Project title:	Carrot and parsnip; intervention studies to assess the effect of consumption on biomarkers of human health
Project number:	FV 420
Project leader:	Dr Kirsten Brandt, Newcastle University
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Key staff:	Sarah Warner (PhD student)
Location of project:	Newcastle University
Industry Representative:	Martin Evans, Freshgro
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The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

[Name]

[Position]

[Organisation]

Signature ..... Date .....

[Name]

[Position]

[Organisation]

Signature ..... Date .....

Report authorised by:

[Name]

[Position]

[Organisation]

Signature ..... Date .....

[Name]

[Position]

[Organisation]

Signature ..... Date .....

CONTENTS

GROWER SUMMARY

Headline

This Research will

- examine any association between carrot and parsnip consumption and cancer in people over 85 years old
- measure the effect of preparation and cooking on polyacetylenes,
- measure the bioavailability of polyacetylenes in different vegetables and
- measure the effect of a diet rich in polyacetylenes on biomarkers of cancer risk in humans.

Background

Nutritional factors have been shown to affect the risk of cancer. It is well known that the intake of fruit and vegetables is inversely correlated to cancer risk and there is increasing evidence that certain fruit and vegetable groups have a protective effect against particular cancers. It is thought that the fibre, antioxidant, vitamin and mineral content of fruit and vegetables are the main factors that contribute to the anti-cancer effect, but current evidence has shown that these common constituents alone cannot explain the effect. Observational studies have found a negative correlation between carrot consumption and cancer (Boggs *et al.*, 2010; Larsson *et al.*, 2010) and there is *in vitro* evidence to suggest the polyacetylene (PA) class of compounds, first investigated in herbal medicines such as ginseng, but also found in root vegetables including carrots, have anti-cancer (Zidorn *et al.*, 2005) and anti-inflammatory properties (Alanko *et al.*, 1994). Animal studies have also shown reduced levels of intestinal cancer when diets are supplemented with carrot (Kobaek- Larsen *et al.*, 2005; Saleh *et al.*, 2013). However, so far there have been no studies on the health effects of polyacetylene intake in humans.

The objective of the present study is to determine if consumption of PA-rich vegetables can affect the biomarkers of cancer and inflammation in humans, by examining:

(i) The effect of a vegetable intake rich in polyacetylenes on cancer incidence and inflammation in a group of older people;
(ii) The effect of cooking techniques on polyacetylene concentration in carrots, when consumed,
(iii) The bioavailability of polyacetylenes from various vegetables.

(iv) The effect of a diet rich in polyacetylenes on biomarkers of cancer risk *in vivo* in humans (dietary intervention study).

Summary

The Newcastle 85+ study

This is an observational trial that collected dietary data and other health and lifestyle information from a group of participants aged 85 years old at the start of the study in 2006. They were followed until death and their cause of death ascertained from medical records (Collerton *et al.*, 2007). The aim of this research will be to find out whether there is a link between PA-rich vegetable consumption and cancer mortality in this group of people.

There was a delay while a request for permission to access the 85+ study was considered. Although permission has now been granted, lack of staff to deal with data handling on the 85+ study has delayed progress.

Vegetable preparation and polyacetylene availability

Carrots will be artificially digested in the laboratory to determine the polyacetylene content when eaten raw (batons or diced), boiled or fried. Different cooking times will be compared.

Bioavailability of polyacetylenes

The polyacetylene content of different vegetables is unknown. MSc students will freeze dry a range of foods, extract the polyacetylenes with solvents and then use high powered liquid chromatography (HPLC) to measure the quantities. This data will inform the dietary intervention study, since the food given to the volunteers will be that highest in polyacetylenes. If time permits, the students will measure vegetables where the leaf is eaten, such as parsley and coriander, as well as other roots such as celeriac.

When the polyacetylene content has been determined, it is planned to publish the results. The hope is that other researchers will use it to re-evaluate their existing data from diet and health studies, to show if there is any correlation between polyacetylene consumption and outcome in terms of health or disease. (This is additional to the PhD work.)

Dietary Intervention Study

Biomarkers can be used to measure the risk of certain diseases. For cancer, it is possible to measure DNA lymphocyte damage from blood samples using the COMET assay, while inflammation can be measured by inflammatory biomarkers in the blood such as TNF-α and IL-6. This project will carry out a human intervention trial to test the effects of consumption of root vegetables on biomarkers for cancer and inflammatory disease, similar to a study on a dietary intervention with watercress and the effects on lymphocyte DNA damage and inflammatory markers carried out by Gill *et al*. in 2007.

SCIENCE SECTION

Introduction

Epidemiological studies have shown that fruit and vegetable intake is inversely correlated to cancer risk (Zhang *et al.,* 2009; Leenders *et al*., 2013) and there is convincing evidence that certain fruit and vegetable groups have a protective effect against particular cancers (Riboli & Norat, 2003; American Institute for Cancer Research, 2007). It was thought that the fibre, antioxidant, and vitamin content of fruit and vegetables are the main factors that contribute to the anti-cancer effect but current evidence has shown that these common constituents alone cannot explain it. Some studies have shown that dietary alpha and beta carotene intake is inversely correlated to cancer risk (Peto *et al*., 1981), but beta carotene and antioxidant supplement intervention studies in healthy populations have shown no effect (Gallichio *et al*., 2008) and some studies have even shown an increase in risk of cancer (Omenn *et al*., 1996). Other health promoting compounds which have previously been overlooked or under-studied could be contributing to the health promoting effect of fruits and vegetables.

Some studies suggest that carrot consumption as a specific food is inversely associated with cancer risk (Boggs *et al*., 2010; Larsson *et al*., 2010), and beta carotene concentrations in blood plasma could be considered a biomarker for overall carrot consumption in Western populations and would therefore also correlate to any other constituents inside the carrot. There is evidence to suggest the polyacetylene (PA) class of compounds, found in the Apiaceae family of plants including carrots, parsnips, parsley, celery, celeriac and fennel (Christensen and Brandt, 2006), can have anti-cancer (Zidorn *et al*., 2005) and anti-inflammatory (Shiao *et al*., 2005) effects. However, this evidence comes from laboratory studies rather than from studies of humans.

PA-Rich Vegetable Intake and Cancer Incidence in a Cohort of the Elderly

Cancer initiation mainly occurs as a result of multiple DNA damage events that are retained and accumulated in the cell until it is able to multiply uncontrollably. Factors both from the environment, such as diet and UV radiation, and processes that happen naturally inside the body, including oxidative damage and inflammation, can lead to DNA damage throughout the lifetime of cells and tissues. As a person ages, they will naturally acquire more DNA damage, so cancer usually manifests in the older person (AICR, 2007). Furthermore, inflammation not only causes oxidative DNA damage but can also

encourage proliferation and angiogenesis (blood vessel formation) of cancer cells, thereby promoting cancer metastasis. Once cancer has been diagnosed, higher inflammation can lead to more aggressive cancer and higher mortality in cancer patients (Coussens and Werb, 2002). Chronic inflammation is therefore implicated in the development of various cancers as well as other disease states such as cardiovascular disease and arthritis.

Older age is associated with a greater risk of developing cancer and could potentially have a lower cancer survival rate than a younger cohort (Il'yasova *et al.*, 2005). Therefore it is more appropriate to study the oldest old populations to determine if there is an association between consumption of carrot and other polyacetylene-rich vegetables and the risk of cancers in this age group.

Investigating Preparation of the Vegetables, including Cooking and Delivery Methods using HPLC Analysis and In Vitro Digestion

Whilst there have been studies investigating the preparation of PA-rich vegetables in industrial processing (Rawson *et al.*, 2010; Koidis *et al.*, 2012), there has been little work on domestic cooking so investigation into the effects of a range of cooking conditions on the preservation of PA is warranted.

Some phytochemicals are seen to increase in bioavailability after cooking and others decrease. For example, some observational studies show raw carrots to be more protective than cooked carrots (Chan *et al.*, 2005) but the converse seemed to be true in other studies (Longnecker *et al.*, 1997; Zeegers *et al.*, 2001). This could be due to the different factors thought to affect the retention of bioactives, including: length of cooking time, size and shape of carrot and cooking in either water or oil. Heating of vegetables can affect the availability of phytochemicals in both a positive and negative way. A decrease in availability is often seen due to heat degradation of compounds (Hansen *et al.*, 2003) but an increase can be seen due to the breakdown of the cell matrix of the vegetable allowing release of the compounds which would otherwise be bound inside the cell (Miglio *et al.*, 2007). The length of cooking time will affect this balance.

Determining the bioavailability of** polyacetylene **and establishing the 'optimal' dose of polyacetylenerich vegetables in order to achieve relevant concentrations in humans in vivo

Polyacetyleneshave previously been shown to be bioavailable in humans in two small trials using carrot juice (Hansen-Møller *et al.*, 2002; Haraldsdóttir *et al.*, 2002). Whilst carrot juice is a convenient way to administer an accurate dose, carrots are more commonly consumed as a whole vegetable in a typical diet. This study will investigate the bioavailability from carrot, including fibre and other components of the whole vegetable that may affect the release of phytochemicals.

In epidemiological studies, carrot intakes have been associated with a lower risk of colorectal (Franceschi *et al.*, 1998; Slattery *et al.*, 2000), breast (Boggs *et al.*, 2010), and bladder cancer (Zeegers *et al.*, 2001), therefore blood, urine and faecal samples will be analysed to see if PA are present in these bio-fluids. *In vitro* studies have shown that PA can inhibit the growth of colon cancer cells (Sun *et al.*, 2010) and inhibit leukaemia cells at much lower concentrations than carotenoids (Zaini *et al.*, 2012), and it has been demonstrated that these compounds can be absorbed by Caco-2 cells (Lee *et al.*, 2013). The anti-cancer effect of carrots has also been demonstrated *in vivo* in mouse (Saleh *et al.*, 2013) and rat models (Kobaek-Larsen *et al.*, 2005) that are predisposed to intestinal cancer. It is therefore possible that one or several carrot components may be affecting risk of colon cancer and thus it is worth examining faeces/faecal water to allow us to determine whether epithelial cells in the digestive tract are exposed to concentrations of PAs that have been shown to affect the viability of colon cancer cells *in vitro*.

Assess the effect of polyacetylene -rich diet on biomarkers of cancer risk in vivo in humans (Dietary Intervention Study)

Biomarkers can be used to measure the risk of certain diseases. For cancer, it is possible to measure DNA lymphocyte damage from blood samples using the COMET assay while inflammation can be measured by inflammatory biomarkers in the blood such as TNF-α and IL-6.

The effect of PA on humans has not been tested *in vivo* and whilst it has been shown to be bioavailable in two small studies, it is unknown whether the biological actions seen *in vitro* will also be seen *in vivo*. This project aims to carry out a human intervention trial to test the effects of consumption of root vegetables on biomarkers for cancer and inflammatory

disease, similar to a study on a dietary intervention with watercress and the effects on lymphocyte DNA damage and inflammatory markers (Gill *et al*., 2007).

Materials and methods

PA-rich vegetable intake and cancer incidence in a cohort of the elderly:

The data will be analysed to determine how much PA-rich vegetable is consumed, both alone and in mixed meals, in the cohort. For the mixed meals, foods will be disaggregated using McCance and Widdowson's 'The Composition of Foods' (McCance and Widdowson, 2002) as well as manufacturer's Nutritional Information and homemade recipes to determine how much of a dish is comprised of PA-rich vegetable. The PA-rich vegetable intake will be used to determine (using linear regression) whether there is an association between consumption and cause of death by cancer and other health parameters. Logistic regression coefficients will be interpreted in terms of odds ratios and associated 95% confidence intervals to ascertain the protective effects of diet.

Further work will involve the retrospective testing of a selection of commonly eaten foods and dishes for amounts of PA. This will allow an estimate of PA content in the diet which can also be correlated to the health parameters in the same way, as done for the vegetable intake.

Preparation of the vegetables, including cooking and delivery methods using HPLC analysis and in vitro digestion

The size and shape of the carrot during cooking will be investigated to determine how surface area and volume affect retention of phytochemicals. A higher surface area is thought to encourage leaching of compounds out of the vegetable and into the cooking fluid due to the mechanical breakdown of cell structures during peeling and cutting as well as a larger surface area coming into contact with the cooking fluid. Carrots will be peeled then cut into disks, batons or left whole to determine the best shape for highest retention. Another variable thought to affect the retention of bioactives in vegetables is the medium in which they are cooked. Depending on the solubility of the phytochemicals in different solvents, leaching may be encouraged. This experiment will investigate cooking in both water and oil to analyse the effect of vegetable preparation on PA and carotenoid content.

Both the resulting cooked vegetable and the cooking fluid will be analysed by HPLC for PA concentration and the carrot samples will undergo *in vitro* digestion.

In vitro digestion uses physiological amounts of digestive fluids and enzymes to simulate digestion in the stomach and intestine (Gleize *et al.*, 2013). PA, being fat soluble, will be in the resulting digest in an oil-water emulsion which will give an indication of the amount of PA available for absorption by the intestine. *In vitro* digestion will be used to both predict PA availability and to determine the amount of oil needed to dissolve the PA for absorption from the digestive tract (Hedren *et al.*, 2002).

Results from the cooking and *in vitro* digestion studies will be used to determine the preparation and amount of carrot to serve, in a bioavailability study.

Bioavailability of polyacetylenes and determining the optimum dose of vegetables

Healthy adults will be recruited to take part in the bioavailability study. Participants will be asked to exclude PA-rich foods from their diet for 3 days prior to the test day. In the 24 hours prior to the test day they will be asked to collect 24 hour urine and will be provided with a standardised meal for the night before. They will be asked to collect a faecal sample on the morning of the test and will come to the test centre fasted for 12 hours (water only) where a fasting blood sample will be taken. They will then consume an acute 'dose' of carrots as part of a mixed meal. Further blood samples will be taken at 0.5, 1, 2, 4, 6 and 8 hours after consumption to determine the appearance of PA in blood following the carrot containing meal. A standard PA-free lunch will be provided after 4 hours and a standard dinner will also be provided for the participant to take away. Urine will be collected into timed containers throughout the next 24 hours (0-4, 4-8 and 8-24 hours) and afurther 24 hour urine to be collected during the following 24 hours (24-48hours). A final fasting blood sample will be taken the following morning ('post 24 hrs') and the all faecal samples after the PA 'dose', up to 48 hours, will also be collected. The full bioavailability will be repeated on two separate days, with a week wash out in between, with two different carrot doses administered in a random order.

The blood, faecal water and urine samples will be analysed by LCMS (liquid chromatography-mass spectrometry) to determine if a biologically relevant concentration may be achieved and to optimise the dose for the subsequent intervention study. The

absorption profile can also be determined i.e. the time at which it first appears in the blood and how long it remains there before clearing. This will give an indication of the length of exposure to the PA.

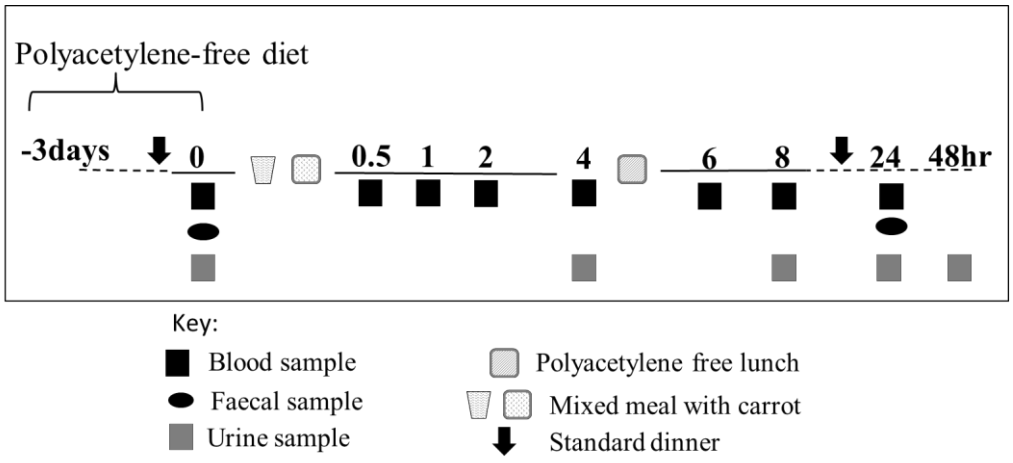

Figure 1: Bioavailability Study Design

Randomised dietary intervention trial to determine the effects of PA-rich vegetables on markers of DNA damage and chronic inflammation

A randomised cross-over design will be used, involving an amount of carrots per day for about 8 weeks (determined in the previous studies) and alternated with a control period for the same duration where an amount of other comparable vegetables with no PA will be consumed (Fig 2). A wash-out period of 6 weeks will separate the two periods when the normal diet of the participant will be eaten. At the beginning and end of the intervention and control period, blood and faecal samples will be collected to see the effect on inflammatory markers, DNA damage in lymphocytes and epithelial cells of the colon. Dietary intake will be monitored throughout to check compliance to the intervention and background diet. Measured biomarkers will be compared between control and intervention periods and correlated with changes in PA concentration as a result of the intervention.

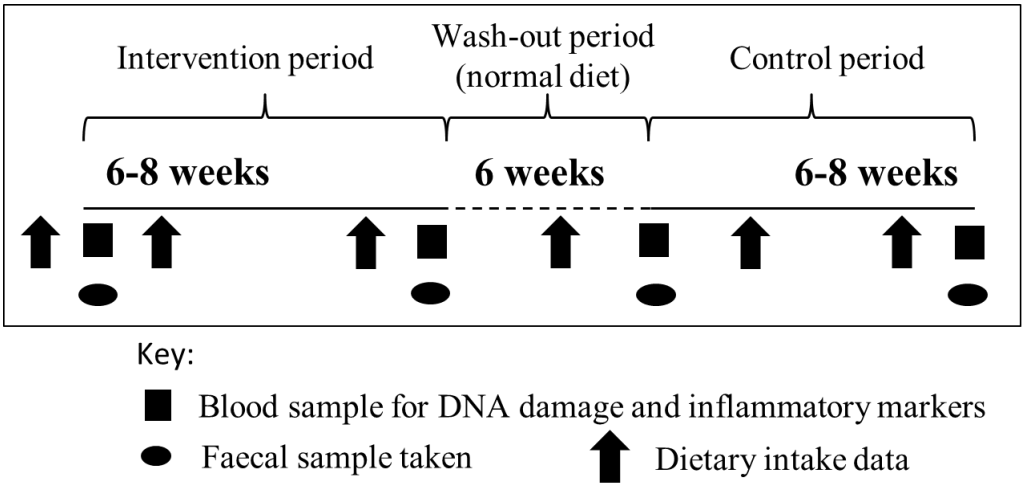

Figure 2: Dietary Intervention Study Design

Results

No results have yet been analysed and therefore none are yet available for presentation.

Knowledge and Technology Transfer

Press release entitled 'Exciting New Study to Measure Effects of Eating Carrots, Parsnips and Celeriac on biomarkers for Cancer and Inflammatory Diseases' issued at the start of June 2014 and subsequently taken up in the front page headline of the Daily Express as 'Carrots Cure Cancer'.

Poster presentation at International symposium of Carrots and other Apiaceae; Angers, France 17-19 Sept 2014.

Paper in the proceedings of the above symposium in Acta Horticulturae (in publication)

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