



Evaluation of the antimutagenic and anticarcinogenic effects of inulin *in vivo*

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ABSTRACT. The incidence of colorectal cancer is growing worldwide. The characterization of compounds present in the human diet that can prevent the occurrence of colorectal tumors is vital. The oligosaccharide inulin is such a compound. The aim of this study was to evaluate the antigenotoxic, antimutagenic and anticarcinogenic effects of inulin *in vivo*. Our study

is based on 3 assays that are widely used to evaluate chemoprevention (comet assay, micronucleus assay, and aberrant crypt focus assay) and tests 4 protocols of treatment with inulin (pre-treatment, simultaneous, post-treatment, and pre + continuous). Experiments were carried out in Swiss male mice of reproductive age. In order to induce DNA damage, we used the pro-carcinogenic agent 1,2-dimethylhydrazine. Inulin was administered orally at a concentration of 50 mg/kg body weight following the protocols mentioned above. Inulin was not administered to the control groups. Our data from the micronucleus assay reveal antimutagenic effects of inulin in all protocols. The percentage of inulin-induced damage reduction ranged from 47.25 to 141.75% across protocols. These data suggest that inulin could act through desmutagenic and bio-antimutagenic mechanisms. The anticarcinogenic activity (aberrant crypt focus assay) of inulin was observed in all protocols and the percentages of damage reduction ranged from 55.78 to 87.56% across protocols. Further tests, including human trials, will be necessary before this functional food can be proven to be effective in the prevention and treatment of colon cancer.

Key words: Inulin; Fiber; Chemoprevention

INTRODUCTION

The malignant tumors that affect the colon and rectum are the 3rd most common incidence of cancer in the world in both males and females, and the second most common incidence of cancer in developed countries (INCA, 2008). Among the variety of factors that are implicated in the etiology of colorectal tumors are genetic factors as well as environmental factors such as a diet rich in fat and meat and poor in fruits, vegetables, and cereals (Stevens et al., 2007).

The most common form of growing cancer cells is the transmission of cellular abnormalities that occurs mainly by morphological and molecular changes, also known as clonal selection. Such changes are related to alterations in the expression of oncogenes and tumor suppressor genes, which regulate cellular activities, such as proliferation, differentiation, and apoptosis (Pool-Zobel et al., 2002).

There is a need for discovering ways to prevent cancer through dietary supplementation with specific types of fibers, which act directly or indirectly by inhibiting the induction and/or development of this type of cancer (Rao et al., 1998).

Probiotic foods, such as fiber, are defined as non-digestible foods that act beneficially by stimulating the growth and/or activity of the bacterial flora in the host (Roberfroid, 2005) and that may prevent cancer. These foods are cheap, naturally present in the human diet (Verghese et al., 2002), and are related to the anticarcinogenic action of the oligosaccharide inulin. Inulin is a non-digestible carbohydrate that is derived from a glycosidic β (2 \rightarrow 1) fructan found in plants, such as chicory, garlic, onion, asparagus, bananas, wheat, barley, and rye. Once these oligosaccharides are fermented inside the bowel, they release chemicals that can regulate physiological functions. However, their mode of action is not properly understood. Scientific data suggest that the ability of inulin to modulate genetic and cytological factors important for colon function may contribute to its ability to prevent or reduce the growth of tumors (Pool-Zobel et al., 2002; Roberfroid, 2005).

This proposed mechanism of action has increased scientific interest in probiotic foods, which is reflected in the increase in the number of studies related to the role of fiber in the prevention of colon and rectal cancer. *In vivo* experimental models have been widely used to study this relationship (Verghese et al., 2002). An assay used to study the effects of inulin is the aberrant crypt focus (ACF) assay, which demonstrated the anticarcinogenic effect of inulin by showing reduced numbers of aberrant crypts in the intestinal lumen of mice treated with inulin as compared to control mice (Femia et al., 2002). Bird (1987) suggested that ACF lesions, also known as biomarkers, may develop into cancer. This biomarker is useful for quickly identifying the action of substances with mutagenic, carcinogenic, and genotoxic potential (Ward and Henderson, 1996). Therefore, the present study aims to evaluate the antigenotoxic, antimutagenic, and anticarcinogenic effects of inulin in response to damage caused by 1,2-dimethylhydrazine (DMH) in Swiss male mice.

MATERIAL AND METHODS

Chemical agents

DMH

DMH (Sigma, USA, CAS No. 306-37-6) at a dose of 20 mg/kg body weight (b.w.) was diluted at the time of use in an aqueous solution of ethylenediaminetetraacetic acid (EDTA) (0.37 mg/mL) and administered to the animals intraperitoneally (*ip*). Treatment was performed according to the protocol in Rodrigues et al. (2002). Briefly, 4 doses of DMH were administered in total, 2 doses each week for 2 weeks.

Inulin

The functional food was donated by ORAFITI, Belgium. Inulin at a concentration of 50 mg/kg b.w. was administered orally (*vo*) to the animals in an aqueous solution.

Animals and maintenance conditions in the vivarium

Swiss male mice (*Mus musculus*) (N = 77, 11 animals/group), of an average weight of 30 g, of reproductive age, and at the beginning of the adaptation period (7 days), were used for this study. The animals were housed in boxes made of propylene lined with white pine sawdust. They were fed a basal commercial ration (Nuvital, Brazil) and given filtered water *ad libitum*. Light was controlled using a photoperiod of 12 h (12-h light/12-h dark) and the temperature was maintained at $22 \pm 2^\circ\text{C}$ with a humidity of $55\% \pm 10$. The experiment was approved by the Committee of Ethics in Animal Experimentation of Universidade Federal do Mato Grosso do Sul (UFMS), under protocol No. 240/2009.

Experimental design

The animals were treated for 12 weeks according to the protocols suggested by Bolognani et al. (2001) and Ishii et al. (2011), as described below.

The control group received standard commercial ration *ad libitum* for 12 weeks. Dis-

tilled water (0.1 mL/10 g b.w., *vo*) was given every day until the end of the 12th week. In the 3rd and 4th weeks of experimentation, the animals received 4 doses of EDTA (0.1 mL/10 g b.w., *ip*). After the last administration, samples of peripheral blood were collected by puncturing the tail vein and were used for evaluating genotoxicity, mutagenicity, antigenotoxicity, and antimutagenicity. Mutagenicity was evaluated via the micronucleus assay (MA) and was carried out twice, once at 24 h (T1) and once at 48 h (T2) after the last administration of EDTA (0.1 mL/10 g b.w., *ip*). Genotoxicity was evaluated by the comet assay (CA) only at T1.

The DMH group was subjected to the same protocols as the control group for obtaining blood samples, for water administration, and for diet. However, instead of EDTA, the animals received DMH (20 mg/kg b.w., *ip*).

The inulin group received an aqueous solution of inulin (50 mg/kg b.w., *vo*) and commercial ration every day for all 12 weeks. In the 3rd and 4th weeks of the experiment, the animals were treated similarly to those in the control group, and blood samples were taken as described previously.

The pre-treatment group received an aqueous solution of inulin (50 mg/kg b.w., *vo*) every day during the first 2 weeks of the treatment. They received commercial ration for all 12 weeks. They were treated with DMH (20 mg/kg b.w., *ip*) in the 3rd and 4th weeks as described above, and blood collection was the same as for the control group.

The simultaneous group received DMH (20 mg/kg b.w., *ip*) in the 3rd and 4th weeks of experimentation, and during these 2 weeks, they also received an aqueous solution of inulin (50 mg/kg b.w., *vo*) every day until the last day of the 4th week. They received commercial ration for all 12 weeks. Blood collection took place in the same way as described for the control group.

The post-treatment group received DMH (20 mg/kg b.w., *ip*) in the 3rd and 4th weeks of experimentation. They also received distilled water (0.1 mL/10 g b.w., *vo*) every day. Twenty-four hours after the last DMH administration, the distilled water was substituted with an aqueous solution of inulin (50 mg/kg b.w., *vo*) until the last day of treatment. Commercial ration was also provided all 12 weeks.

The pre + continuous group received an aqueous solution of inulin (50 mg/kg b.w., *vo*) as well as commercial ration every day during the treatment, until the end of the 12th week. In the 3rd and 4th weeks, they were treated with DMH (20 mg/kg b.w., *ip*). Blood was collected in the same manner as described for the control group.

In the 12th week of experimentation, all of the animals from each group were sacrificed by cervical dislocation in order to collect intestines to test for ACF.

CA

The alkaline CA was performed as described by Singh et al. (1988), with modifications and under indirect light. Briefly, 20 μ L blood cell suspension was embedded into 120 μ L 0.5% low melting point agarose and layered on a pre-coated slide with a thin layer of normal melting point agarose. The slide was covered with a glass coverslip and cooled to 4°C for 20 min. The slides were immersed in lysis solution for 1 h. Next, the slides were transferred to an electrophoresis buffer for 20 min for denaturation and then subjected to electrophoresis in a buffer with pH > 13.0 at 4°C for 20 min. The slides were then neutralized, air-dried, and fixed in absolute ethanol for 10 min.

The slides were stained with 100 μ L 20 x 10⁻³ mg/mL ethidium bromide and evaluated

using a fluorescence microscope (Bioval, Brazil) at 40X, using an excitation filter of 420-490 nm and a barrier filter of 520 nm.

Three independent repetitions were performed, and 100 cells were scored per treatment, classifying the comets as follows: class 0, cells without a comet tail; class 1, cells with a tail less than the diameter of the nucleus; class 2, cells with a tail 1 to 2 times the diameter of the nucleus; and class 3, cells with a tail greater than 2 times the diameter of the nucleus. Apoptotic cells that showed a fragmented nucleus were not counted (Kobayashi et al., 1995).

The total score was calculated by adding the resultant values after multiplying the total number of cells observed in each class of lesion by the number of the class. Statistical analysis was performed using analysis of variance (ANOVA) followed by the Tukey-Kramer test ($P < 0.05$ was the criterion for statistical significance).

MA

The MA was performed using the original protocol described by Hayashi et al. (1990), with the modifications described by Oliveira et al. (2009). The slides were warmed to 70°C and covered with a layer of 20 μ L 1.0 mg/mL acridine orange in an aqueous solution. After the preparation of the slides, a drop of peripheral blood was deposited on the slide and covered by a coverslip. Analysis was performed with a fluorescence microscope (Bioval) at 40X magnification, with a 420-490-nm excitation filter and a 520-nm barrier filter. Approximately 2000 cells were analyzed per animal, and the data were analyzed by ANOVA or the Tukey-Kramer test ($P < 0.05$ for statistical significance).

ACF assay

After sacrificing the animals, a laparotomy was performed to remove the colon, which was opened along the insert mesenteric edge and stored in a 10% formalin buffer solution. For analyses, each segment of the colon was stained with 5% methylene blue solution for 10 min and placed on a slide with the mucosa facing up for analysis under a light microscope (DBG, Brazil) at 10X magnification.

The identification of ACF was based on the criteria utilized by Bird (1987). For the statistical data analysis of the ACF assay, the total number of ACF, aberrant crypts per focus and the relationship between crypt/focus in the different groups were compared. The statistical analysis was performed using ANOVA or the Tukey-Kramer test ($P < 0.05$ for statistical significance).

Percentage of damage reduction (DR%)

The DMH DR% upon inulin administration was calculated as follows: [DMH group mean - the mean of an associated group (pre- and post-treatment, simultaneous, and pre + continuous)] / (DMH group mean - control group mean). The result was multiplied by 100 to obtain the DR%. This procedure was performed to evaluate the DR% in the CA, MA, and ACF assays.

RESULTS

Table 1 shows the mean and the standard error of the initial weight, final weight,

and weight gain of the mice during the experimental period. Initial weights were statistically different and varied from 41.27 ± 0.82 to 47.09 ± 0.99 g. However, there was no statistical difference in final weights, which ranged from 36.80 ± 1.40 to 40.40 ± 0.98 g. There was a significant difference in weight gain between the groups with the inulin-treated animals showing the greatest loss of body mass.

Table 1. Initial weight, final weight, and weight gain of animals during the experimental period.

Experimental groups	Initial weight (g)	Final weight (g)	Weight gain (g)
Control	41.27 ± 0.82^a	40.40 ± 0.98^a	0.20 ± 0.63^a
DMH	41.27 ± 0.78^a	$38.10 \pm 0.79^{b,c}$	$-2.20 \pm 0.85^{a,b}$
Inulin	$44.72 \pm 1.10^{a,b}$	40.00 ± 1.12^a	$-5.60 \pm 1.02^{b,c}$
Pre-treatment	$47.09 \pm 0.99^{a,b}$	40.40 ± 0.98^a	-7.00 ± 1.27^c
Simultaneous	$43.27 \pm 0.90^{a,b}$	36.80 ± 1.40^c	-7.40 ± 1.08^c
Pos-treatment	$42.72 \pm 1.48^{a,b}$	$38.60 \pm 1.36^{b,c}$	$-4.00 \pm 0.79^{a,b,c}$
Pre + continuous	46.00 ± 1.75^b	$39.40 \pm 2.17^{a,b}$	$-6.40 \pm 1.07^{b,c}$

Data reported as means \pm SE. Control (negative control) = 0.1 mL/10 g EDTA (*ip*); DMH = 20 mg/kg 1,2-dimethylhydrazine (*ip*) for 2 weeks (positive control); Inulin = 50 mg/kg inulin (*vo*) for 12 weeks; Pre-treatment = 50 mg/kg inulin (*vo*) in the first 2 weeks + 20 mg/kg DMH (*ip*) for 2 weeks; Simultaneous = 50 mg/kg inulin (*vo*) + 20 mg/kg DMH (*ip*) both administrated simultaneously; Post-treatment = 20 mg/kg DMH (*ip*) for 2 weeks + 50 mg/kg inulin (*vo*) until the end of the experiment; Pre + continuous = 50 mg/kg inulin (*vo*) for 12 weeks + 20 mg/kg DMH (*ip*) for 2 weeks. Different letters indicate statistically significant differences ($P < 0.05$, ANOVA/Tukey).

The total and relative weights of the animals are shown in Table 2. Statistical analyses indicated significant differences for absolute heart weight between the inulin group (0.2797 ± 0.015) and the simultaneous group (0.1884 ± 0.012). There were no significant differences for any of the other organs, such as liver, kidneys, and lungs, or for relative weights.

The total frequency of damaged cells, damage class distribution, and CA score showed that inulin at a dose of 50 mg/kg had genotoxic activity and showed no antigenotoxic action (Table 3). The mean number of damaged cells in the control group was 0.81 ± 0.32 . The mean numbers of damaged cells in the DMH group and the inulin group were 98.36 ± 0.43 and 38.73 ± 1.35 , respectively. Yet, for the antigenotoxic experiments, values across the protocols ranged from 88.63 ± 1.20 to 98.90 ± 0.31 .

Table 4 shows the data on the mutagenic and antimutagenic mechanisms evaluated by the MA. This analysis showed that fructan had no mutagenic action. On the other hand, it had high antimutagenic potential under all experimental protocols. The inulin DR% of the different experimental groups at T1 was 111.54% for the pre-treatment group, 118.67% for the simultaneous group, 47.25% for the post-treatment group, and 103.80% for the pre + continuous group. At T2, the inulin DR% was 121.93% for the pre-treatment group, 77.46% for the simultaneous group, 141.75% for the post-treatment group, and 108.21 for the pre + continuous group.

From the ACF assay data shown in Table 5, it can be seen that the control group and the inulin group showed no ACF in the distal colonic mucosa, a phenomenon that is consistent with the absence of inulin carcinogenic action. On the other hand, inulin showed a potential anticarcinogenic action. The DR% based on this assay was 69.12% in the pre-treatment group, 87.56% in the simultaneous group, 55.78% in the post-treatment group, and 72.89% in the pre + continuous group. However, the aberrant crypt ratios in each focus were similar in the DMH group (1.66) and others: 1.36 (pre-treatment), 1.48 (simultaneous), 1.60 (post-treatment), 1.78 (pre + continuous).

Table 2. Absolute and relative weights of animal organs after the period of experimentation.

Experimental groups	Total weight (g)				Relative weight (g)			
	Heart ²	Lungs ¹	Liver ¹	Kidneys ¹	Heart ²	Lungs ¹	Liver ¹	Kidneys ¹
Control	0.2634 ± 0.0340 ^{a,b}	0.2690 ± 0.029 ^a	2.0461 ± 0.0630 ^b	0.0502 ± 0.0250 ^b	0.0064 ± 0.0008 ^a	0.0064 ± 0.0006 ^a	0.0495 ± 0.001 ^a	0.0122 ± 0.0006 ^a
DMH	0.2560 ± 0.0320 ^{a,b}	0.2994 ± 0.032 ^a	1.9092 ± 0.0660 ^a	0.5399 ± 0.0460 ^{a,b}	0.0061 ± 0.0007 ^a	0.0073 ± 0.0009 ^a	0.0463 ± 0.002 ^a	0.0130 ± 0.0011 ^a
Inulin	0.2797 ± 0.0150 ^a	0.2665 ± 0.007 ^a	2.1663 ± 0.0920 ^a	0.6403 ± 0.0140 ^{a,b}	0.0062 ± 0.0003 ^a	0.0059 ± 0.0002 ^a	0.0484 ± 0.006 ^a	0.0144 ± 0.0005 ^a
Pre-treatment	0.2351 ± 0.0130 ^{a,b}	0.2471 ± 0.009 ^a	2.2356 ± 0.0850 ^a	0.6356 ± 0.0220 ^{a,b}	0.0050 ± 0.0003 ^a	0.0052 ± 0.0002 ^a	0.0475 ± 0.001 ^a	0.0135 ± 0.0005 ^a
Simultaneous	0.1884 ± 0.0120 ^b	0.2320 ± 0.0120 ^b	2.1103 ± 0.1000 ^a	0.5808 ± 0.0170 ^{a,b}	0.0043 ± 0.0002 ^a	0.0054 ± 0.0003 ^a	0.0490 ± 0.003 ^a	0.0134 ± 0.0005 ^a
Post-treatment	0.2417 ± 0.0160 ^{a,b}	0.2570 ± 0.014 ^a	2.1380 ± 0.0960 ^a	0.5505 ± 0.0290 ^b	0.0056 ± 0.0003 ^a	0.0060 ± 0.0003 ^a	0.0504 ± 0.002 ^a	0.0129 ± 0.0006 ^a
Pre + continuous	0.4247 ± 0.1850 ^{a,b}	0.2870 ± 0.028 ^a	2.1065 ± 0.2160 ^a	0.5740 ± 0.0420 ^b	0.0093 ± 0.0004 ^a	0.0064 ± 0.0008 ^a	0.0468 ± 0.005 ^a	0.0124 ± 0.0009 ^a

Data reported as means ± SE. Different letters indicate statistically significant differences (P < 0.05; ¹ANOVA/Tukey; ²Kruskal-Wallis/Dunn). For group explanations, see legend to Table 1.

Table 3. Frequency of damaged cells, distribution between the classes of damage, and score related to the comet assay (CA) in mice peripheral blood.

Experimental group	Damaged cells	Classes of DNA damage - DNA migration in the CA				Score
		0	1	2	3	
Genotoxicity						
Control	0.81 ± 0.32 ^a	98.18 ± 0.32	0.82 ± 0.32	0.00 ± 0.00	0.00 ± 0.00	0.81 ± 0.32 ^a
DMH	98.36 ± 0.43 ^c	1.54 ± 0.45	5.64 ± 2.34	14.90 ± 1.55	77.82 ± 3.49	268.90 ± 6.35 ^c
Inulin	38.73 ± 1.35 ^b	61.27 ± 1.34	30.82 ± 1.72	6.81 ± 1.59	1.09 ± 0.39	47.73 ± 4.68 ^b
Antigenotoxicity						
Pre-treatment	97.36 ± 0.70 ^e	2.64 ± 0.70	7.19 ± 1.34	17.18 ± 1.49	73.00 ± 2.71	260.54 ± 4.68 ^e
Simultaneous	88.63 ± 1.20 ^e	11.81 ± 1.09	35.81 ± 3.23	48.27 ± 3.35	4.54 ± 1.15	146.00 ± 3.97 ^d
Post-treatment	98.90 ± 0.31 ^c	1.09 ± 0.31	11.54 ± 1.17	51.90 ± 2.11	35.45 ± 1.69	221.73 ± 2.39 ^c
Pre + continuous	94.54 ± 1.00 ^d	5.45 ± 1.00	23.00 ± 2.68	36.72 ± 3.35	40.91 ± 4.81	200.90 ± 7.80 ^f

Data reported as means ± SE. Different letters indicate statistically significant differences ($P < 0.05$, ANOVA/Tukey). For group explanations, see legend to Table 1.

Table 4. Frequency of micronucleus (MN), means ± SE, and damage reduction percentage (DR%) related to the micronucleus (MN) assay in mice peripheral blood.

Experimental groups	Frequency of MN		Means ± SE		DR%	
	T1	T2	T1	T2	T1	T2
Mutagenicity						
Control	96	61	8.72 ± 1.11 ^{ab}	5.54 ± 0.73 ^a	-	-
DMH	278	193	25.27 ± 2.07 ^d	17.54 ± 1.14 ^c	-	-
Inulin	165	81	15.00 ± 2.15 ^{bc}	7.63 ± 0.88 ^{ab,c}	-	-
Antimutagenicity						
Pre-treatment	75	81	6.81 ± 0.94 ^a	5.09 ± 0.99 ^{ab,c}	111.54	121.93
Simultaneous	62	56	5.63 ± 0.79 ^a	12.45 ± 1.76 ^a	118.67	77.46
Post-treatment	192	137	17.45 ± 1.32 ^c	1.81 ± 1.88 ^{b,c}	47.25	141.75
Pre + continuous	89	130	8.09 ± 2.15 ^a	7.36 ± 1.14 ^b	103.80	108.21

Different letters indicate statistically significant differences ($P < 0.05$, ANOVA/Tukey). For group explanations, see legend to Table 1.

Table 5. Number, distribution, and damage reduction percentage (DR%) of aberrant crypt foci (ACF) in the colon of mice treated with inulin.

Experimental group	Total of ACF		DR%	Total of AC	VA of AC				Relation AC/focus
	AV	VA ± SE			1 AC/focus	2 AC/focus	3 AC/focus	4 AC/focus	
Carcinogenicity									
Control	0.00	0 ^a	-	0.00	0.00	0.00	0.00	0.00	0.00
DMH	450	40.91 ± 1.63 ^d	-	748	226	145	48	22	1.66
Inulin	0.00	0 ^a	-	0.00	0.00	0.00	0.00	0.00	0.00
Anticarcinogenicity									
Pre-treatment	139	12.63 ± 0.85 ^c	69.12	190	98	32	8	1	1.36
Simultaneous	56	5.09 ± 0.68 ^b	87.56	83	39	10	4	3	1.48
Post-treatment	199	18.09 ± 1.06 ^c	55.78	319	122	47	17	13	1.60
Pre + continuous	122	11.09 ± 0.67 ^b	72.89	218	57	38	19	7	1.78

AV = absolute values; VA = average values; AC = aberrant crypts. Different letters indicate statistically significant differences ($P < 0.05$, ANOVA/Tukey). For group explanations, see legend to Table 1.

DISCUSSION

The consumption of foods rich in fiber is associated with a reduced risk of chronic

diseases, such as intestinal cancer (Schneeman, 1999). *In vivo* experiments show that the benefits of fiber in the diet depend upon the composition and the physical and chemical properties of the fiber (Reddy, 1999). Indeed, it has been established that certain types of fiber are more beneficial than others, with fibers having the greatest potential for solubility in water being more beneficial (Rao, 1999).

The physical properties of the fructooligosaccharide inulin appear to be similar to those of the fibers that benefit the gastrointestinal tract. It is known that the inulin is soluble in water and cannot be hydrolyzed by human enzymes. It is metabolized almost exclusively by bacteria in the intestinal microflora. These features make this fiber a functional food with probiotic potential (Haully and Moscatto, 2002). Thus, we hypothesized that a possible antimutagenic and anticarcinogenic effect of the fiber against damage caused by DMH, which is a pro-carcinogenic substance that must be metabolized by the intestinal flora, became active (LaMont and O’Gorman, 1978).

Data from several studies show that carcinogenicity and mutagenicity are related processes because chemical carcinogens may interact with genetic material causing mutations. It is known that cancer can be caused by a mutational event (Vogel, 1982) such as the alkylation caused by DMH. The objective of this study was to evaluate the antimutagenic effect of inulin and determine whether this effect was mediated through an anticarcinogenic pathway.

Upon assessing mutagenicity and antimutagenicity we found that that the DR% of this fructooligosaccharide is distinct between time points T1 and T2. At both these time points, the DR% was greater than 100%, except for T1 in the post-treatment group and T2 in the simultaneous group, suggesting not only a possible protection against DMH-induced damage but also basal DNA damage. Data from various protocols used to test inulin activity suggest that this compound, being both bioantimutagenic and desmutagenic, acts on two chemopreventive fronts.

In order to identify antimutagenic chemical compounds and elucidate the molecular mechanisms of their action, it is necessary to test these compounds using different treatment protocols (Ferguson, 1994; Flagg et al., 1995; De Flora and Ferguson, 2005; Oliveira et al., 2006, 2007). Of the various protocols used in the literature, we chose the pre-treatment, simultaneous, post-treatment, and pre + continuous treatment protocols. While the simultaneous treatment protocol indicates both desmutagenic and bioantimutagenic activities, pre- and post-treatment protocols demonstrate bioantimutagenic activity (Ferguson 1994; Flagg et al., 1995; De Flora and Ferguson 2005; Oliveira et al., 2006, 2007). Our results from the post-treatment group showed an increased DR% that ranged from 47.25 to 141.75% at T1 and T2, respectively. This chemopreventive effect may be explained by the increased bioavailability of inulin. Although the metabolites of DMH were expected to cause damage, the administration of the fructooligosaccharide reduced damage and promoted chemoprevention.

In the pre-treatment group our results showed a high DR%, ranging from 111.54 to 121.93% at T1 and T2, respectively. The DR% values between the two time points of blood sample collection are similar and indicate the continuous protective action of inulin, well after stopping inulin administration. The continued chemopreventive activity of inulin can be explained by its ability to modulate DNA repair enzymes, making them capable of acting faster and/or more efficiently to prevent the damage caused by DMH metabolites.

By analyzing the simultaneous group, we found that there was a reduction in the DR% between T1 and T2. This may be due to the reduced chemopreventive capacity of inulin when administered along with DMH, which in turn may be due to reduced fructan bioavailability

and the short timespan (only 48 h) available for modulating DNA repair enzymes. This reduction may also be due to the excretion of inulin metabolites or the loss of the bioantimutagenic activity of inulin metabolites upon interacting with DMH metabolites.

The analysis of the pre + continuous group showed that DR% in this group was similar at T1 and T2. The chemopreventive action of this group is based on two forms of action, bioantimutagenic and desmutagenic, as this protocol includes all the different methods of inulin administration used in the other protocols. In addition to acting through both pathways, the increased timespan of fructan exposure in this protocol makes the protocol the most effective.

The above results suggest that inulin has high antimutagenic activity through both the desmutagenic and bioantimutagenic mechanisms of prevention. However, it seems to act mainly through the bioantimutagenic mechanism.

When analyzing inulin anticarcinogenic activity through the ACF assay, we found that the highest DR% was in the simultaneous group (87.56%). Based on this, we infer that the increased timespan available for the metabolism of inulin by bifidobacteria in this protocol may have led to lower levels of the β -glucuronidase enzyme. This enzyme is responsible for activating DMH carcinogenic activity (Haully and Moscatto, 2002).

In the liver, the DMH hydrolysis product methylazoxymethanol interacts with β -glucuronic acid and is transported to the lumen intestine, where it is released as the active DMH metabolite azoxymethane (LaMont and O'Gorman 1978).

In rodents, DMH administration causes lesions in the intestinal mucosa and increases cell proliferation (Krutovskikh and Turosov, 1994). The repeated exposure of the colon mucosa to this pro-carcinogen causes crypt hyperplasia and hypertrophy of the intestinal mucosa (Wiebecke et al., 1973), which can later develop into an adenoma.

Pool-Zobel et al. (2002) showed that inulin is preferentially metabolized by the bifidobacteria present in the intestines of humans and mice. Because bifidobacteria, unlike other species present in the human intestine, produce little or no β -glucuronidase, they cause a decrease in DMH metabolism and consequently reduce the carcinogenicity of DMH in the colorectal mucosa. Another potential mechanism for reduced carcinogenicity in the presence of inulin may be competition among intestinal floral species, which leads to a decrease in the growth of bacterial species that produce enzymes that can metabolize and activate DMH (Haully and Moscatto, 2002).

In the pre-treatment group, the DR% of 69.12% for preventing neoplastic lesions indicates that this fiber reduces the occurrence of mutations in DNA by preventing DNA damage. The pre + continuous group had a DR% of 72.89%, making it the protocol in which inulin showed the second largest anticarcinogenic activity by acting through both the bioantimutagenic and desmutagenic mechanisms as previously discussed.

Other factors contribute to inulin anticarcinogenic activity. An example of such factor is the reduction of serum glucose levels. Roberfroid (2005) showed that the proliferation of tumor cells depends on the availability of glucose since most of the energy produced by these cells is through the glycolytic pathway. Therefore, tumor cells that consume inulin may have a reduced capacity for growth. Another important factor is the production of lactic acid by bifidobacteria, which reduces fecal pH and creates a bactericidal environment for putrefactive bacteria in addition to reducing ammonia absorption (Gibson and MacFarlane, 1995). Inulin also affects the intestinal epithelium by improving mucosal morphology and the composition of mucins (glycoproteins that protect the mucous membranes from environmental damage).

Consequently, inulin improves the resistance to colonization, prevents bacterial translocation, and finally contributes to improve the chemical and enzymatic functions that protect the gastrointestinal tract (Roberfroid, 2005). Furthermore, constipation is among the risk factors for colorectal cancer since prolonged contact of feces with the colon and rectum lumen increases the probability of developing this disease (Roberfroid, 2005). Inulin fiber, which increases the motility of the large intestine and shortens the bowel transit time in the colon, also relieves constipation symptoms and may thus reduce cancer risk through this mechanism.

When examining the number of crypts per focus, we found that there was no difference in this measure among groups. Rudolph et al. (2005) argued that a greater number of crypts per focus was associated with the progression of these lesions into tumors. In our study, there were no major injuries. The reason for this may be related to the length of this experiment. Twelve weeks are perhaps only sufficient to identify biomarkers and not to observe the reduction of ACF that could progress to an adenoma, which is visible only from the 2nd to 3rd week after DMH treatment (Zalatnai et al., 2001).

The CA, which tests inulin genotoxic activity, showed that the fiber does not have any antigenotoxic activity, but that it instead has genotoxic activity. We did not find any evidence in the literature consistent with this type of activity when using the dose and protocols used in this study. This genotoxic effect may be explained by the weight loss of the animals in the experimental groups who ingested the fiber. The products of lipid degradation, such as free radicals and hydrogen peroxide, are reactive chemical species that cause cellular damage primarily to DNA (Beltrão and Oliveira, 2007). However, since this type of damage is refractory to repair it may not have been detected by the MA. The MA cannot detect a genotoxic event; it only has the ability to detect a mutation after it has already occurred (Salvadori et al., 2003).

According to the study by Roberfroid (2005), the recommended intake of inulin in Europe is 3-11 g. By correlating the daily intake of inulin in g and the weight of the adult population in mg/kg, we estimate that insulin consumption in Europe is between 42.86 to 157.14 mg/kg per day and that in the United States is between 14.28 to 57.14 mg/kg per day. Since the dose tested in this study is within the recommended range for consumption in the human population, we did not expect a genotoxic effect of inulin. This finding warns of the need for further studies focused primarily on the genotoxicity of this compound.

The weight loss of the animals that consumed inulin may be due to certain properties of inulin, such as causing increased intestinal motility, which reduces fecal transit time (consequently reducing food absorption, especially that of fat). In addition, it is known that inulin degradation in this region of the intestine reduces the calorific value of fat and the glycemic rate (Roberfroid, 1999). These caloric losses are also related to the fact that a part of this energy is used for the synthesis of microbial biomass. Only a fraction of the original energy value is stored in the form of short-chain fatty acids. However, the host tissue uses only a part of the short-chain fatty acids whereas the rest are excreted (Bernier and Pascal, 1990; Roberfroid, 1991).

When analyzing the total and relative weights of the animals' organs, no significant difference was observed across the groups, except for the total weight of heart in the simultaneous group when compared to the inulin group, suggesting a lack of toxicity. However, this difference in total heart weight was not reflected in the relative heart weight. Therefore, the difference in total heart weight is likely due to the variation in animal size, which can affect organ size. Therefore, the relative weight is a better measure of weight changes.

In conclusion, based on the high DR% values calculated from MA and ACF assay,

we suggest that inulin is an effective antimutagenic and anticarcinogenic oligosaccharide. Protocols used suggest that it has both desmutagenic and bioantimutagenic activity. A better understanding of these chemopreventive models of action may assist in the discovery of new applications of inulin. However, further studies are necessary to investigate the interaction of DNA with inulin, particularly in terms of the genotoxicity of inulin and its effect on body weight. After a more thorough study of the effects of inulin, this oligosaccharide, which is already consumed as part of human diets, might be useful as an effective antimutagenic and anticarcinogenic compound. Inulin should be on the list of functional foods that are used to prevent adverse effects of chemotherapies and preserve the quality of life in patients.

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