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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 (H_2O_2) on *Escherichia coli K-12* by preliminary enrichment of culture media by carbon dioxide (CO_2). For this purpose, the microbes sensitivity to H_2O_2 or/and $Ca(ClO)_2$ at normal and CO_2-enriched medium was studied by spectrophotometric, radioisotopic, and electronmicroscopic methods. Ten-minute preincubation in CO_2-enriched medium enhanced the toxic effect of both H_2O_2 or/and $Ca(ClO)_2$ on bacteria as a result of induced growth inhibition, compared to no-CO_2 enriched group. Additionally, changes in cell morphology and proliferation were observed. It was demonstrated that the preliminary incubation of microbes in CO_2-enriched culture media in nonsupercritical concentration elevate the toxic effect of H_2O_2 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, H_2O_2 and supercritical carbon dioxide ($SCCO_2$) are being used as tools for water purification but separately. While the molecular mechanism of "killing" effect of H_2O_2 on bacteria is well documented [9], the mechanism of "killing" effect of CO_2 on bacteria still remains discussable [10]. Although it is already documented that the combination of $SCCO_2$ and H_2O_2 inactivates spores to high degree [11], the potential mechanism by which this inactivation takes place is still unknown. Moreover, this combination of $SCCO_2$ and H_2O_2 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, CO_2, 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 H_2O_2 or/and $Ca(ClO)_2$ of microbes. Thus, it was suggested that CO_2 could elevate the toxic effect of H_2O_2 or/and $Ca(ClO)_2$ on cell.

Therefore, the use of the activation of microbial metabolism induced by comparatively low concentrations of CO_2 to enhance the toxic effect of H_2O_2 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 CO_2 vacuum tank, the airline tubing with regulator valve and the CellStar CO_2 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-CO_2 controls were made the same way, except that the test tubes were not flushed by carbon dioxide. After 10 min of incubation, the H_2O_2 and/or $Ca(ClO)_2$ in corresponding concentrations was added to the tubes, and then samples were closed and removed to thermostat 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 H_2O_2 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 ЛОМО 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 Lufft. 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 [^{3}H]-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 (10^{10} cells) was added to the 30 mL NEB (containing 40 mL [^{3}H]-thymidine (1.3 μCi/mL) ([6-^{3}H]-thymidine, PerkinElmer, Boston, MA, 14.4 Ci/mmol specific activity)). [^{3}H]-thymidine containing samples were treated by CO_2, H_2O_2, CO_2/H_2O_2, and $CO_2/Ca(ClO)_2$ and then incubated in thermostat at 37°C, for 18 h. [^{3}H]-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 CO_2/H_2O_2 Combination on the Growth of E. coli K-12. The response of *E. coli* to H_2O_2 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

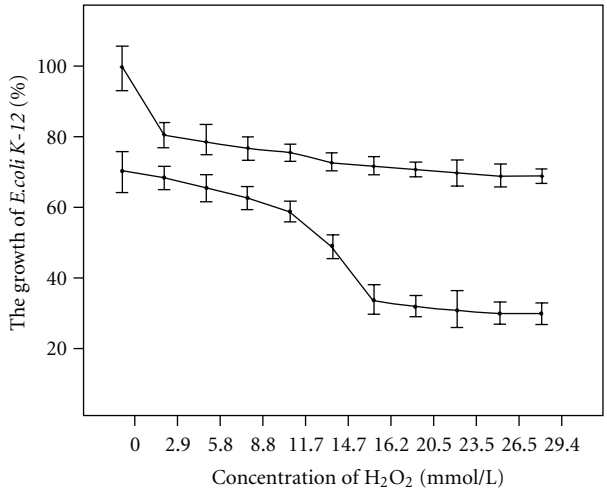


Figure 1: The effect of different concentrations of H_2O_2 (mmol/L) on the growth of *E. coli K-12* (%) in 10 min CO_2-enriched bathing medium (•) and in case of non-CO_2-enriched bathing medium (⧫). The samples were compared with control one (100% growth), which does not contain the carbon dioxide and hydrogen peroxide. $P = 0.01$ (**), $n = 3$.

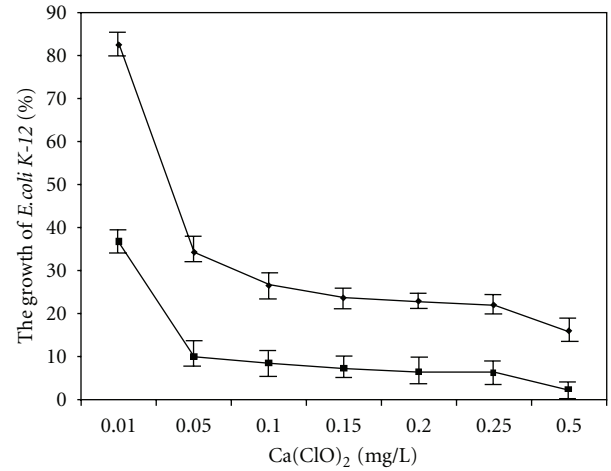


Figure 2: The dose-dependent effect of $Ca(ClO)_2$ (mg/L) on the growth of *E. coli K-12* (%) in 10 min CO_2-enriched bathing medium (■) and in case of non-CO_2-enriched bathing medium (⧫). The P value of the treatments was 0.01 (**). The samples were compared with control one (100% growth), which does not contain the chlorine and carbon dioxide. The number of experiments was three.

checked the potential of elevation of the toxic effect of comparatively low doses of H_2O_2 on microbes by preliminary increase of the metabolic activity of microbes using preincubation of the latter in the aqua medium enriched with CO_2 (10 min). Since CO_2 dissolves in aqueous solutions, forming an acid and lower 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* [20] by addition of NaOH (0.1 mol/L) during CO_2 treatment.

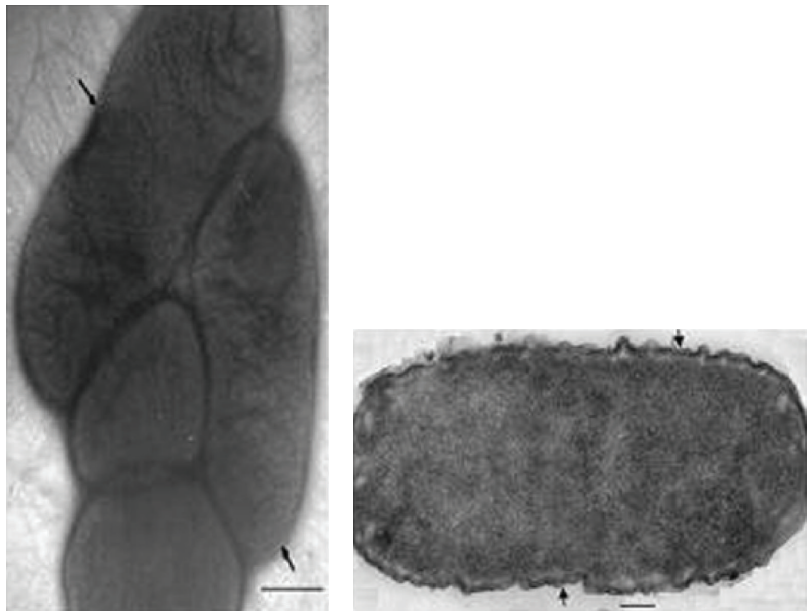
The toxic effect of low concentrations of H_2O_2 on *E. coli K-12* was increased in cell growth medium enriched with CO_2 for 10 min (maximum) (Figure 1, (•)), compared to non-CO_2 enriched medium (Figure 1, (⧫)).

3.2. Effect of the Antibacterial Properties of CO_2/$Ca(ClO)_2$ 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 CO_2 on the toxic effect of relatively low doses of chlorine on microbes, the dose-dependent effect of $Ca(ClO)_2$ (mg/L) on the growth of *E. coli K-12* in normal and after 10 min incubation microbes in CO_2-enriched culture medium was studied (Figure 2).

Preliminary treatment by CO_2 enhanced the "killing effect of $Ca(ClO)_2$ on the bacteria observed in all checked $Ca(ClO)_2$ concentrations. However, this effect was more pronounced at low concentration of $Ca(ClO)_2$ (>0.1 mg/L) and decreased parallel to the concentration increase up to constant level, about 15%. These data suggests the existence of the minimum two pathways through which the CO_2 could potentiate the toxic effect of chlorine. The observed CO_2-induced enhancement of $Ca(ClO)_2$ toxicity at low doses can be explained by CO_2-induced stimulation of oxidative metabolisms, which is a target for poisoning effect of $Ca(ClO)_2$. Probably the CO_2-induced poisoning effect of $Ca(ClO)_2$ is a reason for the increase of the number of cellular targets (receptors) for $Ca(ClO)_2$ 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 CO_2 is metabolic poison, there is theoretical possibility that CO_2-induced cell swelling could serve as a mechanism responsible for the increase of microbes' sensitivity to $Ca(ClO)_2$.

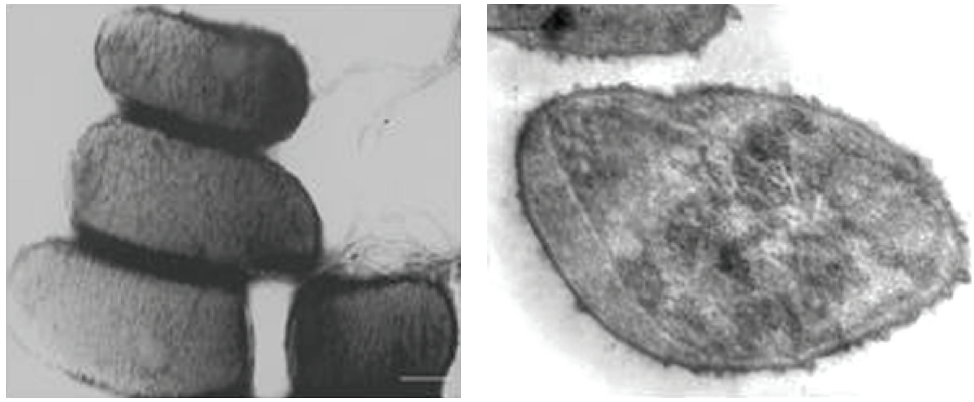
3.3. Electronmicroscopic Study of CO_2, H_2O_2, and $Ca(ClO)_2$ Effects on E. coli K-12. Electron-microscopic study of the untreated *E. coli K-12* culture showed that cells have an ultrastructure, typical for gram-negative bacteria (Figures 3(a) and 3(b)). Ultrastructural analysis of *E. coli K-12* has showed that effect of different biocides, such as CO_2, H_2O_2, or/and $Ca(ClO)_2$, leads to the ultrastructural changes in membranes, cytoplasm, and nucleotide. Treatment by CO_2 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 H_2O_2 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 $Ca(ClO)_2$ destructs the cellular wall, which leaves from citoplasmatic membrane. Moreover, the cytoplasm had small granular structures (Figures 7(a) and 7(b)).

3.4. Radioactive Labeled [^{3}H]-Thymidine-Based Cell Proliferation Assay. The measurement of the cell proliferation using radioactive [^{3}H]-thymidine assay has shown inhibition effects in all cases of treatments. The CO_2 treatment alone inhibited the cell proliferation by 49% compared to the



(a) (b)

Figure 3: Transmission of electronmicroscopy (TEM). Intact cell of *E. coli K-12:* (a) negative staining and (b) ultrathin section. Scale bar (a, b) = 1 μm.



(a) (b)

Figure 4: TEM. The action of CO_2 on the *E. coli K-12*. Negative staining (a). Ultrathin section (b): the external membrane of the cellular wall with expansion of the periplasmatic dimension has been taken. Scale bar (a, b) = 1 μm.

control group. H_2O_2 alone inhibited the proliferation by 27% and its combination with CO_2 (CO_2/H_2O_2) showed the proliferation decrease by 68%, $Ca(ClO)_2$ inhibited the proliferation by 19%, but the combined effect of $CO_2/Ca(ClO)_2$ couple showed higher inhibition of 30% inhibition effect (Figure 8).

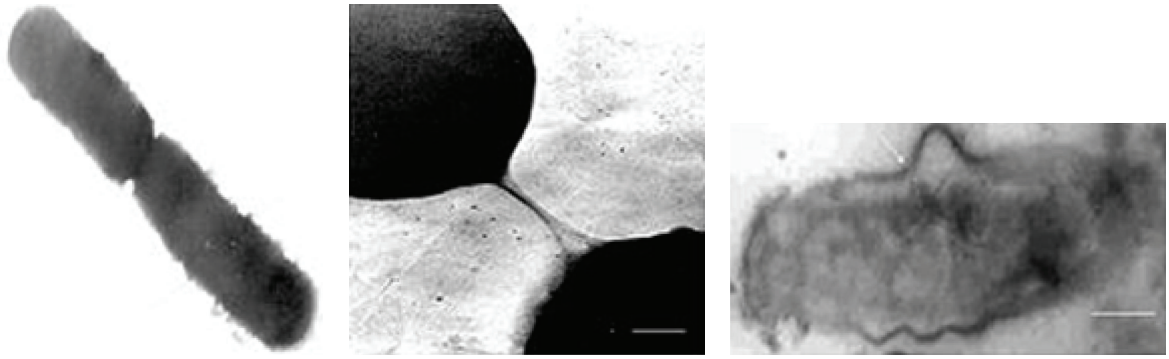
4. Discussion

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_2O_2 and $Ca(ClO)_2$ by combing them with CO_2.

Our data clearly showed that CO_2 increased the toxic effect of H_2O_2 and/or $Ca(ClO)_2$ on microbes, and it could be used as a promising tool for water purification.

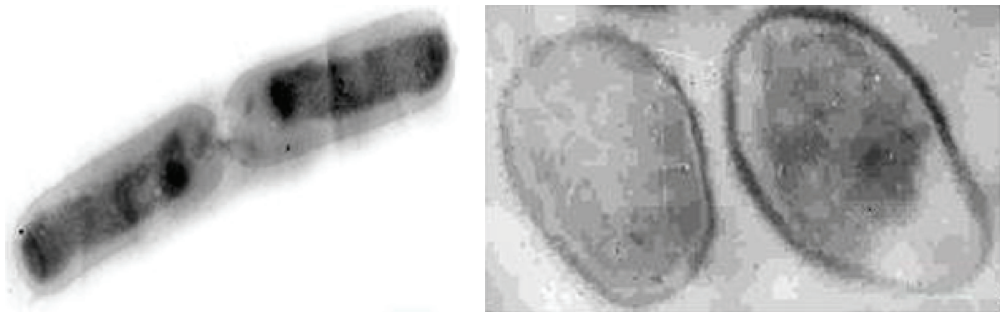
As the toxic effect of H_2O_2 [19] and $Ca(ClO)_2$ [23] is realized by its oxidative properties, consequently, their inhibition effects depend on the initial metabolic activity of microbes. It is suggested that the factor having stimulatory effect on cell metabolism could enhance the toxic effect of H_2O_2 and/or $Ca(ClO)_2$ on microbes.

On the basis of the fact that CO_2 at comparatively low concentrations [10] has strong stimulatory effect on cell metabolism, it could be suggested that cells pretreated by CO_2 could have high sensitivity to toxic effect of H_2O_2 (Figure 1) and $Ca(ClO)_2$ (Figure 2). It should be mentioned that stimulation of growth occurs because some anabolic



(a) (b) (c)

Figure 5: TEM. Action of CO_2 on *E. coli K-12*: (a) negative contrasting, (b) the inner cellular contact with the external membrane of cellular wall of *E. coli*, (c) ultrathin section. Scale bar = 1 μm (a, b, c).



(a) (b)

Figure 6: TEM. The effects of H_2O_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.

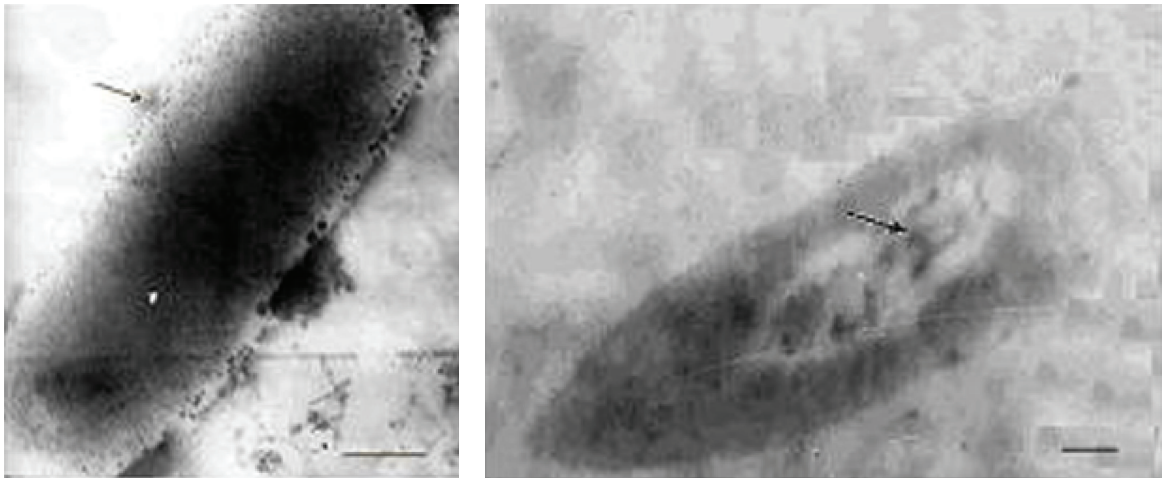
reactions involve CO_2 fixation, and in the absence of an external source of the gas, CO_2 concentration in cell can be rate limiting for these reactions, with resultant decreased growth rates [10]. The basis of CO_2 inhibition has not been clearly established.

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 microbicidal activity of carbon dioxide. CO_2 dissolves in water to form carbonic acid. Thus, dissolved CO_2 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 CO_2, 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 CO_2-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 H_2O_2 and/or $Ca(ClO)_2$.

The data obtained in the current study demonstrated that the toxic effect of H_2O_2 and/or $Ca(ClO)_2$ on *E. coli K-12* increased significantly when cell bathing medium was enriched with CO_2 10 min beforehand. The combination effect of CO_2/H_2O_2 and $CO_2/Ca(ClO)_2$ was higher than in case of CO_2, H_2O_2, and $Ca(ClO)_2$ treatments separately. It is also important to mention that combined inhabitation effect of CO_2/H_2O_2 (68%) is more higher than combined inhibition effect of $CO_2/Ca(ClO)_2$ (30%).

Although our data did not allow us to make final conclusion on the nature of the mechanism through which the CO_2 poisoning metabolism could increase cell membrane sensitivity to H_2O_2 and/or $Ca(ClO)_2$, it was comparable to the reported CO_2-induced swelling of microbes [27]. This suggests that the CO_2-induced cell hydration could serve as one of fundamental mechanisms responsible for CO_2-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 CO_2/H_2O_2 and $CO_2/Ca/ClO)_2$. It is suggested that the ultrastructural visualizations could provide valuable data for correction of



(a) (b)

Figure 7: TEM. The effects of $Ca(ClO)_2$ 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.

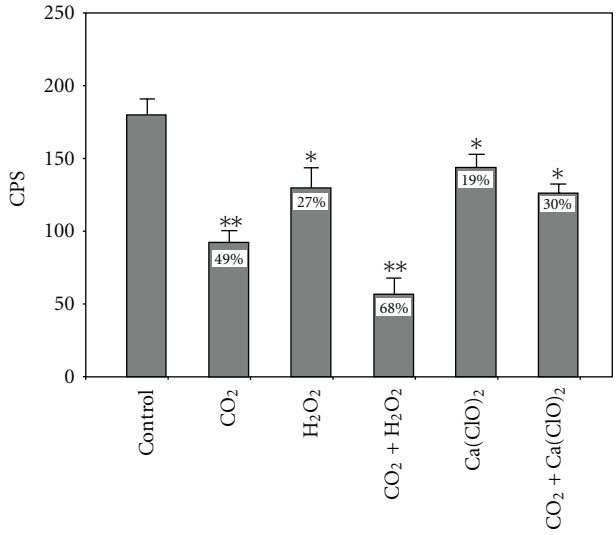


Figure 8: The measurement of the bacterial cell proliferation in different treatments by using radioactive labeled [^{3}H]-thymidine. Values shown in the graph are the means of three experiments ± Std. Error.

cytopathologic characteristics of mechanism of actions of CO_2, H_2O_2, and $Ca(ClO)_2$ biocides on the bacterial culture.

The main conclusion that is drawn from the conducted studies so far is that the noncritical concentration of CO_2 elevate the toxic effect of H_2O_2 and/or $Ca(ClO)_2$ 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_2/H_2O_2 and $CO_2/Ca(ClO)_2$ 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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