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Research paper

Poly D,L-lactide-co-glycolide (PLGA) nanoparticle-encapsulated honeybee (*Apis melifera*) venom promotes clearance of *Salmonella enterica* serovar Typhimurium infection in experimentally challenged pigs through the up-regulation of T helper type 1 specific immune responses

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ABSTRACT

Honeybee (*Apis melifera*) venom (HBV), which includes melittin and lipid-soluble ingredients (chrysin and pinocembrin), elicited increases in the $CD4^+/CD8^+$ T lymphocyte ratio, relative mRNA expression levels of the T helper type 1 (Th 1) cytokines (interferon-γ and IL-12) and reinforced viral clearance of an experimental porcine reproductive and respiratory syndrome (PRRS) virus infection in our previous study. On the basis of that previous study, we have now developed poly-D,L-lactide-co-glycolide (PLGA)-encapsulated HBV nanoparticles (P-HBV) for longer sustained release of HBV. We administered P-HBV to pigs *via* the rectal route, and then evaluated the potential immune-enhancing and bacterial clearance effects of P-HBV against *Salmonella enterica* serovar Typhimurium. The $CD4^+/CD8^+$ lymphocyte ratio, proliferative capacity of peripheral blood lymphocytes and relative mRNA expression levels of IFN-γ and IL-12 (produced mainly by Th1 lymphocytes) were significantly increased in the P-HBV group up to 2 weeks post-administration of P-HBV. After *S.* Typhimurium infection, the P-HBV group showed a marked reduction in microbial burden in feces and all tissue samples (including the ileum, cecum, colon, and mesenteric lymph node (MLN)), a significant increase in Th 1 cytokines (IFN-γ, IL-2, and IL-12) and a marked decrease in a Th 2 cytokine (IL-4) in

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all tissue samples and peripheral blood lymphocytes. Thus, P-HBV may be a promising strategy for immune enhancement and prevention of *S.* Typhimurium or other bacterial infections.

1. Introduction

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is the most commonly isolated *Salmonella* serotype in pigs, and a major source of zoonotic strains of *S. enterica* responsible for human food poisoning mostly associated with the consumption of contaminated pork (Brumme et al., 2007). Typical clinical signs of *S.* Typhimurium infection in pigs are increased body temperature lasting for 2 days post infection, accompanied by diarrhea and enterocolitis (Volf et al., 2012). Pigs of all ages are susceptible to *S.* Typhimurium infection; however, weaned and growing-finishing piglets are most commonly affected and show severe clinical signs (Boyen et al., 2008). After they recover from illness, the pigs are often stunted and grow slowly, causing considerable economic losses to pig farmers (Collado-Romero et al., 2010). In addition, infected but apparently healthy pigs remain asymptomatic carriers, in which bacteria may persist without triggering any clinical signs. Such animals cannot be detected easily, and thus serve as a source of contamination (Boyen et al., 2008; Callaway et al., 2008). Therefore, *Salmonella* infection in pigs is of concern as it has an important impact on the swine industry, negatively affecting the efficiency and leading to economic losses in porcine production systems (Fosse et al., 2009).

Honeybee (*Apis melifera*) venom (HBV) has long been widely used as a traditional alternative remedy for the alleviation of pain, inflammation and some immune-related diseases such as rheumatoid arthritis and multiple sclerosis (Oršolić, 2012). Numerous studies have also reported that a component of whole HBV has several beneficial therapeutic properties, such as immune-stimulating (Son et al., 2007), anticancer (Oršolić, 2012) and radioprotective (Gajski et al., 2009) activities. HBV contains at least 18 active components, including enzymes, biogenic amines, and several biologically active peptides, including melittin (Oršolić, 2012). Melittin, the principal component extracted from the water-soluble fraction of HBV, is a well-recognized antibacterial peptide which acts rapidly and has a broad spectrum of activity against infectious agents including bacteria, fungi, viruses and parasites (Mataraci and Dosler, 2012; Liu et al., 2013). Melittin also has numerous other pharmacological effects, such as immune-stimulating and anti-cancer properties (Son et al., 2007). Hence, many commercial products containing HBV ingredients are manufactured with an emphasis on the melittin content, either as the sole index compound, or as one of a mixture of water-soluble components. The lipid-soluble fraction of HBV has received less attention from HBV manufacturers. Interestingly, some lipid-soluble components of HBV such as chrysin and pinocembrin, classified as flavonoids, have been reported to have anticancer, antioxidant and antimicrobial effects (Schnitzler et al., 2010; Rasul et al., 2013). We have previously demonstrated that our new HBV-derived product, which combines melittin, chrysin and pinocembrin enhances the $CD4^+/CD8^+$ T lymphocyte ratio, increases the relative levels of mRNA of the Th1 cytokines interferon gamma (IFN-γ) and interleukin (IL)-12, and reinforces viral clearance in pigs with experimental porcine reproductive and respiratory syndrome (PRRS) virus infection. However, these immune-enhancing effects of HBV only persisted for 7 days after HBV administration in that study. Hence, we have now developed poly-D,L-lactide-co-glycolide (PLGA)-encapsulated HBV nanoparticles (P-HBV), which are designed to achieve longer sustained release of HBV. PLGA is biocompatible, biodegradable, and is an FDA-approved agent that has been used for drug, protein, and gene delivery applications because of its sustained-release properties, which are due to the protection of entrapped materials from protease-mediated degradation (Dwivedi et al., 2013). PLGA containing hepatitis B, influenza, or PRRS viruses have been reported to effectively induce protective immune responses against several viral diseases (Thomas et al., 2011; Dwivedi et al., 2013). In addition, a study by Dube et al. (2013) demonstrated that PLGA-coated rifampicin could deliver four times rifampicin 4 times the amount of more into alveolar-like macrophages than rifampicin solution. Therefore, innovative nanotechnology using PLGA-based delivery systems could be an attractive approach for a variety of bioactive molecules, such as antibodies (Bicho et al., 2010), aptamers (Farokhzad et al., 2006), peptides (Geldenhuys et al., 2011), and prophylactic agent (Yang et al., 2013). Nanoparticle-based delivery to mucosal sites is advantageous, because nanoparticles are easily recognized and passively phagocytized by professional antigen presenting cells (APCs) (Inaba et al., 1993). Mucosal regions (ocular, nasal, oral, pulmonary, vaginal and rectal) collectively contain 80% of the body immune cells (Holmgren and Czerkinsky, 2005). Particulate antigens delivered *via* mucosal routes are recognized by microfold cells (M cells) and are then presented to APCs which strongly stimulate differentiation of T cells and effectively induce specific adaptive immune responses (Renukaradhya et al., 2012).

The aims of the current study were to (1) investigate the immune-enhancing efficacy of PLGA-encapsulated HBV delivered to pigs *via* the rectal route, and its duration period compared to non-encapsulated HBV, and (2) evaluate the bacterial clearance effect of PLGA-encapsulated HBV delivered *via* the rectal route in pigs experimentally challenged with *S.* Typhimurium, as an initial step toward using PLGA-encapsulated HBV for protection against bacterial diseases and elucidation of host cellular immune responses.