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Evaluation of Antitumor Activity of Sodium Hypochlorite

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ABSTRACT

The present study aimed to evaluate the antitumor activity of newly synthesized oxidized form of sodium hypochlorite. Different cell lines were susceptible to Sodium hypochlorite cytotoxicity at acute, LD₅₀ and therapeutic doses of NaOCl. Therapeutic dose of sodium hypochlorite modulated the significantly reduced level of Hb, IL-12, TNF and the increased levels of creatinine, GPT and GOT towards the normal values. The histopathological study of liver, kidney and spleen groups showed a very significant improvement compared to normal and positive control groups. Sodium Hypochlorite exhibited an anticancer effect on different cell lines *in vitro* and against EAC in mice.

Key words: HEPG2, HEP2 and Vero cell lines, Ehrlich Ascites Carcinoma (EAC), NaOCl, albino mice, cytotoxicity

INTRODUCTION

Sodium hypochlorite (NaOCl) is a compound that can be effectively used for water purification. It is used on a large scale for surface purification, bleaching, odor removal and water disinfection.

Tumor Necrosis Factor- α (TNF- α) plays several therapeutic roles within the body which include immune stimulation, resistance to infection agents and resistance to tumors (Aggarwal and Vilcek, 1992). TNF- α may contribute towards resistance of infection through activation of neutrophils and platelets, enhancement of macrophage/NK cell killing abilities and stimulation of the immune system (Fiers, 1991). Additionally, (TNF- α) shows an anti-malignant cell cytotoxicity especially in combination with interferons (Gruss and Dower, 1995). TNF- α can induce necrotic or apoptotic cell death (Steller, 1995).

Interleukin-12 (IL-12) is a very exciting cytokine. It is a heterodimeric protein that promotes NK and T cell activity and is a growth factor for B cells. It has demonstrated antitumor activity in mouse models. It can activate NK cells, generate lymphokine-activated killer cells and induce interferon- γ (IFN- γ) production and T-cell only, when adaptive immunity is induced. However, because of the need for expansion of antigen-specific T and B cell populations with clonally distributed receptors, an efficient

adaptive response is induced only approximately a week after a primary infection. Innate resistance and adaptive immunity are not simply sequential and complementary mechanisms of resistance to pathogens, they regulate each other, through cellular contacts and the secretion of soluble mediators. In particular, the cytokine milieu that is established during the inflammatory innate response to pathogens sets the stage for the migration of antigen-specific T cells to lymph nodes, where they meet antigen-presenting cells. T helper 1 or T helper 2 responses which are effective against intracellular and extracellular pathogens, respectively are then induced, depending on the pattern of cytokines that are present during the clonal expansion of antigen-specific T cells (Mossmann and Coffman, 1989).

IL-12 is a pro-inflammatory cytokine that induces the production of IFN- γ , favors the differentiation of T helper 1 cells and forms a link between innate resistance and adaptive immunity. Dendritic cells and phagocytes produce IL-12 in response to pathogens during infection (Trinchieri, 2003). The present study was planned to evaluate the antitumor activity of newly synthesized oxidized form of sodium hypochlorite.

MATERIALS AND METHODS

Cell lines: The larynx carcinoma cell line Hep₂ (ATCC No. CCL-23, from ATCC (American Type Culture

Collection, Manassa, VA) and the hepatoma cell line HepG₂ (ATCC No. HB-8065) from ATCC were maintained in RPMI 1640 growth medium (Invitrogen) supplemented with 10% heat-inactivated Fetal Calf Serum (FCS), 2 mmol L⁻¹ L-glutamine, 100 units mL⁻¹ penicillin and 100 units mL⁻¹ streptomycin (all from Sigma/Aldrich, USA). The Vero Cell line (kidney Monkey cells, ECACC No. 84113001) from European Collection of Animal Cell culture, Salisbury, UK) was maintained in DMEM (Invitrogen) supplemented with 10% heat-inactivated FCS, 2 mmol L⁻¹ L-glutamine, 100 units mL⁻¹ penicillin and 100 units mL⁻¹ streptomycin (all from Sigma/Aldrich, USA). All cell lines were tested regularly for contamination. The recovery period bioassay was carried out to determine the therapeutic and acute doses according to lethal dose (LD₅₀) of sodium hypochlorite active ingredient.

Culture methods: All monolayer cells of cell lines were harvested at log phase of growth and seeded in 96-well microtiter plates (100 µL/well) (NuncA/S, Roskilde, Denmark) and incubated at 37°C in 5% CO₂ and 95% humidity incubator for 24 h to reach complete monolayer. Sodium hypochlorite was diluted to reach 1X concentration then, 100 µL was added to wells of the first row. Serial dilutions were made in triplicates, wells containing no active ingredient were used as controls. Microtiter plates were incubated at 37°C in 5% CO₂ and 95% humidity incubator for 24 and 72 h, to investigate LD₅₀ dose and cytopathic effect. For recovery period bioassay, cell culture plates were incubated for 7 days in which growth media was renewed every 2 days (Freshney, 2005).

In vivo studies: Swiss female albino mice, purchased from Theodore Bilhariz Research Institute, Ministry of Scientific Research, Giza, Egypt, with an average body weight of 25 g. Mice were housed at the animal house of Genetic Engineering and Biotechnology Research Institute (GEBRI), Sadat City, Sadat City University in steel mesh cage (5 mice/cage) and maintained for two weeks acclimatization period on commercial standard diet and tap water ad libitum. Mice were randomly divided into 4 groups, 10 animals each according to the following scale: Normal control group, Positive control group (mice bearing-solid tumor untreated), Mice bearing-solid tumor treated with distilled water and treated Group; injected with therapeutic dose of sodium hypochlorite according to the predetermined dose with *in vitro* study.

Ehrlich Ascites Carcinoma (EAC) cells: The initial inoculum of EAC cells was purchased from National Cancer Institute, Cairo University. EAC cells were propagated by weekly intraperitoneal injection of 0.2 mL of freshly drawn ascetic fluid diluted in saline solution to contain (5×10⁶ EAC cells mL⁻¹) from a donor mouse bearing 6-8 days old ascetic tumor, into three mice to ensure that the ascetic fluid will be still propagated and can then be drawn from at least one life mouse.

Induction with EAC cells: EAC cells are resuspended in phosphate buffer saline solution at a concentration of

5×10⁶ cell mL⁻¹, 0.2 mL of cell suspension containing 1×10⁶ cells, viability ≥95% injected intraperitoneally for 24 hrs before treatment.

Dose preparation: NaOCl therapeutic dose (0.02 mg/100 g body weight) was prepared using 0.9% NaCl saline solution, doses were injected subcutaneously three times/week for 1 month.

Biochemical parameters study: Alanine and aspartate amino transferases (ALT), (AST) were done according to Reitman and Frankel (1957). Kidney function tests; serum creatinine was done according to Bartels *et al.* (1972) Haemoglobin (Hb.) was determined according to Tiez (1976).

Tumor necrosis factor-α assay: TNF assay was done according to the Manufacturer's recommendations (RayBio_Rat TNF-alpha ELISA Kit, Cat#: ELR-TNF alpha-001C).

Interleukin-12 assay: IL-12 was done according to the manufacturer's recommendations (Human IL-12 (P70) ELISA Kit, Catalogue No.: EL10032, ANOGEN 2355 Derry Road East, Unit 23 Mississauga, Ontario CANADA L5S 1V6).

Histopathological study: Experimental animals each of control and treated groups were sacrificed by cervical dislocation after one month of dose administration. Liver, kidney and spleen organs were immediately excised and processed for histopathological study; the organs were fixed in 10% normal formalin saline. Paraffin sections of 5 µ thickness were prepared; stained with Hematoxylin and Eosin (H and E) and examined microscopically for histopathological changes graded according to Portmann *et al.* (1975).

Statistical analysis: Statistical analysis was done using the statistical package SPSS version 10. Comparison of mean values of studied variables among different groups was done using ANOVA test. p<0.05 was considered to be significant.

RESULTS

Cytotoxicity study: Sodium hypochlorite acute dose exert a high cytopathic effect compared to LD₅₀ and therapeutic dose in HepG2, Hep2 and Vero cell lines after 24 h exposure time, HepG2 cell line was more susceptible to the growth inhibition by cytotoxic acute, LD₅₀ and therapeutic dose of sodium hypochlorite at percentages; 79.1, 71.5 and 67.3%, respectively followed by cells recovery after 72 h at percentages of 23.6, 25.7 and 32.4%, respectively. In contrast, the Hep2 and Vero cell lines exposure time 24 h exhibited growth inhibition by acute, LD₅₀ and therapeutic dose at percentages of 54.6, 43.1 and 38.05% in Hep2, 58.2, 41.6 and 36.2% in Vero cell line, respectively, while the recovery cells after 72 h was evident for differential cytotoxicity of acute, LD₅₀ and therapeutic dose confirmed by evaluating cell viability at percentages of 69.0, 73.0 and 76.1% in Hep2, 72.1, 76.4 and 81.04% in Vero cell line compared to control

culture which showed complete monolayer cells after 24 h of exposure by saline solution.

Mean survival time study: Table 1 shows that the mean survival time increased from 16 ± 0.2 days for tumor-bearing mice untreated group to 35 ± 2.0 days for tumor bearing mice treated with therapeutic dose of sodium hypochlorite, respectively.

Tumor volume study: Table 2 shows that the therapeutic dose of sodium hypochlorite exert regression in tumor volume of mice given inoculations of EAC cells from (2.068 ± 0.15) for tumor bearing mice untreated group to (0.92 ± 0.196) for tumor bearing mice treated group.

Biochemical study: Data represented in Table 3 shows that treatment of mice implemented with EAC cells showed a significant increase in Hb level from 6.72 ± 0.1 to 11.26 ± 0.30 compared to normal saline treated group (10.46 ± 0.1), at the meantime a non-significant decrease is exhibited in creatinine level from 0.32 ± 0.01 to 0.30 ± 0.02 compared to normal saline treated group 0.28 ± 0.01 . With regard to GOT also a non-significant decrease is exhibited from 115.40 ± 0.01 to 103.52 ± 0.44 compared to normal saline treated group (110.24 ± 0.1), Glutathione peroxidase level also exhibited a non-significant decrease from 79.81 ± 0.01 to 72.87 ± 0.01 compared to normal saline treated group 68.24 ± 0.01 .

Interleukin-12 and tumor necrosis factor study: Data showed that positive control animal group implemented with (EAC) exhibited a highly significant decrease in IL-12 level (2.98 ± 0.5) compared to normal control group (6.32 ± 1.0). While NaOCl treated group exhibited a significant increase in IL-12 level (4.78 ± 1.0) compared to positive control animal group.

The present study showed that positive control animal group implemented with (EAC) exhibited a highly significant decrease in TNF level (2.98 ± 0.43) compared to normal control group (6.24 ± 1.0). While NaOCl treated group exhibited a

significant increase in TNF level (4.621 ± 1.0) compared to positive control group (Table 4).

Histopathological study: The histopathological examination of liver, kidney and spleen of female mice belonging to different groups (Control, Positive Control and Positive Control treated with the therapeutic dose of NaOCl) revealed that the NaOCl induced different histological amelioration in the studied tissues as obvious in Fig. 1-3.

Table 1: Effect of sodium hypochlorite on survival of tumor-bearing mice

Groups	Mean survival time/days
Tumor untreated	16 ± 0.2
Tumor treated	$35 \pm 2.0^*$

*Significant ($p < 0.05$) compared with treated and untreated tumor-bearing mice

Table 2: Effect of sodium hypochlorite on tumor volume

Groups	Tumor volume (mm^3)
Tumor untreated	2.068 ± 0.15
Tumor treated	$0.92 \pm 0.196^*$

*Significant ($p < 0.05$) compared with treated and untreated tumor-bearing mice, values are Means \pm SD

Table 3: Effect of sodium hypochlorite on the GPT and GOT activities as well as the levels of creatinine and hemoglobin among different studied groups

Groups	GPT (U L^{-1})	GOT (U L^{-1})	Creatinine (mg dL^{-1})	Hb (g dL^{-1})
Normal saline	68.24 ± 0.01	110.24 ± 0.1	0.28 ± 0.01	10.46 ± 0.1
Tumor untreated	79.81 ± 0.01	115.40 ± 0.01	0.32 ± 0.01	$6.72 \pm 0.1^*$
Tumor treated	72.87 ± 0.01^s	103.52 ± 0.44^s	0.30 ± 0.02^s	11.26 ± 0.30^s

Results are expressed as Mean \pm SD of ten mice ($N = 10$). *Significant ($p < 0.05$) compared to normal control group. ^sNon-Significant ($p > 0.05$) compared to normal control group

Table 4: Interleukin-12 and tumor necrosis factor concentrations ($\text{pg}/100 \text{ mL}$) in control mice group compared to all other groups

Groups	IL-12 (Mean \pm SD)	TNF- α
Normal saline	6.32 ± 1.0	6.240 ± 1.0
Tumor untreated	$2.98 \pm 0.5^{**}$	$2.980 \pm 0.43^{**}$
Tumor treated	$4.78 \pm 1.0^*$	$4.621 \pm 1.0^*$

Results are expressed as Mean \pm SD of ten mice ($N = 10$). *Significant ($p < 0.05$) compared to normal control group. **Highly Significant ($p < 0.01$) compared to normal control group

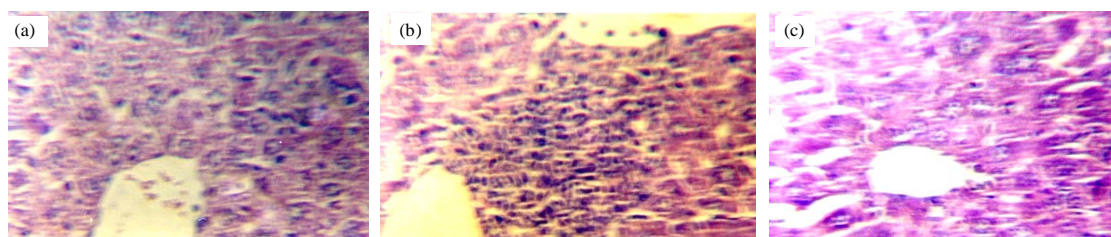


Fig. 1(a-c): Photomicrograph of (a) Normal control mice showing normal pattern of central vein, hepatic lobules, hepatic cells, kupffer cells, binucleated cells and blood sinusoids, (b) A liver of mice bearing-tumor treated with H_2O solvent showing dilated central vein, enlarged swollen hepatocytes and hepatic lobules, dilated blood sinusoids and (c) Liver mice bearing-tumor treated with tested active ingredient showing normal central vein, normal hepatic lobules and enlarged blood sinusoids (E and H.X:400)

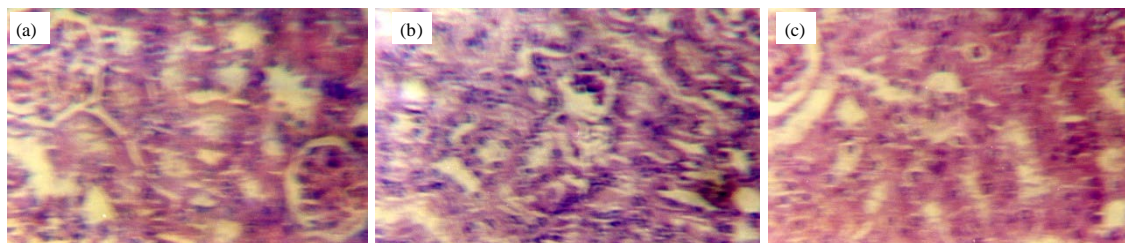


Fig. 2(a-c): Photomicrograph of (a) Normal control kidney of mice showing normal pattern of glomeruli with normal subcapsular space, (b) Kidney mice bearing-tumor carcinoma untreated showing dilated blood vessels and dilated subcapsular spaces of glomeruli and (c) Kidney mice bearing-tumor treated with NaOCl showing normal glomeruli with normal subcapsular spaces, dilated blood vessels (E and H.X:400)

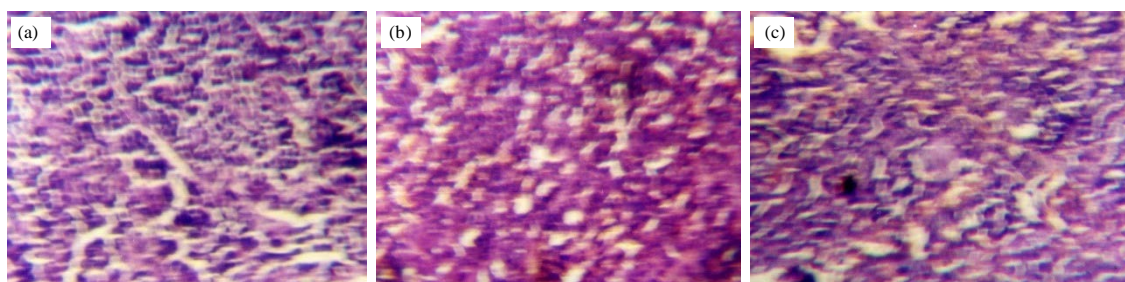


Fig. 3(a-c): Photomicrograph of (a) Normal control mice showing normal pattern of splenic capsule with red pulp, (b) Mice bearing-tumor induced with ehrlich ascites carcinoma untreated showing more reduction in the lymphocytic content accompanied with vacuolization and (c) Mice bearing-tumor induced with ehrlich ascites carcinoma treated with NaOCl showing moderate improvement of the splenic capsule as the red pulp with remarkable appearance of lymphocytes (E and H.X:400)

DISCUSSION

Oxidative species, including hypochlorous acid and hydrogen peroxide, are typically produced by inflammatory cells, this provides an example of cooperation between innate and adaptive immunity. Oxidation of tumor cells as antigens might therefore be a useful strategy for cancer immunotherapy (Chiang *et al.*, 2008). Many patients enter periods of clinical remission (Colombo *et al.*, 2006), during which immunologic function seems to return to normal. Most patients relapse subsequently, due to the persistence of microscopic and undetected tumors and these tumors offer an attractive target for immunotherapeutic approaches. A further advantage is that tumor cells can frequently be obtained directly from ascetic fluid as a single-cell suspension, thus minimizing the need for tissue disruption and manipulation and facilitating *in vitro* re-stimulation assays. Studies of the immune micro environment in ovarian tumors show convincing evidence for a natural immunoprotective response, which may be capable of enhancement by appropriate vaccination strategies (Zhang *et al.*, 2003).

Chiang *et al.* (2008) have examined responses to the well characterized ovarian epithelial cell line SK-OV-3, treated with the oxidizing agent hypochlorous acid. The results suggest a generic method to stimulate an antitumor T cell

response that will be capable of targeting autologous tumor in patients with ovarian cancer. The study verifies the predictions of the hypothesis that dendritic cell presentation of an established ovarian derived cell line, which has been oxidized by exposure to hypochlorous acid, induces a T-cell response that recognizes cross-reactive tumor antigens and autologous tumor cells isolated from ovarian cancer patients.

Khan *et al.* (2010) added sodium hypochlorite in drinking water to female Japanese quail (*Coturnix japonica*) at different doses. They showed no clinical signs were observed in quail given low doses. In addition, those given high exhibited the histopathological picture in mucosa of oviduct consisted of degenerated glandular cells. In some cases, glandular tissue was replaced by cord of cells and fibroblast.

It is well known that there are significant elevations in serum GPT, GOT in hepatocellular damage caused by a number of toxic agents (Osama, 2013). An increase in the GOT level is observed in patients with cardiac damage due to myocardial infarction and with liver disorders (Sadhu *et al.*, 1994). Biochemical measurements of these parameters in this study showed that no significant hepatotoxicity was associated with sodium hypochlorite as values remained near the normal values in all treated groups. An increase in Creatinine level was noted in cases of renal disease and damage disorders (Sadhu *et al.*, 1994). Since, Creatinine values in the treated

groups remained within the normal level. The present study revealed that GPT and GOT activities exhibited a decrease in EAC group treated with sodium hypochlorite compared to saline treated normal mice. Creatinine exhibited a decrease in EAC group treated with sodium hypochlorite compared to saline treated EAC mice group and Hb levels exhibited a significant decrease in EAC group treated with sodium hypochlorite compared to saline treated EAC mice group. These results are in agree with Khanam *et al.* (1997) who investigated the effect of copper benzohydroxamic acid complex (CU-BHA) against Ehrlich Ascites Carcinoma (EAC) in Swiss albino mice.

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