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A hypothetical oxidative stress regulatory role of alpha giardins in the protozoan parasite *Giardia intestinalis*

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Abstract. Giardiasis is an intestinal infection caused by the protozoan parasite *Giardia intestinalis*, affecting hundreds of millions of people worldwide. This microaerophilic protozoan is capable of surviving in the host intestine in the presence of both oxygen and reactive oxygen species (ROS), despite the lack of conventional ROS-scavenging enzymes. The understanding of how *G. intestinalis* tolerates free radicals could help to identify essential biological processes that protect themselves against oxidative stress within the human gut. This review outlines the antioxidant mechanisms that are utilized by *G. intestinalis*, with an emphasis on the potential novel role of alpha giardins. The comprehensive understanding of the processes involved in oxidative stress management may provide new insights into improved treatments for giardiasis, and other medically important protozoan parasitic diseases.

INTRODUCTION

Giardiasis, a gastrointestinal disease caused by the protozoan parasite *Giardia intestinalis* (synonymous *G. duodenalis* and *G. lamblia*), has a global distribution with over 250 million cases per year (Ankarklev *et al.*, 2010). This places a significant concern on worldwide public health systems. Due to the high prevalence and heavy disease burden of the infection, World Health Organization (WHO) has added *Giardia* into the 'Neglected Disease Initiative' in 2004 as a comprehensive approach to combat giardiasis (Savioli *et al.*, 2006).

Giardiasis presents with a broad range of clinical manifestations in humans, ranging from asymptomatic carriage to self-limited acute giardiasis, and may occasionally progress to the chronic stage of infection. The typical clinical symptoms of giardiasis include abdominal pain, nausea, diarrhoea, fever, flatulence, bloating, malaise, weight loss and vomiting (Robertson, 2014). The severity of giardiasis and variability among patients may be due to co-infections, immunological development and nutritional status of the host as well as the virulence of the parasite strain (Carmena, 2010; Robertson, 2014).

Despite the fact that giardiasis is a commonly identified parasitic disease, the incidence of the infection in humans remains greatly underreported (Feng & Xiao, 2011). The prevalence of *Giardia* infection in human is approximately 2-5% in industrialized countries, and 20-30% in developing countries (Feng & Xiao, 2011). In Malaysia, human giardiasis is more prevalent in rural communities than urban communities with no regard of ethnicity due to the low socio-economic status and improper sanitation (Mohammed Mahdy *et al.*, 2007).

To date, there are six species of *Giardia* that have been recognized (Table 1). *G. intestinalis* that can infect a large variety of mammalian hosts, including humans, has been intensively studied. The mammalian isolates of *G. intestinalis* have been genotyped based on *Giardia* "housekeeping" genes (Monis *et al.*, 1999), namely the small subunit of ribosomal RNA (ssu rRNA), glutamate dehydrogenase (*gdh*), triose phosphate isomerase (*tpi*) and elongation factor 1-alpha (*ef-1*), and also other gene sequences such as b-giardin (*bg*) (Volotão *et al.*, 2007), GLORF-C4 (*C4*) (Yong *et al.*, 2002) and the inter-genomic rRNA spacer region (IGS) (Lee *et al.*, 2006). The advent of gene sequencing has further classified *G. intestinalis* into eight different assemblages (designated A-H) (Heyworth, 2016). Two genetic assemblages, A and B, cause infection in both human and animal hosts, suggesting the zoonotic potential of *Giardia* (Ryan & Cacciò, 2013).

Reactive oxygen species (ROS)

Reactive oxygen species (ROS) are mainly comprised of radical compounds such as superoxide ($O_2^{-\bullet}$), hydroxyl radicals ($^{\bullet}OH$), lipid hydroperoxyls, and reactive non-radical compounds including singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), hypochlorous acid (HClO), chloramines (NH_2Cl), and ozone (O_3) (Bedard & Krause, 2007). ROS can cause permanent damage to different cellular components through the oxidation of proteins, carbohydrates, lipids and nucleic acids, followed by the modification of membranes, receptors, cytoskeleton proteins, enzyme inactivation and genome damages (Chiumiento & Bruschi, 2009).

There are various sources that contribute to the production of ROS in the body (Bhattacharyya *et al.*, 2014). ROS are by-products of aerobic cellular metabolism, arachidonic acid metabolism, and auto-oxidation of biological molecules as well as of biotransformation processes of exogenous compounds including toxins, drugs, and antibiotics. Specific environmental conditions can also result in elevated levels of cellular ROS such as the exposure to ionising radiation, high concentration of iron salts, high oxygen pressure, ischemia-induced pathologies or epileptic states. In addition, the formation of intracellular ROS can also be induced by macrophages, neutrophil, eosinophil and basophil granulocytes in response to infection by microbial pathogens (Fang, 2004).

The gastrointestinal tract is a key source of ROS. Despite the protective barrier provided by the epithelial layer, ingested materials and pathogens can cause inflammation by triggering the activation

Table 1. Host specificity of different *Giardia* species

Species	Sub-species	Host(s)	Reference(s)
G. intestinalis	Assemblage A	Humans, mammalian	(Monis *et al.*, 1996)
	Assemblage B	Humans, mammalian	(Monis *et al.*, 1996)
	Assemblage C	Canines (dogs)	(Monis *et al.*, 1998)
	Assemblage D	Canines (dogs)	(Monis *et al.*, 1998)
	Assemblage E	Hoofed livestock	(Ey *et al.*, 1997)
	Assemblage F	Felines (cats)	(Vasilopulos *et al.*, 2007)
	Assemblage G	Rats	(Monis *et al.*, 1999)
	Assemblage H	Phinnipeds	(Lasek-Nesselquist *et al.*, 2010)
G. agilis		Amphibians	(Feely & Erlandsen, 1985)
G. ardeae		Great blue herons	(Erlandsen *et al.*, 1990)
G. microti		Voles, muskrats	(van Keulen *et al.*, 1998)
G. muris		Rodents	(Roberts-Thomson *et al.*, 1976)
G. psittaci		Budgerigars	(Erlandsen & Bemrick, 1987)

of polymorphonuclear neutrophils and macrophages to produce inflammatory cytokines and other mediators that contribute further to oxidative stress (Chiumiento & Bruschi, 2009). However, moderate amounts of ROS have beneficial effects on several physiological processes such as the killing of invading pathogens, wound healing, essential signalling molecules in ROS intracellular homeostasis and tissue repair processes (Bhattacharyya *et al.*, 2014). In order to restrict the damage caused by ROS, mammalian cells have developed a series of enzymatic and non-enzymatic antioxidant systems at different defence stages (Chiumiento & Bruschi, 2009).

During the first line of defence, enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), have a fundamental role in limiting damage caused by ROS at the sites of phospholipid membranes and biological macromolecules. However, these enzymes could not provide a complete protective effect since various products generated by their catalytic activity are extremely reactive (Chiumiento & Bruschi, 2009). To resolve this, other redox systems such as non-enzymatic antioxidants (glutathione (GSH), thioredoxin (Trx), and melatonin), and exogenous antioxidants (Vitamin C, Vitamin E, carotenoids, zinc, copper, manganese, iron, and selenium, and flavonoids) are responsible to capture electrons during the propagation reaction of the radicals (Chiumiento & Bruschi, 2009; Bhattacharyya *et al.*, 2014).

Antioxidant mechanisms in *Giardia*

Giardia and other protozoan parasites, such as *Entamoeba histolytica*, *Trypanosoma*, *Plasmodium*, and *Leishmania* are micro-aerophilic, and are able to thrive in environments with low oxygen tension (Mehlotra, 1996). In particular, *G. intestinalis* is only able to tolerate 0-50 µM dissolved oxygen (Lloyd *et al.*, 2000) and is highly vulnerable to both oxygen and ROS due to the lack of conventional ROS-scavenging enzymes (CAT, SOD, GPx) and the presence of ROS-generating enzymes, such as NAD(P)H:menadioneoxidoreductase, and oxygen-labile key metabolic enzymes (pyruvate-ferredoxin oxidoreductase) (Li & Wang, 2006; Mastronicola *et al.*, 2011). Despite highly sensitive to oxidative stress, it is interesting to observe the adaptability of *Giardia* trophozoites in the fairly aerobic mucosa of the human small intestine, which has been measured at 60 µM (Atkinson, 1980). This suggests that the parasite has to adopt mechanisms to protect itself against oxidative stress as well as strategies to detoxify both oxygen and free radical products produced in the human gut to establish its pathogenesis.

Defence systems against O_2, nitric oxide (NO) and $O_2^{-\bullet}$ have been identified recently in *Giardia* (Figure 1): (i) a flavodiiron protein (FDP) which acts as NADH oxidase and could convert O_2 to H_2O (Di Matteo *et al.*, 2008; Mastronicola *et al.*, 2011); (ii) an inducible flavohemoglobin (FlavoHb) which is able to metabolize NO to nitrate (NO_3^-) aerobically (Mastronicola *et al.*, 2010; Rafferty *et al.*, 2010); (iii) a superoxide reductase (SOR) which is able to reduce $O_2^{-\bullet}$ to H_2O_2 (Testa *et al.*, 2011); and, more recently, (iv) peroxiredoxins (Prxs) which are able to reduce H_2O_2 to H_2O, and peroxynitrite ($ONOO^-$) to NO_3^- (Mastronicola *et al.*, 2014).

The role of the Trx redox system against oxidative stress in *G. intestinalis* is not completely elucidated (Leitsch *et al.*, 2016). Although *G. intestinalis* thioredoxin reductase (TrxR) demonstrated significant disulphide reduction and NADPH oxidase activities (Tejman-Yarden *et al.*, 2013), a functional Trx has not been unequivocally identified yet. Furthermore, several enzymes known to be dependent on Trx-mediated redox regulations, as in the case of ribo-nucleotide reductase, are also not found in the parasite (Leitsch *et al.*, 2016).

Potential antioxidant properties of alpha giardins

The ubiquitous family of annexin proteins is widespread throughout the Eukaryota. The discovery of parasite annexins makes the proteins potentially attractive targets for anti-parasitic therapeutic development due to the significant difference from those of the

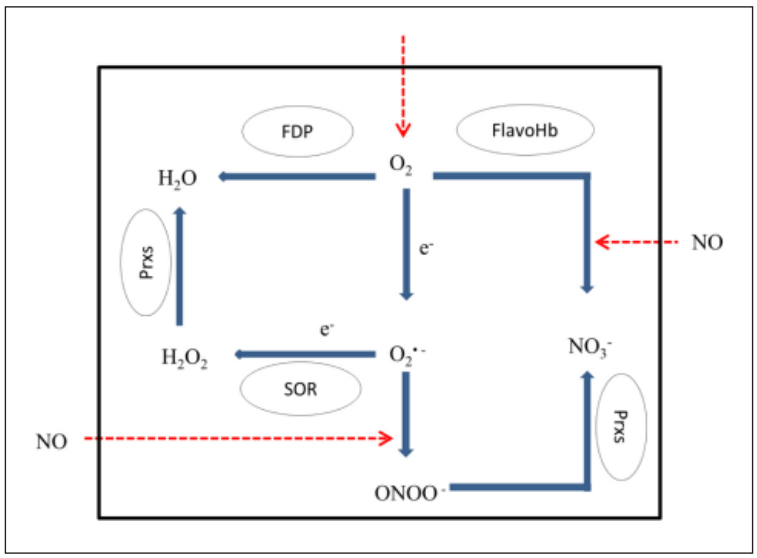

Figure 1. Schematic representation of the antioxidant system in *G. intestinalis*. FDP, flavodiiron protein; FlavoHb, flavohemoglobin; SOR, superoxide reductase; Prxs, peroxiredoxins (Adapted from Mastronicola *et al.*, 2016).

host. The distinct feature of annexin proteins is their calcium-dependent binding ability to phospholipid membranes (Weeratunga *et al.*, 2012). These soluble proteins play essential roles in the regulation of a wide range of molecular and cellular processes, particularly in the regulation of oxidative stress (Monastyrskaya *et al.*, 2009).

The first annexin was identified from the plant model *Arabidopsis thaliana* (AnnAt1), where this protein was shown to play a physiological role in the response to oxidative stress (Gidrol *et al.*, 1996). This finding was then supported by another study which demonstrated that the mammalian cell lines transfected with AnnAt1 were protected from H_2O_2-induced cell death (Gorecka *et al.*, 2005). A recent study also showed that annexin A2 (AnxA2)-depleted cells had elevated ROS levels during oxidative stress, which can cause severe liver and lung oxidative damages in knockout mice deficient in AnxA2 (Madureira *et al.*, 2011). Annexin-homologous proteins, alpha giardins, as parts of *Giardia* cytoskeletal system have been widely studied for its potential roles in the pathogenesis associated with oxidative stress management (Weiland *et al.*, 2005). This is supported by a recent finding that showed both mRNA and protein expression of alpha-2 giardin were significantly up-regulated in *G. intestinalis* under albendazole-induced oxidative stress (Paz-Maldonado *et al.*, 2013). Although there is no published evidence that have demonstrated the involvement of parasite annexins in the regulation of redox activities, all these findings serve as a platform which reveals the unique role of annexin-like molecules, alpha giardins as a potential antioxidant.

Giardins were first described as unique components of the cytoskeleton of *Giardia* (Crossley & Holberton, 1983). The cytoskeleton is responsible for the critical aspects of survival such as in the mediation of mucosal attachment, cell motility and providing resistance against bile salts (Elmendorf *et al.*, 2003). There are four major classes of giardins that have been identified to date: alpha (α) giardins as annexin homologues (Fiedler & Simons, 1995; Morgan & Fernandez, 1997), beta (β) giardins and delta (δ) giardins as striated fibre (SF)-assemblin homologues (Weber *et al.*, 1993; Jenkins *et al.*, 2009), and gamma (γ)

giardins as novel proteins (Nohria *et al*., 1992). Among these, most studies have focused on the alpha giardins proteins which are assigned to the family E annexins. Multiple sequence alignment showed no similarity of redox-thiol conservation domain found in alpha giardins and other parasite annexins. However, alpha-1 giardin and annexins showed similarity in overall three-dimensional molecular folding (Weeratunga *et al*., 2012), suggesting that they may share similar functions which warrants further investigation.

There are 21 different alpha giardins that have been identified in the *Giardia* genome (http://giardiadb.org), with relative molecular masses of 29-38 kDa (Peattie *et al*., 1989; Hofmann *et al*., 2010). Giardins are often associated with the membrane systems and involvement in the cytoskeletal movement, cellular signal transduction, growth regulation, proliferation, and the formation of atypical Ca^{2+} channels (Weiland *et al*., 2005). Annexin-like alpha giardins are key proteins which display important roles in the encystation and excystation process of *Giardia* by inhibiting the breakdown of the cell membrane and disintegration of the cellular structure under extremely harsh environmental conditions (Peattie *et al*., 1989; Weiland *et al*., 2005).

Recent studies on the cellular localization of different alpha giardins have been described. Previous findings showed that only a few alpha giardins (alpha-3, -5, and -17) localized to the adhesive disc, while the other giardins (alpha-1, -2, -6, -7.2, -7.3, -9, -10, and -14) were mainly distributed along the flagella, adhesive disc, cytoplasm, or plasma membrane (Weiland *et al.*, 2005). Alpha-15 and -16 giardins showed a patchy distribution in the plasma membrane (Weiland *et al*., 2005). The same pattern of localization of alpha-1 giardin was observed in Assemblages A, B and E of *G. intestinalis* (Feliziani *et al*., 2011). Alpha-8 giardin was found to be located in the plasma membrane and flagella (Wei *et al*., 2010), whereas alpha-4 giardin was localized mainly along the flagella (Weiland *et al*., 2005). The immunofluorescence assays showed that the main localization of alpha-11 giardin was within the plasma membrane and the basal body of the anterior flagella of *G. intestinalis* trophozoite (Kim *et al*., 2013). In addition, alpha-18 and -12 giardins were found primarily in the flagella and cytoplasm, respectively (Wu *et al*., 2016). The immunofluorescence assays in a recently published study revealed the localization of alpha-13 giardin in the cytoplasm of *Giardia* trophozoite (Yu *et al*., 2017).

Among all the 21 members of the annexin-like alpha giardin gene familly, alpha-1 giardin has been shown recently to be a potential vaccine target that provides protection against *G. intestinalis* in murine model (Jenikova *et al*., 2011). This highly immunogenic protein contains an epitope between amino acids 160 and 200 that can stimulate the production of anti-*Giardia* antibodies (IgA and IgG2a) (Jenikova *et al*., 2011; Emery *et al*., 2015). More than 97% amino acid sequence conservation was reported from the analysis of alpha-1 giardin from divergent assemblage A and B isolates of *G. intestinalis*, further suggesting the potential utility of alpha-1 giardin as a promising tool for immunodiagnosis of human giardiasis (Jenikova *et al*., 2011).

CONCLUSION

The spread of human giardiasis is an increasing global health issue despite the advancement in giardiasis vaccine research as efficient vaccine is still not available currently. This situation might go beyond control and thwarts the efforts to combat giardiasis due to the emergence of drug-resistant *Giardia* and recurrence of the infection. The understanding of how *G. intestinalis* survives in the unavoidable ROS-rich environment will serve as a fundamental platform to unveil novel potential drug targets to control the disease. Given that annexins of other organisms have been shown to protect cells from oxidative stress, additional information on the less studied molecule, alpha giardin, will be necessary to fully comprehend the importance of this

protein in the regulation of oxidative stress in *G. intestinalis*. An effective and promising drug against this unique alpha giardin might be selective for the cytoskeleton of parasite without affecting host cells due to its high distinctiveness from host genome.

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