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EFEITO DO CHÁ PRETO

SOBRE A CARCINOGÊNESE ESOFÁGICA INDUZIDA

POR DIETILNITROSAMINA EM CAMUNDONGO.

ANÁLISE HISTOPATOLÓGICA

Tese de Doutorado apresentada ao Curso de Pós-Graduação em Cirurgia da Universidade Federal do Rio Grande do Sul

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SUMÁRIO

Resumo

O câncer de esôfago prossegue uma doença de distribuição mundial que, em áreas de maior risco, está relacionada a hábitos regionais, em particular alimentares. No sul do Brasil, da Argentina e do Uruguai, há zonas de maior incidência, relacionada ao uso de tabaco, de álcool, de churrasco (comida típica) e de uma infusão de erva-mate, chamada chimarrão, bebida a temperaturas elevadas. Ao uso destas infusões foi atribuído no passado um grande poder carcinogênico, que parece estar mais relacionado a irritação térmica do esôfago do que às suas propriedades químicas. Gradativamente, no entanto, algumas destas ervas e chás, têm demonstrado uma apreciável capacidade inibidora da carcinogênese, que poderia ser empregada na quimioprofilaxia do câncer em diversas regiões, devido à grande aceitação de seu consumo. No presente trabalho, visando avaliar o efeitos do chá preto sobre a carcinogênese esofágica experimental, foi utilizado o método de RUBIO (1987), que emprega a indução tumoral do esôfago pela administração oral de dietilnitrosamina (DEN), aplicado a uma população de 120 camundongos fêmeas durante 160 dias. Os animais foram distribuídos em 2 grupos controles (6 animais cada) e 3 grupos de tratamento (36 animais cada), nestes últimos foi efetuada a indução tumoral com dietilnitrosamina (DEN) em concentração de 0,04ml/L e, a dois deles, foi administrado chá preto a 1%, de duas diferentes formas. Após 160 dias foi efetuado a eutanásia dos animais e seus esôfagos foram analisados macroscopicamente e à histopatologia. A comparação entre os resultados obtidos quanto a presença de tumores (macroscopia) e a intensidade das lesões encontradas (histopatologia) revelou uma incidência significativamente menor de tumores ($p < 0,0001$) e uma maior proporção de lesões de menor gravidade nos grupos que receberam chá preto ($p < 0,001$), em relação ao grupo que não o recebeu, revelando um acentuado efeito quimioprofilático da substância

Effect of black tea on diethylnitrosamine-induced esophageal carcinogenesis in mice

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Abstract

In the south regions of Brazil, Argentina and Uruguay there are areas with a higher incidence of esophageal cancer related to the use of barbecue and *chimarrão* – an infusion of maté leaves drank at high temperatures. A great carcinogenic power was once ascribed to the use of such infusions, an effect that today seems to be rather associated with the thermal irritation of the esophagus than with the chemical properties of the beverages. Indeed, some of these herbs and teas present considerable anticarcinogenic potential, which may be used in cancer chemoprevention. This work was designed to assess the effect of black tea on experimental esophageal carcinogenesis induced by oral administration of diethylnitrosamine (DEN). The method was applied to a population of 120 female mice which were divided in 2 control groups (Water and Tea), with six animals each, and 3 experimental groups (DEN I, DEN II and DEN III), with 36 animals each, treated for 160 days. The animals in the three experimental groups were given DEN for three consecutive days. On the four subsequent days, group DEN I received water, group DEN II received 1% black tea, and group DEN III received water for two days followed by black tea on the other two days. Also, the DEN administered to the latter group was diluted in black tea instead of water. After 160 days the animals were sacrificed and their esophagi were submitted to macroscopic and histopathologic analyses. The comparative analysis of the groups regarding tumor incidence (macroscopy) and lesions found at histopathology showed a significantly smaller incidence of tumors ($p < 0.0001$), as well as a greater proportion of low-grade epithelial lesions ($p < 0.001$) in the groups that received black tea, revealing a marked inhibitory effect of this substance on chemical carcinogenesis in this experimental model.

Introduction

The accumulated knowledge produced by population epidemiological studies (12,13), by the development of genetic science (19), and by experimental research (2,28,29) has afforded a progressive understanding of esophageal carcinogenesis (31). Thus, the theories of viral etiology deserve less credit today, while the thesis of genetic origin, related to the successive transformations undergone by DNA has been strengthened (45).

Esophageal cancer occurs universally, but for certain regions of the East it represents a scourge (14). In these regions it is easier to evidence the connection between the disease and local habits that may participate as risk factors (13, 34, 42). Peoples of northeast Iran, Kashmir Valley in India, and north China and of certain countries in south old Russia, owing to unique feeding habits (24, 30, 8), and Asian and African peoples owing to severe nutritional deficits (34), have an elevated incidence of esophageal cancer, constituting a living history of the origin of this disease in these regions.

Brazil is the fifth country in mortality by esophageal neoplasia. The state of Rio Grande do Sul – southernmost region of the country – presents the greatest incidence of the disease ($26{:}10^4$ people/year), which is also associated with local habits such as barbecue (typical food) and *chimarrão* (an infusion of maté leaves drank at high temperatures) (42).

Other carcinogenic factors related to feeding habits exert a direct action on the esophageal mucosa or inhibit the action of protective factors, such as very hot beverages, certain herbs and N-nitroso compounds (8).

Modern strategies of cancer prevention extend the focus of prevention to the precursory alterations at cellular level, as an attempt to inhibit or delay cell mutations or revert the incipient process of the disease at one or more stages of carcinogenesis (1, 10, 16, 17, 18, 41, 46). The ideal agent for that should be not only effective but also natural, accessible, stable, nontoxic, well-absorbed by oral route, well-tolerated, and have a low cost and well-known action mechanism (38). Among the studied inhibitors are the polyphenols, chemically active substances to which the anticarcinogenic effect of the tea is ascribed in

experimental and epidemiological studies (3, 5, 11). In locations where tea is widely consumed at temperatures below 55°C, there is significant reduction in the cases of esophageal cancer, suggesting an inhibitory action on this type of tumor (7).

Polyphenols in tea have various pharmacological effects: antioxidant activity (22, 36), clearance of active radicals (27, 28), inhibition of enzymes participating in carcinogenic metabolism (37, 49), increase of enzymes catalyzing inhibitory reactions of carcinogenic activity (21, 25), inhibition of the formation of the final carcinogen (6), and inhibition of the active carcinogen binding to DNA, preventing its methylation (47).

Despite representing 80% of the world consumption of tea, black tea is scarcely studied, since research on the pharmacological effects of tea are more valued in the East, where the consumption of green tea predominates. At any rate, differently from previous research, recent studies in human volunteers suggest that black tea presents an *in vivo* anticarcinogenic effect equivalent to that of green tea, because the condensation undergone by its polyphenols during fermentation can be reverted under the action of the low gastric pH and of metabolism (36).

In the present study we investigated the effect of dietary black tea on experimental esophageal carcinogenesis induced by oral administration of diethylnitrosamine (DEN) to a female mice population (*Mus musculus*, CF1 strain).

The work was performed in three stages: first, the animals were randomly assigned to 2 control groups and 3 experimental groups submitted to tumor induction by DEN (according to Rubio), and two of the latter received black tea on the diet as well, while the third did not receive tea; the second stage consisted in the macroscopic evaluation of the esophagi of the animals, taking notice of tumors $\geq$ 1 mm linearly, and histopathological examination of these tumors and remaining pieces; lastly, a comparative analysis was made of the groups in order to determine the level of significance of the influence of black tea on the chemical carcinogenesis in this experimental model.

Material and methods

Animals and experimental setting

A total of 120 female mice *Mus musculus* (CF1 strain) were used. The animals were 60 days old and had mean weight of 31.37g at the beginning of the experiments. They were breastfed for the first 21 days of life and then were fed on standard rat chow (GERMANY, composed of wheat bran, soy flour, oyster flour, bone flour, ground corn, meat flour, salt and kaolin). The animals were randomly placed in 20 plastic cages, 6 specimens in each, and were kept there until sacrifice. The two control groups comprised one cage each, while the three treatment groups comprised 6 cages each. Kept in an experimental animals' facility, the mice were submitted to a 12h light/dark cycle in an air-conditioned room with continuos air flow, humidity between 57 and 84%, and temperature between 14 and 27°C. For health control the animals were weighed again in electronic scales at 60, 90, 120 and 160 days. There were 6 deaths in the course of the work, which were excluded from the analysis.

Research outline

Stage 1 – Experimental chemical carcinogenesis

The first stage of the study comprised the induction of chemical esophageal carcinogenesis by oral route using Rubio's method (32), for macroscopic study of morphological alterations of the esophagus. The carcinogenic agent used was an aqueous solution of *diethylnitrosamine* - DEN (SIGMA CHEMICALS COMPANY - density 0.95 g/ml, molecular weight 102.1, and chemical formula C_4H_{10} - N_2O), at a concentration of 0.04 ml/L of water or 1% black tea, depending on the group. Black tea *Camellia sinensis (*FLEISCHMANN and ROYAL LTDA) - was used always at a concentration of 10 g/L of water (1%).

The solutions were prepared at a toxicology laboratory (PRÓ-AMBIENTE ANÁLISES QUÍMICAS E TOXICOLÓGICAS) and were always fresh and at room temperature when offered to the animals. The substances were offered *ad libitum* during the week as follows:

the Water control group received only water everyday; the Tea control group received only 1% black tea daily; group DEN I received water for 4 days and DEN aqueous solution for the next 3 days; group DEN II received 1% black tea for 2 week days, water on the next 2 days, and DEN solution in black tea in the remaining 72 hours; group DEN III received black tea for 4 days and DEN aqueous solution on the 3 subsequent days.

Stage 2 – Macroscopic and microscopic analysis

The animals were sacrificed after 160 days (33) through inhalation of sulphur ether. Necropsies and macroscopic examination were performed immediately after euthanasia. After dissection the esophagus was sectioned longitudinally, exposing the mucosa in its entire length, and its measures were recorded. After fresh analysis of the piece with a stereoscopic microscope (4x), it was spread on a polystyrene plate and fixed in 10% formalin solution. It was previously defined that esophageal masses $\geq$ 1 mm linearly would be considered as tumors, and the examiner was unaware of the origin of the pieces.

Each tumor-containing segment of esophagus was transversally sectioned keeping a 1mm visual margin of normal tissue in both the cranial and caudal directions. Subsequently it was included in a paraffin block and cut into 2μm-thick transversal sections which were mounted onto glass slides and stained with hematoxylin and eosin (HE). These slides were then examined in light microscopy in blind fashion. In order to standardize the analysis the findings were sorted into four types: normal (no grade of dysplasia); low-grade dysplasia (increased cellularity failing to reach the epithelial surface layer, frequent mitoses, or modest nuclear atypia); high-grade dysplasia (accumulation of squamous cells along the epithelial surface or in the basal lamina, without surpassing it [*in situ* carcinoma], hyperchromatism or moderate nuclear atypia); and invasive carcinoma (great cellular disarray with intense nuclear atypia or squamous cells below the basal lamina).

Stage 3 - Analysis of Results

The analysis of the results was first carried out on the basis of all tumors and all collected esophageal pieces. Later on a single identity was ascribed to each animal corresponding to the highest grade of histopathologic injury presented.

Qualitative variables were described by absolute frequencies and, as ordinal variables were involved, we also used the median and the minimum and maximum values as descriptors of the series. Using the *Rank* transformation proposed by Montgomery (26), in comparing the groups we applied the one-way ANOVA on Ranks with localization of differences by DUNCAN *post-hoc* test. Box-plot graphs were prepared for visual evaluation of the differences found (4). $P \leq 0.05$ was adopted as the significance level. The data were processed and analyzed using the Statistical Package For Social Sciences (SPSS V11.0) and SigmaPlot (V7.0).

Results

General observations

No aggression displays were observed among the mice, but there were six early deaths, two in each treatment group, due to physical debility secondary to the carcinogen's action.

The five groups presented similar initial weight, but along the experiment, groups Water, Tea and Den III showed a similar, positive weight variation, while group DEN I was the only one showing negative weight variation, significantly different from the previous ones. Group DEN II remained in an intermediate range of statistical similarity to these two sets (Table I).

Table I. Weight variation and tumor incidence across the different groups

Variables	Groups *					P (ANOVA)
	Water	Tea	DEN I	DEN II	DEN III	
Initial weight (g)	31.70 ± 1.44	32.10 ± 1.48	31.28 ± 1.44	31.42 ± 1.07	31.25 ± 1.32	0.617
Final weight (g)	38.90^{b} ± 3.45	39.00^{b} ± 1.73	30.92^{a} ± 6.23	$32.96^{a.b}$ ± 4.93	36.02^{b} ± 2.16	< 0.0001
Mean number of tumors	00	00	2.25^{a} ± 1.40	0.79^{b} ± 0.95	1.06^{b} ± 1.01	< 0.0001

* Values are expressed as means ± standard deviation. Superscripts represent statistically significant differences (a≠b).

Macroscopy

The mean number of tumors was significantly greater in group DEN I and similar between groups DEN II and DEN III (Table I). Figure 1 shows in box plot form the frequency behavior of esophageal tumors in the different groups.

The control groups do not present tumors and their esophagi serve as normality standard (Figure 2). There were no gross differences between the macroscopic features of the tumors in the treatment groups (Figures 3 and 4).

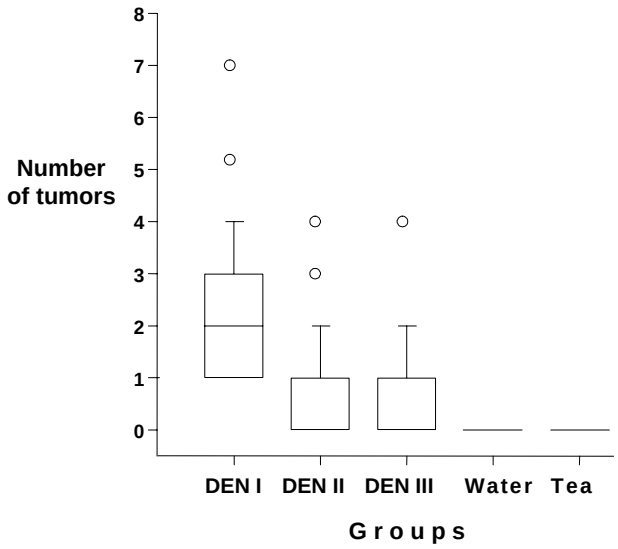

Fig. 1. Number of tumors at macroscopic level (Box plot graph)

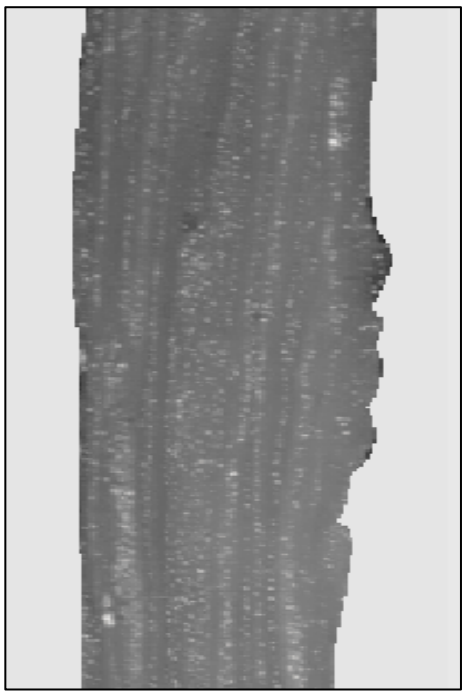

Fig. 2. Normal esophagus (specimen from group Water - 10x).

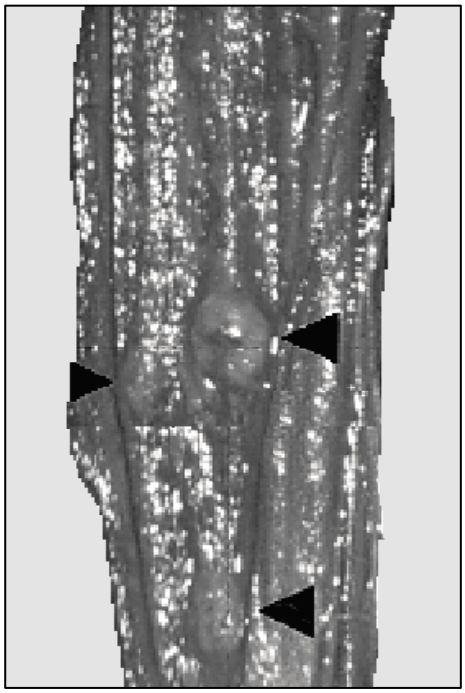

Fig. 3. Esophagus with tumors in the distal third (specimen from group DEN I - 25x).

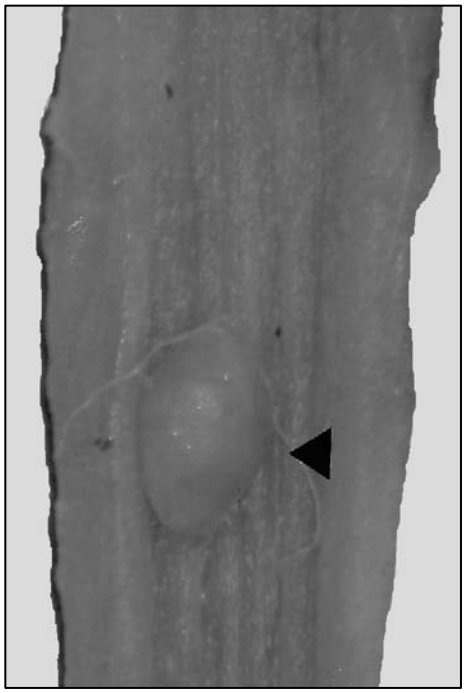

Fig. 4. Excrescent tumor in esophagus (specimen from group DEN II - 40x).

General Microscopy

Differently from what happened in groups DEN II and DEN III, in group DEN I there were no normal esophagi at macroscopic or microscopic level, all presented tumors, often more than one, and histopathologic alterations corresponding to these lesions were detected in all of them.

There were 6 cases of low-grade dysplasia detected in tissue samples which were macroscopically considered as normal (no tumor), all of them in the groups treated with tea. The reverse did not occur, i.e., there were no tumors that did not present corresponding microscopic alterations. The incidence of invasive carcinoma was small and exclusive to group DEN I (Table II).

Table II. Distribution of all macroscopic and microscopic findings

Lesions	Treatment Groups			Control Groups		Total
	DEN I n = 76	DEN II n = 42	DEN III N = 47	Water n = 6	Tea n = 6	n = 177
1. Macroscopy (tumors)	76	27	36	00	00	139
2. Histopathology	76	30	39	00	00	145
a. normal	00	12	08	06	06	32
b. low-grade dysplasia	48	26	29	00	00	103
c. high-grade dysplasia	24	04	10	00	00	38
d. invasive carcinoma	04	00	00	00	00	04

In the distribution of frequencies the data appear as absolute counts

Specific Microscopy

The control groups did not present histopathologic alterations in the randomly collected tissue samples, since there was no tumor at macroscopic level. In the general evaluation of all lesions found and other esophageal specimens (Table II), as well as in the analysis based on the highest grade lesion presented by each specimen, which ascribed to it

a single histopathologic identity (Table III), there was statistic similarity between groups DEN II and DEN III, treated with tea, and a significant difference between these and group DEN I, regarding tumor frequency and severity of epithelial lesions.

Table III. Histopathologic analysis considering the highest grade lesion in each specimen

Lesions	Treatment Groups			Control Groups	
	DEN I n = 34	DEN II n = 34	DEN III n = 34	Water n = 6	Tea n = 6
Normal	00	12	08	06	06
Low-grade Dysplasia	11	18	20	00	00
High-grade Dysplasia	19	04	06	00	00
Invasive Carcinoma	04	00	00	00	00
Median	2.0^{a}	1.0^{b}	1.0^{b}	0.0^{c}	0.0^{c}
Minimum and Maximum	1 - 3	0 - 2	0 - 2	0 - 0	0 - 0

In the distribution of frequencies the data appear as absolute counts. Comparison of groups by one-way ANOVA on ranks, $p < 0.001$. Superscripts represent differences at Duncan *post-hoc* test ($a \neq b; b \neq c; a \neq c$).

In groups DEN II and DEN III (Figure 5), which presented less severe cell alterations than those of group DEN I, the lesions were distributed in similar fashion concerning grade of epithelial lesion. There was a strong correlation between greater frequency of tumors at macroscopy and greater histopathologic severity of lesions, attributes present in group DEN I (Figures 6 and 7). This group presented lesions with greater epithelial injury than those detected in groups receiving black tea (DEN II and DEN III). These findings were analyzed by the one-way ANOVA test on ranks, which indicated three strata of differences concerning lesion severity (groups water and tea; group DEN I; groups DEN II and DEN III), as shown in Table III. Figure 8 is a graphic representation of this effect.

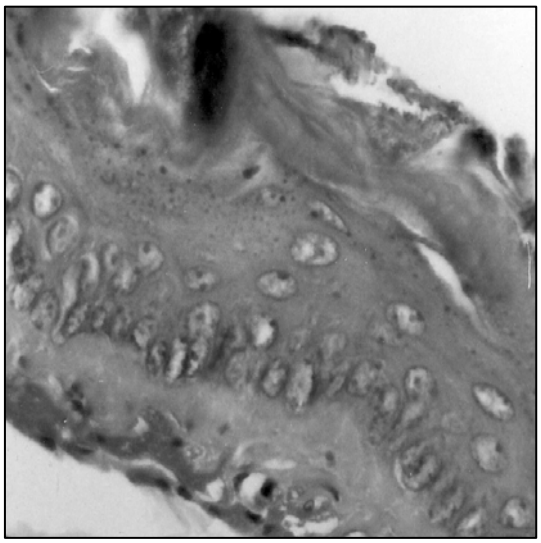

Fig. 5. Esophagus with Low-grade dysplasia: increased cellularity and presence of frequent mitoses (specimen from group DEN III - 100x)

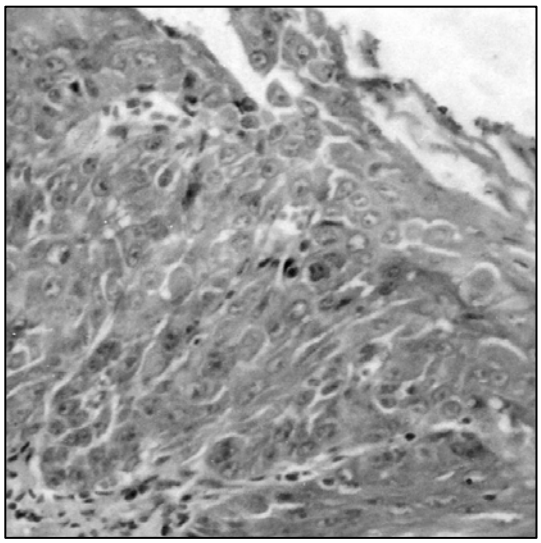

Fig. 6. Esophagus with High-grade dysplasia: squamous cells across the entire width of the epithelium and intense nuclear atypia (specimen from group DEN I - 200x)

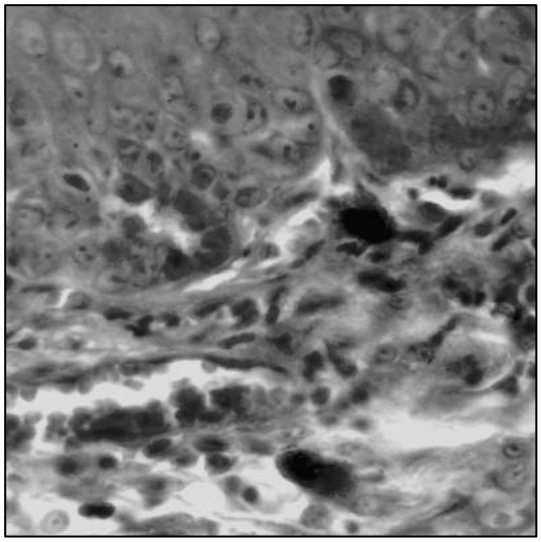

Fig. 7. Invasive carcinoma: intense nuclear atypia and epithelial cell beyond basal lamina (specimen from group DEN I - 250x).

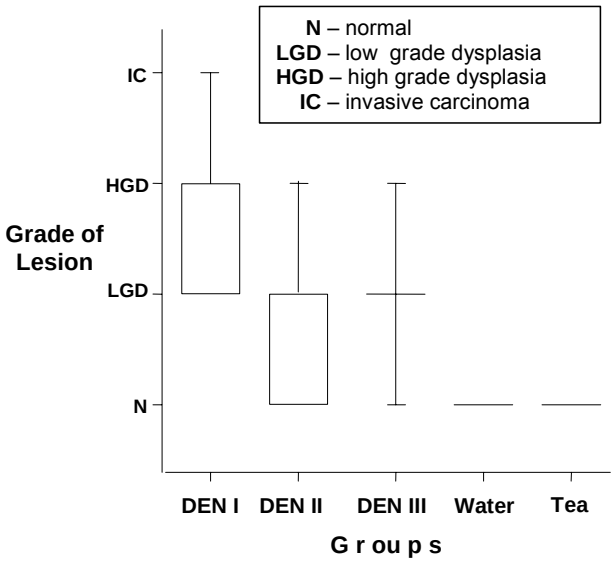

Fig. 8. Distribution of lesions according to intensity (Box plot graph).

Discussion

Other animals susceptible of tumor induction can be employed for studying esophageal carcinogenesis, such as rats (40), canids (35), rabbits (20) and gallinaceans (50), but they imply higher operational costs, especially when one wants to work with larger samples. In the present work we used mice *Mus musculus,* CF strain, which were appropriate to the proposed study (32). We chose to use females in order to avoid frequent deaths due to conflicts over territorial demarcation, typical of males (23), which could compromise the research, since other less controllable losses were expected in this experiment.

The experiment was designed to last 160 days, because by the precepts of experimental chemical carcinogenesis and by the chosen tumor size (1 linear millimeter), the lesions would be well characterized at this time, since invasive carcinoma arises 120 days after DEN administration is started (33). This criterion employed for the macroscopic analysis proved to be effective in the evaluation of carcinogenesis, since the tumors $\geq$ 1 mm were strongly correlated with histopathological alterations at the microscopic analysis.

Concerning the weight progress of animals, from similar initial measures between the groups ($p = 0.617$) weight loss was observed only in group DEN I ($p < 0.0001$), which did not have the attenuating effect of black tea on the process of carcinogenesis. Although this result appears to be logic, it is not the rule in similar studies, where DEN compromises weight gain even during chemoprophylaxis (5).

The concentration of DEN used for inducing carcinogenesis (0.04 ml/L) was the one recommended in other experiments (32). Oral administration is effective, minimizes the handling of animals, and reduces hepatotoxicity and the adverse, debilitating occurrence of tumors in other organs. The concentration of black tea used was arbitrarily set as 1% because of its similarity with that of human consumption, since there no established standard in this regard (5, 15, 25, 43, 44, 51).

The way of administering black tea, i.e. mixed with the carcinogen (group DEN II) or alternating with it (group DEN III) had no influence in this study, as in both conditions the effects were similar. The finding that DEN mixed with black tea results in a similar number of tumors as that obtained by alternating it with tea can be taken as absence of inhibition of the anticarcinogenic effect of the tea due to its dilution in DEN, *in vitro*. The existing studies in this regard are unclear as to the differences in the effect of tea related to the way of administering the substances (39, 43, 48). Works with their use in mixed form were not found in the literature.

The treatment groups that drank black tea presented less tumors than group DEN I did ($p < 0.0001$), which represented an important change in the response to tumor induction. Group DEN I, as already highlighted, was the only one to present tumors in all examined specimens, some animals with as many as seven tumors. Further studies are needed to clarify the mechanisms involved in this outcome, but there is overall evidence that the smaller incidence of tumors in groups DEN II and DEN III ($p < 0.0001$) can be ascribed to black tea.

In the macroscopic analysis in this work we have not used the traditional technique of Swiss roll, whose longitudinal sectioning provides an ample sampling of the esophagus, using instead the individual sectioning of the lesions described at macroscopy, which allowed to exclude other lesions from the analysis, afforded a reliable confrontation of macroscopy and microscopy, and allowed a meticulous classification of each tumor by a thorough investigation of it.

Dysplasia, considered as the main precursor of epidermoid carcinoma of the esophagus and the most frequent pathological finding in at-risk populations (9), was the parameter used in the histopathological analysis to compare the groups. For greater reliability of analysis a strict criterion for histological classification of lesions was previously established, based on cellularity relative to epithelial surface and basal layer, intensity and extension of damage to cell structure, presence of nuclear mitoses and atypias, and chromatin characteristics at staining.

From this work we can conclude that the incidence of tumors was significantly smaller in both groups treated with black tea than it was in group DEN I; in the histopathological analysis there was a significantly greater incidence of normal esophagi and a smaller incidence of severe dysplastic lesions and invasive carcinoma in groups DEN II and DEN III than in group DEN I. There was a strong correspondence between the macroscopic and microscopic findings, which allows to consider the presence of tumors $\geq$ 1 mm at macroscopy as an affirmative element of chemical carcinogenesis in this experimental model. Also, the similarity between the groups treated with black tea concerning the incidence of tumors and the distribution of lesions at the histopathologic analysis indicates that there was no previous chemical neutralization of diethylnitrosamine when it was diluted in tea (group DEN II).

In this study, therefore, black tea had a significantly anticarcinogenic effect, resembling a true chemoprophylactic agent, attenuating the esophageal carcinogenesis process induced by diethylnitrosamine.

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