The influence of phytic acid in wheat bran on early biomarkers of colon carcinogenesis

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A colon cancer protective effect of wheat bran has been observed in animal studies, but it is unclear whether the effect is due to fiber or other components, such as phytic acid (PA). Thus the objectives of this study were to determine if wheat bran alters early biomarkers of colon cancer risk, e.g. aberrant crypt foci (ACF) characteristics and indices of colonic cell proliferation, whether PA is the component responsible and whether there is a difference between endogenous and exogenously added PA. Five groups of azoxymethane-treated male Fischer 344 rats were fed for 100 days on a basal control diet (BD) or BD supplemented with either 25% wheat bran (WB), 25% dephytinized WB (DWB), 25% DWB plus 1.0% PA or 1.0% PA. All the WB-containing diets reduced the number of sialomucin-producing ACF and the degree of aberrant crypt luminal alterations in the whole colon. The WB and PA diets lowered the labeling index (LI) and the position of the uppermost labeled cell in the distal colon. Dephytinization caused an increase in the overall LI, LI in the top 40% and the position of the topmost labeled cell. Exogenous PA also reduced the number and size of ACF, the number of ACF per unit length colon as well as the number of sialomucin-producing ACF in various colon sections. It is concluded that WB, partly due to its endogenous PA, and exogenous PA when added to a low fiber diet can reduce early biomarkers of colon cancer risk.

Introduction

Epidemiological studies have shown that high fiber foods, such as fruits, vegetables, whole grains and cereals, may be protective against colon cancer (1–5). Specifically, many animal studies have shown that wheat bran (WB*) has a colon cancer protective effect (6–11), attributed mostly to its high fiber content. Interestingly, many of the proposed protective mechanisms of WB fiber, such as decreased transit time (12), increased bulk (13) and fermentation (14), are analogous to those suggested for the protective effects of phytic acid (myo-inositol 1,2,3,4,5,6-hexakis-dihydrogen phosphate; PA), which is a major fiber-associated component of WB (15). In some epidemiological studies a colon cancer-protective effect has been observed for fiber foods rich in PA, such as WB, but not for low PA fiber foods (15,16).

Therefore, the purpose of this study was to determine: (i) if WB can reduce early biomarkers of colon cancer risk, such as ACF characteristics and indices of colonic cell proliferation; (ii) if PA is the component of WB responsible for any observed effects; (iii) if there is a difference between endogenous and pure exogenous PA added to the diet. A secondary objective was to test the use of the high iron diamine alcian blue (HIDAB) staining method for sialomucin-producing ACF along with a grading system to measure the degree of luminal alterations of AC as a means of further characterizing AC and ACF.

Materials and methods

Experimental design

Seventy five male Fischer 344 rats (40 days old; Charles River Inc., Montreal, Canada) were maintained in individual stainless steel cages at an ambient temperature of 22–24°C on a 12 h light–dark cycle. They were acclimatized for 2 weeks on the AIN-93G basal diet (BD) (28) and then injected i.p. with 15 mg/kg body wt of the colon carcinogen AOM (Sigma Chemical Co., St Louis, MO). One week later they were randomized into five groups with 15 rats per group, such that the mean weight of each group was equal. They were fed ad libitum either BD (BD group) or BD supplemented with either 25% WB (WB group), dephytinized WB (DWB group), 25% DWB plus 1.0% PA (DWBPA group) or 1.0% PA (PA group). All diet ingredients were ordered from Dyets Inc., except the WB (King Milling Co., MI). The 25% WB level was chosen so as to provide a 1.0% level of PA in the diet. All the diets were based on the AIN-93G diet (28) containing 39.75% corn starch, 20.00%...
casein, 13.20% dextrinized corn starch, 10.00% sucrose, 7.00% soybean oil, 5.00% dietary fiber, 3.50% mineral mix, 1.00% vitamin mix, 0.30% L-cystine, 0.25% choline bitartrate and 0.0014% t-butyldihydroquinone. The WB-containing diets were corrected for the protein, fat, fiber and moisture content contributed by the added WB. The WB was dephytinized according to the method of Morris and Ellis (29), i.e. activating the bran’s natural phytases by incubating a 15% dispersion in de-ionized water at 37°C for 18 h in a pasteurization vat followed by freeze drying. The PA content was determined by the Association of Official Analytical Chemists method (30) and found to be 4.10% for undephytinized WB and 0.12% for BD. Thus the 25% level of WB in the WB diet provided a 1.0% PA concentration. The level of added PA in the DWBP diet was adjusted for the residual PA in DWB such that the overall concentration of PA in the diet was 1.0%. The food cups were supplied with fresh diet every 2 days. Fresh diet was prepared b-weekly and stored at -20°C. In use diets were refrigerated at 4°C. Food intake and weight were monitored weekly.

After 100 days treatment the rats were killed by CO2 gassing. All the major organs (liver, heart, lungs, kidneys, small intestine, colon and spleen) were weighed and examined for pathological alterations. The colons were collected for examination of ACF.

**Aberrant crypt formation**

ACF formation was analyzed according to the method of McLellan and Bird (31). Briefly, the colons were split open lengthwise, washed with saline, flushed with physiological saline (0.9%, pH 7.0), cut into two equal length sections (proximal and distal) and fixed flat between two layers of filter paper in 10% buffered formalin for a minimum of 3 days. The fixed colon sections were dehydrated in a graded series of ethanol from 100 to 70% and then distilled water. Endogenous peroxidase activity was blocked by immersing the slides in 3% H2O2 in water for 10 min with a subsequent double washing in distilled water. To expose the PCNA protein tissue sections were microwaved for 30 min in citric acid buffer, pH 6.0. The slides were allowed to cool for 30 min while immersed in the buffer and then incubated with 0.025% Triton in phosphate-buffered saline (PBS). The primary antibody (PCNA Ab-1; Calbiochem Inc., CA) was placed onto the tissue sections and incubated in a humid chamber for 2 h. The slides were again treated, in succession, three times with 0.025% Triton in PBS, the secondary links antibody (Dako Inc., Carpenteria, CA) in a humid chamber for 60 min, three times with 0.025% Triton in PBS, a streptavidin complex (Dako Inc., Carpenteria, CA) for 30 min, three times with distilled water, AEC substrate/chromagen (Dimension Labs, Mississauga, ON) for 30 min in a warm humid chamber, three times with distilled water, haematoxylin for 20 s, distilled water, tap water and eventually mounted with crystal mount. For the labeling index (LI) evaluation 24 crypts were counted along the entire distal colon section of each rat. All counting was performed at 400× magnification. Only whole longitudinally sectioned crypts that showed the entire column length from the lumen down to the muscularis mucosa were counted. Incomplete crypts or those with more than two missing cells were not counted. Only cells with a strong dark brown color, indicating a cell in S phase, were considered labeled (33). The numbers of these labeled cells and the total number of cells in each crypt were counted. In addition, the position and height of each cell was recorded. LIs were calculated as percent number of labeled cells for the whole crypt and the top 40% of the crypt. The 0th index, which is the ratio of labeled cells in the top 40% of the crypt to the labeled cells in the entire crypt and which is thought to be the best discriminator of increased colon cancer risk (34), was also calculated for each crypt.

**Statistical analyses**

All data were analyzed by one way analysis of variance followed by the Tukey’s pairwise multiple comparisons test or Dunn’s pairwise non-parametric multiple comparisons test using the SigmaStat statistical software package (Jandel Scientific, San Rafael, CA). ACF data were also analyzed for two way analysis of variance followed by the Tukey’s pairwise multiple comparisons test.

**Results**

**Aberrant crypt formation**

No significant differences were seen in the total number of ACF in the proximal and distal colons (Figure 1), although in both sections all treatment groups tended to have lower total numbers of ACF than the BD. However, when considering the whole colon, i.e. proximal plus distal sections (Figure 1), a significant decrease ($P < 0.01$) in the number of ACF was observed in the PA group versus BD.

The size of ACF in the proximal section did not differ significantly amongst the groups (Figure 2). In the distal section the PA group had significantly smaller ACF than both the BD ($P < 0.02$) and WB ($P < 0.04$) groups but did not differ significantly from the other groups.

Because there are differences in colon lengths which approached significance ($P < 0.08$), the concentration of ACF per cm colon length was estimated. The number of ACF per cm colon length was less in all the treatment groups versus BD in both the proximal and distal colon sections, but the difference did not reach significance (Figure 3). However, the WB and PA groups had significantly lower numbers of ACF per cm than the BD group ($P < 0.03$ and $P < 0.02$ respectively) in the whole colon.

Figure 4 shows the number of sialomucin-producing ACF. When compared with Figure 1 it is clear that with the BD group over 50% of all the ACF are sialomucin-producing, while the treatment groups have less than a third of the total ACF producing sialomucins. PA significantly decreased the number of sialomucin-producing ACF in both the distal and proximal colon sections, while all the treatment groups significantly decreased the number of sialomucin-producing ACF in the whole colon.

All the WB-containing diets as well as the PA diet signific-
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Fig. 1. Number of ACF in the whole colon and in the proximal and distal colon sections. For definitions of abbreviations see Table I.

Cell proliferation

Table II shows the indices of cell proliferation in the distal colon. The BD group had the highest rate of cell proliferation, which was significantly higher than all the treatment groups with the exception of DWBPA. The LI of the DWB and PA groups, although not significantly different from each other, were significantly higher than that of the WB group. All the treatment groups had significantly lower positions of the topmost labeled cell versus the BD, particularly the WB group, which also had significantly lower values than the DWB and PA groups.

The LI in the top 40% of the crypt (Table II) followed a similar pattern, again with the BD diet having a rate of cell proliferation significantly higher than all the treatment groups. In addition, the WB group was significantly lower than the DWB and PA groups. In the distal colon all the treatment groups with the exception of PA had a significantly lower $\phi_h$ index versus the BD group (Table II).

Discussion

This study has shown that WB, DWB and DWBPA can reduce some early biomarkers of colon cancer, such as the number of sialomucin-producing ACF in the whole colon and the degree of luminal alterations, i.e. the degree of observed deformity of the ACF lumen. These treatment diets can significantly decrease cell proliferation in the entire crypt and in the top 40% of the crypt as well as generate a smaller proliferative zone and a lower $\phi_h$ index, which is thought to be the best cell proliferation discriminator for determining colon cancer risk (34). This study has also shown that PA added to a low fiber diet can significantly decrease the number of ACF (distal and whole colon) as well as the size of ACF (distal and whole colon) versus BD, suggesting a potential reduction in colon cancer risk. If PA was the only active component in WB it would be expected that dephytinization of WB would cause an increase in ACF parameters. However, such an effect was not observed, while addition of exogenous PA had a significant effect only when added to a low fiber diet. Since no differences were observed in the initial weight, final weight, weight gain and food intake of the rats, none of the diets was likely more palatable than the others and thus the effects seen are not due to variations in food intake or body weights.

ACF formation was used as an early indicator of reduction in cancer risk since ACF are believed to be valid preneoplastic markers of colon cancer (35). Although there are some conflicting opinions on whether ACF are reliable end-points in experimental colon carcinogenesis, they have been shown to have much increased proliferative activity compared with normal adjacent crypts (36) as well as mutations in the $p53$ tumor suppressor gene (37) and in the $k-ras$ oncogene (38), both of which are common to many colon cancers.

It has been suggested that WB fiber has some colon cancer protective effects (39), based on many experiments over the
past few decades (6–11, to list but a few). In this experiment the WB-containing diets lowered the number and size of ACF, but the changes were not significant, although the WB diet did decrease ACF concentration (number of ACF per cm) over the length of the colon. However, the WB-containing diets significantly reduced the number of sialomucin-producing ACF, which have been shown to be linked to colon tumorigenesis (32). The shift to sialomucin production by ACF is important, since sialomucins are produced by a large percentage of colon tumors and are linked to increased aggressiveness, increased probability to metastase and a generally poorer prognosis for the patient (40). Thus sialomucin-producing ACF are likely further advanced along the stepwise adenoma–carcinoma sequence of colon cancer and thus probably have a higher likelihood of becoming colon tumors. Hence, the fact that all the WB-containing diets as well as PA decreased this parameter suggests that these diets may potentially modulate colon cancer risk from an early stage.

The differential staining of ACF by methylene blue can also provide information on tissue structural changes which are features of precancerous lesions. Thus it has been shown that the severity of the degree of luminal alteration may relate to the degree of dysplasia present in a particular AC (32). In this study we found a strong reduction in luminal deformity and alterations by all the treatment groups in the proximal and distal sections and in the colon as a whole. Thus the WB diets likely slowed down the development of deformity and consequently the advancement towards neoplasticity for the ACF observed.

**Table I.** Degree of luminal alterations of the AC in the various colon sections

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<th>Proximal</th>
<th>Distal</th>
<th>Whole</th>
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<tbody>
<tr>
<td>BD</td>
<td>2.50 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.62 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.57 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>WB</td>
<td>2.06 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.32 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.27 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DWB</td>
<td>1.97 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.26 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.21 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DWBPA</td>
<td>1.97 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.40 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.32 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PA</td>
<td>2.04 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.34 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.29 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
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Values are means ± SEM, n = 14 per group; values with different superscripts are significantly different, P < 0.05. BD, basal diet, control group; WB, 25% wheat bran diet; DWB, 25% dephytinized wheat bran diet; DWBPA, 25% dephytinized wheat bran plus 1.0% added PA; PA, 1.0% added PA. For determination of the degree of luminal alterations each AC was given a grade from 1 to 3 based on whether the luminal shape was mildly altered (enlarged and round, grade 1), moderately altered (elliptical, grade 2) or severely altered (slit-like, grade 3).

**Fig. 3.** Number of ACF per cm colon length in the whole colon and in the proximal and distal colon sections. For definitions of abbreviations see Table I.

**Fig. 4.** Number of sialomucin-producing ACF in the whole colon and in the proximal and distal colon sections. For definitions of abbreviations see Table I.

WB is thought to exert its colon cancer protective effect by several mechanisms, including physical dilution of gut contents, shortening transit times, alterations in the mutagenicity of intestinal contents, alterations in mucosal cytokinetics, increased fermentation producing butyrate and effects on the production, absorption and excretion of putative carcinogens (41). Interestingly, most of these mechanisms appear to be analogous to those suggested for the protective effects of PA, adding to the speculation that PA may be the component of WB which is contributing to its colon cancer protective effects. For example, WB fiber increases the stool bulk considerably (a point observed in this experiment; data not shown), causing...
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dilution and adsorption of any mutagens and carcinogens present (41). Like WB fiber, PA also increases stool bulk (observed in this study; data not shown). PA is a negatively charged molecule and is thus capable of binding proteins and starches (18), leading to their malabsorption and subsequent fermentation to SCFA in the colon, increasing bacterial and fecal bulk and acting like a soluble fiber. Since WB is a rich source of PA, it is difficult to distinguish between the effects of the WB fiber and those of the PA associated with it. In this study very little difference in effect was seen upon dephytinization of the WB used or upon addition of exogenous PA back to DWB, even though exogenous PA added to the low fiber diet was very effective in reducing the biomarkers of colon cancer risk. This suggests that both endogenous and exogenous PA probably interact with the fiber in WB making them less available for binding with other dietary components. On the other hand, in the low fiber diet PA may be freer to interact with dietary components other than fiber, such as starches and proteins, leading to a more pronounced protective effect as described above.

WB may be exerting its protective effects by reducing the rate of cell proliferation. Increased cell proliferation has been linked to the genesis of a number of neoplasias (42), including colon cancer (43). Measurement of the LI of cell proliferation may be considered a valid intermediate biomarker for colon cancer risk. In fact, a stepwise increase in cell proliferation rate of cell proliferation indicates a reduced effectiveness of WB and that this effect is conditional on the role of PA in its natural matrix, since pure PA added to the DWB diet did not decrease LIs measured down to the level of WB. Interestingly, although LI and the position of the top-most labeled cell for pure PA added to a low fiber diet (PA group) were lower relative to the BD group, they were significantly higher than the WB group, but not different to either the DWB or DWBPA groups. The differential effect of endogenous PA and pure PA added to the low fiber diet is also evident in values of φ index, thought to be the best discriminator of colon cancer risk (34), which were decreased relative to BD by all the WB-containing diets but not by the PA diet. Thus with respect to these results and the decreases observed in the ACF parameters with the PA group it is possible to suggest that endogenous PA and PA added to the low fiber diet, although both effective, may be so due to different mechanisms, which require further elucidation. In addition, the significant decreases in overall LI and of LI in the top 40% of the crypts brought about by the WB-containing diets is further indication of a decreased risk of colon carcinogenesis associated with their consumption, although all these data need to be confirmed in colon tumorigenesis studies.

Although generally showing a protective effect of WB fiber, experiments to date have not studied the effect of PA within the matrix of WB as a possible contributor to the effects observed. Pure PA added to the drinking water has been shown to be protective in a number of experiments (21–24), but since the major source of intake of PA is in the natural matrix of fibers in which it is present and not as a pure additive in drinking water, studies such as this one are necessary. From our results it can be concluded that, based on some of the methylene blue ACF characteristics, WB does not seem to be protective of early biomarkers of colon cancer, but based on decreases in the degree of luminal alterations, the number of sialomucin-producing ACF and indices of cell proliferation, WB appears to be protective, WB, with or without endogenous or exogenous PA, along with exogenous PA added to the low fiber diet, reduced the level of cell proliferation, the proliferative zone and φ index, suggesting a distinct protective effect.

It can also be concluded that, while having a role, endogenous PA is not the sole active component in WB and that exogenous PA is most effective when added to a low fiber diet. Although with dephytinization of WB significant changes in the ACF parameters were not observed, significant changes in the indices of cell proliferation indicate a reduced effectiveness of WB upon removal of PA. Furthermore, it appears that measurement of sialomucin-producing ACF and grading of luminal alterations may be effective ways of further characterizing ACF as preneoplastic markers of colon cancer.

Table II. Whole crypt LI, LI of the top 40% of each crypt, position of the top-most labeled cell and φ index in the distal colon

<table>
<thead>
<tr>
<th>Group</th>
<th>LI</th>
<th>LI top 40%</th>
<th>Position of top-most labeled cell</th>
<th>φ index</th>
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<tr>
<td>BD</td>
<td>58.74 ± 1.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.45 ± 1.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.95 ± 0.39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.107 ± 0.007&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>WB</td>
<td>48.41 ± 1.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.97 ± 0.99&lt;sup&gt;d&lt;/sup&gt;</td>
<td>22.62 ± 0.38&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.052 ± 0.008&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DWB</td>
<td>54.35 ± 0.93&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.71 ± 1.48&lt;sup&gt;c&lt;/sup&gt;</td>
<td>19.79 ± 0.39&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.081 ± 0.009&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DWBPA</td>
<td>55.89 ± 1.01&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>9.69 ± 0.91&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>21.47 ± 0.39&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.063 ± 0.005&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PA</td>
<td>53.61 ± 0.96&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.69 ± 1.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19.88 ± 0.36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.085 ± 0.007&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>

Values are means ± SEM on a sample size of 24 crypts/rat with 6 rats/group; values with different superscripts are significantly different, P < 0.05. Position of top-most labeled cell is denoted as a percentage of all cells that are unlabeled from the top of the crypt to the first labeled cell. Thus a high value indicates a greater percentage of unlabeled cells at the top of the crypts and thus a lowered position of the top-most labeled cell. The φ index is defined as the ratio of labeled cells in the top 40% of the crypt to the labeled cells in the entire crypt. For definitions of abbreviations see Table I.
References


