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Boric acid enhances in vivo Ehrlich ascites carcinoma cell proliferation in Swiss albino mice

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Abstract

The influence of boric acid, a boron carrier, on Ehrlich ascites carcinoma (EAC) cell-bearing mice was investigated in view of its importance in the boron neutron capture therapy and the influence of boron on proliferation and progression of cancer cells mediated by proteoglycans and collagen. The present study included the evaluation of boric acid for the effects on total count and viability of EAC cells in addition to their non-protein sulfhydryls (NP-SH) and malondialdehyde (MDA) contents as parameters for conjugative detoxication potency and possible oxidative damage. The EAC cell-bearing animals were also observed for the effect on survival, body weight changes, and histopathological evaluation of the tumors grown at the site of inoculation. The treatment with boric acid significantly increased the total number of peritoneal EAC cells and their viability. A significant increase in the body weight was observed that dose-dependently reached plateau levels by 20 days of treatment. Conversely, a reduction in the duration of survival of these animals was evident with the same protocol. Boric acid treatment resulted in a decrease in NP-SH contents with a concomitant increase in MDA levels in EAC cells as revealed by the results of the biochemical analysis. These data are supported by our results on histopathological investigations, which apparently showed fast growth, in addition to several mitotic figures and mixed inflammatory reaction, after treatment with boric acid. It seems likely that a particular combination of properties of boric acid, rather than a single characteristic alone, will provide useful information on the use of this boron carrier in neutron capture therapy.

Keywords: Boric acid; Ehrlich ascites carcinoma; Proliferation; Sulfhydryls; Malondialdehyde; Histopathology

1. Introduction

Boric acid occurs in nature as the mineral sassolite. It is widely used in industrial, agricultural, and cosmetic applications, besides its traditional use in the health care system. Boric acid is fairly rapidly absorbed after its administration and appears to be rapidly distributed through the body

Abbreviations: CHO, Chinese hamster ovary; CP, cyclophosphamide; EAC, Ehrlich ascites carcinoma; LET, linear energy transfer; MDA, malondialdehyde; NP-SH, non-protein sulfhydryl; PF, peritoneal fluid; TCA, trichloroacetic acid.

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water via passive diffusion. Following administration of boric acid the concentration of boron in the blood and tissue is reported to be in the ratio of 1:1 in rats and humans. In contrast, both in rodents and humans this boron concentration exceeds by four times in bones (Murray, 1998). Boric acid is not metabolized and is excreted from the body via urine. The half-life ($t_{1/2}$) of boric acid is approximately in the order of one day and it does not appear to accumulate in soft tissue, but in bones (Moseman, 1994). Normal levels of boron in soft tissue, urine, and blood range from 0.05 to 10 ppm. In one study, Ku et al. (1991) demonstrated an increase in boron levels in the tissue (testis, epididymis, accessory sex organs, hypothalamus, and rest of the brain) that appeared to reach steady-state boron levels (12–30 μg/g) by three to four days after daily administration of 9000 ppm boric acid (≅ 1575 ppm boron) to adult Fischer rats for up to seven days. All the other tissues showed no appreciable accumulation of boron over plasma levels. Reports in the literature have also indicated that rodents fed with 9000 ppm boric acid had as high as 2000 ppm boric acid in brain but few days after cessation these levels in the tissue quickly reached to a plateau of 15 ppm (Moseman, 1994).

As a boronated compound, boric acid has been extensively used as a tool in the boron neutron capture therapy of different types of cancer (Martin et al., 1989; Gregoire et al., 1993; Primus et al., 1996). The tumor-selective delivery of boron by liposomes or other biodistribution techniques has been demonstrated as a very useful means for increasing boron concentrations in the tumor tissues (Hawthorne and Shelly, 1997). In the process of neutron capture therapy the high thermal neutrons result in the high LET fission products of the neutron capture reaction. Following the capture reaction, an α-particle and a ^{7}Li ion are emitted that carries a 2.79 MeV energy and destroy all molecular structures along their path close to 10 μm (Pingol et al., 1996). So the resulting radiochemical damage is confined to boron containing cells, which allow the boron neutron capture reaction, generating highly penetrating γ-rays and thereby initiating a lethal event within the cancer cell (Martin et al., 1989; Dorn, 1994). Additionally, most of the boronated moieties and their derivatives (trimethylamine carboxyboranes, their amides and esters, thymidine derivatives of borane, ribo- and arabinoside boron nucleosides) by themselves possess cytotoxic activity in murine and human tumors and different cell lines (Hall et al., 1990, 1996; Sood et al., 1994; Burnham et al., 1995; Hudecz, 1995). Nevertheless, little is known about the toxicity and cytodestructive nature of boric acid, despite its toxic potential due to its boron contents alone. Major toxicity of boric acid is reproductive and developmental (Linder et al., 1990; Fail et al., 1991; Treinen and Chapin, 1991; Heindel et al., 1992; Ku et al., 1993). It is reported to potentiate the mutagenicity of thermal neutron irradiation in Chinese hamster ovary (CHO) cells (Kinashi et al., 1997) and to increase the intensity of β-galactosidase synthesis induced by aflatoxin B_1 in *Escherichia coli* (Odunola, 1997).

Benderdour et al. (1997) observed boron to increase the release of proteoglycans and collagen, which are known to stimulate tumor progression and metastasis in the presence of the transforming growth factor beta (Adany et al., 1990; McBain et al., 1990; Kovalszky et al., 1993; Tang and Honn, 1994; Flug and Kopf-Maier, 1995; Robert, 1996; Auvinen et al., 1997; Price et al., 1997; Schamhart and Kurth, 1997; Sun and Chen, 1997; Ricciarcdelli et al., 1998). Furthermore, collagen matrix and growth factors such as epidermal growth factor and the fibroblast growth factor have been reported to stimulate the proliferation of cells and progression of tumors (Laduca and Sinha, 1993; Yuspa et al., 1993; Reinbach et al., 1995).

Keeping in view the importance of boric acid in the boron neutron capture therapy the present study examined the influence of boric acid treatment on Ehrlich ascites carcinoma (EAC) cells, and the influence of boron per se on in vivo EAC-cell proliferation and progression of cancer cells mediated by proteoglycans and collagen.

2. Materials and methods

2.1. Animal stocks

The animals used and the experimental design had the prior approval of the Animal Care and

Use Committee, King Saud University, Riyadh. Female Swiss albino mice (SWR) 5–6 weeks old, weighing 24–26 g, were obtained from the Experimental Animal Care Center, King Saud University, Riyadh, Saudi Arabia. The animals were fed on Purina chow diet and water ad libitum and were maintained under standard conditions of humidity, temperature, and light (12 h light/12 h dark cycle).

2.2. Drugs and administration procedures

Boric acid (Sigma Chemical Co., St. Louis, MO, USA) was used as the test compound. All the other chemicals and reagents were of analytical reagent grade purchased from commercial sources. Cyclophosphamide (CP) (Endoxan-Asta®, Asta Medica AG, Frankfurt, Germany) was used as a standard drug.

The doses of boric acid (62.5, 125, and 250 mg/kg/day) were determined on the basis of previous literature (Fail et al., 1991; Heindel et al., 1994) and a trial experiment, which showed it to be pharmacologically effective at a dose of 100 mg/kg. Freshly prepared sterile aqueous solution of boric acid was administered intraperitoneally (i.p.) daily for five days. The intra-tumoral route of administration was adopted in view of its reported efficacy in the acquisition and retention of the drug by the EAC cells (Adamietz et al., 1990; Unnikrishnan and Kuttan, 1990; Hall et al., 1992; Devi et al., 1994). Cyclophosphamide (CP) (10 mg/kg/day, i.p., for five consecutive days) was used as a reference drug.

2.3. Experimental design

Ehrlich ascites carcinoma cells supplied by Dr C. Benckhuijsen (Amsterdam, Holland) were maintained by serial transplantation in female Swiss albino mice every eight days. A total of 150 female mice were randomly allotted to different control and treatment groups (25 mice in each group divided as 10 and 15 animals in two separate sets). Ten mice in each group (set 1) were used for the evaluation of parameters on cytotoxicity, biochemistry, and histopathology; the remaining 15 mice in each group (set 2) were used for the study on the changes in body weight and survival. The EAC cells (2.5×10^6 cells/mouse) were implanted (i.p.) into all the experimental mice except those in the negative control group. The treatment was initiated six days after tumor implantation and continued for five consecutive days.

The experimental groups of mice consisted of the following: group 1, negative control (devoid of EAC-cell implant) (sterile water); group 2, positive control (EAC-cell bearing) (sterile water); group 3, as in group 2, followed by cyclophosphamide (10 mg/kg/day); groups 4–6, as in group 2, followed by boric acid (62.5, 125 or 250 mg/kg/day, respectively). In each treatment, five animals were killed 24 h after the last dosage (12th day); their ascitic fluid was collected and tabulated. The peritoneal fluid (PF) sample from each mouse was collected in two different vials. One of these samples was immediately processed for parameters on cytotoxicity and viability, while the second vial was stored at -70°C until used for the determination of lipid peroxides, non-protein sulfhydryls (NP-SH), nucleic acids, and proteins. Tumors that developed at the site of inoculation of EAC cells (by using 17-gu age needle to allow EAC-cell penetration into the skin tissue and to develop into a solid tumor) were obtained from the same animals and preserved in buffered formaline and processed by the routine procedures for histopathological investigations.

2.4. Evaluation of body weight and survival

The sets of animal groups allotted for observations on body weight changes and survival were maintained separately. These animals were observed for their weight and mortality daily until their death or up to a maximum of 35 days.

2.5. Cytotoxicity and viability

The aliquots of PF collected were immediately processed for the observation of total count, cytotoxicity, and viability of EAC cells with a hemocytometer using a dye-exclusion technique (Qureshi et al., 1993).

2.6. Biochemical studies

Frozen PF samples were used for the estimation of malondialdehyde (MDA) and NP-SH.

2.6.1. Determination of MDA contents

The method described by Ohkawa et al. (1979) was followed. Peritoneal fluid containing EAC cells was homogenized in aqueous KCl solution and incubated with thiobarbituric acid reagent. After centrifugation, the optical density of the clear pink supernatant was read at 532 nm. Malondialdehyde bis(dimethyl acetal) tetraammonium was used as an external standard.

2.6.2. Quantification of NP-SH

The levels of NP-SH were determined according to the method described by Sedlak and Lindsay (1968). Peritoneal fluid containing EAC cells was homogenized in ethylenediaminetetraaceticacid disodium (EDTA). Aliquots of homogenate were treated with 50% w/v TCA and centrifuged at $3000 \times g$. The supernatant fractions were mixed with Tris buffer and 5,5-dithiobis-(2 nitrobenzoic acid) (DTNB) and the absorbance was read at 412 nm against a reagent blank with no homogenate.

2.7. Histopathological procedure

The tumors that developed at the site of injection of EAC cells were excised and fixed in 10% formaldehyde. The preserved tumor tissue was dehydrated, cleared, and processed for routine paraffin-block preparation using an American Optical® rotary microtome. Sections of about 5 μm thickness were cut, stained with hematoxylin, and counter-stained with eosin (Al-Harbi et al., 1995). The slides were examined for histopathological changes such as inflammatory reaction, necrosis, number of hair follicles, mitotic figures, and size of the tumor by an observer who was blind with respect to the treatment groups.

2.8. Statistical analysis

The observations were calculated as mean ± S.E. in a group and inter-treatment comparison for a certain parameter was performed by using Student's *t*-test.

3. Results

The results of our present study are presented in Tables 1–3 and Figs. 1–4.

3.1. Body weight changes

The EAC cell-bearing mice (group 2) showed a significant increase in body weight as compared with the normal mice in group 1 (negative control). Preliminary experiments revealed that i.p. injection of boric acid at 62.5 or 125 mg/kg dose for five days had no significant effect on body weight when compared to the untreated control group (data not shown). However, boric acid treatment increased the body weight of EAC cell-bearing mice, as compared with mice in the positive control (group 2), but the increase was statistically significant only between days 10 and 20 and only at the highest dose (250 mg/kg, group 6). Only the CP treatment group survived at days 25 and 30 and showed a significant reduction in the body weight of EAC cell-bearing mice at days 10–30 after implantation as compared with mice in the positive control (group 2, Fig. 1).

3.2. Effect on rate of survival

The percentage of survival in EAC cell-bearing mice in the control group (group 2) was 40% (six animals out of 15) on the 20th day after implantation (Fig. 2) and no animal survived beyond 25 days. The mean duration of survival in this group was 16 days (Fig. 3). The percent survival after treatment with CP (group 3) was 80% (12 animals out of 15) on 20th day after implantation and no animal survived beyond 33 days. The mean duration of survival in this group was 25 days. Boric acid treatment showed 26 (four animals out of 15), 13 (two animals out of 15) and 6% (one animal out of 15) survival at the 62.5, 125.0, and 250 mg/kg doses, respectively (groups 4, 5, and 6) (Fig. 2) at the 20th day. No animal survived beyond 22 days at 62.5 mg/kg (group 4), 21 days at 125.0 mg/kg (group 5), and 20 days at 250 mg/kg (group 6). The mean duration of survival was 15, 14, and 12 days in groups 4, 5, and 6, respectively (Fig. 3).

3.3. Effect on cell proliferation, viability and ascitic fluid contents

Boric acid treatment for five consecutive days increased the ascitic fluid contents and total number of peritoneal EAC cells at different doses on the 12th day after inoculation. However, the increase in EAC-cell number was statistically significant only at the high dose (250 mg/kg, group 6) as compared with the number of EAC cells obtained from mice in the positive control group (group 2) (Table 1). The number of viable EAC cells was also significantly increased at this dose. Cyclophosphamide treatment (group 3) significantly reduced the total count and number of viable EAC cells as compared to the values obtained in the positive control (group 2).

3.4. Effect on the MDA and NP-SH contents

Administration of boric acid resulted in an increase in MDA levels accompanied by a decrease in NP-SH contents in the EAC cells of mice that were statistically significant at all the doses when compared to the values obtained in untreated EAC cell-bearing mice (Table 2). Also, the CP treatment group demonstrated a significant increase in the MDA contents concomitant to a decrease in NP-SH contents in EAC cells as compared with the positive control value.

3.5. Effect on histopathological changes

The tumors induced by EAC cells at the site of injection in the positive control were very prominent and revealed fast growth in addition to several mitotic figures and hair follicles (Table 3, Fig. 4). Boric acid treatment (125.0 mg/kg) showed very fast growth of tumors as compared to those grown in the positive control (group 2). The tumors almost reached to the skin (Fig. 4) with increased number of mitoses and mixed inflammatory reaction (Table 3, Fig. 4). At 250 mg/kg, the treatment with boric acid revealed mixed inflammatory reaction around the increased growth of the tumor (Table 3, Fig. 4). On the other hand, CP treatment shows a reduced number of hair follicles and marked hyperkeratosis of the skin (Table 3, Fig. 4) as well as a reduction in the frequency of mitoses (Table 3, Fig. 4).

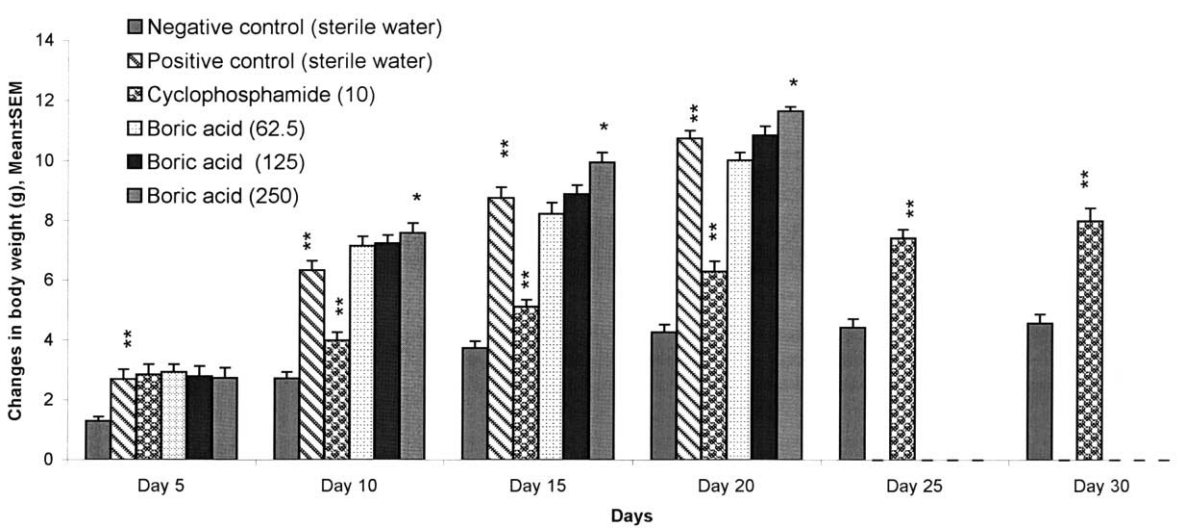


Fig. 1. Effect of boric acid treatment on body weight changes in mice implanted with EAC cells. Fifteen mice were used in each group. Negative control group was not implanted with EAC cells. The positive control (EAC-cell implanted) group was compared to the negative control group. Cyclophosphosphamide and boric acid treatment groups were statistically compared with the positive control group. $^{*}P < 0.05$; $^{**}P < 0.001$ (Student's t-test).

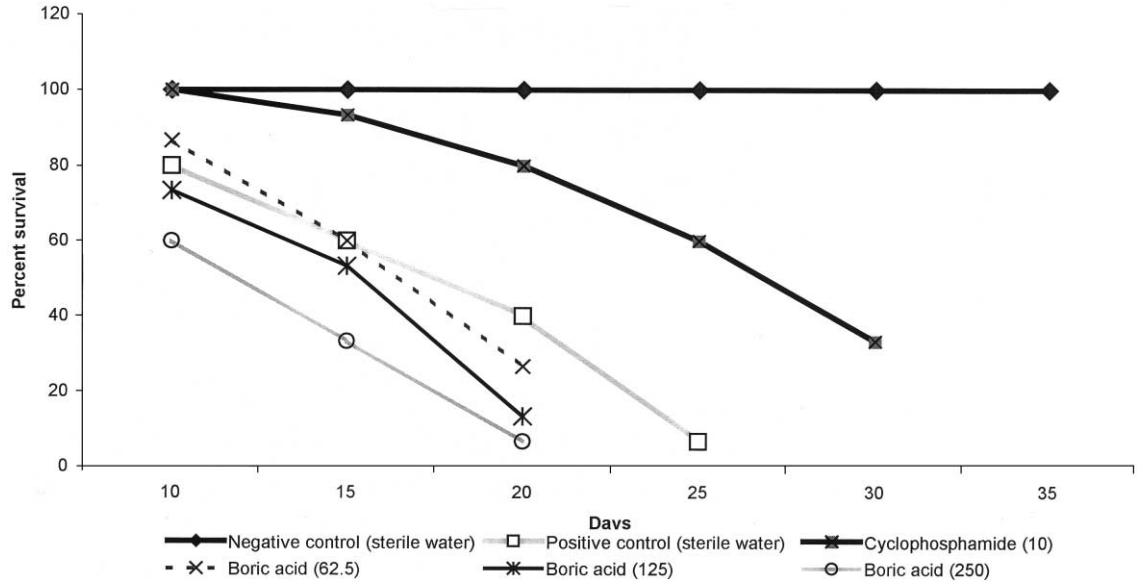


Fig. 2. Effect of boric acid on the percentage of survival of EAC cell-bearing mice. Fifteen mice were used in each group. EAC cells were implanted in all the mice except those in the negative control group. Treatments were started six days after implantation of EAC cells and continued for the next five days. Survival was observed up to 35 days.

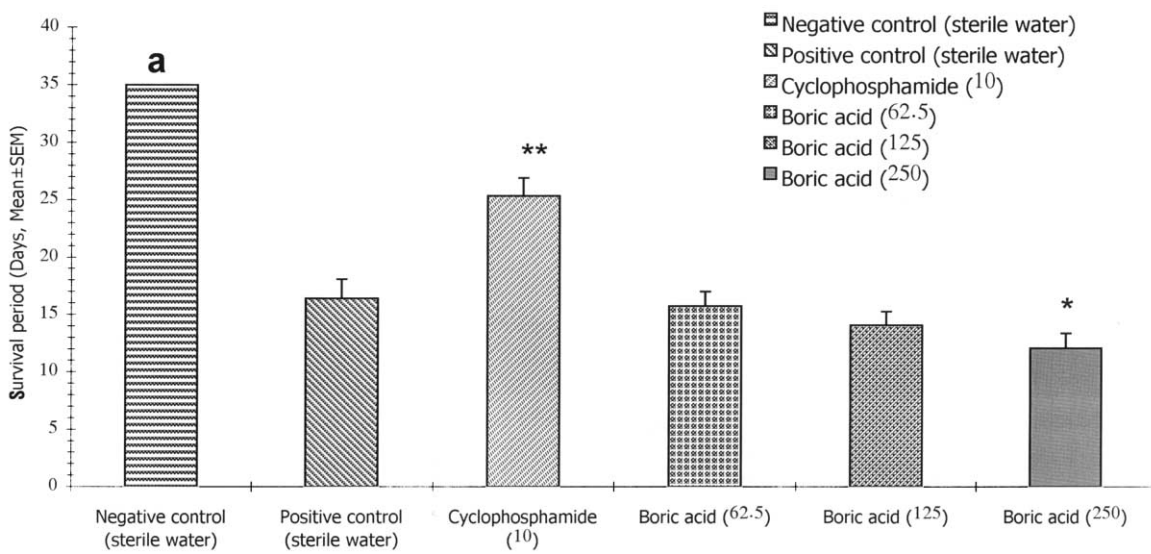


Fig. 3. Effect of boric acid on survival (days) of EAC cell-bearing mice: (a) survival was observed up to a maximum of 35 days. $^{*}P < 0.05$; $^{**}P < 0.001$ (Student's t-test).

Table 1
Effect of boric acid on the ascitic fluid contents, proliferation, and viability of EAC cells in mice[a]

Group	Treatment/dose (mg/kg/day)	Ascitic fluid volume (ml, mean ± S.E.)	Total cells counted ($\times 10^6$/ml, mean ± S.E.)	% Viable cells (mean ± S.E.)
1	Negative control (sterile water)	–	–	–
2	Positive control (sterile water)	4.3 ± 0.25	428 ± 20.22	86.98 ± 2.21
3	Cyclophosphamide (10)	2.5 ± 0.20*	189 ± 23.26**	75.89 ± 3.39*
4	Boric acid (62.5)	4.25 ± 0.79	432 ± 23.95	88.67 ± 2.81
5	Boric acid (125)	4.4 ± 0.83	449 ± 19.13	87.35 ± 3.33
6	Boric acid (250)	4.9 ± 0.56	498 ± 21.07*	94.29 ± 2.22*

[a] Five mice were used in each group. Groups 3–6 were statistically compared to group 2. *$P<0.05$; **$P<0.001$ (Student's t-test). *Note:* treatments were started six days after inoculation with EAC cells and continued for the next consecutive five days. Cell counts were made on 12th day. The negative control group was devoid of EAC-cell implant.

Table 2
Effect of boric acid on NP-SH and MDA contents in EAC cells[a]

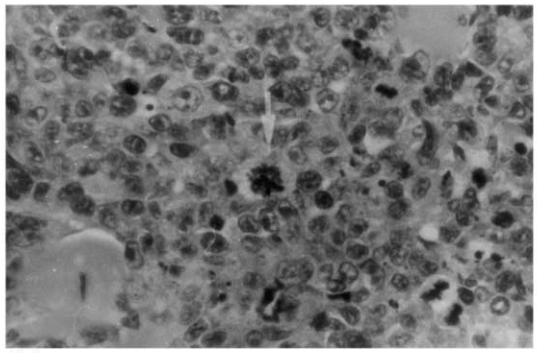
Group	Treatment/dose (mg/kg/day)	NP-SH (pmol/ml PF) (mean ± S.E.)	MDA (pmol/ml PF) (mean ± S.E.)
1	Negative control (sterile water)	–	–
2	Positive control (sterile water)	1175.30 ± 20.95	828.11 ± 17.5
3	Cyclophosphamide (10)	980.4 ± 24.85***	740.26 ± 12.39**
4	Boric acid (62.5)	1104.55 ± 11.44*	939.85 ± 20.6**
5	Boric acid (125)	1040.8 ± 25.55**	946.75 ± 14.4***
6	Boric acid (250)	1030.96 ± 15.68***	968.73 ± 11.68***

[a] Five mice were used in each group. Groups 3–6 were statistically compared to group 2. *$P<0.05$; **$P<0.01$; ***$P<0.001$ (Student's t-test). PF, peritoneal fluid. *Note:* treatments were started six days after inoculation with EAC cells and continued for the next consecutive five days. Peritoneal fluid samples were collected on the 12th day. The negative control group was devoid of EAC-cell implant.

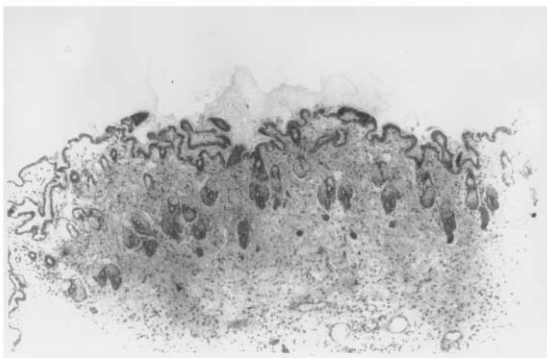
Table 3
Effect of boric acid on the histopathological changes in tumors grown at the site of inocculation of EAC cells in mice[a]

Group no.	Treatment/dose (mg/kg)	Histological indices				
		Inflammatory reaction	Mitosis	Hair follicles	Tumor size	Hyperkeratosis of skin
1	Negative control (sterile water)	–	–	–	–	–
2	Positive control (sterile water)	++	++	++++	+++	++
3	Cyclophosphamide (10)	+	+	+	++	++++
4	Boric acid (62.5)	++	++	+++	+++	+
5	Boric acid (125)	++	+++	++	+++	++
6	Boric acid (250)	+++	+++	++	++++	++

[a] (–), normal; (+), mild; (++), moderate; (+++), severe; (++++), intensely severe. *Note:* treatments were started six days after inoculation with EAC cells and continued for the next consecutive five days. Tissue specimens were excised off the body, from the place of inoculation, day after the last dosage. The negative control group was devoid of EAC-cell implant.



(a)



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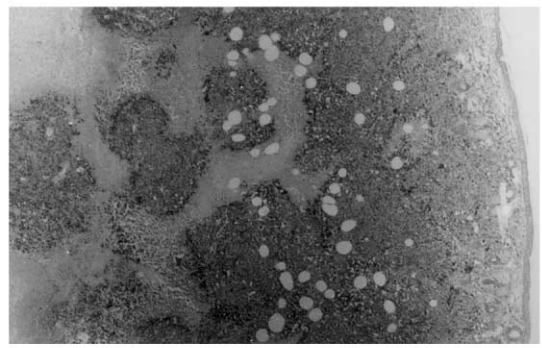

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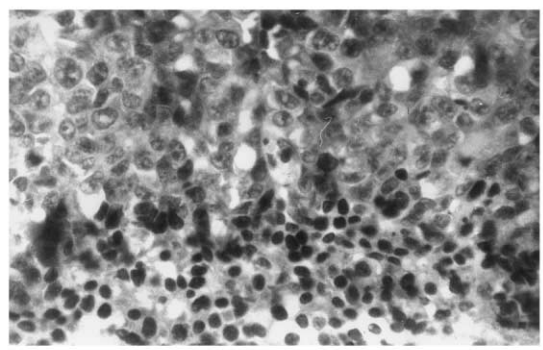

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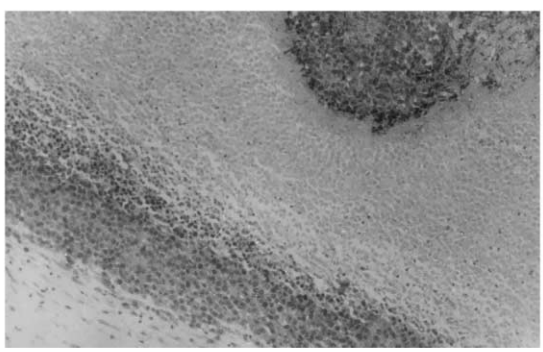

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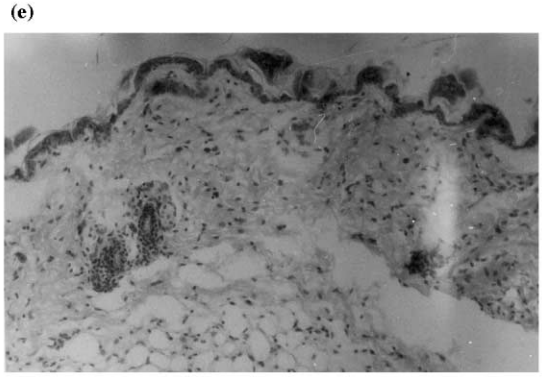

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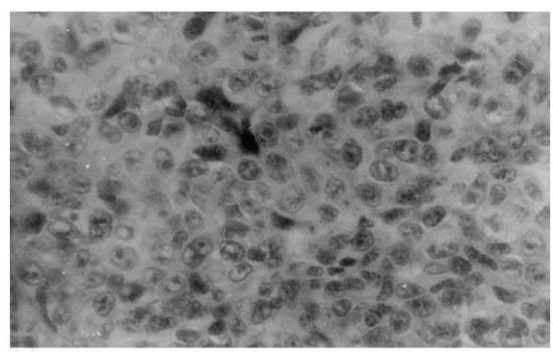

(g)

Fig. 4.

4. Discussion

The results obtained in the present study on EAC cell-bearing mice reveal that treatment with boric acid induces significant increase in the body weight with an evident increase in the quantity of ascitic fluid and reduces the duration of survival in these animals. These data are substantiated by our results on cytotoxicity and viability, which show a significant increase in the number of EAC cells and viability and the results of biochemical analysis accompanied by a decrease in the levels of NP-SH and elevated contents of MDA in EAC cells after boric acid treatment. These observations find a further support from histological assessment of the tumor tissue, which apparently showed fast growth, increased frequency of mitotic figures, and mixed inflammatory reaction in contrast to the reduced number of mitoses, hair follicles, and hyperkeratosis of the skin observed in the CP-treated EAC cell-bearing animals. In contrast, most of the other boronated compounds and their derivatives (trimethylamine carboxyboranes, their amides and esters, thymidine derivatives of borane, ribo- and arabinoside boron nucleosides) by themselves are found to be cytotoxic in murine and human tumors and different cell lines (Hall et al., 1990; Sood et al., 1994; Burnham et al., 1995; Hudecz, 1995; Hall et al., 1996).

Literature reports reveal that the difference in boron reaching the cell nucleus or getting trapped in the cellular membranes may be responsible for the differential response (Hall et al., 1996; Gedda et al., 1997). Nevertheless, our results of proliferative action of boric acid on EAC cells are in contrast to the effects of CP, which is quite well known for its cytotoxic properties (Oleinik, 1985, 1986; Berrigan et al., 1987; Patel, 1987). In addition, our data are also compatible with the reports on boron and boric acid to potentiate mutagenicity (Odunola, 1997) with a higher degree of mutagenic response after thermal neutron irradiation or γ-irradiation in the presence than in the absence of boron (Kinashi et al., 1997). The effect on biochemical changes observed in the present study and the reports on mutation promoting activity clearly delineate the reactive nature of boric acid, which may be attributed to its metabolism to boron responsible for the release of proteoglycans and collagen (Benderdour et al., 1997). Previous studies have demonstrated proteoglycans and collagen to stimulate tumor progression and metastasis in presence of transforming growth factors (Adany et al., 1990; McBain et al., 1990; Kovalszky et al., 1993; Tang and Honn, 1994; Flug and Kopf-Maier, 1995; Robert, 1996; Auvinen et al., 1997; Price et al., 1997; Schamhart and Kurth, 1997; Sun and Chen, 1997; Ricciarcdelli et al., 1998). McBain et al. (1990) found stimulation of proteoglycan synthesis in human colon cancer cells to cause progression of colon cell into changes stimulating the terminal differentiation. Furthermore, collagen and the growth factors such as epidermal growth factor and the fibroblast growth factor have also been reported to stimulate the proliferation of cells and progression of tumors (Laduca and Sinha, 1993; Yuspa et al., 1993; Reinbach et al., 1995).

From our present investigation it appears that the tumor promoting effects of boric acid result from its ability to lower the intracellular concentration of NP-SH with an anticipated rise in MDA. It can be speculated that the influence of boric acid, which is based on an increase in the concentration of free radicals, may be one of the

Fig. 4. (a) Microphotograph through a tumor induced by EAC cells at the site of inocculation showing fast growth indicated by several mitotic figures in a control animal (H & E $\times$ 40). (b) Microphotograph showing several hair follicles, patchy skin, and normal sebacious glands in a control animal (H & E $\times$ 100). (c) Microphotograph showing fast growth in tumor size evident by the tumor bulging out of the skin at the site of inoculation with EAC cells after treatment with 62.5 mg/kg boric acid (H & E $\times$ 40). (d) Section through a tumor showing increased mitoses, enlarged tumor size, and mixed inflammatory reaction in a mouse treated with 125 mg/kg boric acid (H & E $\times$ 400). (e) Section through a tumor showing mixed inflammatory reaction around the fast growing core in a mouse treated with 250 mg/kg boric acid (H & E $\times$ 100). (f) Section through the skin covering tumor showing reduced hair follicles, hyperkeratosed skin, and atrophic sebacious glands after treatment with 10 mg/kg cyclophosphamide (H & E $\times$ 100). (g) Section through a tumor showing few mitoses and inflammatory reaction after treatment with 10 mg/kg cyclophosphamide (H & E $\times$ 400).

possible mechanisms of its cancer promoting activity. Despite the modifying nature of boric acid on several cellular mechanisms, there is a paucity of literature on its carcinogenic potentials. Recent experiments on carcinogenicity revealed boric acid to be non-carcinogenic in rodents (Dieter, 1994; Fail et al., 1998). The discordance between the results obtained in studies on tissue of the normal animals and those of EAC cell-bearing mice may have been due to the sensitivity of the EAC cells, and interplay of a large number of factors, e.g. metabolism, autoimmune status, and stress. Earlier reports have also suggested that drug sensitivity may reflect differences in the intracellular concentration of enzymes and mediators of various target biochemical processes or repair mechanisms (Buick, 1984).

The exact mode of action of boric acid is not known. However, it appears to be due to the influence of boric acid on stimulation of lipid peroxidation and consequent proliferation of cells and progression of tumor under the influence of proteoglycans and collagen released in response to boron

Our present studies suggest special caution in the use of boric acid as a boronated compound in the neutron capture therapy.

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