Hyperbaric oxygen therapy increases the effect of 5-fluorouracil chemotherapy on experimental colorectal cancer in mice

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Abstract

Tumor hypoxia may compromise the results of chemotherapy for treating colorectal cancer because it stimulates angiogenesis and the release of tumor growth factors. Hyperbaric oxygen (HBO) supplementation may potentiate the effects of chemotherapy in such cases. This study aimed to assess the effect of HBO therapy combined with chemotherapy on the treatment of colorectal cancer in mice. C57BL6 mice were submitted to the intrarectal instillation of N-methyl-N-nitrosoguanidine (MNNG) and treated with 5-fluorouracil (5FU) and/or HBO therapy. The MNNG group presented the highest dysplastic crypt rate. The 5FU + HBO group presented the highest rate of apoptotic cells per dysplastic crypt. The 5FU group presented the highest expression of hypoxia-inducible factor-1 alpha and CD44. HBO therapy increased the effect of 5FU on the treatment of the experimental colorectal neoplasia in mice.

Key words: 5-fluorouracil; adjuvant chemotherapy; carcinogenesis; colorectal neoplasias; experimental cancer; hyperbaric oxygen therapy; hypoxia; N-methyl-N-nitrosoguanidine

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Introduction

Adenocarcinoma is the most common malignant neoplasia of the digestive tract. It is important worldwide because it is the third most commonly diagnosed malignity and the fourth leading cause of death by cancer.[1] While most new cases (55%) occur in more developed countries, the highest proportion of deaths (52%) occurs in less developed countries, reflecting the low survival in such regions.[2]

In patients diagnosed with colorectal cancer (CRC), early treatment may decrease the impact of the disease.[3] Currently, CRC may be treated through a combination of surgery, chemotherapy, and radiotherapy. The primary goal of the surgery is to completely remove the primary tumors with regional lymphadenectomy (en bloc) of the lymph nodes that drain the region of the neoplasia.[4-6]

In CRC, chemotherapy may have three purposes: neoadjuvant, adjuvant, or palliative treatment. 5-Fluorouracil (5FU) is the first line of treatment and most common chemotherapy for metastatic CRC.[7] It is most often used in association with modulating drugs such as folinic acid. The adjuvant treatment aims to eradicate hidden metastases or micrometastases.[7-9] Despite the multimodal treatment, the maximum global survival in 5 years is 67%, and only 14% when there are distant metastases.[10] Therefore, the efforts to seek new adjuvant therapies with the purpose of increasing global survival are justified. Among such measures, the role of hyperbaric oxygen (HBO) therapy in combination with chemotherapy has been investigated.[11]

HBO therapy is a type of treatment that has been used in inflammatory, ischemic, and infectious conditions for over fifty years and consists of breathing pure oxygen (100%) at an ambient pressure above the absolute atmospheric pressure (1.01325 bar).[12] Specific therapeutic effects are obtained through high tissue oxygen concentrations, namely the stimulation of bacterial lysis by leucocytes, the increase in fibroblast and collagen proliferation, and the neovascularization of ischemic or irradiated tissues.[13] Due to the high cell replication rate and irregular and tortuous vascularization, there are several hypoxic areas in a single tumor, which may be a factor stimulating angiogenesis and tumor growth and the cause of resistance to chemotherapy and radiotherapy.[14,15] To improve treatment efficacy, studies are being carried out to eliminate the hypoxic environment of the tumor, in which it is adapted to develop.[16]

Studies on the effect of HBO therapy on tumor growth have shown contradicting effects. Some have reported a tumor-stimulating effect after HBO therapy sessions, with an increase in metastases in patients with cervical neoplasias.[17-19] However, other studies have shown an inhibitory effect of HBO therapy on the tumor or no effect.[20-22] Feldmeier et al.[23] suggested that HBO therapy has neither a stimulating nor an inhibitory effect on malignant tumors. They also stated the likely benefit of the adjuvant therapy on cancer but that more studies would be necessary.[23] An experimental study with

the aim of measuring tumor growth, proliferation, apoptosis, and microvasculature demonstrated that HBO therapy had no tumor-stimulating effect and did not promote distant metastases.[14] Hence, this study aimed to analyze the effect of HBO therapy on chemotherapy treatment in a murine model.

Materials and Methods

Animals

The project was approved by the Ethics Committee on Animal Experimentation of the USP Ribeirão Preto Medical School, protocol 185/2014, on April 9, 2015 and the animals used were kept according to the standards of this committee.

Sixty female C57BL/6 mice with an initial age of 8 weeks and body mass of around 20 g from the Central Vivarium of the Ribeirão Preto Medical School of the University of São Paulo were used in the experiment. These animals were maintained at the Department of Surgery and Anatomy Vivarium under the following conditions: a constant ambient temperature of 24 ± 1°C, a night-day cycle of 12:12 hours, and relative air humidity ranging from 60% to 70%. The animals were fed with a standard Purina® (São Paulo, SP, Brazil) diet and tap water ad libitum and lodged in groups of five animals per cage, maintained under these conditions for an adaptation period of 1 week. After the adaptation period, the animals were randomly divided into the following groups: Control group (n = 15), N-methyl-N-nitrosoguanidine (MNNG) group (n = 15), 5FU group (n = 10), HBO group (n = 10), and 5FU + HBO group (n = 10). The experimental design is presented in **Figure 1**.

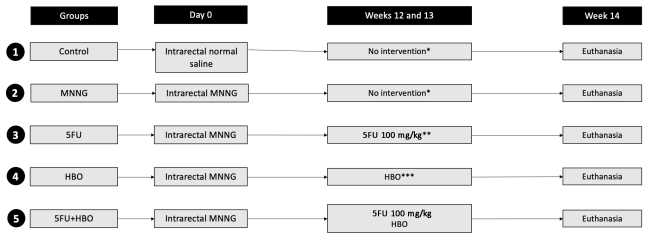

Figure 1: Experiment design.
Note: * Normal saline solution was applied intraperitoneally at weeks 12 and 13, and the mice were confined in the hyperbaric chamber without pressurization for the time corresponding to one HBO therapy session. ** They were confined in the hyperbaric chamber without pressurization. 5FU: 5-Fluorouracil; HBO: hyperbaric oxygen; MNNG: N-methyl-N-nitrosoguanidine.

Carcinogen

MNNG (Sigma-Aldrich, Louis, MO, USA) was applied to all animals except of the control group on day 0 of the experiment at the 100 mg/kg dose. It was diluted in distilled water. The animals were kept awake in the prone position, and 100 µL was applied intrarectally using a size six catheter.[24]

Chemotherapy application

5FU (Sigma-Aldrich) was applied to the animals of the 5FU and 5FU + HBO groups once a week at weeks 12 and 13. 5FU was used at the 50 mg/mL dose, diluted in a 0.9% normal saline solution, and applied intraperitoneally at the 100 mg/kg dose. The animals of the control groups received a normal saline solution intraperitoneally once a week at weeks 12 and 13.[25]

HBO therapy

The experimental hyperbaric chamber used was constructed according to the international standards (ASME VIII26), with pressurization reaching 7.0924 bar and a capacity for 20 animals per session (**Figure 2**). It has 70 cm of length and 45 cm of diameter. The ambient pressure was deemed 1.01325 bar, considered at the beginning of the sessions. The HBO therapy was initiated from week 12 of the experiment for the 5FU and 5FU + HBO groups. After sealing the chamber, the pressurization with oxygen for 15 minutes began until the chamber pressure reached 2.0265 bar. The treatment under this pressure lasted for 60 minutes. Next, the decompression was initiated by opening the depressurization valve for 15 minutes. The animals were kept under observation for 30 minutes after each session. The sessions were repeated in 24-hour intervals, totalizing 10 sessions.

Figure 2: Experimental hyperbaric chamber.
Note: a: Animal entrance opening; b: manometer; c: tube to connect to the oxygen; d: oxygen outlet valve; e: display for observing the session measuring 15 cm in diameter.

The control group animals were confined in the hyperbaric chamber with no pressurization for the time corresponding to one HBO session, once a day.

Material collection

The animals were euthanized at 14 weeks of the experiment according to the chosen group by applying a lethal dose of intramuscular xylazine and ketamine. Next, they were submitted to a midline laparotomy with the posterior isolation and removal of the distal colon. The animals were frozen at –8°C and sent to the Central Vivarium of the University of São Paulo Ribeirão Preto Medical School, where they were incinerated. After removing the specimen, the colon was opened from its mesenteric margin, cleaned with saline solution (0.9% normal saline), and carefully extended on a glass plate to cut the sections for storage in the cassettes. The colons were then placed between filter papers inside previously identified cassettes that were immediately immersed in a 10% formalin solution to fix the material within a period of 48 to 72 hours. After the fixation, the samples were submitted to dehydration by immersion in increasing alcohol concentrations. The materials were then submitted to 100% xylol baths for diaphanization. The processed colon was included in paraffin to cut the histological sections to make the slides. Through a rotating microtome, the paraffin blocks containing the colon samples were cut at a thickness of 4 µm, adhered to the salinized slides, and taken to an oven heated at 60 °C to optimize the adhesion of the material

to the slide. Before the immunohistochemical or hematoxylin and eosin coloration technique, the blades were deparaffinized and hydrated. Lastly, the antigen retrieval of the material was performed through a physicochemical method by exposing the material to vaporization in a citrate buffer solution with pH 6.0.

Microscopic assessment

The specimens were fixed in 10% buffered formalin and later included in paraffin blocks (5 µm thick) and identified, and the slides were stained with hematoxylin and eosin for the histological assessment.

The microscopic analysis was performed by one and the same qualified and experienced pathologist with a microscope (Axiostar plus, Zeiss, Oberkochen, Germany). All pieces and slides were kept with their identifications hidden so as not to know to which group the animal belonged.

In the microscopic analysis, the index of dysplastic crypts and index of apoptotic cells per dysplastic crypt were determined.

Immunohistochemistry

The immunohistochemistry was carried out in transverse histological colon sections heated at 60°C for 75 minutes and occurred through an antigen-antibody reaction, followed by a reaction with a marker visible under the microscope. To mark the histological sections, they were adhered to salinized slides, deparaffinized in xylol, and hydrated in decreasing concentrations of ethanol to water. The antigen retrieval was carried out in a citrate buffer with pH 6.0 (in a vaporizer) for 20 minutes. To inhibit the reactions with tissue peroxidases, 3% hydrogen peroxide was added to the slides. To block nonspecific tissue connections that could cause nonspecific marking, normal horse serum 10-bio100 Eq (LGC Biotechnology, SP, Brazil) diluted in a phosphate buffer solution pH 7.4 at the concentration of 10% was used. Between each step, the slides were washed three times with phosphate buffer solution to prevent the formation of precipitates. The slides were incubated at 24°C with their respective primary antibodies: hypoxia-inducible factor-1 alpha (HIF-1α; mouse, monoclonal, 0.5 mg/dL, Biocare Medical, Pacheco, CA, USA, Cat# ESEE122, RRID: AB_11217480) and CD44 (NCL-CD44-2 clone, mouse, 500 µg/mL, Novocastra, IL, USA, Cat# PA594934, RRID: AB_2806740) in a dark and humid chamber for 2 hours. After the washing, the slides were incubated at 24 °C with biotinylated secondary antibody (mouse IgG (H + L) secondary antibody, 0.8 mg/mL, Thermo Fisher Scientific, Minneapolis, MN, USA, Cat# 31430, RRID: AB_228307) for 30 minutes, and, after a new washing, incubated with conjugated polymer also for 30 minutes. For the revelation, the slides received a reagent solution containing chromogen 3,3′-diaminobenzidine for 1 minute to 3 minutes until the emergence of a brown precipitate attributed to the reduction of the 3,3′-diaminobenzidine by the reaction. All reagents are available with the PicTureTM Max PolymerDetection Kit (Invitrogen, Waltham, MA, USA). The slides were counterstained with Harris hematoxylin diluted for about 1 minute, dehydrated, and clarified again. Entelan (Merck, Rahway, NJ, USA) was used to configure the slides. The positively marked cells (that presented browns) were counted manually using a Zeiss optical microscope with 400× magnification, and the entire extent of each section was considered. All immunohistochemical assessments were carried out in cells from the colon crypt.

Statistical analysis

A sample calculation was performed to verify the number of animals in each group. The data were analyzed through the statistical program GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA, www.graphpad.com). The D'Agostino-Pearson test was applied to verify the normal distribution of the data, presented as mean ± standard error of the mean (SEM). For the data with normal distribution, the one-way analysis of variance followed by Bonferroni's *post-hoc* test was used. To compare the macroscopic and microscopic scores among the groups, the non-parametric Mann-Whitney test with reduced variability was used. The probability of $P < 0.05$ was considered statistically significant, with a 5% significance level to demonstrate the difference among the data.

Results

Histological examination

In the MNNG group, the dysplastic crypts formed over 14 weeks, which was not found in the control group ($P < 0.05$), showing that the carcinogenesis was effective.[24]

The MNNG group presented the highest number of dysplastic crypts among the four groups (one-way analysis of variance, $P < 0.0001$). HBO therapy could significant reduced the dysplastic crypt rate compared with HBO group (Bonferroni's *post-hoc* test, $P < 0.05$; **Figure 3**).

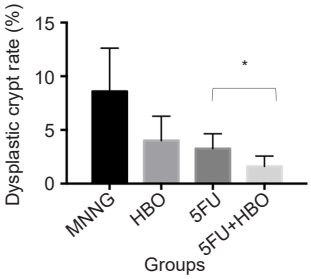

Figure 3: HBO therapy reduces the dysplastic crypt rate in 5FU-treated colorectal cancer mice.
Note: Data are expressed as mean ± SEM (n = 15, 10, 10, 10). *$P < 0.05$ (one-way analysis of variance followed by Bonferroni's *post-hoc* test). 5FU: 5-Fluorouracil; HBO: hyperbaric oxygen; MNNG: N-methyl-N-nitrosoguanidine.

The MNNG group presented the lowest rate of apoptotic cells per dysplastic crypt among the four groups (one-way analysis of variance, $P < 0.0001$). The apoptotic cell rate in the 5FU + HBO group was higher compared with the 5FU group ($P < 0.0001$; **Figure 4**).

Immunohistochemistry

The MNNG group did not present a significant difference in the rate of HIF-1α- and CD44-positive cells compared with those in the HBO and 5FU + HBO groups. The 5FU group presented the higher rate of HIF-1α- and CD44-positive cells when compared with the 5FU + OHB group ($P < 0.0001$). There was no significant difference between the HBO and 5FU + HBO groups (**Figure 5**).

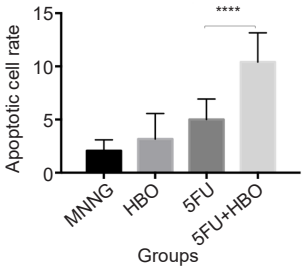

Figure 4: HBO therapy increases the apoptotic cell rate in 5FU-treated colorectal cancer mice.
Note: Data are expressed as mean ± SEM (n = 15, 10, 10, 10). ****P < 0.0001 (one-way analysis of variance followed by Bonferroni's *post-hoc* test). 5FU: 5-Fluorouracil; HBO: hyperbaric oxygen; MNNG: N-methyl-N-nitrosoguanidine.

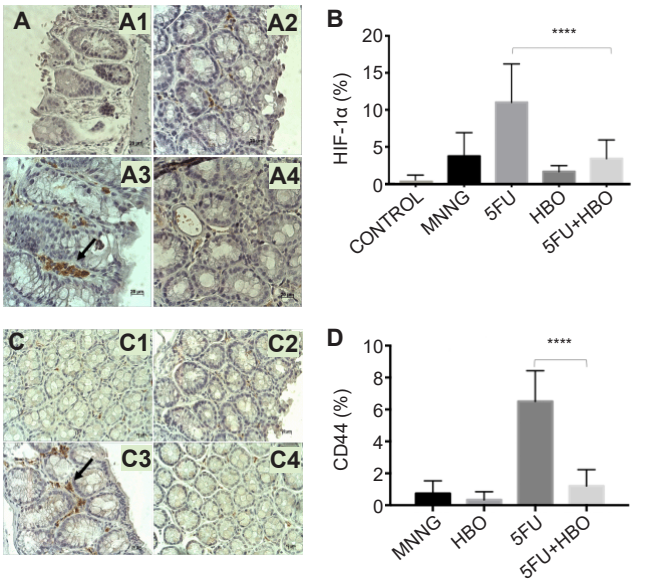

Figure 5: HBO therapy reduces the rate of HIF-1α- and CD44-positive cells in 5FU-treated colorectal cancer mice.
Note: (A) HIF-1α-positive cells (arrow) in rectal neoplasias. A1–4: MNNG, 5FU, HBO, and 5FU + HBO groups. (B) Quantitative results of HIF-1α-positive cells in A. (C) CD44-positive cells (arrow) in rectal neoplasias. Scale bars: 20 μm. C1–4: MNNG, 5FU, HBO, and 5FU + HBO groups. (D) Quantitative results of CD44-positive cells in C. Data are expressed as mean ± SEM (n = 15, 15, 10, 10, 10). ****P < 0.0001 (one-way analysis of variance followed by Bonferroni's *post-hoc* test). 5FU: 5-Fluorouracil; HBO: hyperbaric oxygen; HIF-1α: hypoxia-inducible factor-1 alpha; MNNG: N-methyl-N-nitrosoguanidine.

Discussion

Despite the advances in CRC treatment, adjuvant therapy still causes many side effects and has a high cost and tumor resistance index. Chemotherapy with 5FU is the first line for the adjuvant treatment of CRC. Even so, the response rate for advanced CRC is only 5% to 10%,[27] while, when combined with oxaliplatin or irinotecan, this rate increases to 40% to 50%.[28] Hence, HBO therapy is an option, and this study is a pioneer in analyzing the adjuvant effect of hyperbaric 100% oxygen on the treatment of colorectal tumors. The murine model was chosen because the use of rodents as experimental CRC models has been widely accepted due to their similarity to humans.[28] There are several advantages of inducing neoplasias for studying carcinogenesis in these animals, including the fast and reproducible induction of tumors and the possibility of studying the adenoma-carcinoma sequence, as occurs in humans.[29]

The application of MNNG on the colon mucosa induces inflammation, which predisposes to the appearance of adenomas and dysplasia. In the colon, the initial process is characterized by the appearance of aberrant or dysplastic crypts and, later, aberrant crypt foci. From these initial changes, the interaction of cellular and molecular components in the tumor microenvironment allows tumors to progress and develop.[30]

Dysplastic crypts are considered the most initial phase of the adenoma-adenocarcinoma sequence, which allows their use for colorectal neoplasia studies.[31] Maurin et al.[32] carried out a colorectal carcinogenesis study with MNNG and observed a significant increase in the number of dysplastic crypts, which showed that the carcinogen is a promising agent for studying CRC. After the treatment, a beneficial effect was observed in the animals who received 5FU in isolation and those for which the 5FU was associated with HBO therapy, made evident by the decrease in the dysplastic crypt rate, more prominent in the group with the combined treatment. It is believed that the smaller number of dysplastic crypts is due to the higher rate of apoptosis per dysplastic crypt, which was more evident in the 5FU + HBO group. No studies were found in the literature associating 5FU and HBO therapy for colorectal neoplasia; however, Refaat et al.[33] and Patyar et al.[34] demonstrated that, in experimental groups of CRC treated with 5FU, there was a significant reduction in the number of dysplastic crypts and an increase in apoptosis compared to the control groups.

Cell damage and death, as occurs during chemotherapy, are known for causing hypoxia.[35] In turn, the decrease in the oxygen tension in the tumor tissue is the primary stimulus to rises in the HIF-1α levels. In high concentrations, the HIF-1α induces angiogenesis, which may be one of the factors of treatment resistance or recurrence of the tumor.[35] Wu et al.[36] showed in an experimental study that HIF-1α is present in 75% of patients with CRC and that its expression increases significantly in hypoxia conditions. The fact that hypoxia modulated HIF-1α is one of the reasons for using HBO therapy for treating intestinal neoplasias and inflammations.[37,38]

In neoplasias, HBO therapy changes the hypoxic state of the tumor and consequently alters the stimulus for tumor growth and angiogenesis. Little is known about the effects of the combination of HBO therapy and 5FU on the HIF-1α levels; however, the HBO therapy may reduce the tumor hypoxia induced by radiotherapy and chemotherapy.[39]

In another experimental study, several authors showed that radiation increases the level and activity of HIF-1α within 12 to 24 hours after treatment, with a peak after 48 hours. They also showed that the increase in HIF-1α is dose-dependent. In animals genetically modified or with pharmacological inhibition of the HIF-1α synthesis before radiotherapy, significantly slower growth was observed in several studied tumors after the end of the treatment.[40,41]

In this study, there was an increase in the HIF-1α production after the start of the treatment with 5FU compared to the MNNG group, which is explained by the chemotherapy-induced cell death. Another finding was that the combination of HBO therapy and 5FU prevented the increase in HIF-1α

levels, which may be understood as an antineoplastic factor.

Stem cells express some stromal cell markers (e.g., src-2, -3, and -4 homologs), cytokine receptors (e.g., interleukin-1 receptor and tumor necrosis factor-α receptor), and a large number of adhesion molecules, including CD44.[42] The discovery in recent years that stem cells have tumor tropism was remarkable. Stem cells are not merely recruited from the bone marrow to the tumor; they arrive at the tumor site and may differentiate into cells such as fibroblasts, macrophages, or perivascular cells, which perform an important role in tumor progression.[43] Therefore, CD44 works as a marker of the stem cell migration to the tumor tissue, which in turn is associated with a worse therapeutic efficacy.

In this study, in the group treated with 5FU, there was a significant increase in the CD44 rate relative to the MNNG group, demonstrating a stem cell migration to the tumor site. In the groups that received HBO therapy (alone or combined with 5FU), there was no significant alteration to the CD44 rate. In a study on invasive breast carcinoma, Marangoni et al.[44] noticed that, upon associating an anti-CD44 antibody to the treatment, the tumor growth was reduced. They also showed that, in breast cancer, cells marked with CD44 were identified in more vascularized peripheral areas with the progressive and significant increase in hypoxic areas with central necrosis, showing that cell death promotes the migration of stem cells marked with CD44. Therefore, stem cell migration may be related to hypoxia.[44] In our study, the reversion of the hypoxia through the HBO therapy was able to inhibit this displacement of stem cells to the tumor site, which may demonstrate an antineoplastic effect of the adjuvant treatment with HBO therapy.

The adjuvant HBO therapy was first tested in head and neck neoplasia and the treatment of actinic lesions with satisfactory results.[39] In patients with head and neck tumors submitted to the combined treatment, better local control and an increase in global survival were observed.[20] Similar results were obtained in several other locations, including the bladder, bronchi, gastric, prostate and cervix.[45-50]

The use of HBO therapy adjuvant to 5FU has already been tested to treat sarcoma.[51] The combination of 5FU and HBO therapy significantly reduced the progression of the tumor growth compared to the group submitted to treatment with 5FU only. In a breast cancer murine model, it was observed that the combined standard was able to promote tumor regression.[52,53] Albeit transitory, the HBO therapy effect is important, and a single session increases the tissue capture of 5FU immediately after the oxygenation.[52,54]

This is an experimental study carried out in murine and its correlation with clinical practice may be debitable since there are numerous other factors that interfere in human carcinogenesis. Only the effect of one type of chemotherapy has been studied, perhaps the study of other chemotherapy agents may have different effects.

In summary, tissue hypoxia plays an important role in tumor growth and treatment resistance. Recent results have shown that HBO therapy does not induce tumor growth, recurrence, or metastases. The use of HBO therapy as an adjuvant therapy has shown promising results.

Several studies on HBO therapy and the treatment of neoplasia have shown the constant search for new therapeutic modalities. This study presents unprecedented and promising results. However, such results may be related to the fact that we established the treatment at the initial phase of the tumor (dysplastic crypts). Perhaps other studies assessing treatment with well-established tumors may have different results. Moreover, this is an experimental study, and we cannot extrapolate these results in humans. New studies must be carried out for HBO therapy to be included as an adjuvant treatment for CRC.

Author contributions
VFM: conception and design of study, acquisition of data and manuscript writing. JJRR: Critical revising and final approval of the version to be published. RSP: Manuscript review. MRF: manuscript review and statistical analysis. CAL: acquisition of data. SBM: acquisition of data. SBG: acquisition of data and critical revising. TMC: Acquisition of data. OF: Critical revising and final approval of the version to be published. All authors read and approved the final manuscript for publication.

Conflicts of interest
All authors report no conflict of interest.

Data availability statement
No additional data are available.

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