

Hypercarotenaemia in Wistar rats and ICR mice and correlation to humans

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Received on : 15 - 05 - 2010

Accepted on : 03 - 06 - 2010

Abstract

Hypercarotenaemia can occur at any age but it is more commonly seen in infants and young children due to the excessive intake of carotenoid bearing food. The objective of this study was to induce hypercarotenaemia and trace the fate of excess carotenoids in Wistar rats and ICR mice.

Wistar rats (n=20) and ICR mice (n=28) were used. Rats and mice were divided into two groups (Test and Control). The controls were fed with standard rat/mice pellets while test group was fed with freeze-dried carrot incorporated standard rat/mice feed with boiled carrot. After a month and 2.5 months, blood was drawn for analyses of carotenoids and metabolites and after 2.5 months liver, adipose and digesta of rats were collected. Faeces were freeze dried and then analyzed for carotenoids of metabolites (RP-HPLC). Serum, adipose, liver and bile of test and control mice were also analyzed as above.

Wistar rats and ICR mice fed on excess carrot and papaw did not show outward signs of hypercarotenaemia. Their serum, adipose tissue, liver, digesta (in the case of rats) and bile (in the case of mice) did not show detectable amounts of carotenoids or their metabolites. However the faeces of both rat and mice had high levels of α and β carotenes. This indicates that one method of control of hypercarotenaemia may be at the level of absorption.

Key words: Hypercarotenaemia, Induction of, Wistar rats and ICR mice, papaw, carrot diet

Introduction

Hypercarotenaemia can occur at any age but it is more commonly seen in infants and young children in Sri Lanka (Wageesha, et al., 2008 and Priyadarshani, et al., 2009). The cause for this condition in Sri Lanka is mainly due to excessive intake of carotenoid bearing food such as carrot, pumpkin and papaw (Wageesha, et al., 2008). It can also be associated with the ingestion of many other yellow and green leafy vegetables and citrus fruits. There is some evidence that not all infants fed in this way develop hypercarotenaemia. There is probably a genetically based metabolic factor at play (Lindqvist, et al 2007 and Wageesha, et al., 2009). Due to ethical reasons no detailed study has been done to identify the metabolic fate of these excess carotenoids in humans. That is, to induce hypercarotenaemia and trace the fate of carotenoid metabolized in the humans. Thus the objective of this study was to induce hypercarotenaemia in Wistar rats and Institute of Cancer Research (ICR) mice and trace the fate of carotenoids in these animals.

Materials and methods

Materials

Animal models

Three weeks old inbred Wistar rats (n=20; animal model 1) and inbred male Institute of Cancer Research (ICR) mice (n=28; animal model 2) were purchased from the Medical Research Institute (MRI), Borella and housed in the Animal House, University of Sri Jayewardenepura under standard conditions.

After a week of acclimatization the rats and mice were divided into two equal mean weight (90g and 13 g for rats and mice respectively) groups as Test and Control (10 rats and 14 mice in each).

Oven dried wood shavings were used to cover the bottom of the cage and wood shavings were removed once in 4 days time. The weights of the food placed on the cage were recorded. Each day solid material at the bottom of the cage was removed and faeces, and residual feed were separated and residual feed weighed.

Feed

The control animal feed was prepared according to the standard MRI food composition by a modified version of Sabourdy (1998), while the test feed was prepared by incorporating freeze-dried carrot (30g/ Kg⁻¹) into the standard rat/mice feed.

Ethical Clearance

Ethical clearance was obtained from Ethics Committee of the University of Sri Jayewardenepura, Nugegoda, Sri Lanka (Approval number 369/7).

Methods

Inducing hypercarotenaemia in rats and mice

Test groups of both animal models (animal model 1 and animal model 2) were fed with freeze-dried carrot incorporated (30 g/ Kg^{-1}) standard rat/mice feed (instead of grass powder). At the same time boiled carrot with butter was given to the test group daily (1 g/day for rats and 0.5 g/day for mice). This diet was continued up to $2 \frac{1}{2}$ months in an attempt to induce hypercarotenaemia. The amount of diet/carrot consumed was noted.

The controls were fed with standard rat/mice pellets while same amount of butter was incorporated in the diet of control group. Thus the diets were isocaloric. The amount of diet consumed was noted.

Isolation of carotenoids from blood, faeces, liver, adipose and digesta – Wistar rats

The blood samples (1 mL) of both test and control groups were drawn from the tail vein of each Wistar rat at the end of 1 month and at the end of $2 \frac{1}{2}$ months under mild anesthesia and serum separated. Faeces samples of each rat was collected into a teflon tube separately at the end of $2 \frac{1}{2}$ months. After $2 \frac{1}{2}$ months twenty rats (10 tests and 10 controls) were euthanized and dissected and their liver and adipose collected into containers and stored under N_2 in freezer (-20°C) until analyses of carotenoids and their metabolites were carried out.

Carotenoid and vitamin A analysis - serum

Serum carotenoids their metabolites, internal standards both β -apo-8'-carotenal and retinyl acetate which were added prior to the extraction and vitamin A were extracted into hexane, under the dim light after addition of ethanol (1:1 base on serum) (Barua, and Olson, 1998 and Bieri, et al., 1979). The hexane layer was pipetted out and extracted with a fresh portion of hexane. The above procedure was repeated until there was no colour left in the hexane layer. The hexane layers were pooled.

For analysis of carotenoids and metabolites, a known volume of hexane from above was evaporated under a stream of nitrogen and re-dissolved in $200 \mu\text{L}$ of RP-HPLC mobile phase (58% Acetonitrile: 35 % Methanol: 7% Tetrahydrofuran) while the other portion was re-dissolved in $200 \mu\text{L}$ of 100%

methanol for vitamin A analysis and separately introduced to Reversed Phase High Performance Liquid Chromatography (RP- HPLC) equipped with C₁₈ column. Peaks were detected at the wavelength of 450 nm, while vitamin A detection was at 325 nm. Flow rate was 1 mL/min. The retention times for monohydroxy and polyhydroxy derivatives of carotenoids were determined according to Priyadarshani et al., 2009.

Carotenoid analysis of faeces

The faeces collected at the end of the study (2 ½ months) were first freeze dried and approximately 1 g weighed. The weighed samples were extracted into the HPLC mobile phase, and concentrated using a stream of nitrogen and subjected to RP-HPLC to obtain the chromatograms. Another set of faeces were extracted into CHCl₃ and a thin layer chromatography (TLC) performed.

(a) Thin layer chromatography of faeces

A same volume of CHCl₃ extracts of test and control group rat faeces were spotted along with β-carotene standards on activated prepared glass backed plates (20 x 20 cm) of silica gel G 250 with a thickness of 300 µm. TLC was run in a mobile phase of 5% methanol in toluene (Rodriguez-Amaya, and Kimura, 2004), and R_f values calculated. The spots were visualized under the UV illuminator. The spots which were UV active were scraped out and dissolved in Petroleum ether (PE), concentrated using a stream of nitrogen and subjected to RP-HPLC.

Carotenoid analysis of liver, adipose tissue and digesta

The excised livers were freeze dried. The tissues were then crushed and then ground in to a powder by using a pestle and mortar. The mixture was vortexed for 5 minutes following addition of 5 mL of HPLC mobile phase. The supernatant was pipetted out into a clean teflon tube. The procedure was repeated 4 times with 5 ml portions of mobile phase. The volume of the extracts were pooled and reduced to a small volume by using a stream of nitrogen and filtered with 0.45 µm sample filter and injected into RP-HPLC. Adipose tissue (500 mg) from around the kidney was first saponified using 10 mL of 0.16M methanolic KOH and then extracted into petroleum ether (PE). The PE fraction was evaporated using a stream of nitrogen and re-dissolved in a volume of 200 µL of mobile phase, filtered with 0.45 µm sample filter and injected into RP-HPLC.

The digesta was collected from rats by rinsing the contents of the small intestine (duodenum to just above the ileum) using a syringe with cold normal saline into teflon tubes and stored in freezer until analysis. The stored

saline digesta (12 to 15 mL) were freeze dried and extracted into HPLC mobile phase and concentrated using a stream of nitrogen. Finally the concentrated extract was filtered using 0.45 µm sample filter and injected to RP-HPLC.

Isolation of carotenoids from blood, faeces, liver, adipose and bile – ICR mice

In ICR mice experiments at the end of 2 ½ months blood of the two groups (test and control) were obtained by cardiac puncture and collected separately. The faeces, liver, adipose tissues were collected and stored, and qualitatively and quantitatively analyzed as described above in Wistar rat experiment.

Isolation of carotenoids from bile of ICR mice

The gall bladders of mice (both test and control) were punctured using an insulin syringe needle in order to collect the bile into an Eppendorf tube. The bile collected from the animals of the test group was pooled in to 4 separate samples. Same procedure was followed with the control group, since the volume of bile was small to be analyzed separately. The samples were then freeze dried, and then extracted into mobile phase (58 acetonitrile: 35 methanol: 7 THF). The extract was concentrated and injected into RP-HPLC.

Results and Discussion

Studies on Wistar rats

Despite feeding an average of six grams of carrot per day with butter for 2 ½ months, none of the 10 test Wistar rats showed any external features of hypercarotenaemia. There was no significant difference in the body weights and vitamin A levels of the rats of test and control groups ($p= 0.41$ and $p= 0.15$ respectively) and peaks corresponding to β-carotene, α-carotene, polyhydroxy metabolites and monohydroxy metabolites were not observed in serum carotenoid profiles.

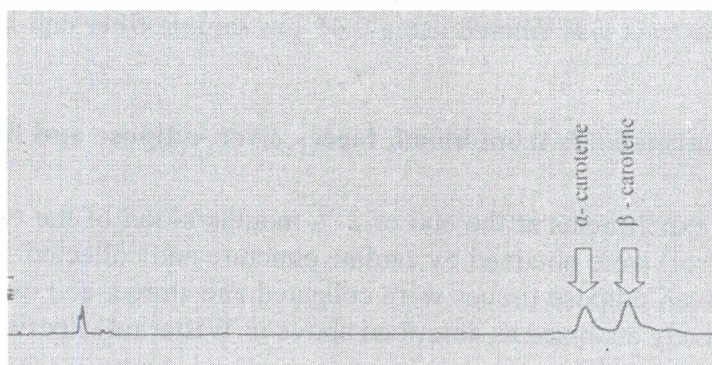


Figure 1. RP-HPLC profile of carotenoids in a fecal extract of test group rat

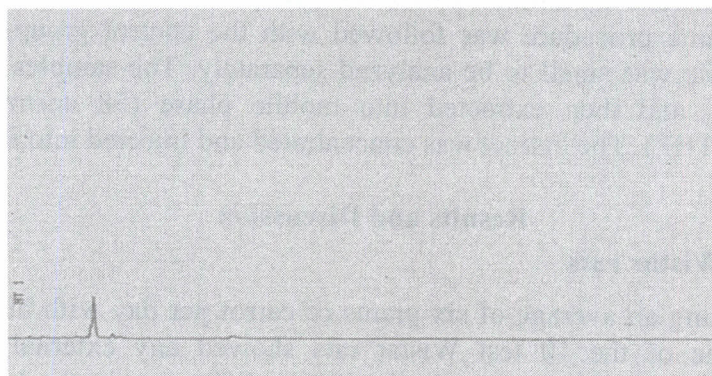


Figure 2. RP-HPLC profile of carotenoids in a fecal extract of test group rat

The digesta, liver and adipose extractions of the rats of test group did not contain major carotenoids or any of their metabolites. However the faeces of test rats had high amounts of α and β carotenoids while carotenoids were not present in the control faeces (Figures 1 and 2).

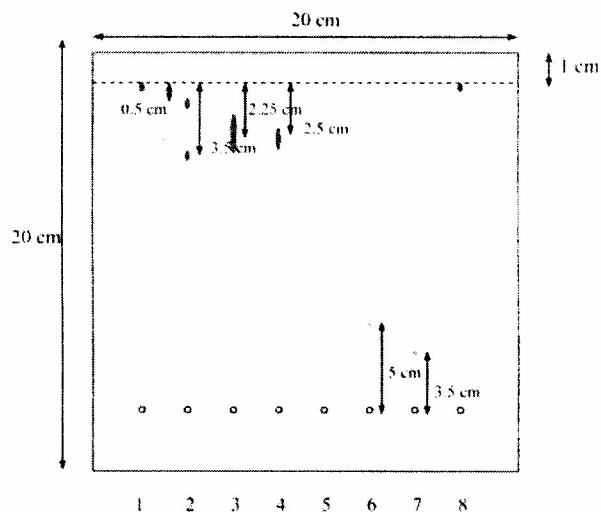


Figure 3. Diagrammatic representation of TLC of fecal of rats

Key:

1 – Standard β -carotene, 2 – Test 01, 3 – Test 02, 4 – Test 03, 5 – Control 01, 6 – Control 02, 7 – Control 03, 8 – Standard β -carotene

● - UV active spots

○ - Non UV active spots

The fraction of faeces which were extracted into CHCl_3 and subjected to thin layer chromatography (TLC) showed UV active spots of which one spots' R_f value corresponded to standard β -carotene (Figure 3). The RP-HPLC chromatograms of the UV active spots give rise the typical peak pattern of β -carotene and α carotene. Thus the presence of β -carotene and α carotene were confirmed by the UV absorption and RP-HPLC.

Studies on ICR mice

As in Wistar rats described above the ICR mice also did not show hypercarotenaemia externally. However literature (Hessel, et al., 2007) reports that a mutant mouse had shown hypercarotenaemia. The average carrot consumed by the rats of test group was five grams per day. The results show that there was no significant difference ($p=0.27$) between the body weights of the two groups at the end of a 2 ½ months period.

The adipose tissue extracts and bile extracts of test and control mice did not have major carotenoids or their metabolites and both the test and control groups showed similar results to Wistar rat model. The vitamin A levels of the test and control groups were not significantly different ($p=0.18$).

As in the case of test faeces of Wistar rats the faeces of test mice had α and β carotenes but not in the control group.

The TLC results of CHCl_3 extract shows that there is β - carotene present in the test group mice faeces and were confirmed by the RP- HPLC chromatogram of the scraped UV active spots.

Thus studies with Wistar rats and ICR mice resulted in no outward features of hypercarotenaemia. Serum samples from both animal models showed no evidence of major carotenoids or their metabolites. This could be due to rapid metabolism of any absorbed carotenes. The main adipose tissue around the kidney and the liver tissues yielded no detectable carotenoids, which suggests that carotenoids have not been stored to a significant extent. In other words Wistar rats and ICR mice could not be induced with hypercarotenaemia. But carotenoids were excreted in the faeces of test rats and mice suggesting controlled absorption. This was the key to the thinking that the same process may be occurring in humans. A study is underway to test this hypothesis. Preliminary results shows that hypercarotenaemic's faeces do not show α and β carotenes or any other carotenoids or their metabolites while non hypercarotenaemic's faeces similarly to faeces of rats and mice shown the presence of α and β carotenes (Unpublished data).

The above animal studies clearly indicate that neither Wistar rats nor ICR mice can be induced with hypercarotenaemia even when they are fed high amount of carotenoid bearing foods for an extended period (2 ½ months). Although the results are negative the fact that their faeces contain high amounts of carotenoids points to the fact that control at the level of intestinal absorption being one way of controlling development of hypercarotenaemia.

Acknowledgements

The authors thank International Programme in the Chemical Sciences (IPICS), Uppsala University, Sweden for financial support (Sri: 07).

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