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SURGICAL ENDOSCOPY
and Other Interventional Techniques

Carbon dioxide inhibits the growth rate of *Staphylococcus aureus* at body temperature

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Abstract
Background: Since the 1930s, carbon dioxide (CO_2) has been combined with cold storage for the preservation of food. However, its use for the prevention of surgical wound infection was long considered to be impractical. Now CO_2 is widely used during laparoscopic procedures, and a method has been developed to create a CO_2 atmosphere in an open wound. The aim of this study was to investigate the effect of CO_2 on the growth of *Staphylococcus aureus* at body temperature.
Methods: First, *S. aureus* inoculated on blood agar were exposed to pure CO_2 (100%), standard anaerobic gas (5% CO_2, 10% hydrogen, 85% nitrogen), or air at 37°C for a period of 24 h; then a viable count of the bacteria was made. Second, *S. aureus* inoculated in brain–heart infusion broth and kept at 37°C were exposed to CO_2 or air for 0, 2, 4, 6, and 8 h; then the optical density of the bacteria was measured.
Results: After 24 h, the number of *S. aureus* on blood agar was about 100 times lower in CO_2 than in anaerobic gas ($p = 0.001$) and about 1,000 times lower than in air ($p = 0.001$). Also, in broth, there were fewer bacteria with CO_2 than with air ($p < 0.01$). After 2 h, the number of bacteria was increased with air ($p < 0.001$) but not with CO_2 ($p = 0.13$). After 8 h, the optical density had increased from zero to 1.2 with air but it had increased only to 0.01 with CO_2 ($p = 0.001$).
Conclusion: Pure CO_2 significantly decreased the growth rate of *S. aureus* at body temperature. The inhibitory effect of CO_2 increased exponentially with time. Its bacteriostatic effect may help to explain the low infection rates in patients who undergo laparoscopic procedures.

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Key words: Surgery — Laparoscopy — Wound infection — Carbon dioxide — Bacteriostatic effect

In 1889, Fränkel conducted the first systematic investigation of the effect of carbon dioxide (CO_2) on microorganisms [4]. Four decades later, further studies led to the use of CO_2 in modified atmosphere packaging to prolong the shelf life of fresh food [5, 8, 12]. The bacteriostatic effect of CO_2 was found to be especially marked in fresh meat [9]. As a result, it was first used as a preservative for shipments of beef, by 1938, 60% of all beef sent from New Zealand to Britain was transported in this manner [5]. Because most transport took place in cold storage, food-related studies focused on CO_2's effect on bacterial growth at low temperature.

Other applications were not considered of practical value, and clinical application of CO_2's bacteriostatic effect was never seriously contemplated. The main reason was, of course, that it seemed impossible to maintain a CO_2 atmosphere in an open wound. Now, however, CO_2 is daily insufflated intraabdominally in numerous laparoscopic procedures. Moreover, a method has been developed to create a CO_2 atmosphere in a wound without sealing it off [13, 14, 16–18]. Surprisingly, these innovations have not stimulated any great interest in the question of whether or not CO_2 retains its bacteriostatic potential at body temperature—i.e., the optimal temperature for bacterial growth. If it does, all the blowing of CO_2 gas that at present takes place in hospitals throughout the world could have an unexpected beneficial side effect. As a first step, we exposed *Staphylococcus aureus* to 100% CO_2 at body temperature and studied its subsequent growth rate.

Materials and methods

Blood agar experiment

S. aureus (Newman) inoculated on blood agar (5% horse blood) was allowed to grow overnight at 37°C. With the use of a sterile inoculating loop, bacteria from the colonies were transferred to the surface of 24 blood agar plates. The inoculated plates were divided into three groups, each with eight plates, which were incubated in pure CO_2 (100%), standard anaerobic gas (5% CO_2, 10% hydrogen, and 85% nitrogen), and air, respectively. The CO_2 and anaerobic atmospheres were created in boxes in which oxygen-free atmospheres were maintained via continuous gas supply. As a control measure, the oxygen content in the boxes was checked at the beginning and end of the incubation using an oxygen sensor (CheckMate 9900; Dansensor ApS, Ringsted, Denmark). The oxygen content was always $< 0.1\%$. After 24 h of incubation at 37°C, colonies could be seen on the agar plates in all groups, and the agar plates were taken out of the atmospheres. Three detached colonies were removed from each plate with a sterile inoculating loop and suspended in 1 ml phosphate-buffered saline (PBS, pH 7.3). Then 100 µl of different dilutions was plated on blood agar plates to make a viable count. In addition, the surface pH of the agar was measured (PerpHecT LogR meter, model 320; ATI ORION, Boston, MA, USA) on eight noninoculated agar plates before and after the gas exposure.

Broth culture experiment

S. aureus (Newman) inoculated on blood agar (5% horse blood) was allowed to grow overnight at 37°C. Bacterial colonies were then taken with a sterile inoculating loop and resuspended in PBS to an optical density of 1.0 at 550 nm (Novaspec II; Pharmacia Biotech, Cambridge, England, United Kingdom). Two bottles were filled with 300 ml of brain–heart infusion broth (BHI). One bottle was insufflated with CO_2 and one with air. The gases were filtered through a 0.2-µm gas filter and supplied at a flow of 3.5 L/min via a sterile pumice stone that was immersed in the broth. To obviate any foaming during the insufflation, one drop of polyglycol, which does not influence bacterial growth, was added to each bottle. After an initial 20 min of gas insufflation, 3 ml of the bacterial suspension was added to each bottle. During the gas insufflation, the bottles were kept at 37°C in a heated water tank with a shaker. After 0, 2, 4, 6, and 8 h of gas exposure, broth culture samples were taken and the optical density was measured. In addition, after 4 and 8 h, 100 µl of different dilutions of the samples were plated on standard blood agar plates so that a viable count could be made. The pH in the broth samples was measured (744 pH Meter; Metrohm, Herisau, Switzerland) before and after the 8 h gas exposure. This experiment was repeated eight times.

Statistical analysis

This is an analysis of variance (ANOVA) design, but due to unsuitable distribution characteristics a more simple and conservative nonparametric analysis was chosen. The Mann-Whitney *U* test and the Wilcoxon test were used when appropriate. Data are presented as medians with quartiles. Differences were considered to be statistically significant at $p < 0.05$.

Results

On blood agar, bacterial growth was slowest in CO_2 and fastest in air (Fig. 1). After 24 h of growth, the median number of bacteria per colony in CO_2 was $\sim$2-$\log_{10}$ lower than in anaerobic gas ($p = 0.001$) and $\sim$3-log lower than in air ($p = 0.001$). The median surface pH on eight noninoculated blood agar plates after the 24-h exposure to air, anaerobic gas, and CO_2 were 7.2, 7.2, and 6.8, respectively. Before gas exposure, the median surface pH was 7.4 in all three groups.

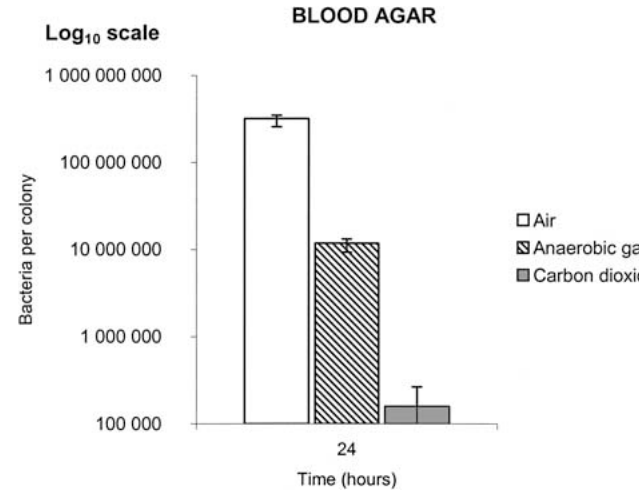

Fig. 1. Median number of bacteria in a colony grown on blood agar plates that were incubated in air, anaerobic gas, and carbon dioxide at 37°C for 24 h. Error bars represent quartiles.

In the broth cultures after 4 h, the median number of colony-forming units (CFU) per milliliter was $\sim$2-log lower with CO_2 than with air ($p = 0.002$) (Fig. 2A). After 8 h with air, the median number of CFU/ml had increased further with $\sim$1-log ($p = 0.01$). By contrast, with CO_2 after 8 h, there was no significant increase in the median number of CFU/ml ($p = 0.12$), which was then $\sim$3-log lower than with air ($p = 0.003$). With air insufflation, the optical density had already increased significantly after 2 h ($p < 0.001$), whereas with CO_2 insufflation it did not increase statistically until after 4 h ($p = 0.01$) (Fig. 2B). After 2, 4, 6, and 8 h, the median optical density was significantly lower with CO_2 than with air ($p = 0.001$). After 8 h, the optical density had increased from an initial zero value to 1.2 with air but it increased only to 0.01 with CO_2. The median pH values in the broth cultures were 7.3 before exposure to air and CO_2 and decreased to 6.3 and 6.5, respectively, after 8 h.

Discussion

At low temperature, high concentrations of CO_2 have been found to have an inhibitory effect on both aerobes and anaerobes [7, 12]. This effect has been attributed to two main mechanisms: suffocation and a specific CO_2 effect that acts directly on the bacterial cell but is not yet completely understood [5, 11].

The pivotal question now is whether or not CO_2 retains its inhibitory effect at body temperature—i.e., the optimal temperature for bacterial growth. In the present experiments, the growth temperature was kept at 37°C and the CO_2 concentration was kept at 100%. To analyze the results of the blood agar experiment, the incubation time had to be long enough (24 h) for the bacteria colonies in the CO_2 atmosphere to become visible. Growth in the broth cultures made shorter incubation times possible.

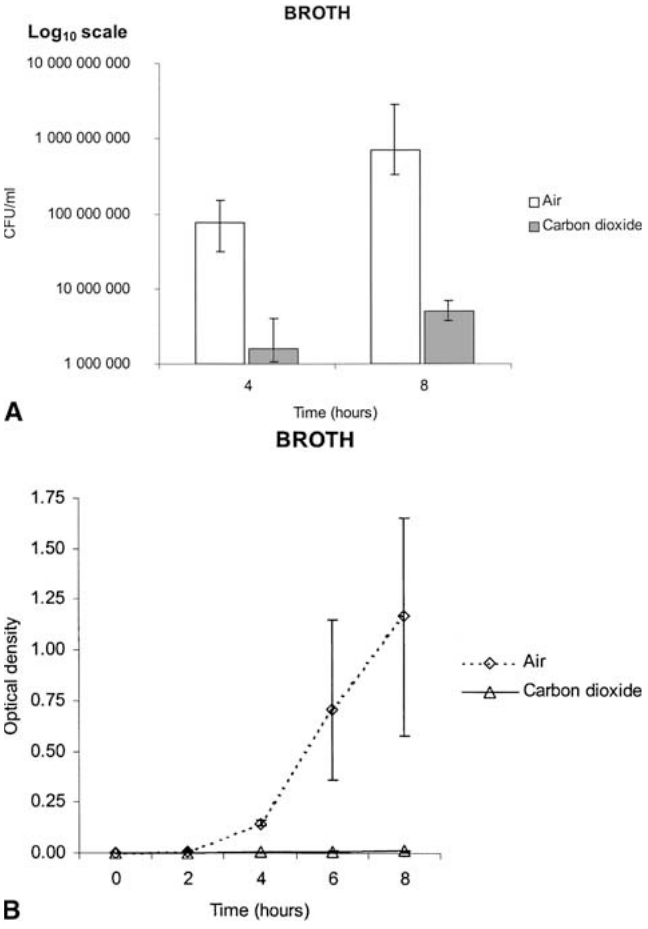

Fig. 2. Bacteria grown in broth cultures at 37°C during 8 h under exposure to air and carbon dioxide. The bacterial content is expressed as **A** the median number of colony-forming units per milliliter (CFU/ml) and **B** the median optical density of the broth cultures. Error bars represent quartiles.

Because the addition of CO_2 to an aqueous medium may cause the pH to drop [5], we measured the pH on the agar plates and in the broth culture before and after gas exposure. Our measurements showed that all of the gases caused only a slight decrease in the pH of the growth media. Coyne [4] has shown that this slight decrease does not appreciably affect the degree of bacterial growth inhibition. On blood agar, bacteria grow in colonies; whereas in broth culture, they appear as single cells detached from each other. This difference did not affect the results because in both experiments the growth rate in CO_2 was much lower than the aerobic growth rates. Figure 1 confirms that the bacteriostatic effect of CO_2 was due not only to oxygen deficit but also to a specific CO_2 effect that turned out to be mainly responsible for inhibiting bacterial growth. The difference between the growth rate in CO_2 and the growth rate in an anaerobic atmosphere containing only 5% CO_2 also shows that high CO_2 concentrations are required for the CO_2 effect to occur [7, 12]. As shown in Fig. 2A, the number of bacteria was 100 times lower with CO_2 than with air after 4 h and 1,000 times lower after 8 h. As measured in terms of optical density, the number of bacteria was already significantly higher in air than in CO_2 after 2 h, and this difference increased exponentially with time (Fig. 2B). This finding indicates that the antibacterial effect of CO_2 is relevant within the time frame of many surgical procedures. The strength of the effect increases exponentially with the duration of an operation. Patients undergoing long procedures are thus more likely to benefit from continuous CO_2 exposure. There may also be a residual postoperative effect, given that abdominal CO_2 has been seen in 19% of patients as long as 3 days after their laparoscopic procedures [6].

Recently, Hanly et al. [10] induced perioperative sepsis in rats by ligating and puncturing the cecum. They performed the operation either by the open approach or laparoscopically using CO_2 or helium (an inert gas) as insufflation gases. Twenty-four hours later, the acute-phase inflammatory response was less marked in rats treated with CO_2 than in animals who received helium or underwent open operation. Hanly et al. ascribed the attenuated inflammatory response to the abdominal insufflation with CO_2. The specific bacteriostatic effect of CO_2 could serve as an explanation for these interesting findings.

The same goes for the difference in infection risk between laparoscopic and open cholecystecomy [15] and between laparoscopic and open colorectal surgery [1]. In the former study [15], the overall risk of surgical site infection was significantly lower with laparoscopic cholescystectomy than with open cholecystectomy (0.62% vs 1.82%; relative risk, 0.3; $p = 0.001$). Also, in the latter study [1], fewer patients had infectious complications in the laparoscopic (11.0%) than in the open colectomy group (23.3%) ($p = 0.01$). The lower rates of infection after laparoscopic procedures may also be due, at least in part, to the bacteriostatic effect of 100% CO_2. It is possible that CO_2 inhibits not only bacteria but also immunological active cells, such as macrophages in a wound. However, the total sum of all effects can only be tested in clinical trials, which do indeed show a lower infection rate after laparoscopic operation using CO_2 [1, 15].

If this hypothesis is correct, it can certainly be called a lucky coincidence that during laparoscopy CO_2 is being insufflated not only at the right spot—the place where the contamination occurs—but also at the right moment. The first few hours after bacterial contamination constitute a critical period during which a wound infection becomes established [2, 3]. It is during this time that the wound is exposed to CO_2. By another happy chance, laparoscopists mostly use 100% CO_2 for insufflation. Thus, they unknowingly fulfill the requirement that high CO_2 concentrations be used for the CO_2 effect to occur. To top it all off, the CO_2 effect increases with time and thus is strongest when most needed—i.e., in protracted procedures, which are notorious for their propensity to foster infection.

When a clinical hypothesis is proposed, it is customary to recommend its testing in a clinical trial, In this particular case, the laparoscopists are in an unusual position in that the trials have been partly made before the hypothesis has been advanced. Trials have shown that laporoscopic procedures have lower infection rates than their open counterparts [1, 15]. But that is not

enough. To link the difference firmly to the CO_2 effect, a trial should compare the various insufflation gases. As for open surgery, trials are needed to determine whether CO_2 insufflation into the open surgical wound actually decreases postoperative infection rates.

To sum up, 100% CO_2 significantly decreased the growth rate of *S. aureus* at body temperature. The inhibiting effect of CO_2 increased exponentially with the duration of the exposure. The bacteriostatic effect of CO_2 could help to explain the low infection rates after laparoscopic procedures.

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