Modulation of transforming growth factor *beta*2 (TGF-*beta*2) by inositol *hexaphosphate* in colon carcinogenesis in rats[1]

Modulação do fator transformador de crescimento *beta*2 (TGF*beta*2) pelo inositol hexafosfato na carcinogênese colônica em ratos

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ABSTRACT

Purpose: To evaluate modulation in the expression of Transforming growth factor *beta*2 (TGF-*beta*2) in short-term colon carcinogenesis. **Methods:** 64 male rats was used, comprising 4 groups of 16 animals each: group 1 received Inositol hexaphosphate (IP6) and azoxymethane (AOM); group 2, AOM alone; group 3, IP6 alone; group 4 was used as control. Groups 1 and 3 were given 1% IP6 in drinking water for 6 weeks. AOM was administered subcutaneously at weeks 3 and 4 of the experiment at 20 mg/kg of body weight each week. Immunohistochemical processing was performed with the use of anti-TGF-*beta*2 primary antibodies in right colon samples and quantitation of TGF-*beta*2 as percentage of expression, through computer-assisted image processing. **Results:** mean values of TGF-*beta*2 expression were $9.0 \pm 3.9\%$ for group 4 (control), $12.7 \pm 4.0\%$ for group 3 (IP6), $19.3 \pm 6.2\%$ for group 2 (AOM), and $13.1 \pm 5.3\%$ for group 1 (IP6+AOM). The value of p was calculated as 0.0001 for a 5% or lower significance level. **Conclusion:** the experiment revealed a significant increase in TGF-*beta*2 expression in right colon with the administration of AOM, and a significant decrease in TGF-*beta*2 expression when IP6 was administered with AOM.
Keywords: Transforming Growth Factor Beta. Phytic Acid, Azoxymethane. Rats.

RESUMO

Objetivo: Avaliar a modulação da expressão do TGF-*beta*2 na carcinogênese colônica de curta duração em colon direito de ratos. **Método:** foram utilizados 64 ratos Wistar, machos divididos em 4 grupos de 16 animais. Grupo 1: recebeu Inositol hexafosfato (IP6) e azoximetano (AOM). Grupo 2 recebeu somente AOM. Grupo 3: recebeu somente IP6. Grupo 4: grupo de controle não recebeu nem IP6 nem AOM. O azoximetano (AOM) foi ministrado na dose 20mg/kg, por via subcutânea na 3ª e 4ª semanas do experimento. Foi realizada imunoistoquímica utilizando-se anticorpo primário TGF*beta*2. Utilizou-se processamento de imagem computadorizada para quantificação da expressão do TGF*beta*2. **Resultados:** a média da expressão do TGFbeta2 foi de $9.0 \pm 3.9\%$ para o grupo 4 (controle), $12.7 \pm 4.0\%$ para o grupo 3 (IP6), $19.3 \pm 6.2\%$ para o grupo 2 (AOM), e $13.1 \pm 5.3\%$ para o grupo 1 (IP6+AOM). **Conclusão:** ocorreu aumento significante a da expressão de TGF-*beta*2 no cólon, com a administração de AOM, e uma diminuição significante na expressão de TGF-*beta*2 quando IP6 IP6 foi administrado com AOM.
Descritores: Fator Transformador de Crescimento Beta. Ácido Phytico acid, Azoximetano. Ratos.

Introduction

Transforming growth factor *beta* (TGF-*beta*) is a member of a family of proteins that regulate numerous biological activities, including cell proliferation, differentiation, adhesion, and apoptosis, extracellular matrix (ECM) production, and early embryo development and immunity. The involvement of members of this family in carcinogenesis is a complex one, but a predominantly suppressing activity is observed in normal tissues. In tumorigenesis, however, changes in cell expression and response favor oncogenic activity[1,2]. TGF-*beta* is secreted by subepithelial myofibroblasts[3] and is stored in the extracellular matrix as a latent complex, which can be cleaved by the following alterations: (a) physicochemical changes (microalterations in

cell acidity, extreme pH values, gamma radiation, reactive oxygen species—ROS); (b) enzymatic changes and with the interaction of nonspecific proteins (proteases, metalloproteinases type 2 and 9, glycosidases, trombospondin, and mediators of integrin); (c) induction by drugs (antiestrogens, retinoids, vitamin D3 derivatives, and glucocorticoids)[4]. Cleavage of the latent complex promotes TGF-*beta*2 activation, generating strong cell signals after bonding with its cell membrane receptor—TGFBR2[5]. Carcinogenesis is composed of a sequence of steps—initiation, promotion, and tumoral progression—and TGF-*beta* action has been identified during cancer progression in the following functions: (a) limiting normal epithelial growth and early tumors; (b) causing loss of growth-inhibitory response by the loss of TGF-*beta* receptors or SMAD proteins, or by the specific loss of response of cytostatic genes, selected by more aggressive tumoral growth, facilitated by the additional acquisition of oncogenic mutation; (c) altering tumoral cells that display impaired cytostatic response, while preserving components of the TGF-*beta* signaling pathway, making them more invasive; (d) developing an environment of immunosupression for the function of T cells, evading cytotoxic removal mediated by T lymphocytes—an event that takes place when a tumor is already established; (e) mediating induction of angiogenic response, facilitating the recruitment of new tumor-nourishing blood vessels; (f) promoting tumoral cell adhesion to the endothelium and/or extravasation of tumoral cells at methastatic sites; and (g) stimulating gene expression of osteoclast differentiation factor (interleukin 11) and connective tissue growth factor[6]. A number of animal studies have demonstrated that inositol hexaphosphate (IP6) inhibits tumoral growth in diverse types of cancer, including that of colon. Proposed action mechanisms are: increased activity of natural killer cells, changes in signal transduction with PI3-K blockade, stimulation of genes responsible for cell differentiation, apoptosis, and antioxidant activity[7]. Because changes in tissue oxygenation levels accompany several pathological states, including cancer, a mechanism must exist that activates latent TGF-*beta*, whose redox sensitivity is within the range of that displayed by the precursor peptide LTGF-*beta*. LTGF-*beta* redox sensitivity is presumably directed toward recovery of homeostasis; however, oxidation may also be a mechanism of LTGF-*beta* activation that can be deleterious during disease mechanisms involving chronic ROS production[8]. These aspects led us to raise doubts on the role and ability of IP6 in modulating the expression of TGF-*beta*2 upon administration of a free-radical-generating substance that is carcinogenic to colon. In order to clarify these aspects, we developed the present study, whose purpose was to investigate experimentally the modulation of TGF-*beta*2 expression with the administration of IP6 in short-term colon carcinogenesis in rats.

Methods

Sixty four male Wistar rats were used. They were bred in the Central Animal Facilities of UFMS and had a mean weight of 152 ± 26 g. The study was evaluated by the Animal Ethics Committee of UFMS, approved, and certified by Protocol 01/2001 of April 25, 2001. The experiment was carried out in the Laboratory of Experimental Carcinogenesis, an addition to the Central Animal Facilities of UFMS. Polypropylene cages

(standard size for 5 rats) with galvanized wire lids were used, each housing four animals randomly selected by draw. The animals were then acclimated to the housing conditions for 7 days under artificial light (130 to 325 lux) with light/dark cycles of 12 h each, mean temperature of $24 \pm 2\,°C$, and mean humidity of $52 \pm 8\%$. They were fed *ad libitum* on rat chow (Nuvilab CR1, Nuvital Nutrientes e Produtos Veterinários Ltda, Curitiba, Brazil) and filtered water. The 64 rats were distributed into four groups by draw. Tail tattoos, made with an indelible black ink pen, were used to identify groups (1 through 4) and individual animals (A through Q) within each group. Groups distribution was as follows: group l (IP6+AOM): 16(n) animals receiving IP6 and AOM; group 2 (AOM): 16(n), receiving AOM alone; group 3 (IP6): 16(n), receiving IP6 alone; group 4 (control): 16(n) untreated animals.

Procedures

Administration of substances

IP6 (anti-tumoral substance, $C_6H_6O_{24}P_6Na_{12}$. Sigma, cat. P3168) was administered as a 1% solution in drinking water *ad libitum* to groups 1 and 3, for six weeks. AOM (carcinogenic substance, $C_2H_6N_2O$. Sigma, cat. A9517, lot 70K0847) was administered subcutaneously at 20 mg/kg of body weight (prepared as 100 mg AOM/0.1 ml + 49.9 ml 0.9% physiological solution, resulting in 2 mg AOM/ml) to groups 1 and 2 in the beginning of the third and fourth weeks of the six-week period, at the same times when groups 3 and 4 were given 0.9% saline solution subcutaneously at 10 ml/kg of body weight—in volumes equivalent to those of diluted AOM administered to groups 1 and 2 (Figure 1).
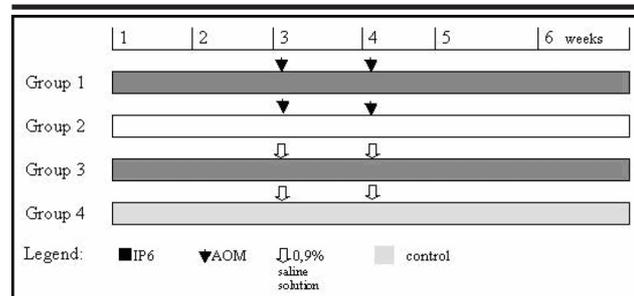
FIGURE 1 - Protocol for the administration of substances.

Euthanasia

At the end of the six-week period, the animals were identified, weighed, and subjected to euthanasia by intraperitoneal thiopental at 150 mg/kg of body weight.

Harvesting of material for study

Each rat was positioned in dorsal decubitus and submitted to midline laparotomy. The ileocecal region was then identified and the terminal ileum and right colon were harvested en bloc.

Preparation of material for study

Each colon specimen was opened along the antimesenteric

border and the mucosa was rinsed with Ringer's solution. A 1-cm long segment was then resected from the right colon, distally from the ileocolic region. The segment of colon wall was sandwiched between the plates of a hinged perforated double holder in order to be maintained straightened, and the resulting set was immersed in 10% buffered formol solution for 24 hours.

Preparation of blocks and slides

Each sample was embedded in paraffin (with sample identification being preserved) and sectioned with a microtome (4-micrometer-thick sagittal sections of colon wall). The resulting sections were mounted on silanized glass slides (first cut for histology; second cut for immunohistochemistry).

Histological analysis

The tissue sections were stained with hematoxylin-eosin and viewed under an optical microscope at 400X magnifications for identification of crypts and aberrant crypt foci (ACF). The following histological abnormalities of ACF were taken into account: increased nucleus-cytoplasm ratio, nuclear stratification, loss of nuclear polarity, structural abnormality, and number of crypts in the ACF (Figure 2).
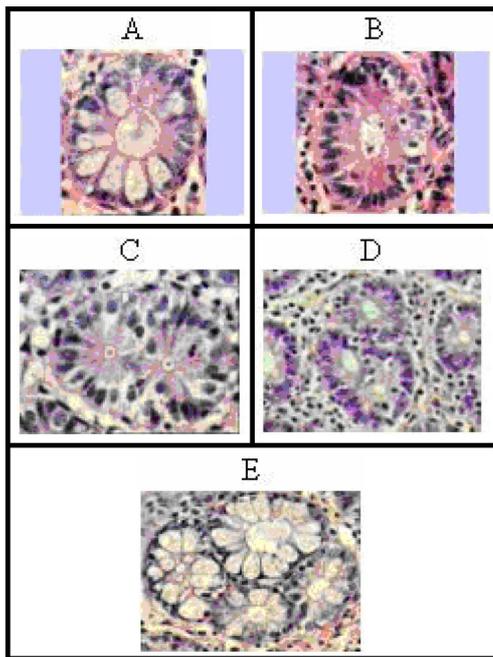
FIGURE 2 - Photomicrographies showing: (A) normal crypt, (B) Aberrant crypt focus (ACF) with 1 crypt, (C) ACF with 2 crypts, (D) ACF with 3 crypts, (E) ACF with 4 crypts. Magnification 400x.

Immunohistochemistry

Anti-TGF-*beta*2 primary antibodies (SantaCruz Biotechnology Inc., cat. SC90, lot 60303; 1:500 dilution), anti-rat secondary antibodies (Dako, cat. KO-609, lot 09371; 1:1 500 dilution), and diaminobenzidine (DAB) color developer (Dako, cat. K34767-1, lot 09381; 1:20 dilution) were used for immunohistochemistry processing. TGF-*beta*2 expression was obtained as brown color areas.

Microscopy and image capture

Optical microscopy was performed with a microscope (Nikon, mod. Elipse E200) equipped with 10X binocular lenses and a 40X/0.65 objective. The microscope was coupled to a video camera (Samsung, mod. SCC131) connected to a microcomputer (Duron 750-MHz processor, 128-MB RAM, 20-GB hard disk, Microsoft Windows 98 SE) through a videoboard (Pinnacle Studio PCTV, USB).

Immunohistochemistry quantitation

TGF-*beta*2 was quantified by computer-assisted image processing with ImageLab software. One single image from each sample was captured, which was centered either on the crypt or the ACF. For quantitation purposes, the ACF were examined and selected so that when more than one focus was available, a focus containing two crypts was chosen. In the absence of ACF with two, three, or four crypts, a random selection of crypts or ACF with one single crypt was made. Each image captured was saved (for documentation purposes) and codified according to a previously designed scheme. The quantified brown coloration density corresponds to TGF-*beta*2 expression in area percentage (using a RGB filter, blue background, 0-to-147 color interval) in the selected crypt or ACF (Figure 3).

FIGURE 3 - Computer-assisted image processing. TGF-beta2 was quantified by computer-assisted image processing with ImageLab software. One single image from each sample was captured, which was centered either on the crypt or the ACF. The quantified brown coloration density corresponds to TGF-beta2 expression in area percentage (using a RGB filter, blue background, 0-to-147 color interval) in the selected crypt or ACF.

Statistical analysis

A 95% confidence interval and a 5% significance level ($p \leq 0.05$) were adopted. ANOVA was used for comparing: (a) the mean weights of animals per group across all groups at the beginning of the experiment; (b) the mean differences between the initial and final weights across groups (Table 3); (c) the mean values, in percentage, of TGF-*beta*2 expression as revealed by immunohistochemistry processing. Paired comparison of the mean values obtained through ANOVA was then performed by applying the Fisher's Least Significant Difference (LSD) test.

Results

TABLE 1 - Immunohistochemistry results. Percentages of the captured areas of TGF-*beta*2 expression in aberrant crypt foci in colon, as quantified through computerassisted image processing (ImageLab software) from immunohistochemistry-processed slides. Campo Grande, Brazil 2004.

Groups	1	2	3	4
			%	
	6.5	13.6	12.2	8.5
	6.7	25.8	19.9	13.4
	17.8	23.7	15.2	5.3
	8.5	23.3	10.3	13.3
	14.1	21.5	11.6	6.6
	12.0	19.5	22.1	9.6
	16.4	9.7	10.1	11.5
	11.9	13.6	7.4	7.5
	14.7	16.8	10.5	6.4
	10.4	19.1	15.0	---
	8.3	26.4	13.0	7.3
	9.1	23.6	8.0	---
	19.5	19.4	14.6	---
	19.6	27.0	11.1	5.0
	24.4	20.3	9.1	17.8
	9.6	4.9	12.4	5.3
Mean ± SD	13.1 ± 5.3	19.3 ± 6.2	12.7 ± 4.0	9.0 ± 3.9
Coefficient of variation	40.1	32.4	31.6	43.2

--- Noncollected sample.

TABLE 2 - Paired comparison of TGFbeta2 expression in the groups. Campo Grande, Brazil 2004.

Groups	*p*
1 and 2	0.0009*
1 and 3	0.8051
1 and 4	0.0336*
2 and 3	0.0004*
2 and 4	0.0000*
3 and 4	0.0571

*Significance: $p \leq 0.05$ (*t* test)

TABLE 3 - Weight gain during the experiment. Mean and standart deviation of the difference between final and initial weights. Campo Grande, Brazil, 2004.

| Groups	Substances administered	n	Weight (g) mean	SD	*p**
1	IP6+AOM	16	128	± 20.4	
2	AOM	16	134.6	± 15.4	0.2274
3	IP6	16	130.5	± 29.6	
4	none (control)	16	143.7	± 22.7	

* Significance, $p \leq 0.05$ (ANOVA).

Statistical calculation

The analysis of variance among groups calculated for TGF-*beta*2 expression yielded $p = 0.0001$ (significance: $p \pounds 0.05$). Results of the paired comparison of mean values are shown in Table 2.

Effect on TGF-beta2 with administration of AOM

A significant increase in TGF-*beta*2 expression was found at the end of the fourth week after administration of AOM (group 2). A significant decrease in TGF-*beta*2 expression was found when IP6 was supplied. The administration of IP6 was started two weeks before that of the carcinogenic substance AOM (Figure 4).
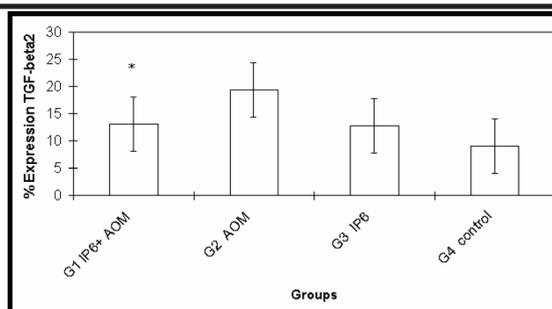
FIGURE 4 - Schematical representation of significant influence of inositol hexaphosphate on TGF*beta*2 expression in aberrant crypt foci in colon (Group 1) at the end of the sixth-week experiment, as compared with group 2, in azoxymethaneinduced colon carcinogenesis model in rats. ***p**=0.0001

Effect on TGF-beta2 with administration of IP6

No significant difference was found for TGF-*beta*2 expression in group 3, which received IP6 alone, as compared with group 4 (control). A significant decrease in TGF-*beta*2 expression (group 1) was found when the antitumoral substance IP6 was supplied in conjunction with AOM, as compared with group 2 (AOM alone).

General observations

No significant differences were found when the mean weight gains were compared across groups ($p = 0.2274$). No deaths occurred in the experimental period. Three samples failed to be collected because of losses during microtomy or because sectioning made them unusable.

Discussion

In this investigation, rats were chosen as experimental animals because ACF have been studied in them. Also, rat gene TGFBR2[9] has been identified as a tumoral suppressor. These facts further increased the reliability in the carcinogenesis model already standardized and recommended by the International Agency for Research on Cancer. The adoption of a short-term colon carcinogenesis model, which

uses ACF identification as an endpoint structure, addresses the need for investigating a phenomenon related to primary prevention of cancer, at a stage when this disturb is not yet phenotypically characterized. When the genetic bases of colorectal cancer and critical signaling pathways of tumorigenesis (APC/*beta*-catenin, DNA mismatch repair (MMR), and TGF-*beta*/SMAD pathways) are considered, a higher prevalence is found for colorectal cancer mutations with genetic instability of gene TGFBR2 in culture cell lines. In sporadic cancer, a prevalence of gene TGFBR2 has been identified in 82% to 90% of cases; in familiar cancer, the prevalence ranged from 78% to 83%[10]. Theses levels point to the need to investigate its potential importance in cancer prevention, and for implementing procedures aimed at modulating TGF-*beta*2 expression in the tumorigenesis process. Because apoptosis is a relevant process—a physiologic condition necessary for the elimination of defective cells—the choice of substances that participate in it and in antioxidation defined the criteria that led to the adoption of TGF-*beta*2 and IP6 in the experiment. Also, IP6 is a natural substance, being available from daily-use foodstuffs, a feature that favors, in economic terms, its wide-scale consumption in nonpurified form. Both the well-establish link between diet and cancer and the new genomic technologies have made it possible to investigate the nutritional modulation of carcinogenic pathways with the use of nutrients, micronutrients, and phytochemicals[11]. In rats, the administration of 1% IP6 promotes a decrease in the appearance of ACF[12], whereas at a concentration of 2% this substance inhibits tumor establishment[13]. Chemoprevention of colon cancer has come to be regarded as an important procedure, so that known substances can be ranked in terms of the potency that leads the a decrease in the frequency of ACF establishment—of 31% with the use of 1% IP6 and of 70% for 2% IP6. It is worth mentioning that 2% IP6 is an alkaline solution, and the choice of IP6 at a 1% concentration and neutral pH favors the oral use of the solution, with preserved effectiveness[14]. The technique for keeping the colon wall segments straightened prevented the retraction caused by immersion in formol, which results in shrinkage of muscle fibers. This procedure made tissue samples more suitable for sagittal microtomy and subsequent optical microscopy and viewing of crypts and ACF. Computer-assisted image analysis was chosen to quantify the brown-tinted areas corresponding to TGF-*beta*2 expression and made visible by immunohistochemical processing. This type of analysis was needed because TGF-*beta*2, having low molecular weight, has intra- and extracellular distribution. With this method, it became possible to quantify TGF-*beta*2 expression in an objective and reproducible manner. This is in contrast with most investigations reported in the literature, which utilize semiquantitative immunohistochemistry, whereas in the present study a quantitative immunohistochemistry methodology was required[15]. An algorithm was developed to deconvolve the color information acquired with red-green-blue (RGB) cameras and to calculate the contribution of each of the applied stains based on stain-specific RGB absorption. The algorithm was tested using different combinations of diaminobenzidine, hematoxylin and eosin at different staining levels. Quantification of the different stains was not significantly influenced by the combination of multiple stains in a single sample. The color deconvolution algorithm resulted

in comparable quantification independent of the stain combinations as long as the histochemical procedures did not influence the amount of stain in the sample due to bleaching because of stain solubility and saturation of staining was prevented. This image analysis algorithm provides a robust and flexible method for objective immunohistochemical analysis of samples stained with up to three different stains using a laboratory microscope, standard RGB camera setup and the public domain program NIH Image[16]. The absence of significant differences in weight gain across groups ($p = 0.2274$) corroborates results from other studies where no weight changes were observed when IP6 was supplied[17] even when the dietary and proteic quality of the animal chow was evaluated. In the present study, food was supplied *ad libitum*, since a calorie-restricted diet has been shown elsewhere to decrease significantly the expression of the proteins TGF-*beta*1/*beta*2 and COX-2 in the mucosa of obese rats[18]. Apoptosis is significantly triggered upon the administration of AOM, to eliminate cells damaged by the administration of this substance[19]. This was evident in group 2, where a significant increase ($p = 0.0000$) of TGF-*beta*2 expression occurred, as compared with group 4 (control). The administration of IP6 alone promoted an increase in TGF-*beta*2 expression, though not in a significant manner, as compared with the control group. This finding, however, can be regarded as relevant, as it contradicts the expectation that IP6 might normalize cellular physiology through one of its actions and promote normal or hypoexpression of TGF-*beta*2. Such increase might be explained by demonstrating that apoptosis is increased by 60% at the lower portion of the crypt when a diet containing IP6 is supplied[20]. As many as 82.6% of mucinous goblet cells present in ACF having 1 to 3 crypts contain labile class III mucin[21]. In the present experiment, no TGF-*beta*2 expression was observed in the mucin of goblet cells in those groups receiving AOM (Fig.3, groups 1 and 2). Evidence for a significant increase in TGF-*beta*2 expression at the end of the fourth week after the initial administration of AOM, in ACF-type lesions regarded as nonadenomatous and premalignant, has not been reported in the literature. This allows the occurrence of early alterations in TGF-*beta*2 to be regarded as preceding the phase of tumorigenesis progression; this finding differs from the previously accepted view on the genetic events related to colon cancer tumorigenesis, where TGF-*beta*2 is increased from the adenoma to the carcinoma phase (Chung DC, 2000). The present study has demonstrated that in group 1 (IP6+AOM) the administration of IP6 provides a modulating action, significantly decreasing TGF-*beta*2 expression, as compared with group 2 (AOM) ($p = 0.0009$). The experiment reveals that the administration of the carcinogenic substance stimulates an increase in TGF-*beta*2, which is maintained at the end of the fourth week following the administration of AOM. It can be inferred that a mechanism must be at play, other than the generation of free radicals and DNA damage, as the latter undergoes repair within 72 hours[22]. The existence of a stimulus promoting sustained high levels of TGF-*beta*2 at the end of the fourth week deserves investigation. The modulation of TGF-*beta*2 expression promoted by the administration of IP6 reinforces the recommended chemopreventive use of selected foods containing IP6, and/or the addition of IP6 to diets. These measures are expected to reduce the incidence of colon cancer, as they should help to

promote, through nutritional re-education, a better selection of foods for daily consumption, leading to a future lower incidence of cancer.

Conclusion

The experiment demonstrated a significant increase in TGF-*beta*2 expression in right colon with the administration of azoxymethane and a significant reduction of TGF-*beta*2 when inositol hexaphosphate was administered in conjunction with azoxymethane.

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