Apoptotic effects of inositol hexaphosphate on biomarker Itpr3 in induced colon rat carcinogenesis[1]

Efeito de apoptose do inositol hexafosfato no marcador biológico Itpr3 em carcinogênese induzida de colo em ratos

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ABSTRACT

Purpose: To study the effect of the modulation of inositol hexaphosphate (IP6) in the biological immunohistochemistry expression of cellular signaling marker apoptosis, in model of carcinogenesis of colon induced by azoxymethane (AOM). **Methods:** Wistar rats (N=112) distributed in 4 groups (n=28): Control; B, AOM (5 mg kg^{-1}, 2x, to break week 3); C, IP6 (in water 1%, six weeks); D, IP6+AOM. Weekly euthanasia (n=7), from week three. Immunohistochemistry of ascendant colon with biological marker inositol 1,4,5 triphosphate receptor type III (Itpr3). Quantification of the immune-expression with use of computer-assisted image processing. Analysis statistics of the means between groups, weeks in groups, groups in weeks, and established significance when $p \leq 0.05$. **Results:** One proved significant difference between groups in the expression of Itpr3, $p<0.0001$; with Itpr3 reduction of BxD group, $p<0.001$. **Conclusion:** Inositol hexaphosphate promotes modulation of biological markers with reduction of Itpr3 in carcinogenesis of colon.

Key words: Apoptosis. Azoxymethane. Cancer. Colon. Inositol 1,4,5 trisphosphate receptors. Computer-assisted image processing. Phytic acid. Rats.

RESUMO

Objetivo: Estudar os efeitos da modulação do inositol hexafosfato (IP6) na expressão imunoistoquímica de marcador biológico de sinalização celular de apoptose, em modelo de carcinogênese induzida pelo azoximetano (AOM). **Métodos:** Ratos Wistar (N=112) distribuídos em 4 grupos (n=28): A, controle; B, AOM (5 mg Kg^{-1}, 2x, a partir semana 3); C, IP6 (em água a 1%, seis semanas); D, IP6+AOM. Eutanásia semanal (n=7), a partir de semana três. Imunoistoquímica de colo ascendente com marcador biológico *inositol 1,4,5 trisphosphate receptor type III* (Itpr3). Quantificação da imunoexpressão com uso de processamento imagem assistida computador. Análise estatística da expressão média entre grupos, semanas em grupos e grupos em semanas, e estabelecido significância quando $p \leq 0.05$. **Resultados**: Evidenciou-se diferença significante entre grupos na expressão de Itpr3, $p<0.0001$; com diminuição Itpr3 de grupo BxD, $p<0.001$. **Conclusão**: O inositol hexafostato promove a modulação de marcador biológico com diminuição Itpr3 em carcinogênese de colo.

Descritores: Apoptose. Ácido fítico. Azoximetano. Câncer. Colo. Receptores de inositol 1,4,5-trifosfato. Processamento imagem assistida computador. Ratos.

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Introduction

Inositol 1,4,5-trisphosphate receptor (Itpr) is the primary cytosolic target responsible for the initiation of intracellular calcium (Ca^{2+}) signaling. To fulfill this function, the Itpr depends on interaction with accessory subunits and regulatory proteins. Specific interactions between these modulatory proteins and the Itpr have been described, making it clear that the controlled modulation of the Itpr by its binding partners is necessary for physiological cell regulation. The functional coupling of these modulators with the Itpr can control apoptosis, intracellular pH, the initiation and regulation of neuronal Ca^{2+} signaling, exocytosis, and gene expression. The pathophysiological relevance of Itpr modulation is apparent when the functional interaction of these proteins is enhanced or abolished by mutation or overexpression. The subsequent deregulation of the Itpr leads to pathological changes in Ca^{2+} signaling, signal initiation, the amplitude and frequency of Ca^{2+} signals, and the duration of the Ca^{2+} elevation. Consequences of this deregulation include abnormal growth and apoptosis1.

Itpr was identified in chromosome 20, localization 20p12, geneID 25679, and participates of the pathway phosphatidylinositol signaling system. The amplitude, frequency, and sub cellular localization of Ca^{2+} signals play an important role in determining cellular responses. Itpr´s require the second messenger inositol 1,4,5-trisphosphate (IP3) for activation but they are also regulated physiologically via cross-talk with other signaling pathways. Among the signaling pathways that modulate Itpr function are those involving kinases and phosphatases. The Itpr3 acts as a messenger for inositol triphosphate, mediating the pathway signalling of calcium, this why isoform Itpr3 loses feedback of inhibition with cytosol calcium2. However, has evidence that the calcium increases the sensitivity of the Itpr3 in unbroken cells and supports the concept that this isoform can act as a target for signaling calcium (hormone-induced)[3].

Failure of apoptosis may be of particular importance in the development of colorectal cancer. Evidence indicates that the regulatory pathways of apoptosis are frequently disabled in colorectal carcinoma (CRC), with an increased threshold for its activation and a progressive disorder of apoptotic homeostasis during carcinogenesis as genomic instability progressively increases4. As a consequence, genetically defective cells escape apoptotic deletion with the possible survival of clones possessing biologically significant mutations. Apoptosis is one Ca^{2+}-mediated event that may be influenced differently by each Itpr isoform. A number of studies have shown that reducing Itpr expression inhibits apoptosis5 however, type Itpr3 was selectively increased during apoptosis6.

Elucidation of the critical events associated with carcinogenesis provides the opportunity to prevent cancer development through induction of apoptosis, particularly by bioactive agents or functional foods7.

Thus, understanding the modulation of death receptors by chemopreventive agents and their implications in chemoprevention may provide a rational approach for using such agents alone or in combination with other agents to enhance death receptor-mediated apoptosis as a strategy for effective chemoprevention of cancer8. This study was conducted to test the ability of inositol hexaphosphate9 (IP6; or phytic acid) to inhibit alteration in biomarkers Itpr3 in azoxymethane (AOM) induced colon tumorigenesis when IP6 was fed to rats during the short carcinogenesis.

Methods

The study was evaluated by the Animal Ethics Committee of UFMS, approved, and certified by Protocol 064/2004 and to authenticate by Research Ethics Committee of UNIFESP-Hospital São Paulo by protocol 0183/06.

Sample

112(N) male Wistar rats (*Rattus norvegicus albinus*), eight-week-old, weighted 117 ± 29g, were selected from the Central Animal Facilities of UFMS.

Environment

The experiment was carried out in the Laboratory of Experimental Carcinogenesis of 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 12h each, mean temperature of 22 ± 3°C, and mean humidity of 58 ± 13%. They were fed ad libitum with rat chow (Nuvilab CR1, Nuvital Nutrientes e Produtos Veterinários Ltda, Curitiba, Brazil) and filtered water.

Experimental design

The 112 rats were distributed into four groups by draw. Tail tattoos, made with an indelible black ink pen, were used to identify groups (A through D) and individual animals (A through Q) within each group. Groups distribution was as follows: group A (control): 28(n) untreated animals; group B (AOM): 28(n), receiving AOM alone; group C(IP6): 28(n), receiving IP6 alone; group D (IP6+AOM): 28(n) animals receiving IP6 and AOM.

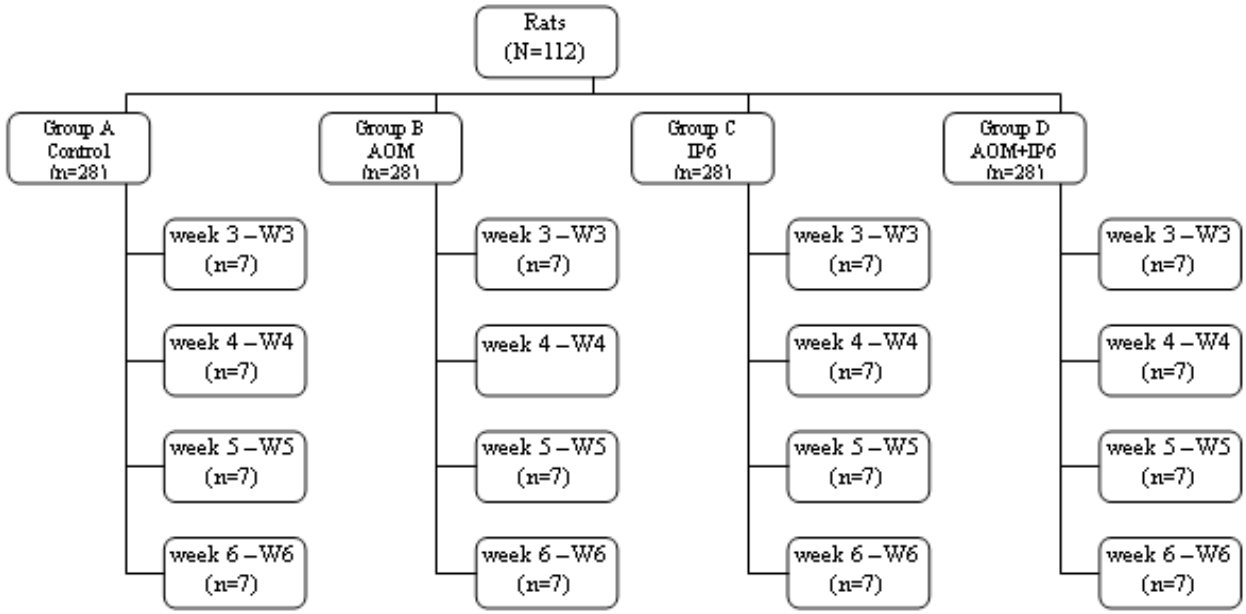


FIGURE 1 - Distribution of animals into four groups.

Administration of substances

IP6 (antitumoral substance, $C_6H_6O_{24}P_6Na_{12}$. Sigma, cat. P3168) was administered as a 1% solution in drinking water *ad libitum* to groups C and D, for six weeks. AOM (carcinogenic substance, $C_2H_6N_2O$. Sigma, cat. A9517, lot 014K0719) was administered subcutaneously at 5 mg/kg of body weight to groups B and D in the beginning of the third and fourth weeks. At the same times to the groups A and C were given 0.9% saline solution subcutaneously at 10 ml/kg of body weight. The volumes were equivalent to those of diluted AOM administered to groups B and D (Figure 2).

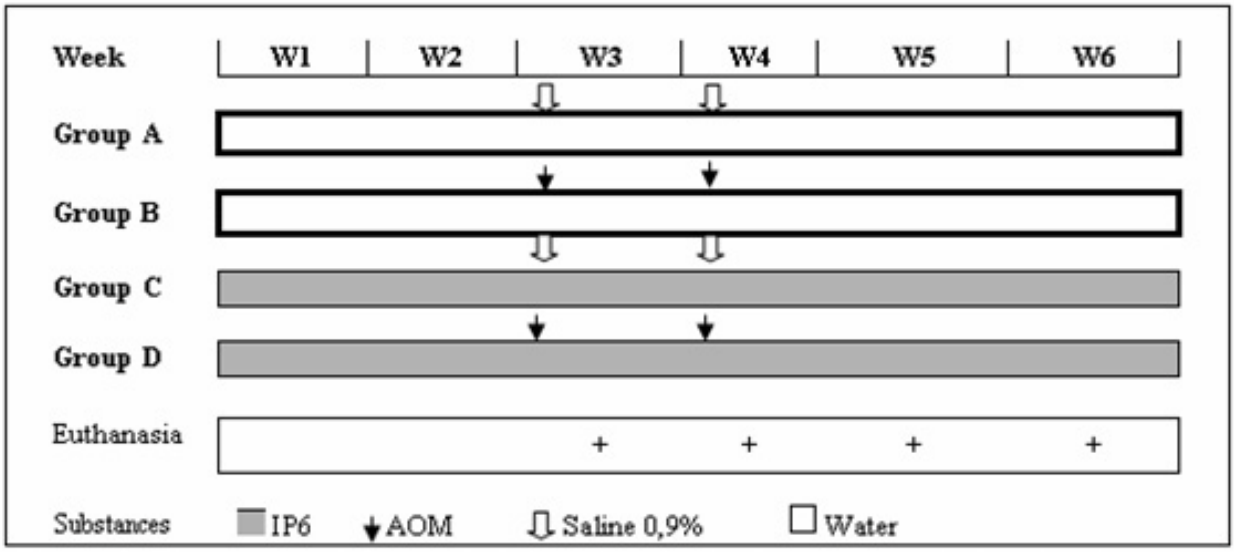


FIGURE 2 – Flux gram of drugs administration in the four groups, during the six weeks of observation period

Euthanasia

At the beginning of the third, fourth, fifth or sixth week, the animals were identified, weighed, and subjected to euthanasia by intraperitoneal thiopental (150 mg/kg of body weight), about 3 hours post-administration of AOM in groups B and D.

Collecting colon samples

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

Histological procedure

Each colon and ileum sample was opened along the antimesenteric border and the mucosa was rinsed with Ringer's solution. A 1-cm long segment was resected from the ascendant colon, inicially in the ileocolic transition. The segment of colon wall was sandwiched between the two plates of a hinged perforated double holder in order to be maintained straightened, and the resulting set was immersed in 10% buffered formaldehyde solution for 24 hours. Each sample was embedded in paraffin (with sample identification being preserved) and sectioned with a microtome (2-micrometer-thick sagital sections of colon wall). The resulting sections were mounted on silanized glass slides.

Immunohistochemistry procedure

The colon was prepared with biological marker inositol 1,4,5 trisphosphate receptor type III (Itpr3, primary antibodies, Santa Cruz Biotechnology Inc., cat. SC7277, lot G1604; 1:100 dilution) and followed by secondary antibody anti-rat (Dako LSAB™, K609 product, lot 15068); revealer of color DAB (diaminobenzidine, Dako™, K3468 product, lot 01317, in 1:10 dilution); Itpr3 expression was obtained as brown color areas.

Microscopy, image capture and photographic optimization

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 video board (Pinnacle Studio™ PCTV, USB). The captured image was saved for in separated archives with previous codification. All arquives were submitted for optimization of the photographic quality with use of Adobe Photoshop™ Software 7.0 / image / adjusted / canal RGB / curves / automatic.

Itpr3 measure

Itpr3 was quantified by computer-assisted image processing with Image Lab™ software. Ten single images from each sample were captured, always centered on the crypt. The optimized image crypts were randomly captured, saved and codified scheme. The measure were done by double-blind method. The quantified brown coloration density corresponds to Itpr3 expression in percentage area (using a RGB filter, blue background, 0-to-147 color interval) in the selected crypt (Figure 3).

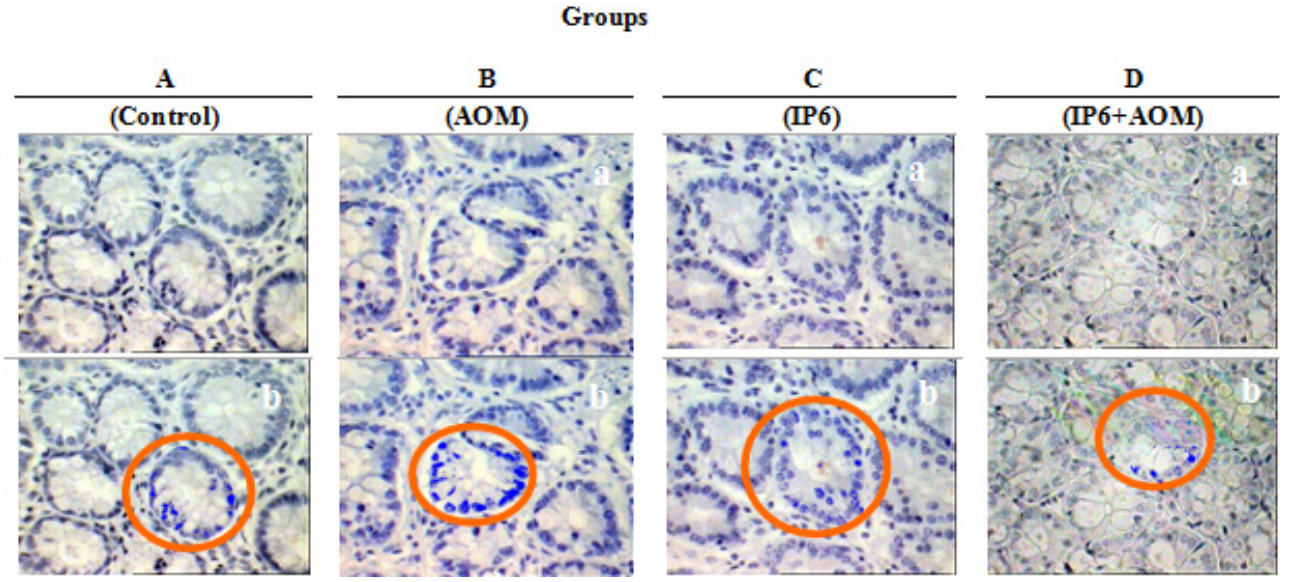


FIGURE 3 – Representative photomicrography of all groups in the week 5. The immunohistochemical expression (a) and the quantification of the expression of Itpr3 (b, in blue coloration - circle).400x.

Statistical analysis

For data analysis was used the statistical program Epi-Info™ and BioEstat™. 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 in each group versus all groups at the beginning of the experiment; (b); the mean values of Itpr3 expression, as revealed by immunohistochemistry processing, is paired comparison .Variance Test the mean differences between the initial and final weights in all groups. Kruskal-Wallis test to comparing: in group, week versus week; in week, group versus group. Dunn test or Student-Newman-Keuls test to comparing: in week, differences between weeks.

Results

General observations

No significant differences were found when the mean weight gains were compared between groups (p=0.2274, ANOVA) and with the weight profit (p=0.9131, Variance test). No deaths occurred in the experimental period. Three samples failed to be collected because of losses during microtomy or because sectioning made them unusable.

Effect on Itpr3 with administration of AOM

In Group B, significant difference occurred between the weeks (Table 1, W3xW4xW5xW6, p=0.0005). There was an increase of Itpr3 expression of week 3 and 4 for the week 5 (Table 1,W3xW5, $p<0.001$; W4xW5, $p<0.001$) and subsequent significant reduction of week 5 for week 6 (Table 1,W5xW6, $p<0.001$). Significant increase in Itpr3 expression was found on week 5 with administration of AOM (Table 1, Group AxB, $p \leq 0.001$).

Effect on Itpr3 with administration of IP6

Significant difference between Groups was showed (Table 1, AxBxCxD, $p<0.0001$); a significant reduction of Itpr3 expression ocurred after AOM+IP6 administration (Table 1, BxD, $p<0.001$). The Itpr3 expression increased significantly in group D (Table 1, AxD, p=0.0164) in comparison with group A (without any drug). The comparison of weeks in each group evidenced: a) in week 5, significant difference between Groups (Table 1, AxBxCxD, $p<0.0001$), with reduction in the Itpr3 expression when is administred IP6+AOM (Table 1, BxD, $p<0.001$); b) in week 6, significant difference between Groups (Table 1, AxBxCxD, p=0.0435), with significant reduction in the Itpr3 expression when the IP6 is administred (Table 1, AxC, p=0.0334).

TABLE 1 - Percentual average of inositol 1,4,5 trisphosphate type III (Itpr3) biomarker quantified in ten crypts of colon, according to four groups (A=control, B=AOM, C=IP6 and D=AOM+IP6) and the weeks of observation (W3, W4, W5 and W6).

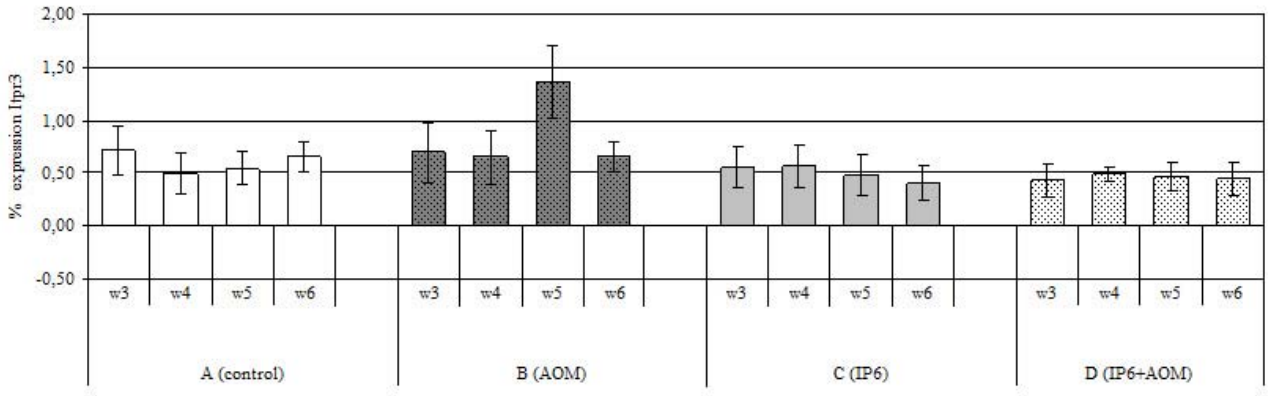
Groups	A (control)				B (AOM)				C (IP6)				D (AOM + IP6)			
Weeks	W3	W4	W5	W6	W3	W4	W5	W6	W3	W4	W5	W6	W3	W4	W5	W6
	0,72	0,87	0,85	0,70	0,33	0,96	1,63	0,93	0,80	0,47	0,61	0,16	0,70	0,75	0,49	0,38
	0,56	0,55	0,57	0,68	0,89	0,67	0,88	0,57	0,74	0,65	0,32	0,49	0,43	0,38	0,58	0,39
	0,48	0,40	0,90	0,35	1,22	0,46	1,20	0,41	0,45	0,83	0,27	0,42	0,44	0,41	-	0,49
	0,64	0,48	0,68	0,67	0,66	0,93	1,37	0,31	0,69	0,28	0,78	0,65	0,42	0,43	0,36	0,60
	0,43	0,51	0,44	0,80	0,51	0,41	1,65	1,19	0,25	0,58	0,51	0,36	0,20	0,36	0,72	0,37
	0,91	0,43	0,47	0,76	0,69	0,31	1,04	0,50	0,46	0,37	0,27	0,27	0,32	0,44	0,35	0,66
	0,66	0,52	0,50	0,60	0,57	0,80	1,79	0,74	0,47	0,74	0,57	0,48	0,49	0,45	0,18	0,20
Mean	**0,63**	**0,54**	**0,63**	**0,65**	**0,70**	**0,65**	**1,37**	**0,66**	**0,55**	**0,56**	**0,48**	**0,40**	**0,43**	**0,46**	**0,45**	**0,44**
+/- DP	**0,16**	**0,16**	**0,19**	**0,15**	**0,29**	**0,26**	**0,34**	**0,31**	**0,20**	**0,20**	**0,20**	**0,16**	**0,15**	**0,13**	**0,19**	**0,17**

Comparisons between:

	Groups	Groups in week 3	Groups in week 4	Groups in week 5	Groups in week 6
AxBxCxD	#<0,0001*	#0,1194	#0,3919	#<0,0001*	#0,0435*
AxB	< 0,001*	-	-	< 0,001*	ns
AxC	ns	-	-	ns	0,0334*
AxD	0,0164*	-	-	ns	ns
BxC	< 0,001*	-	-	< 0,001*	0,0259*
BxD	< 0,001*	-	-	< 0,001*	ns
CxD	ns	-	-	ns	ns
Weeks		**Group A**	**Group B**	**Group C**	**Group D**
W3xW4xW5x W6		#0,4265	#0,0005*	#0,3896	£0,9882
W3xW4		-	ns	-	-
W3xW5		-	< 0,001*	-	-
W3xW6		-	ns	-	-
W4xW5		-	< 0,001*	-	-
W4xW6		-	ns	-	-
W5XW6		-	<0,001*	-	-

(#) Variance test followed of test t-LSD (Least Significance Difference); (£) Kruskal Wallis test followed of Dunn test; (§) Kruskal Wallis test followed of Student-Newman-Keuls test. * $p \leq 0,05$.

GRAPHIC 1 - The inositol 1,4,5-trisphosphate receptor type 3 (Itpr3) expression modulated by the IP6 on the colon carcinogenesis.



Discussion

The use of experimental carcinogenesis model of shortness and average duration does not demonstrate difference in the installation of aberrant crypt foci (ACF) in colon10. AOM is recognized as carcinogen inductor of ACF in colorectal tumor, being this used as structure type endpoint in carcinogenesis of colon. The multiple and repeated injection of carcinogen in rats results in increase in the tumor colorectal incidence.

The biomarker use as endpoint is gaining ample acceptance in the precocious diagnosis in experimentations of short term of chemoprevention of the cancer in the place of traditional endpoints intermediate (ex., ACF) of the cancer. Biomarkers genetic molecular can be used as tools in the risk identification, screening and evaluation of cancer cure. Biomarkers presents high potential to identify involved changes in the development of new boarding's in the chemoprevention, and is promising seek area. Biomarkers in cancer prevention are increasingly important tools in primary prevention and in intervention by chemo preventive agents. Biomarkers can be utilized as indicators of exposures, effects and individual susceptibility to cancer. Sampling of biomarkers in relation to exposure may have a great impact on the reliability of mechanism of action11.

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. 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 prevented12 and were used in the quantification of immunohistochemistry13.

Genotoxic carcinogens, such as azoxymethane, alkylate DNA, forming DNA adducts, induction of mutation of gene K-ras and gene •-catenin (G-to-A transitions). K-ras mutations at codon 12 may contribute to induce hyperplastic changes, while •-catenin mutations seem to be involved in generation of dysplastic lesions. These carcinogens cause DNA adducts, resulting in initiating mutations and subsequent development of tumours14. DNA damage due to carcinogens causes 'nuclear anomalies' in the proliferative compartment of the crypt colon. Some damaged cells are repaired but a few are not. This failure to delete such mutated cells may give rise to an abnormal clone with the potential to progress to cancer. It has been proposed that this reactive apoptotic response to DNA damage is the biological mechanism for protection against tumorigenesis15.

The redox status of the protein is another parameter that seems to be very important for safeguarding the normal functioning of the channel Itpr. It can be concluded that oxidative stress may result in aberrant activation of the Itpr3 channel and depletion of the Ca^{2+} stores, thereby disturbing normal Ca^{2+} signaling16. Although IP3 is necessary to open native Itpr, activation of these channels is complex and their open probability actually depends on the ambient Ca^{2+} concentration. Up to ~500 nM, Ca^{2+} works synergistically with IP3 to activate Itpr. At higher concentrations, cytosolic Ca^{2+} inhibits Itpr-receptor opening. The inhibition of Itpr by Ca^{2+} is thought to be a crucial mechanism for terminating channel activity and thus preventing pathological Ca^{2+} rises.

Although some cells and tissues express a single or predominant isoform of the receptor, most cells instead express two or all three Itpr isoforms. Itpr is an intracellular Ca^{2+} channel that is for the largest part expressed in the endoplasmic reticulum. Its precise sub cellular localization is an important factor for the correct initiation and propagation of Ca^{2+} signals. The relative position of the Itpr´s, and thus of the IP3-sensitive Ca^{2+} stores, to mitochondria, nucleus or plasma membrane determines in

many cases the physiological consequences of IP3-induced Ca^{2+} release. Most cell types express more than one Itpr isoform and their sub cellular distribution is cell-type dependent. Moreover, it was demonstrated that depending on the physiological status of the cell redistribution of Itpr´s and/or of IP3-sensitive Ca^{2+} stores could occur. This indicates that the cell must be able to regulate not only Itpr expression but also its distribution. The various proteins potentially determining Itpr localization and redistribution will therefore be discussed17.

Significant increase of Itpr3 was showed after AOM administration and is concordant with: (a) acute apoptotic reply to the AOM in colon and initialization of tumorigenesis[18]; (b) Itpr3 selectively is increased in apoptosis[6], has active participation of apoptosis in a sample diversity, it stress oxidative results in aberrant activation of the Itpr canal[166] and the selective increase during apoptosis of linphocites[6].

The comparison of weeks in each group evidenced: Group B, rise in the Itpr3 expression of week 3 for 5 week and posterior significant reduction of week 5 to 6. The initial increase is evidences original and justifies it reduction in the Itpr3 expression: a) when apoptosis is induced for the extrinsically or intrinsic pathway, the induction will be more efficient through the inhibition of the Itpr3 and the transmission of calcium signals for mitochondria[19]; b) chronic activation of hydrolysis of phosphoinosídes can reduce Itpr3[20]; c) evidence of that Itpr3 reduction inhibits apoptosis[5].

The comparison between groups to each week evidenced: a) in week 5, with increase in the expression of Itpr3 of the control in comparison to the AOM and reduction in the Itpr3 expression when is administred associated IP6+AOM, demonstrating to be the effective IP6 in neutralizing the AOM; b) in week 6, significant reduction in the Itpr3 expression when administred IP6 alone in comparison to the control however, was showed that the Itpr3 expression is bigger in group AOM in comparison to the IP6. The related results are associated: a) the Itpr3 demands a higher concentration of IP3 (resultant of IP6 hydrolysis to reach the maximum reaction of 3.2 microM against 0.5 microM for the Itpr1, for comparison effect). Increase in the activity in the canal of membrane in the presence of high concentration of IP3 can be important during periods of drawn out stimulation, considering that the alosteric modulation for the ATP can help in the intracellular modulation of the signaling of Ca^{2+}[21]; b) selective expression of a type of particular receiver will influence the sensitivity of Ca^{2+} in accordance with the cellular concentration of IP3; c) evidence that Itpr3 plays the special rolls in induction of apoptosis[19].

A striking anticancer effect of IP6 was demonstrated in different experimental models and because it is abundantly present in regular diet, efficiently absorbed from the gastrointestinal tract, and safe, IP6 holds great promise in our strategies for the prevention and treatment of cancer. Uncontrolled proliferation is a hallmark of malignant cells, and IP6 can reduce the cell proliferation rate of many different cell lines of different lineage and of both human and rodent origin. Although normal cells divide at a controlled and limited rate, malignant cells escape from the control mechanisms that regulate the frequency of cell multiplication and usually have lost the checkpoint controls that prevent replication of defective cells. IP6 can regulate the cell cycle to block uncontrolled cell division and force malignant cells either to differentiate or go into apoptosis. IP6 induces G_1 phase arrest and a significant decrease of the S phase of human breast, colon, and prostate cancer cell lines. However, a cDNA micro array analysis showed a down-modulation of multiple genes involved in transcription and cell-cycle regulation by IP6. Thus, for cancer prevention, prophylactic intake of IP6 may be not only more effective, but also more practical than gorging on large quantities of fiber[9].

The study carried through with biomarkers it anticipates and potencialize the identification of alterations for the carcinogenesis stage of promotion of signaling benefit deriving right-handers of its accomplishment. Beyond adding to the IP6 to the list of Itpr modulators[1].

Conclusion

Inositol hexaphosphate promotes modulation of biological markers with reduction of Itpr3 in carcinogenesis of colon.

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