

## 14 The effects of dietary interventions on carcinogenesis in the colonic mucosa of rats UNew

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NuGO approved

General

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We used the NuGO Standard Operating Procedure (SOP) number 14 produced by the Human Nutrition Research Centre, Newcastle University, UK

Details of the SOP are available via the web link:

<http://www.nugo.org/frames.asp?actionID=28250&action=loginFromPPiIs>

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### Backgrounds

Diet is an important environmental factor in the development of cancers and may account for ~ 30% of cancers at all sites worldwide. Evidence for the role of diet in the aetiology of colorectal cancer is particularly strong with diets in fruit and vegetables and those low in fat being associated with low risk. Dietary intervention can be used to modulate changes in cell proliferation and apoptosis, the maintenance of normal gastrointestinal epithelial integrity requires a balance between cell production and cell loss - cancer represents a disruption of this balance which may result in an accumulation of cells with genetic damage and a failure in apoptotic mechanisms. In the normal colonic mucosa the proliferative compartment is confined to the lower half to two thirds of the crypt, it has been suggested that one of the earliest changes in pre-malignant mucosa may be the extension of the proliferative compartment towards the luminal surface. Several studies have suggested that the whole of the colonic mucosa expresses proliferative abnormalities ie. there is a field change rather than a focal change. In the colon, enhancement of crypt cell proliferation, expansion of the zone of proliferation and a reduction in apoptosis are all considered risk factors for tumour development, hence dietary agents which decrease cell proliferation or stimulate apoptosis are potential strategies for chemoprevention. Genetic mutations such as in the *APC* gene deregulate intestinal differentiation specifically through disturbing the intestinal crypt cycle, this leads to an increase in crypt fission. Increases in crypt fission may allow selection and expansion of mutated clones, the stem cells have also been implicated in controlling crypt fission. Little is known about the effects of nutritional manipulation on crypt fission but it is thought that asymmetrical fission may be influenced by dietary modification, and so bifid crypts have also been identified as a possible biomarker of intestinal neoplasia.

### Overview

The effects of dietary interventions on carcinogenesis in the colonic mucosa of rats using:

#### A. Assessment of cellular status by Microdissection of Whole Crypts.

One of three simple techniques may be used to assess both cell proliferation and cell death (apoptosis) in fixed colonic mucosa from rodents. *Microdissection* allows both mitotic and apoptotic states to be examined in 3-D **whole** crypts whereas assessment of cellular state using simple *Haematoxylin and Eosin (H&E) staining* or *antibody labelling with Ki-67 antigen* will give data on the cellular state in a single plane of a crypt at a given point in time in paraffin wax processed sections and **not** in the whole crypt. Many

criticisms have been levelled at cell kinetic studies because of methodological flaws so using microdissection alongside H&E assessment and/or Ki-67 antigen labelling will give an improved analysis of the changes occurring in the mucosa during carcinogenesis and also any effects of different treatments, as the two techniques identify cells during different phases of the cell cycle (Ki-67/Mib-1 antibody – throughout the cell cycle and microdissection in M phase only). However, comparison of the two techniques does show a significant correlation.

## Materials

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Amount	Name	Supplier	Catalogue Number	Further information
100ml	Carnoy's Fixative	Make in Laboratory	N/A	Prepare as described
100ml	1MHCl	Make in Laboratory	N/A	Prepare as described
500ml	Schiff's Reagent	Sigma	3952016	Buy commercially
100ml	45% acetic acid	Make in Laboratory	N/A	Prepare as described

## Main Procedures

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- Collect and fix tissue.
- Hydrolyse tissue.
- Stain tissue.
- Microdissect tissue.
- Measure and count.

## Sub Procedures

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### 1) Remove mucosal biopsies from gut

Remove mucosal **only** biopsies from the gut (**not** full thickness mucosa containing muscle) and gently place flat onto filter paper strips.

### 2) Fix tissue biopsies: 12 – 24 hours

Tissue biopsies must first be fixed flat in Carnoy's fixative (75ml ethanol / 25ml acetic acid) in 1.5 ml eppendorfs for 12-24 hours, then transferred to 70% ethanol at 4°C for storage until preparation. Samples may be stored in this state for many years.

### 3) Hydrolyse and stain samples

Prior to assessment, samples must be hydrolysed and stained as follows:

- 50% ethanol at room temperature (RT) for 10 minutes
- 25% ethanol at room temperature (RT) for 10 minutes
- 1MHCl at 60°C for 10 minutes
- Schiff's reagent at room temperature (RT) for 45-60 minutes
- 45% acetic acid at 4°C until microdissection

**NB.** Samples may only remain in 45% acetic acid at 4°C for 2-3 days, it is recommended to

prepare small batches of samples that can be counted in 2-3 days.

#### **4) Microdissect the samples using a stereomicroscope**

Use a stereomicroscope with a magnification of x4 - x7 to microdissect the samples. Identify the two opposing surfaces of the colonic mucosa and dissect the tissue with the mucosal lumen (crypt opening) facing downwards and the crypt base facing upwards towards the microscope user.

#### **5) Dissect out individual crypts**

Place tissue biopsy on a glass microscope slide in a **small** drop of 45% acetic acid to prevent drying out. Use two 21 gauge needles to dissect out individual crypts or small groups of crypts, insert the bevelled edge of one needle tip in between crypts whilst stabilising the tissue (gently) with the other needle tip, gently pull the crypts apart until all of the biopsy has been dissected. Take care not to chop crypts in half as only intact crypts may be counted. Several small clumps of tissue should be seen floating in the drop of acetic acid.

#### **6) Place coverglass on dissected tissue**

Gently place a 22mm x 22mm square coverglass over the dissected tissue taking care to avoid air bubbles. Apply gentle pressure to the surface of the coverglass to spread the crypts out – applying too much pressure will ‘burst’ the crypts or may crack the coverglass.

#### **7) Count cells using a good quality light microscope**

The mucosal preparation is now ready for counting. Use a good quality light microscope with a numbered ‘ruler’ or ‘cross’ graticule in the eyepiece. Use a reference slide with the chosen graticule to calculate the exact size of each mark on the graticule, this will give a value in microns ( $\mu\text{m}$ ). Count cells at x40 objective (overall magnification of x400).

#### **8) Count cells**

Ten intact crypts should be counted in each sample, i.e. a crypt in which the base and sides are intact and where the luminal surface may be seen. If required, any bifid crypts may be noted but **not** counted. Count the total number of crypts in the microscope field, at low power (x4 objective) then count the number of bifid crypts in the same field, calculate a percentage.

#### **9) Measure using the graticule the length and width of each normal crypt**

The length and width of each normal crypt is measured using the graticule; the crypt is then divided into ten equally sized compartments beginning at the base of the crypt with compartment 1 and following up the crypt to compartment 10 at the luminal surface. To do this, the ‘zero’ point on the graticule is placed on the base of the crypt then e.g. if a crypt measures 100 marks of the graticule then compartment 1 will be 0-10, compartment 2 10-20 and so on up the crypt to compartment 10 90-100. The number of mitotic figures and apoptotic bodies identified in each of the ten compartments should be noted.

#### **10) Count only the cells in mitosis**

Only cells in mitosis should be counted, ie. cells without a cell membrane and with condensed chromatin – counting only distinct late prophase, metaphase, anaphase and early telophase - and those cells identified as being in an apoptotic state with densely stained clumps of DNA.

## **Safety**

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**Users must comply with COSHH and local safety regulations.**

COSHH forms attached

## Results Analysis

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Data may be entered directly into the computer on an excel spreadsheet (see attachments). Data entry for apoptotic cells is added in the 'comments' column of the spreadsheet e.g. if apoptosis seen in third compartment of second crypt enter C5 in spreadsheet. Formulas in excel will work out the mean values for each sample and these values may be entered into a separate 'summary' excel spreadsheet where the effects of different treatments may be compared. Changes in the distribution of both mitotic and apoptotic cells throughout the colonic crypt compartments may be analysed.

## Additional Information

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Good training in recognition of different cell states is **essential**.

For assessment of cell status in animal tissues only, animals may be injected with Vincristine Sulphate 2 hours before sacrifice to arrest cells in metaphase; this aids in identification and counting of cells in mitosis. Inject animals IP with 1mg/kg of body weight.

Templates for recording data in excel spreadsheets are attached also.

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