lesions in mitochondrial DNA (mtDNA), with resultant mitochondrial dysfunction[1]. Defects in mitochondria can further potentiate the generation of ROS, leading to catastrophic and irreversible damage to the cell. Over the past few decades, oxidative stress has been found to be associated with the pathogenesis of a number of diseases, e.g. cancers, metabolic disorders and neurodegenerative diseases[2-4]. A recent review updating the MFRTA has suggested that a low generation rate of endogenous damage and the presence of macromolecules with high resistance to oxidative modification in mitochondria are likely to increase the longevity of organisms[5]. It therefore follows that the capability of aerobic organisms to cope with unavoidable oxidative challenge is of fundamental importance.

Components and physiological functions of the glutathione antioxidant system

In order to counteract the oxidant-induced injury, all cells are equipped with an antioxidant system. As the chemistry theory goes, the reversal change of oxidation is defined as a reduction reaction. As such, oxidized biomolecules can be reduced by cellular antioxidants with themselves being oxidized. Given that reduction and oxidation occur simultaneously, the co-occurrence of reduction and oxidation is termed redox reaction. Glutathione, a tripeptide containing glutamic acid, cysteine and glycine, accounts for approximately 90% of non-protein low molecular weight thiols in mammalian tissues[6]. The thiol group in the glutathione molecule can serve as an antioxidant moiety to reduce the ROS. As such, reduced glutathione (GSH) is oxidized to form oxidized glutathione (GSSG). Glutathione therefore exists in two redox isoforms - namely, the thiol-reduced form (GSH) and the disulfide-oxidized (GSSG) form. As a primary antioxidant in the cell, glutathione actively participates in antioxidant defense processes in various ways. Firstly, the free radical scavenging activity of GSH is attributable to the thiol group of its cysteine residue[7]. Secondly, the higher reducing potential of GSH also enables the reduction of dehydroascorbate (the oxidized form of ascorbate) to ascorbate (the active form of vitamin C)[8], which in turn regenerates the phenoxyl radical of α-tocopherol back to α-tocopherol (the active form of vitamin E) in cell membranes[9]. The GSH-coupled redox reactions ensure the efficient regulation of cellular antioxidant capacity via the maintenance of an optimal GSH/GSSG ratio. Finally, GSH serves as a co-substrate of several antioxidant enzymes – notably, glutathione S-transferase (GST) and selenium-glutathione peroxidase (GPx). While GPx catalyzes the reduction of both organic and inorganic hydroperoxides into their corresponding alcohols at the expense of GSH, GST inactivates electrophiles by catalyzing the transfer of a glutathiolate group to their reactive molecular moieties.

To maintain the high efficiency of both non-enzymatic and enzymatic antioxidant networks, the availability of GSH is essential. While GSH is synthesized in a two-step reaction catalyzed by γ-glutamyl cysteine ligase (γ-GCL) and glutathione synthetase (GS), GSSG can be regenerated by reduction to GSH by glutathione reductase (GR) at the expense of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH). NADPH is produced in the pentose phosphate pathway [in which glucose-6-phosphate dehydrogenase (G6PDH) is the enzyme catalyzing the rate-limiting step] or isocitrate dehydrogenase II (ICDH2)-catalyzed reactions. Glutathione and glutathione-related enzymes, which are collectively referred to as the "glutathione antioxidant system" (Figure 1), provide a generalized protection against oxidative stress in cytosolic and mitochondrial compartments.

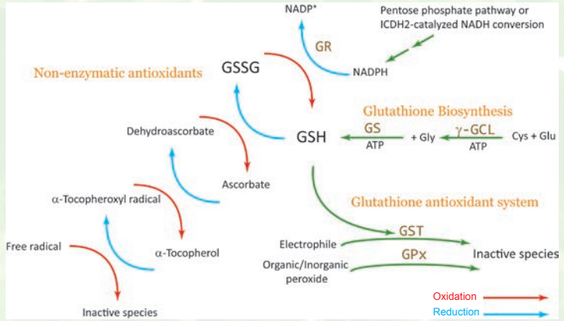

Figure 1. The pivotal role of GSH in glutathione redox cycling and the glutathione antioxidant system2.

Abbreviations: ATP, Adenosine triphosphate; Cys, cysteine; Glu, glutamate; Gly, glycine; GSH, reduced glutathione; GSSG, glutathione disulfide (or oxidized glutathione; GR, glutathione reductase; γ-GCL, γ-glutamate–cysteine ligase, GS, glutathione synthetase; GST, glutathione S-transferase; GPx, glutathione peroxidase; NADH, reduced nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NADP+, oxidized nicotinamide adenine dinucleotide phosphate.

Regulation/Induction of the glutathione antioxidant system – Nrf2/Keap1 redox signaling

Despite the fact that an excessive production of ROS is deleterious, accumulating evidence suggests an important physiological role of ROS as signal transduction molecules (particularly at very low concentration)[10]. The redox-sensitive nuclear factor (erythroid-derived 2)-like 2 (Nrf2)/antioxidant response element pathway, which induces a cytoprotective response against oxidative injury, is primarily activated by pro-oxidant or oxidants. Under normal (i.e., non-stressful) conditions, Nrf2, which is bound by its specific repressor, namely, Kelch-like ECH-associated protein 1 (Keap 1), in the cytosol, is subjected to degradation, resulting in inactivation[11-12]. As such, the dissociation of Nrf2 and Keap1 can promote the nuclear translocation of Nrf2 and the subsequent expression of antioxidant genes. In this respect, it has been shown that the translocation of Nrf2 from the cytosol to the nucleus is facilitated by phosphorylation of Nrf2[13] or oxidative modification of the cysteine residue of Keap1, which can dissociate Nrf2 from Keap1[14]. This leads to a subsequent enhancement in the expression of antioxidant defense genes, including the catalytic and modulatory subunit of γ-GCL, GR, GPx and GST, which encompass the glutathione antioxidant response[15].

Regulation of apoptosis by the glutathione antioxidant system

Apoptosis, also known as programmed cell death, is a highly regulated self-destructive process occurring in cells in response to various stress conditions in order to prevent further damage to the surrounding cells or tissues. Mitochondrial glutathione redox status (i.e. GSH/GSSG ratio) is the determining factor for the activation of apoptosis, as evidenced by a study showing that a transient and rapid oxidation of GSH (i.e. a decrease in the ratio of GSH to GSSG) is sufficient to trigger a mitochondrial apoptotic pathway, which cannot be reversed by a subsequent restoration of glutathione redox status[16]. The depletion of cellular GSH can lead to apoptosis, presumably via the activation of mitogen-activated protein kinase cascades, as demonstrated in various cell models[17-18]. In addition, the depletion of mitochondrial GSH was found to be associated with a range of apoptotic stimuli[19-22]. Therefore, the enhancement of mitochondrial/cellular/tissue GSH recovery capacity during the early phase of oxidative stress represents an effective process in the protection against oxidative stress-induced cell apoptosis.

The reservoirs of GSH in mitochondria and the cytosol serve as critical determinants in maintaining an optimal glutathione redox status. Cellular levels of GSH are determined by two reactions - namely, de novo GSH synthesis and GSH regeneration. The biosynthesis of GSH occurs in the cytosol of all cell types[23]. It involves two anabolic reactions which are catalyzed by 2 ATP-dependent enzymes, namely, γ-GCL and glutathione synthetase. The first step, which is catalyzed by γ-GCL, is considered to be the rate-limiting step in GSH synthesis[24]. GSH regeneration, in which GSSG is reduced into GSH at the expense of NADPH, is catalyzed by GR. Nrf2-regulated glutathione redox cycling, rather than GSH biosynthesis, was found to be crucial for cell survival during oxidative stress[25]. Therefore, the most effective way to confer protection against oxidant-induced cell injury is the enhancement of GSH recovery capacity[26], which is presumably mediated by the induction of Nrf2-regulated cytoprotective proteins such as GR and NADPH-producing enzymes via a redox signaling pathway.

Phytochemicals as inducers of the glutathione antioxidant response and their cytoprotective effects

In view of the increased morbidity and mortality associated with oxidative stress-related diseases, antioxidant interventions are urgently being sought. In an effort to develop safe and effective therapies for oxidant-induced injuries, the use of phytochemicals, with their ability to activate the Nrf2-mediated antioxidant response, has attracted a great deal of interest [27]. It has been suggested that phytochemicals can activate Nrf2/Keap1. In this review, various well-known Nrf2 inducers isolated from plants will be

discussed in relation to their ability to induce a glutathione antioxidant response.

Curcumin

Curcumin is one of the principal curcuminoids obtained from the root of Curcuma longa that has been widely used in ancient Ayurvedic medicine (Figure 2). The ability of curcumin to activate Nrf2 is likely attributed to its electrophilic properties and its capacity to modify critical cysteine residues on Keap1. Recent studies have demonstrated that curcumin induces an antioxidant response via the Nrf2 pathway[28-31]. In this regard, curcumin has been shown to attenuate the extent of GSH depletion via the induction of Nrf2 in various experimental settings, such as glucose oxidase-induced insulin resistance in cultured human hepatocytes[28], high fat diet-induced non-alcoholic steatohepatitis in rats[29] and arsenic-induced hepatotoxicity in mice[30]. Curcumin has also been shown to increase the activity of GST in HepG2 human hepatocytes[31]. The ability of curcumin to increase cellular GSH levels under conditions of oxidative stress was demonstrated in cultured astrocytes[32] and in isolated rat cerebellar granule neurons[33]. The neuroprotection afforded by curcumin was further demonstrated in quinolinic acid-induced neurotoxicity in rats, through the induction of Nrf2 and the activation of GPx[34].

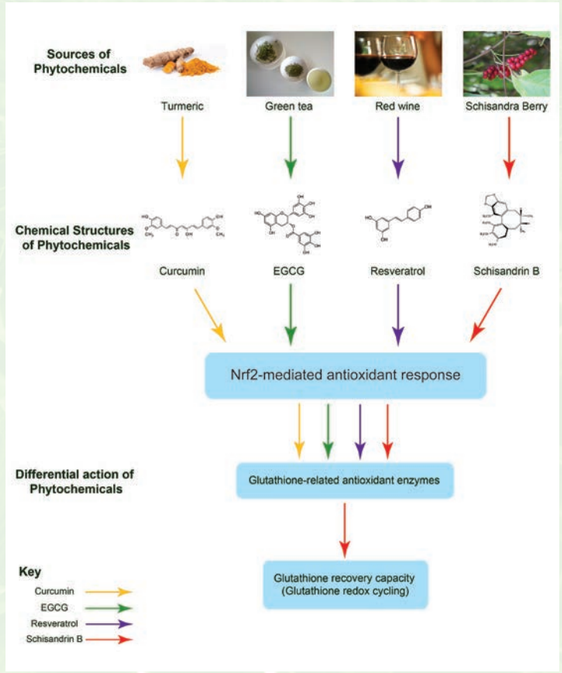

Figure 2. Sources, chemical structures and the differential actions of various Nrf2 inducers isolated from plants.

Abbreviations: EGCG, epigallocatechin gallate; Nrf2, nuclear factor (erythroid-derived 2)-like 2. Sources of figures: The photo of turmeric: by Simon A. Eugster - Own work, CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=31748137; the photo of green tea: By Alessandro Martini - donated by the photographer for sharing, CC0, https://commons.wikimedia.org/w/index.php?curid=15435152; the photo of red wine: By Hungry Girl - originally posted to Flickr as Glass of wine, CC BY 2.0, https://commons.wikimedia.org/w/index.php?curid=5235385; the photo of Schisandrae Fructus: by Vladimir Kosolapov - Own work, CC BY 3.0, https://commons.wikimedia.org/w/index.php?curid=7753805

Epigallocatechin gallate

Epigallocatechin gallate (EGCG), which is the principal active catechin found in green tea, can induce Nrf2 and elicit an antioxidant response (Figure 2)[35-37]. EGCG can confer hepatoprotection against oxidant injury by increasing hepatic GSH levels and GPx activity in concanavalin A-intoxicated mice[38] and bile duct-ligated mice[39]. Cardioprotection against doxorubicin toxicity in mice[40] and neuroprotection against ischemia/reperfusion injury in rat cerebrum[37] afforded by EGCG were associated with the activation of Nrf2 and increases in γ-GCL activity and GSH levels. EGCG can induce the Nrf2-mediated expression of γ-GCL and GPx, with resultant protection against crescentic glomerulonephritis in mice[36]. EGCG has also been shown to increase GSH levels and GPx activity in cisplatin-induced nephrotoxicity in mice[41].

Resveratrol

Resveratrol is a stilbene present in grape skins, peanuts as well as in red wine (Figure 2). Resveratrol was found to increase the Nrf2-mediated expression of GPx and GST in isolated rat hepatocytes[42] and in diabetic rat livers[43]. Resveratrol can also protect against arsenic trioxide-induced cardiotoxicity[44] and myocardial ischemia/reperfusion injury[45] in rats, with associated increases in Nrf2 nuclear translocation, glutathione redox ratios and GPx activity. The nephroprotection afforded by resveratrol in streptozotocin-induced diabetic rats[46] and in young spontaneously hypertensive rats[47] correlated well with the induction of the Nrf2-mediated expression of GST.

Schisandrin B

Schisandrin B (Sch B) is the most abundant dibenzocyclooctadiene lignan found in the fruit of Schisandra chinensis (Figure 2), a Chinese herb traditionally used for the treatment of hepatitis[48]. Recent studies have demonstrated that Sch B can elicit a glutathione antioxidant response via the ERK/Nrf2/ARE pathway[49-50]. The ability of Sch B to activate Nrf2 seems to be related to the transformation of Sch B in cytochrome P450-catalyzed reactions[51]. The resultant metabolite of Sch B can then be oxidized into a corresponding quinone that may give rise to the generation of ROS or modify Keap1, leading to the activation of Nrf2. In this regard, Sch B was found to induce a glutathione antioxidant response (including an elevation in cellular/mitochondrial GSH levels and increased expression of GR, γ-GCL and G6PDH) in rodent brain, heart and liver in vitro and in vivo, with associated neuro/cardiac/hepatic protection against oxidant-induced injuries[49-55].

Comparison among various Nrf2-inducing phytochemicals in the induction of the glutathione antioxidant response

Results concerning the ability of phytochemicals to elicit a Nrf2-mediated glutathione antioxidant response vary among studies, presumably due to variations in experimental conditions, e.g., differences in concentration(s)/dose(s) and cell types being used. In this regard, our laboratory has conducted comparative studies of the four aforementioned phytochemicals with respect to their ability to induce a glutathione antioxidant response in vitro and in vivo, using the same concentrations and/or dose[52,56]. The neuroprotective effects of curcumin, EGCG, resveratrol and Sch B in β-amyloid-intoxicated human neuroblastoma SH-SY5Y cells were investigated[52]. While curcumin and resveratrol did not activate the glutathione antioxidant response or protect neuronal cells against β-amyloid-induced apoptosis, EGCG and Sch B did afford protection, although the mechanisms differed. In this model of injury, EGCG did not activate Nrf2, GR or G6PDH, so that its attenuation of the oxidant-induced depletion of GSH was presumably due to its free radical scavenging activity. In addition to activating Nrf2 and GR, Sch B also elevated G6DPH activity, which would sustain the generation of NADPH for the efficient GR-catalyzed regeneration of GSH from GSSG, with a consequent attenuation of oxidant-induced GSH depletion, resulting in neuroprotection. As the bioavailability of various phytochemicals in vivo may vary, the protective effects of long-term, low-dose oral treatment with curcumin, EGCG, resveratrol and Sch B on oxidant injury was also investigated in rat heart and liver in relation to their ability to increase glutathione recovery capacity[56]. Among the tested phytochemicals, Sch B and resveratrol (but not curcumin or EGCG) could protect the liver against carbon tetrachloride toxicity, whereas treatment with Sch B or curcumin (but not EGCG or resveratrol) conferred cardioprotection against ischemia/reperfusion injury in rats. When the effects of phytochemicals on glutathione recovery capacity in rat heart and liver were compared, only Sch B (but not curcumin, EGCG or resveratrol) increased glutathione recovery capacity in tert-butyl hydroperoxide-challenged heart and liver homogenates. Results obtained from these studies strongly suggest a correlation between the enhancement of glutathione recovery capacity and cyto/tissue protection against oxidant injury following Sch B incubation/treatment. Other phytochemicals also protect against oxidative injury but without an enhancement of glutathione recovery capacity, possibly through other protective mechanisms. It has been reported that the protection against oxidative stress afforded by the four tested phytochemicals was associated with increases in cellular GSH level as well as GST and GPx activities]. However, the effects of curcumin, EGCG and resveratrol on GR and G6PDH activities were not always reported. Conceivably, curcumin, EGCG and resveratrol might confer cyto/tissue protection against oxidative stress via a GST-catalyzed S-glutathionylation of important proteins against irreversible oxidative modification, a GST-catalyzed inactivation of electrophiles or a GPx-catalyzed reduction of organic/inorganic peroxides. In addition, many cellular antioxidant thiols require a reducing equivalent (i.e. NADPH) for the enzymatic regeneration of the respective reduced form. In this regard, the ability of phytochemicals to activate the pentose phosphate pathway in the cytosol or the ICDH2-catalyzed NADH conversion in mitochondria are crucial for conferring tolerance/resistance against acute oxidative stress. Consistent with this, Sch B can increase the activities of G6PDH and ICDH2 (as well as GR) in rodent hearts and livers [unpublished data], suggesting that the cyto/tissue protection afforded by Sch B may be mainly mediated by an enhancement of the glutathione recovery capacity. An in-depth study of the activation of an array of antioxidant genes by different phytochemicals remains to be conducted.

Despite the fact that the four tested phytochemicals are well-known Nrf2 inducers, they belong to different sub-categories of polyphenolics based on their chemical moieties. Given their different molecular structures, curcumin, EGCG, resveratrol and Sch B are likely metabolized by different CYP-catalyzed processes[57], with a resultant generation of stereo-specific electrophiles. The differing structures of the electrophiles might result in differential accessibility to the regulatory cysteine residue of Nrf2/Keap1, resulting in differing extents of Nrf2 activation and antioxidant gene expression. In this regard, the signaling ROS arising from the redox cycling of metabolites of various phytochemicals are likely to be primarily responsible for eliciting antioxidant response in vivo.

Conclusions

The induction of the glutathione antioxidant response, particularly the activation of glutathione redox cycling, plays an important role in protecting against oxidant-induced cell death. Despite the fact that a number of phytochemicals can elicit a Nrf2-mediated antioxidant response, the underlying protective mechanism of a given phytochemical limits its ability to protect against different types of oxidative stress and hence its potential therapeutic application. Sch B primarily induces the glutathione antioxidant response and has been shown to consistently protect against oxidant-induced cell death/injury in various in vitro and in vivo experimental systems, whereas curcumin, EGCG and resveratrol likely confer protection via other antioxidant/cellular actions. Further studies are required to define the protective mechanism, particularly the array of antioxidant enzyme expressions, induced by various phytochemicals. Hopefully, new therapeutic strategy using phytochemicals can be developed for safeguarding health and ameliorating the pathogenesis of oxidative stress-related diseases.

Abbreviations

EGCG, epigallocatechin gallate; GPx, glutathione peroxidase; GR, glutathione reductase; Grx, glutaredoxin; GSH, reduced glutathione; GSSG, Oxidized glutathione; GST, glutathione S-transferase; ICDH2, isocitrate dehydrogenase II; Keap1, Kelch-like ECH-associated protein 1; MFRTA, Mitochondrial Free Radical Theory of Aging; mtDNA, mitochondrial DNA; Nrf2, nuclear factor (erythroid-derived 2)-like 2; PSSG, mixed disulfide protein; ROS, reactive oxygen species; Sch B, Schisandrin B; γ-GCL, γ-glutamyl cysteine ligase.

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Molecular Links between Caloric Restriction and Sir2/ SIRT 1 Activation

Abstract

Ageing is the most significant risk factor for a range of prevalent diseases, including cancer, cardiovascular disease, and diabetes. Accordingly, interventions are needed for delaying or preventing disorders associated with the ageing process, i.e., promotion of healthy ageing. Calorie restriction is the only nongenetic and the most robust approach to slow the process of ageing in evolutionarily divergent species, ranging from yeasts, worms, and flies to mammals. Although it has been known for more than 80 years that calorie restriction increases lifespan, a mechanistic understanding of this phenomenon remains elusive. Yeast silent information regulator 2 (Sir2), the founding member of the sirtuin family of protein deacetylases, and its mammalian homologue Sir2-like protein 1 (SIRT1), have been suggested to promote survival and longevity of organisms. SIRT1 exerts protective effects against a number of age-associated disorders. Caloric restriction increases both Sir2 and SIRT1 activity. This review focuses on the mechanistic insights between caloric restriction and Sir2/SIRT1 activation. A number of molecular links, including nicotinamide adenine di-nucleotide, nicotinamide, biotin, and related metabolites, are suggested to be the most important conduits mediating caloric restriction-induced Sir2/SIRT1 activation and lifespan extension.

Introduction

Life expectancy has remarkably increased during the past century, due mainly to medical and pharmaceutical advances, which help to reduce life-threatening and ageing-associated diseases. For example, the discovery of antimicrobial therapy and vaccines resulted in a huge drop of infectious diseases and a major gain in life expectancy in 1900s. The modern drug development and better treatment contribute to over 50% decrease in death rates for heart disease and stroke since 1972. In 21st century, epidemics of chronic diseases, such as diabetes, obesity, cardiovascular disease, and cancer, are the targets of antiageing therapies. Increased lifespan and ageing-related problems have been brought to the forefront not only because of the financial burden to the health care and government pension system, but also due to the impacts on our society, family, and industry. In fact, many countries are facing the challenges to accommodate older workforce and to extend work lives. Innovations and new conceptions in medicine are inspiring hopes to break and further extend the biological limit of life expectancy, beyond the success achieved for lifespan extension since 1900s, especially to counteract the wide array of contemporary problems in the current century. In order for modern pharmaceutics to break the biological ageing barrier and to reach the ultimate goal of medicine, immortality, a thorough understanding of the biological basis of ageing and lifespan extension is critically urgent and important. Here, the biological pathways mediating caloric restriction-induced lifespan extension will be reviewed and discussed.

Caloric restriction and lifespan extension

The lower intake of calories, the longer lifespan can be achieved.

Caloric restriction without malnutrition is a nongenetic intervention that consistently promotes the extension of maximum lifespan in model organisms including yeast, worms, flies, mice, and nonhuman primates[1-3]. The effect can be robustly achieved by restricting up to half of the typical calorie intake in these model organisms, when malnutrition is avoided. The most striking benefit of caloric restriction is to prevent the development of a broad spectrum of ageing-associated pathological changes, such as tumorigenesis, immunosenescence and cardiometabolic disorders. In humans, long life expectancy of Okinawans is attributed to a low caloric intake and negative energy balance at younger ages, a life-long low body mass index, and a low risk of mortality from age-related diseases[4]. Optimal nutrient composition and feeding regimen of the lifespanextending diets are not yet established. There are also debates on whether the ingested energy, when expressed in per gram body weight of the organisms, is restricted, increased or remain similar during dietary restriction[5]. In fact, the antiageing effect of caloric restriction may be achieved through restriction of certain types of amino acids, carbohydrates, lipids, or vitamins. Thus, the term 'dietary restriction is increasingly utilized when describing limited food intake in relation to the extension of healthspan and lifespan.

The biological basis of caloric restriction remains poorly understood. The involvement of a single gene and pathway has been investigated in non-mammalian systems[3]. For example, removal of ethanol and/or acetic acid extends the chronological longevity (the survival of a population of nondividing cells) of the model organism yeast, whereas their replicative lifespan (the number of daughter cells generated by a single mother cell) is more sensitive to glucose restriction[6]. Down-regulation of Sch9, a serine-threonine kinase that shares high sequence identity with the mammalian Akt/ protein kinase B (PKB) and ribosomal protein S6 kinase (S6K), extends the chronological lifespan by up to 2-fold[7]. Reduction of the TOR complex 1 activity leads to an extension of yeast replicative lifespan that cannot be further promoted by caloric restriction[8]. In the fruit fly Drosophila, reduction of amino acid consumption, but not sugar intake, extends life span substantially with essential amino acids mediating most of the responses[9]. In mammals, although different nutrient contents are sensed by distinctive pathways; however, it is unlikely that one single pathway is responsible for the effect of caloric restriction. Restricted dietary intake triggers the inactivation or activation of a number of nutrient sensing pathways, including insulin-like growth factor (IGF)/insulin, mammalian target of rapamycin/ S6K, and silent information regulator 2 (Sir2)-like protein 1 (SIRT1) signaling pathways. These pathways are also involved in the antiageing effects of a number of chemical compounds and drugs (Figure 1).

In rodents, dietary restriction significantly delays the occurrence of many chronic diseases and increases life span by up to 60%[10]. Attenuated IGF-1 signaling mediates some of the antiageing effects. Excess nutrient intake activates the proageing IGF signaling pathway. Mice that are under restricted dietary intake display hypoinsulinemia, enhanced sensitivity to insulin and reduced glucose levels. In humans, dietary restriction provides similar metabolic and cardiovascular benefits as in rodents, but without reducing IGF-1 levels, unless protein intake is also reduced[11], suggesting that restriction of protein intake provides additional antiageing benefit. Older Okinawans consumed a diet with restricted calorie (10% to 15%) and low saturated fat content, but rich in functional foods (e.g., herbs or spices) that may mimic the biological effects of caloric restriction. However, the caloric restriction mimetics and the related nutrient sensing pathways remain to be characterized. In addition to longevity, caloric restriction leads to additional phenotypes, such as increased resistance to oxidative stress, enhanced repairing of DNA and protein damages, improved glucose homeostasis and insulin sensitivity, lowered serum glucose and cholesterol levels, decreased oxygen consumption and body temperature, all of which contribute to delayed onset of age-related diseases[10,12,13]. In the following part of this review, a more specific molecular mechanism involving caloric restriction-evoked activation of SIRT1 will be discussed.

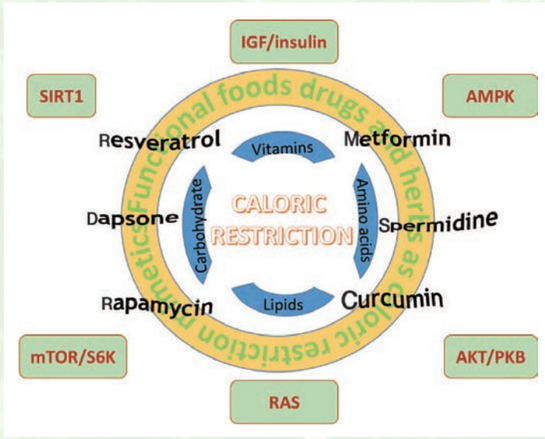

Figure 1. In response to different dietary intake, a number of nutrient sensing pathways are activated or inactivated to modulate the ageing process. IGF, insulin-like growth factor; SIRT1, Sir2-like protein 1; AMPK, AMP-activated protein kinase; mTOR/S6K, mammalian target of rapamycin/ribosomal protein S6 kinase; ROS, reactive oxygen species; AKT/PKB, AKT/ protein kinase B.

Sirtuins and caloric restriction

Sirtuins are a family of nicotinamide adenine dinucleotide (NAD)-dependent protein deacetylases[14]. Sir2, the first gene discovered in this family, was originally shown to regulate transcriptional silencing at cell-mating loci, telomeres, and ribosomal DNA (rDNA) in yeast, through deacetylation of the epsilon-amino groups of lysines in the amino-terminal domains of histones[15]. Sir2 cleaves the glycosidic bond between nicotinamide and adenosine diphosphate (ADP)-ribose in NAD and this reaction requires the presence of acetylated lysine[16,17]. Thus, one molecule of NAD and one molecule of acetyl-lysine are catalyzed to one molecule each of deacetylated lysine, nicotinamide and 0-acetyl-ADP-ribose. There are seven mammalian sirtuins, termed SIRT1-7, which share the sequence homology of catalytic domain with Sir2[18]. SIRT1 is the mammalian ortholog most highly related to Sir2. However, unlike the intra-nuclear localization of yeast Sir2, SIRT1 is not tightly bound to chromatin but shuttles between cytoplasm and nucleus[19]. Thus, in addition to histones, SIRT1 interacts with and mediates the deacetylation of a wide range of signaling molecules, including transcription factors, enzymes and tumor suppressors. The dynamic functions of SIRT1 are largely attributed to the additional domains at its NH2- and COOH-terminus, which allow the regulation of this protein by various post-translational modifications and protein-protein interactions[14,20].

During the past decade, sirtuins have attracted major attention due to their potentials of expanding life span in lower organisms and protecting against age-associated disorders in mammals. In yeast, integration of extra copies of Sir2 extends lifespan by up to 30% and deletion of this gene shortens life span by about 50%[21]. Calorie restriction by limiting glucose availability in the growth medium of the budding yeast Saccharomyces cerevisiae leads to the activation of Sir2 and the extension of replicative lifespan[22]. Sir2 mediates caloric restriction-induced lifespan extension, which requires NAD synthesis. Under conditions of reduced glucose, the metabolism of S. cerevisiae shifts from fermentation to respiration, resulting in elevated NAD or decreased NADH levels. In respiratory deficient yeast cells, on the other hand, caloric restriction could not increase Sir2 activity, but extends the replicative lifespan independent of Sir2[23]. Moreover, the chronological lifespan of S. cerevisiae is not affected by Sir2 deficiency[24,25]. In some long-lived yeast mutants, deletion of Sir2 enhances chronological longevity extension by caloric restriction[24]. Thus, Sir2 regulates longevity in yeast through a pathway related to caloric restriction, but a direct link between these two anti-ageing factors has not been firmly demonstrated. The localization of Sir2 to certain age-related loci, such as the rDNA repeats, promotes its lifespan-sustaining function during caloric restriction[26]. On the other hand, Sir2 function is not a limiting factor for chronological ageing [27].

The role of Sir2 in caloric restriction-induced lifespan extension has subsequently been confirmed in Caenorhabditis elegans and Drosophila melanogaster[28,29]. Unlike yeast, most cells in these fully grown and multicellular metazoan organisms are nondividing. Sir2 mediates the beneficial effects of caloric restriction via mechanisms involving metabolic control and stress responses to genotoxicity, heat shock, and oxidative damage[30,31]. Sir2 promotes genomic silencing either by repressing genomic instability or by preventing inappropriate gene expressions. In mammals, increased SIRT1 expression and function contributes to the beneficial effects of caloric restriction on delaying the onset of age-associated diseases, including cancer, atherosclerosis, and diabetes[32,33]. Mice lacking both copies of SIRT1 fail to show an increased activity and extended lifespan in response to caloric restriction, but display a shorter median lifespan than wild type mice[34,35]. Mice with elevated SIRT1 expression exhibit a beneficial phenotype resembling that of caloric restriction: they are leaner, more metabolically active, more glucose tolerant, and have reduced levels of circulating cholesterol, proinflammatory adipokines, insulin, and fasting glucose[36]. However, mice lacking one allele of SIRT1 still show identical lifespan to that observed in wild-type mice, when subjected to caloric restriction[37]. Small molecule activators of SIRT1 replicate signaling pathways triggered by calorie restriction[38]. SIRT1 plays an important role in adjusting the metabolic processes during caloric restriction, thus having been regarded

as a metabolic regulator of energy homeostasis[14,39-41]. Importantly, SIRT1 is modulated by caloric restriction in a tissue-specific manner[42]. The activity and expression of SIRT1 in liver is reduced by caloric restriction, while those of white adipose tissue and skeletal muscle are enhanced. The systemic regulation of mammalian SIRT1 is mediated, in part, by insulin and IGF-1, two serum factors negatively involved in life-span regulation[32]. It is tentative to speculate that in tissues containing rapidly dividing cells, caloric restriction-induced SIRT 1 expression shifts the balance away from cell death toward cell survival and/or regeneration, whereas in tissues containing mainly postmitotic cells, SIRT1 promotes longevity by regulating metabolic shift from using different carbon source of nutrients.

Mechanistic insights of SIRT1 activation by caloric restriction

When cells have high levels of calories, a substantial portion of the NAD pool is recruited into a high carbon flow of glycolysis by the enzyme glyceraldehyde-3-phosphate dehydrogenase. When calories are restricted, more carbons are oxidized in mitochondria via the electron transport chain-mediated cellular respiration, which produces NAD from NADH[43,44]. Thus, under caloric restriction, the NADH levels are significantly decreased as a result of up-regulated mitochondrial respiration[45,46]. Sir2 depletion does not affect caloric restriction-induced elevation of the intracellular NAD/NADH ratios in yeast. Because the inner membrane of mitochondria is impermeable to NADH and NAD, the malate-aspartate shuttle is used for translocating electrons produced during glycolysis for oxidative phosphorylation. This allows the hydrogen ions of NADH produced in the cytosol to reach the electron transport chain in the mitochondria. Over-expression of the malate-aspartate NADH shuttle components extends yeast replicative life span in a Sir2-dependent manner[47]. Consistently, over-expression of the mitochondrial NADH dehydrogenase specifically lowers NADH levels and extends lifespan[45]. The major modification catalyzed by Sir2/SIRT1 is deacetylation. NADH, nicotinamide adenine dinucleotide phosphate (NADP), or NADPH could not substitute NAD for this reaction. However, NAD levels do not correlate with the lifespan of yeast[46]. During calorie restriction, the NAD levels in yeast are actually decreased, indicating that Sir2 is not primarily regulated by the availability of NAD. In this regard, the deacetylase activity of Sir2 is closely linked to the decreased NADH, as the latter is a competitive inhibitor of Sir2[45]. However, overexpressing the NADH oxidase or alternative oxidase, both of which increase NADH oxidation, could not alter the life span of the wild type yeasts[6]. These information suggest that increased respiration plays a major role in lifespan extension by caloric restriction in yeast. Sir2 acts to facilitate this process by detoxifying oxidized macromolecules, including nucleic acids, proteins and lipids. However, the activity of Sir2 and SIRT1 are not affected by physiological alterations in the NAD/NADH ratio[46].

Unlike mitochondria, the nuclear envelope is permeable to a wide variety of small molecules[48], suggesting that cellular perturbations of NAD/NADH affect their levels in cytoplasmic as well as in nuclear compartments. NAD and NADH in nuclei play active roles in regulating gene transcription and genome stability[49]. Genotoxic stress depletes the nuclear and cytosolic pools of NAD, but not the mitochondrial pools, due largely to the extensive use of this substrate by poly (ADP-ribose) polymerases (PARPs)[50]. PARPs catalyze the polymerization of ADP-ribose units from donor NAD molecules on target proteins, resulting in the attachment of linear or branched polymers. There is a strong positive correlation between the longevity of a species and the polymer synthesis capacity of PARPs in mammalian cells[51]. PARP1 knockout mice age much faster than the wild-type control animals[52]. However, hyperactivation of PARP1 results in the depletion of NAD/adenosine triphosphate (ATP) and increases mitochondrial pore formation and cell death[53]. SIRT1 is a consumer of NAD and competes with other NAD-dependent enzymes for this common substrate. Thus, it has been proposed that inhibition of PARPs can increase NAD availability for SIRT1 to elicit the anti-ageing activity[54]. However, only certain types of tissues in PARP1 knockout mice exhibit increased NAD+ content and enhanced SIRT1 activity[55,56]. Moreover, the relationships between PARP and caloric restriction remain uncharacterized.

Alternatively, caloric restriction may activate Sir2 by regulating the level of nicotinamide, a known inhibitor of Sir2[57,59]. Crystal structures of the conserved sirtuin catalytic domains reveal that NAD and the peptide containing an acetylated lysine residue enter the active site from opposite sides of a cleft between a large Rossmann fold domain and a small Znbinding domain[60]. During the formation of an alkylimidate intermediate between the ADP-ribose 1' position and the acetyl oxygen, nicotinamide dissociates from NAD and occupies a so-called C-pocket. If nicotinamide binds to the C-site before alkylimidate conversion, it will inhibit the deacetylation reaction. Thus, removal of nicotinamide may be as important for the activation of Sir2/SIRT1 as the production of NAD. The salvage pathway for NAD biosynthesis begins with either nicotinamide or nicotinic acid, collectively referred to as niacin or vitamin B3[61]. Nicotinamide is first converted to nicotinamide mononucleotide (NMN) by nicotinamide phosphoribosyltransferase (NAMPT). The production of NAD+ from NMN and ATP is catalyzed by a family of nicotinamide mono-nucleotide adenylyltransferases (NMNATs). In lower eukaryotes, including S. cerevisiae, D. melanogaster, and C. elegans, no NAMPT activity has been found. Nicotinamide is converted to nicotinic acid, which then enters the parallel salvage pathway found in all eukaryotic species. Alternatively, nicotinamide riboside forms a precursor for NAD synthesis, connecting to the nicotinamide salvage pathway through NMN[62]. The predominant form of NMNAT in mammals, NMNAT-1, is a nuclear protein, while other forms, NMNAT-2 and NMNAT-3, are cytoplasmic and mitochondrial, respectively[63]. Over-expression of NMNAT-1 in mammalian cells does not affect total NAD levels, but regulate nuclear NAD-dependent processes[64]. Manipulation of a nuclear NAD salvage pathway delays ageing in yeast, without changing the steady-state levels of NAD[65]. Despite these information, the detailed links between caloric restriction and the NAD salvage pathway in mediating Sir2/SIRT1 activation has not been established. It is also possible that the genes involved in NAD salvage pathway act in a more general manner to promote cell survival[59,66,67].

We have recently shown that SIRT1 is strongly inhibited by biotin, the water-soluble vitamin B7, and its metabolite biotinyl-5'-AMP[39]. Biotin occupies the binding pocket of nicotinamide, which may affect the conformational change from nonproductive to productive SIRT1[68,69]. Biotinyl-5'-AMP competitively occupies the NAD binding site and prevents the breakdown of NAD by SIRT1. In addition, biotin may react with NAD to generate biotinyl-5'-AMP, in turn inhibiting the deacetylase activity of SIRT1. Since NAD also acts as a cofactor permitting SIRT1 to interact with protein substrates, by inhibition of NAD binding, biotinyl-5'-AMP prevents the interactions between SIRT1 and acetylated protein substrates. Adipose tissue represents a major reservoir of biotin in mammals. During ageing, biotin is progressively accumulated in adipose tissues. Chronic biotin supplementation mainly increases adipose biotin contents and abolishes adipose SIRT1-mediated beneficial effects on insulin sensitivity, lipid metabolism, and locomotor activity. In fact, caloric restriction prevents biotin accumulation in adipose tissues. Biotin and nicotinamide were originally discovered as the same class of heat-stable vitamins[70]. However, unlike nicotinamide, nutritional deficiencies of biotin are rare. The role of biotin in metabolism has been established in experimental microorganisms and animals. Biotin functions in mammals as a CO2 carrier for reactions in which a carboxyl group is transferred to one of four biotin-dependent carboxylases. Consequently, biotin participates as an important cofactor in gluconeogenesis, fatty acid synthesis, and branched-chain amino acid catabolism[71]. Based on these information, we speculate that in mammals, caloric restriction may enhance SIRT1 activity by selective depletion of biotin storage in adipose tissue, in turn preventing ageing-associated metabolic disorders and promoting lifespan extension.

Conclusions

Caloric restriction has been considered as a robust means of reducing ageing-related diseases and slowing the ageing process. Sir2 and its mammalian homologue SIRT1 are up-regulated by caloric restriction. Thus, Sir2/SIRT1 proteins sense low calories and mediate the beneficial effects of caloric restriction. However, the mechanism underlying caloric restriction-induced Sir2/SIRT1 activation remains elusive. Here, based on the available literature and our own research data, it is postulated that the induction of Sir2/SIRT1 activity by caloric restriction is an evolutionarily conserved response to decreased availability of certain nutrients, such as B vitamins.

The initial breakthrough of identification of Sir2 as a deacetylase with weak ADP-ribosyltransferase activity came along with the identification of the Salmonella typhimurium CobB protein as a Sir2 homolog[72]. CobB compensates for the lack of CobT mutants during vitamin B12 biosynthesis and possesses nicotinate mononucleotide (NaMN)-dependent phosphoribosyltransferase activity. Thus, CobB catalyze the release of nicotinic acid from NaMN, whereas Sir2/SIRT1 removes nicotinamide from NAD. Taken together, Sir2 family of proteins play important roles in modulating the biosynthesis of B vitamins. As a feedback mechanism, increased B vitamins may negatively regulates the enzymatic activities of Sir2/SIRT1. In this regard, caloric restriction-mediated activation of Sir2/ SIRT1 may at least partly relate to the nutrient availability of B vitamins, including biotin and niacin (Figure 2).

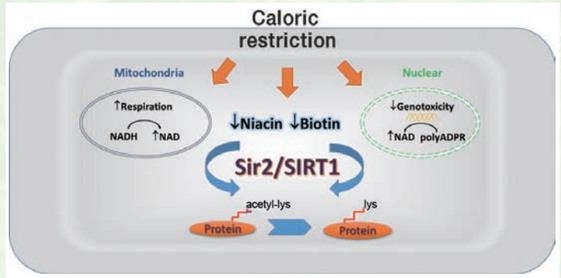

Figure 2. A number of molecular links, including nicotinamide adenine dinucleotide (NAD), niacin, biotin, and related metabolites, are important conduits mediating caloric restriction-induced Sir2/Sir2-like protein 1 (SIRT1) activation and lifespan extension. polyADPR, poly ADP-ribose.

Calorically restricted organisms are protected from ageing-induced damages as a result of heightened defensing and repairing capacity. Various caloric restriction mimetics, including caffeine, curcumin, dapsone, metformin, rapamycin, resveratrol, and spermidine, have been developed or under development. However, none of them elicits consistent effects as caloric restriction on extending lifespan across all different organisms. The mechanisms of ageing are more complex than any single type of ageing-related diseases. It is not known which physiological changes elicited by caloric restriction in mammals are most important for longevity. The belief that many benefits of caloric restriction are due to the induction and activation of sirtuins has led to the search for promising sirtuin activators as dietary supplements to promote health and longevity. In the meantime, limiting the negative regulators of Sir2/SIRT1 by restricted diet intake may be alternative or more effective approaches.

Conflicts of interest

No potential conflict of interest relevant to this article was reported.

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