Research Article

Carbon Dioxide as a Microbial Toxicity Enhancer of Some Antibacterial Agents: A New Potential Water Purification Tool

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The aim of current paper was to investigate the possibility of increasing the toxicity of calcium hypochlorite (Ca(ClO)2) and hydrogen peroxide (H2O2) on Escherichia coli K-12 by preliminary enrichment of culture media by carbon dioxide (CO2). For this purpose, the microbes sensitivity to H2O2 or/and Ca(ClO)2 at normal and CO2-enriched medium was studied by spectrophotometric, radioisotopic, and electronmicroscopic methods. Ten-minute preincubation in CO2-enriched medium enhanced the toxic effect of both H2O2 or/and Ca(ClO)2 on bacteria as a result of induced growth inhibition, compared to no-CO2 enriched group. Additionally, changes in cell morphology and proliferation were observed. It was demonstrated that the preliminary incubation of microbes in CO2-enriched culture media in nonsupercritical concentration elevate the toxic effect of H2O2 or/and Ca(ClO)2 on microbes. This can serve as a novel, effective, inexpensive, and environmentally friendly approach for water purification from bacteria, further improving the protection of the environment and human health.

1. Introduction

One of the global challenges of the 21st century is to find the best method for cleanup of aquatic ecosystems from microbes, which will be cheap, safe for human health, and environmentally friendly [1, 2]. Chlorination is one of the most widely used processes for microbial control [3] in both drinking water and wastewater processing [4]. Chlorine is a powerful antimicrobial substance due to its potential oxidizing capacity [3]. In general, disinfectants like chlorine are used in very high concentrations for the purpose of attaining a rapid rate of killing. It is difficult for microorganisms to survive in such concentrations. However, the use of the chlorine increases the risk of the formation of potentially hazardous by-products or the production of foul tastes and odors, which are the main drawbacks of chlorination [5]. Conversely, at low chlorine levels, microorganisms that survive the treatment may be injured rather than inactivated [6]. Under suitable conditions, injured cells might repair cellular damage and recover [7]. An appreciation of the nature of sublethal injury and repair is, therefore, important in devising chlorination strategies and in developing combination treatments with synergistic actions against the target microorganisms. The mechanisms of action of chlorine on microorganisms have been widely investigated [8]. Nevertheless, the mechanism by which chlorine exerts its lethal effect has never been fully elucidated.

Currently, H2O2 and supercritical carbon dioxide (SCCO2) are being used as tools for water purification but separately. While the molecular mechanism of “killing” effect of H2O2 on bacteria is well documented [9], the mechanism of “killing” effect of CO2 on bacteria still remains discussable [10]. Although it is already documented that the combination of SCCO2 and H2O2 inactivates spores to high degree [11], the potential mechanism by which this inactivation takes place is still unknown. Moreover, this combination of SCCO2 and H2O2 has not yet been used as a water purification tool.

Our previous investigations showed 2 functionally different states of membrane proteins, active and inactive (reserve)
in eukaryotic cells [12]. The ratio of these active and inactive molecules could be changed by the modulation of cell hydration [12–14]. It was also shown, that metabolic poisons cause cell hydration [15] resulting in the increase of the number of functionally active receptors in the membrane [12]. On the one hand, CO₂, as a strong metabolic poison, that leads to the increase of cell hydration, could serve as a potential factor able to elevate membrane sensitivity to H₂O₂ or/and Ca(ClO)₂ of microbes. Thus, it was suggested that CO₂ could elevate the toxic effect of H₂O₂ or/and Ca(ClO)₂ on cell.

Therefore, the use of the activation of microbial metabolism induced by comparatively low concentrations of CO₂ to enhance the toxic effect of H₂O₂ or/and Ca(ClO)₂ on microbes was checked. This fact and data obtained from our published patents [16, 17] suggest that a certain combination of both of “killer” molecules may allow the development of a novel and safe method for water purification.

2. Materials and Methods

2.1. Experimental Setup and Apparatus. The processing apparatus used in this work contained 50 liter CO₂ vacuum tank, the airline tubing with regulator valve and the CellStar CO₂ incubator (USA, Model: SWJ500TV BA). The test tubes containing 100 mL bacterial culture were placed in the CellStar incubator at 20°C. Carbon dioxide (air liquid, 99.7% purity) was injected into the individual test tubes in the incubator at the selected pressure (atmospheric pressure) for 10 min. The corresponding non-CO₂ controls were made in the same way, except that the test tubes were not flushed by carbon dioxide. After 10 min of incubation, the H₂O₂ and/or Ca(ClO)₂ in corresponding concentrations was added to the tubes, and then samples were closed and removed to the incubator at 37°C for 18 h for the future processing.

2.2. Bacterial Strain, Culture Media, Chemicals, and Estimation of the Bacterial Growth. Wild-type Escherichia coli K-12 (obtained from the Armenia Collection of Microorganisms (Institute of Microbiology of the Center of Microbiology and Microbial Depository of Armenian NAS, Yerevan)) was used for experiments. Prior to each test, the bacterial cultures (previously stored at 4°C in nutrient-enriched agar slants (NEA, containing: nutrient enrich broth (NEB) + 1.5% agar (Difco), final pH 7.1 ± 0.2 at 37°C) were grown in nutrient-enriched broth (NEB, containing: peptone 15 g/L, sodium chloride 6.0 g/L, yeast extracts 3.0 g/L, final pH 7.5 ± 0.2 at 37°C), at 37°C in thermostat overnight (18 h) and used for the treatments.

For H₂O₂ and Ca(ClO)₂ treatments, fresh hydrogen peroxide and calcium hypochlorite solutions were prepared immediately before the experiments and added to the bacterial cultures in the corresponding concentrations of the substances in aqua media. Microbial growth was evaluated spectrophotometrically at a wavelength of 600 nm using a СФ-46 ЈTOMO spectrophotometer (Russian Federation) for 18 h.

2.3. Electronmicroscopy Assay. The damage of microbial cells was visualized by electron-microscopy. Negative contrasting was performed using 2% phosphoric-tungsten acid solution at pH 6.8–7.0. Microscopic preparations of bacterial suspensions were made after the centrifugation of bacterial suspension at 3000 rpm (CLR-1) for 10 min, then bacterial sediment was fixed by 2.5% solution of glutaraldehyde in 0.1 M cacodylate buffer under pH 7.4 during 2 h under room temperature. After three-phase washing in 0.1 M cacodylate buffer, the cells were fixed under room temperature by 1% of solution tetroxid osmium in 0.1 M cacodylate buffer under pH 7.4 during not less than 1 h. After washing in the same buffer, dehydration of sample was conducted in the ethanol increasing concentrations for 30, 50, 70, 96 and 100% or acetone increasing concentrations and potting by mixture of Araldite on Luft. Then the bacterial sediment was polymerized in thermostat at 37°C and 59°C in Araldite preparations. Ultrathin sections were prepared using the ultra cut “Reichert” “Ultracut-Young” and stained in the uranyl acetat and lead citrate. The sections were checked by electronic microscope Tesla BS-500 (Czechoslovakia). The photomicrographies were scanned in permit of 900 pixels for inch and processed on computer programme Corel Draw versions 11 and Photoshop versions 8. Computer morphometric and stereometric analysis of electron microscopic imaging was performed according to the program “Video Test Structure-5, nanotechnology.”

2.4. Radioactive Labeled [³H]-Thymidine-Based Cell Proliferation Assay. For the measurement of the cell proliferation, the bacteria cultures were grown in 100 mL of NEB at 37°C overnight. One mL of overnight culture (10¹⁰ cells) was added to the 30 mL NEB (containing 40 mL [³H]-thymidine (1.3 μCi/mL) ([6-³H]-thymidine, PerkinElmer, Boston, MA, 14.4 Ci/mmol specific activity)). [³H]-thymidine containing samples were treated by CO₂, H₂O₂, CO₂/H₂O₂, and CO₂/Ca(ClO)₂ and then incubated in thermostat at 37°C, for 18 h. [³H]-thymidine uptake was stopped by precipitation of each portion with ice-cold 10% trichloroacetic acid (TCA). The cells were harvested by centrifugation for 10 min at 5000 g and washed 2 times by TCA and then again harvested by centrifugation. Then 3 mL of Bray’s scintillation cocktail was added to each sample, and the radioactivity was measured by using a Wallac 1450 liquid scintillation counter.

2.5. Statistical Analysis. Statistical analyses were conducted by SPSS 17 software. The differences among the means of treatment were tested by using the paired sample t-test. All experiments were repeated at least three times. Values are shown as means ± std. error.

3. Results

3.1. Effect of the Antibacterial Properties of CO₂/H₂O₂ Combination on the Growth of E. coli K-12. The response of E. coli to H₂O₂ is dose dependent, with production of different oxidative species as a result of cell exposure to different concentrations of the oxidant. Two different mechanisms of cell death are possible: at low concentrations microbes are forming filaments, while at higher concentrations microbes are shrinking [9, 18, 19]. In the current study, we have
checked the potential of elevation of the toxic effect of comparatively low doses of H₂O₂ on microbes by preliminary increase of the metabolic activity of microbes using preincubation of the latter in the aqua medium enriched with CO₂ (10 min). Since CO₂ dissolves in aqueous solutions, forming an acid and lowering the pH, to avoid the additional effects of these, the pH was adjusted to the constant 6.0, supporting the maximal growth of E. coli by addition of NaOH (0.1 mol/L) during CO₂ treatment.

The toxic effect of low concentrations of H₂O₂ on E. coli K-12 was increased in cell growth medium enriched with CO₂ for 10 min (maximum) (Figure 1, (♦)), compared to non-CO₂ enriched medium (Figure 1, (♦)).

3.2. Effect of the Antibacterial Properties of CO₂/Ca(ClO₂)₂ Combination on the Growth of E. coli K-12. The response of E. coli to chlorine highly depends on its concentration [21, 22]. As the aim of present work is to find out the possibility of the modulation of the effect of CO₂ on the toxic effect of relatively low doses of chlorine on microbes, the dose-dependent effect of Ca(ClO₂)₂ (mg/L) on the growth of E. coli K-12 in normal and after 10 min incubation microbes in CO₂-enriched culture medium was studied (Figure 2).

Preliminary treatment by CO₂ enhanced the "killing effect" of Ca(ClO₂)₂ on the bacteria observed in all checked Ca(ClO₂)₂ concentrations. However, this effect was more pronounced at low concentration of Ca(ClO₂)₂ (>0.1 mg/L) and decreased parallel to the concentration increase up to constant level, about 15%. These data suggest the existence of the minimum two pathways through which the CO₂ could potentiate the toxic effect of chlorine. The observed CO₂-induced enhancement of Ca(ClO₂)₂ toxicity at low doses can be explained by CO₂-induced stimulation of oxidative metabolisms, which is a target for poisoning effect of Ca(ClO₂)₂. Probably the CO₂-induced poisoning effect of Ca(ClO₂)₂ is a reason for the increase of the number of cellular targets (receptors) for Ca(ClO₂)₂ attack. Our previous work showed that cell swelling, probably the result of cell metabolism poisoning, could serve as one of such pathways [13]. Therefore, according to the fact that CO₂ is metabolic poison, there is theoretical possibility that CO₂-induced cell swelling could serve as a mechanism responsible for the increase of microbes' sensitivity to Ca(ClO₂)₂.

3.3. Electronmicroscopic Study of CO₂, H₂O₂, and Ca(ClO₂)₂ Effects on E. coli K-12. Electron-microscopic study of the untreated E. coli K-12 culture showed that cells have an ultrastructure, typical for gram-negative bacteria (Figures 3(a) and 3(b)). Ultrastructural analysis of E. coli K-12 has showed that effect of different biocides, such as CO₂, H₂O₂, or/and Ca(ClO₂)₂, leads to the ultrastructural changes in membranes, cytoplasm, and nucleotide. Treatment by CO₂ leads to the destruction of cellular wall and the removal of external membrane of wall of bacteria (Figures 4(a), 4(b), 5(a), 5(b), and 5(c)). An addition to the similar effect of H₂O₂ and its derivatives, for example, removal of external membrane of wall, increase of the transparency of citoplasmatic membrane was also demonstrated (Figures 6(a) and 6(b)). The treatment of E. coli K-12 by Ca(ClO₂)₂ destructs the cellular wall, which leaves from citoplasmatic membrane. Moreover, the cytoplasm had small granular structures (Figures 7(a) and 7(b)).
control group. H\textsubscript{2}O\textsubscript{2} alone inhibited the proliferation by 27\% and its combination with CO\textsubscript{2} (CO\textsubscript{2}/H\textsubscript{2}O\textsubscript{2}) showed the proliferation decrease by 68\%, Ca(ClO\textsubscript{2}) inhibited the proliferation by 19\%, but the combined effect of CO\textsubscript{2}/Ca(ClO\textsubscript{2}) couple showed higher inhibition of 30\% inhibition effect (Figure 8).

4. Discussion

The search for novel alternatives of water disinfection is of immense interest, and much effort is concentrated to address this issue. The current paper suggests a novel tool for enhancement of the efficiency of the two most common disinfecting agents, such as H\textsubscript{2}O\textsubscript{2} and Ca(ClO\textsubscript{2}) by combing them with CO\textsubscript{2}.

Our data clearly showed that CO\textsubscript{2} increased the toxic effect of H\textsubscript{2}O\textsubscript{2} and/or Ca(ClO\textsubscript{2}) on microbes, and it could be used as a promising tool for water purification.

As the toxic effect of H\textsubscript{2}O\textsubscript{2} \cite{23} and Ca(ClO\textsubscript{2}) \cite{19} is realized by its oxidative properties, consequently, their inhibition effects depend on the initial metabolic activity of microbes. It is suggested that the factor having stimulatory effect on cell metabolism could enhance the toxic effect of H\textsubscript{2}O\textsubscript{2} and/or Ca(ClO\textsubscript{2}) on microbes.

On the basis of the fact that CO\textsubscript{2} at comparatively low concentrations \cite{21} has strong stimulatory effect on cell metabolism, it could be suggested that cells pretreated by CO\textsubscript{2} could have high sensitivity to toxic effect of H\textsubscript{2}O\textsubscript{2} (Figure 1) and Ca(ClO\textsubscript{2}) (Figure 2). It should be mentioned that stimulation of growth occurs because some anabolic
reactions involve CO₂ fixation, and in the absence of an external source of the gas, CO₂ concentration in cell can be rate limiting for these reactions, with resultant decreased growth rates [10]. The basis of CO₂ inhibition has not been clearly established.

Carbon dioxide has a dual physiological role in microorganisms since it can both stimulate and inhibit cell development [24]. Various hypotheses have been proposed to explain the antimicrobial activity of carbon dioxide. CO₂ dissolves in water to form carbonic acid. Thus, dissolved CO₂ acts by lowering the pH of the medium, and the resulting acidity leads to a disturbance of some biological systems within cells. It was, therefore, suggested that microbial inhibition was due to an alteration in the properties of cell (membrane, cytoplasm, enzymes, etc.) [25]. However, the reduction in the pH of the medium is not sufficient to account for the antimicrobial action of CO₂, since it shows a specific inhibitory effect, which is greater than that of the other acids used to lower medium acidity (hydrochloric acid, phosphoric acid, etc.). These acids do not penetrate the microbial cells as easily as carbon dioxide [26]. Therefore, it was suggested that the comparatively low concentration of CO₂-induced activation of the metabolism of microbes could serve as a convenient method for the increase of the sensitivity of microbes to the toxic effect of low concentration of H₂O₂ and/or Ca(ClO)₂.

The data obtained in the current study demonstrated that the toxic effect of H₂O₂ and/or Ca(ClO)₂ on E. coli K-12 increased significantly when cell bathing medium was enriched with CO₂ 10 min beforehand. The combination effect of CO₂/H₂O₂ and CO₂/Ca(ClO)₂ was higher than in case of CO₂, H₂O₂, and Ca(ClO)₂ treatments separately. It is also important to mention that combined inhabitation effect of CO₂/H₂O₂ (68%) is more higher than combined inhibition effect of CO₂/Ca(ClO)₂ (30%).

Although our data did not allow us to make final conclusion on the nature of the mechanism through which the CO₂ poisoning metabolism could increase cell membrane sensitivity to H₂O₂ and/or Ca(ClO)₂, it was comparable to the reported CO₂-induced swelling of microbes [27]. This suggests that the CO₂-induced cell hydration could serve as one of fundamental mechanisms responsible for CO₂-induced enhancement of the toxic effect of these substances which can be realized in two ways: increasing the number of membrane chemoreceptors [12] and activating intracellular macromolecules (folding) activity [14].

Additionally, the electronmicroscopic measurement of cell volume in the above-mentioned conditions demonstrated that the cell swelling is not the only mechanism through which the cell inhibition is realized by CO₂/H₂O₂ and CO₂/Ca(ClO)₂. It is suggested that the ultrastructural visualizations could provide valuable data for correction of
The effects of Ca(ClO)\textsubscript{2} on *E. coli* K-12. Negative contrasting (a) and ultrathin section (b): the external membrane cellular wall with expansion of periplasmatic space has been taken. Scale bar (a, b) = 1 μm.

The main conclusion that is drawn from the conducted studies so far is that the noncritical concentration of CO\textsubscript{2} elevate the toxic effect of H\textsubscript{2}O\textsubscript{2} and/or Ca(ClO)\textsubscript{2} on *E. coli* K-12. The current paper proves grounds for a new approach for decreasing microbial pollution in water, as well as for better understanding of the fundamental mechanisms controlling the microbial growth inhibition depending on environmental factors. We believe that this work will represent the first systematic investigation of the effect of CO\textsubscript{2}/H\textsubscript{2}O\textsubscript{2} and CO\textsubscript{2}/Ca(ClO)\textsubscript{2} combination at comparatively low concentrations on the reduction of microbial pollution of water. The suggested method will be free from the most negative properties of water purification methods, which are currently in use.

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**References**

[1] A. Mokrini, D. Ousse, and S. Esplugas, "Oxidation of aromatic compounds with UV radiation/ozone/hydrogen peroxide," *Water Science and Technology*, vol. 35, no. 4, pp. 95–102, 1997.

[2] J. Wesley Eckenfelder, "The role of chemical oxidation in waste water treatment process, chemical oxidation technologies for the nineties," *Water Environment and Technology*, pp. 1–4, 1992.

[3] K. L. Kotula, A. W. Kotula, B. E. Rose, C. J. Pierson, and M. Camp, "Reduction of aqueous chlorine by organic material," *Journal of Food Protection*, vol. 60, no. 3, pp. 276–282, 1997.

[4] M. Sisti, A. Albano, and G. Brandi, "Bactericidal effect of chlorine on motile *Aeromonas* spp. in drinking water supplies and influence of temperature on disinfection efficacy," *Letters in Applied Microbiology*, vol. 26, no. 5, pp. 347–351, 1998.

[5] S. D. Richardson, A. D. Thruston, T. V. Caughran, T. W. Collette, K. S. Patterson, and B. W. Lykins, "Chemical by-products of chlorine and alternative disinfectants," *Food Technology*, vol. 52, no. 4, pp. 58–61, 1998.

[6] J. T. Lisle, S. C. Broadaway, A. M. Prescott, B. H. Pyle, C. Fricker, and G. A. Mcteters, "Effects of starvation on physiological activity and chlorine disinfection resistance in Escherichia coli O157:H7," *Applied and Environmental Microbiology*, vol. 64, no. 12, pp. 4658–4662, 1998.

[7] B. Mackey, "Injured bacteria," in *The Microbiological Safety and Quality of Foods*, B. Lund, T. Baird-Parker, and G. Gould, Eds., pp. 315–341, Aspen Publishers, Gaithersburg, Md, USA, 2000.
[8] C. Venkobachar, L. Iyengar, and A. V. S. P. Rao, “Mechanism of disinfection: effect of chlorine on cell membrane functions,” *Water Research*, vol. 11, no. 8, pp. 727–729, 1977.

[9] G. Brandi, M. Fiorani, C. Pierotti, A. Albano, F. Cattabeni, and O. Cantoni, “Morphological changes in Escherichia coli cells exposed to low or high concentrations of hydrogen peroxide,” *Microbiology and Immunology*, vol. 33, no. 12, pp. 991–1000, 1989.

[10] H. Kumagai, C. Hata, and K. Nakamura, “CO₂ sorption by microbial cells and sterilization by high-pressure CO₂,” *Bioscience, Biotechnology and Biochemistry*, vol. 61, no. 6, pp. 931–935, 1997.

[11] J. Zhang, N. Dalal, M. A. Matthews et al., “Supercritical carbon dioxide and hydrogen peroxide cause mild changes in spore structures associated with high killing rate of Bacillus anthracis,” *Journal of Microbiological Methods*, vol. 70, no. 3, pp. 442–451, 2007.

[12] S. N. Ayrapetyan and V. L. Arvanov, “On the mechanism of the electrogenic sodium pump dependence of membrane chemosensitivity,” *Comparative Biochemistry and Physiology*, vol. 64, no. 4, pp. 601–604, 1979.

[13] S. Ayrapetyan, “On the physiological significance of the pump-induced cell volume changes,” *Advances in Psychological Science*, vol. 23, pp. 67–82, 1980.

[14] V. A. Parsegian, R. P. Rand, and D. C. Rau, “Osmotic stress, crowding, preferential hydration, and binding: a comparison of perspectives,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 8, pp. 3987–3992, 2000.

[15] S. N. Ayrapetyan and M. A. Sulejmanian, “On the pump-induced cell volume changes,” *Comparative Biochemistry and Physiology*, vol. 64, no. 4, pp. 571–575, 1979.

[16] S. Ayrapetyan, Eh. Moosavi, and V. Martirosyan, “New method for water purification from microbes,” Patent # 2357A, 2010, http://www.aipa.am/upload/File/Texekagir/20103.pdf.

[17] V. Martirosyan and S. Ayrapetyan, “Water purification method,” Patent AM20100077, 2011.

[18] N. R. Asad, L. M. B. O. Asad, A. B. Silva, I. Felzenszwalb, and A. C. Leitão, “Hydrogen peroxide effects on *Escherichia coli* cells,” *Acta Biochimica Polonica*, vol. 45, no. 3, pp. 677–690, 1998.

[19] A. Hegde, G. Bhat, and S. Malliya, “Effect of exposure to hydrogen peroxide on the virulence of *Escherichia coli*,” *Indian Journal of Medical Microbiology*, vol. 26, no. 1, pp. 25–28, 2008.

[20] K. A. Presser, D. A. Ratkowsky, and T. Ross, “Modelling the growth rate of *Escherichia coli* as a function of pH and lactic acid concentration,” *Applied and Environmental Microbiology*, vol. 63, no. 6, pp. 2355–2360, 1997.

[21] R. Virta, P. Mañas, I. Álvarez, S. Condon, and J. Raso, “Membrane damage and microbial inactivation by chlorine in the absence and presence of a chlorine-demanding substrate,” *Applied and Environmental Microbiology*, vol. 71, no. 9, pp. 5022–5028, 2005.

[22] M. Cho, J. Kim, J. Y. Kim, J. Yoon, and J. H. Kim, “Mechanisms of *Escherichia coli* inactivation by several disinfectants,” *Water Research*, vol. 44, no. 11, pp. 3410–3418, 2010.

[23] J. A. Imlay, S. M. Chin, and S. Linn, “Toxic DNA damage by hydrogen peroxide through the fenton reaction in vivo and in vitro,” *Science*, vol. 240, no. 4852, pp. 640–642, 1988.

[24] C. Wei, M. Balaban, S. Fernando, and A. Peplow, “Bacterial effect of high pressure CO₂ treatment on foods spiked with Listeria or Salmonella,” *Journal of Food Protection*, vol. 54, pp. 189–193, 1991.

[25] N. M. Dixon and D. B. Kell, “The inhibition by CO₂ of the growth and metabolism of micro-organisms,” *Journal of Applied Bacteriology*, vol. 67, no. 2, pp. 109–136, 1989.

[26] J. King and L. Mabbrit, “Preservation of raw milk by the addition of carbon dioxide,” *Journal of Dairy Research*, vol. 49, pp. 439–447, 1982.

[27] E. Dehs-Louka, N. Louka, G. Abraham, V. Chabot, and K. Allaf, “Effect of compressed carbon dioxide on microbial cell viability,” *Applied and Environmental Microbiology*, vol. 65, no. 2, pp. 626–631, 1999.
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