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Ovicidal activities of supercritical CO_2 and N_2O on *Ascaris suum* eggs

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ABSTRACT

This study examined whether supercritical CO_2 and N_2O fluids are effective in inactivating *Ascaris suum* eggs, which were chosen as a model for parasite eggs. The treatments were carried out in a multibatch apparatus, in which, the eggs could be placed atop a solid surface (non-immersed condition) or in aqueous solution (immersed condition). Various CO_2 and N_2O phases—including gas (6 MPa, 30 °C), liquid (8 MPa, 30 °C), subcritical (10 MPa, 30 °C), and supercritical (10 MPa, 37 °C)—were tested with exposure times ranging from 1 to 20 min. Supercritical CO_2 and N_2O both showed a similar, strong ovicidal effect, requiring only 1 min in non-immersed conditions and 5 min in water-immersed conditions to achieve a 2.4-log inactivation. Subcritical CO_2 and N_2O showed a weaker ovicidal effect. The effect was significantly reduced for the gas and liquid phases, compared with the supercritical phases. This study reports that supercritical CO_2 and N_2O can be effectively employed as a non-thermal treatment technique to control parasite egg contamination in fecal matter and food.

1. Introduction

Ascaris lumbricoides is one of the most prevalent parasitic intestinal nematodes implicated in infections, and an estimated 1.4 billion people are infected annually worldwide, mostly in developing countries [1]. *A. lumbricoides* infection occurs through the ingestion of food contaminated with fecal matter containing *A. lumbricoides* eggs [2–4]. Therefore, although the use of fecal material as agricultural fertilizer offers a beneficial recycling route, it has the potential risk of parasitic infection [5]. Furthermore, *Toxocara canis* or *Toxocara cati* eggs from pets or wild animals can contaminate the soil at public sites, such as playgrounds and parks; and because these eggs can infect humans, this also raises public health concerns [6–9]. For these reasons, fecal materials, food, and soil need to be appropriately disinfected in order to prevent parasite infection.

Current methods for inactivating *A. lumbricoides* eggs in sludge and fecal materials include high temperature with anaerobic or aerobic digestion, or high pH with ammonia, both of which are known to be ineffective [7,10,11]. However, they require temperatures of over 52 °C, relatively lengthy treatment, and adequate mixing [7]. Even though hydrostatic pressure treatments have been shown to have ovicidal effects on *A. suum* eggs, achieving more than a 2-log reduction in 1 min [12], their high capital and operating costs are serious drawbacks for commercialization [13], prompting the need for less costly disinfection technologies.

Over the past 2 decades, supercritical carbon dioxide (SC CO_2) treatment has been recognized as a promising non-thermal treatment technology because of its non-toxic, non-flammable, chemically inert properties, and its high inactivation effect for a large variety of microorganisms, from bacteria to viruses and yeast [14–16]. Recently, supercritical nitrous oxide (SC N_2O) has been suggested as an alternative supercritical fluid; it has a microbicidal effect similar to that of SC CO_2 without causing a pH change [17–19]. In addition, the supercritical fluids have been widely applied not only to inactivate microorganism, but also to decontaminate the hazardous substances from surface and water as an environmental friendly solvent [16,20].

Various microorganisms can be inactivated by SC CO_2 and SC N_2O treatments; however, little information is available regarding the effectiveness of these treatments on parasite eggs that are present in soil and water.

Therefore, this study attempted to examine whether supercritical fluid treatment using CO_2 and N_2O is effective in inactivating parasite eggs. *A. suum* eggs were chosen as a model for *A. lumbricoides* eggs because these two are known to have similar morphological and physiological characteristics [21]. We

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investigated the effects of the CO_2 and N_2O phase (gas, liquid, subcritical, or supercritical) and the egg location (on a solid surface or immersed in aqueous solution) on the egg inactivation efficiency.

2. Experimental

2.1. Preparation of A. suum eggs

A. suum eggs in this study were extracted from the uterus of female adult nematodes which were collected from the intestine of naturally infected pigs [22]. The nematodes were kindly supplied from the National Institute of Health (Seoul, Korea), and these were washed twice with 150 mM of commercial phosphate-buffered saline (PBS; pH 7.2) (Sigma, Co., MO) before dissection. The extracted eggs were resuspended in PBS and washed twice with 0.5% formalin solution, which is a common procedure for reducing the growth of fungi and bacteria as described by Bowman et al. [23]. The washed eggs were filtered through a 75–38 μm metallic mesh (Chung Gye Industrial Mfg., Seoul, Korea) in order to collect pure eggs. The pure *A. suum* eggs were transferred to a 50 mL Falcon tube with 20 mL of PBS. The number of unembryonated eggs in the samples was counted using an optical microscope (Eclipse 80i, Nikon, Japan) under ×200 magnification. The eggs were stored at 4 °C prior to the inactivation experiments.

2.2. Inactivation experiments by SC CO_2 and SC N_2O

The inactivation experiments were carried out in a multi-batch apparatus as described previously [24] with slight modifications, which includes mechanical mixing (Fig. 1). Pure liquid N_2O or CO_2 (purity >99%) were pumped into three temperature-controlled stainless steel (SS) reactors (50 mL of inner volume) using a high-pressure P-50 pump (Thar Technologies, PA) to reach the desired pressure with a maximum flow rate of 50 g min^{-1}, and this took approximately 1–2 min. The pressure and temperature were continuously monitored with a pressure gauge and thermometer, respectively. After completing the inactivation experiments with SC CO_2 or SC N_2O, it took 2 min to bring the pressure back to atmospheric with a back pressure regulator (TESCOM, Co., MN).

Depending on whether the eggs to be inactivated are located on a solid surface or immersed in aqueous solution, the experiments were divided in two types. First, approximately 1000 eggs in 50 μL of PBS were placed on the surface of a sterilized wood strip (4 cm × 2 cm), which was placed askew on the top of a 3 mm stainless steel mesh basket in the middle of the reactor. The magnetic stirrer was inserted at the bottom of the reactor to provide mixing at 500 rpm (Fig. 1.5a). This was called the non-immersed condition and indicated that water completely solubilized the SC CO_2 in contact with eggs because the solubility of pure water in SC CO_2 has been known to be approximately 35 μL water in 50 mL^{-1} CO_2 at 40 °C and 10 MPa [25]. The same amount of sample volume was used for the SC N_2O experiment, because the solubility of water in compressed N_2O has been reported to be similar to that of the compressed CO_2 [26]. In the non-immersed condition, the inactivation experiments were carried out under a gas (6 MPa, 30 °C), liquid (8 MPa, 30 °C), sub-critical (10 MPa, 30 °C), and SC (10 MPa, 37 °C) phase, for up to 20 min, in order to compare the ovicidal efficiency of these treatments.

Secondly, approximately 1000 eggs in 3 mL of PBS were placed in the bottom of the reactor with a magnetic bar (Fig. 1.5b). This was called the immersed condition. In the immersed condition, the inactivation of the eggs was conducted with the SC phase of CO_2 and N_2O (10 MPa, 37 °C). In order to investigate the ovicidal effect of the gases themselves at high pressure, *A. suum* eggs were treated by CO_2 and N_2O at 37 °C, at atmospheric pressure, for 20 min under both non-immersed and immersed conditions, and at 10 MPa, using fluid N_2 as the pressure medium, at 37 °C for 20 min. Three replicate experiments were conducted to examine the reproducibility, and the results were expressed as averages with their standard deviations.

2.3. Recovery of eggs and enumeration

The level of inactivation of the *A. suum* eggs by SC treatment was expressed as $\log(N/N_o)$, where N is the remaining number of embryonated eggs after treatment and N_o is the initial number of embryonated eggs, after incubation for 21 days at 28 °C. To measure the embryonated eggs, they were recovered, incubated, and enumerated. In the case of the non-immersed experiment, the wood strip taken from the reactor was vertically placed in the 50 mL Falcon tube containing 10 mL of distilled water at the bottom. In this study, the distilled water was used as the incubation solution, because no significant difference was found in the embryonation rate or speed when employed a 0.1 N sulfuric acid solution [22]. Furthermore, this is supported by our previous work that indicated a high embryonation of *A. lumbricoides* eggs (97–98%), which are collected from infected human feces and incubated in sterilized water [9]. The Falcon tube was employed as a humid chamber for incubation.

On the other hand, in the immersed experiment, the 3 mL egg-containing sample taken from the reactor was concentrated by centrifuging at 800 × *g* for 5 min, and, then, approximately 50 μL of the sample with the eggs pellet was loaded on a new wood strip. The strip was placed vertically in the 50 mL Falcon tube as described for the non-immersed experiment.

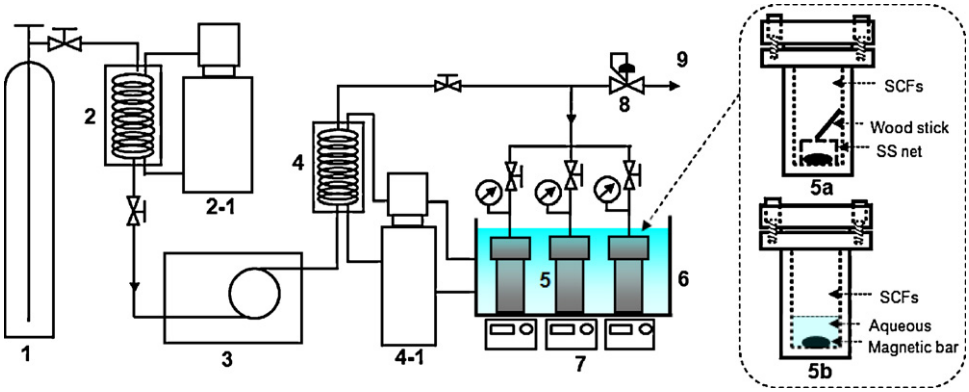

Fig. 1. Schematic of the supercritical treatment apparatus for the inactivation of *A. suum* eggs: (1) CO_2 (or N_2O) cylinder, (2) pre-cooler, (2–1) cooling circulator, (3) high-pressure pump, (4) pre-heater, (4–1) heating circulator, (5) multibatch reactors, (5a) non-immersed condition, (5b) immersed condition, (6) thermostated bath, (7) digital magnetic stirrers, (8) back pressure regulator, and (9) vent.