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Review

Elucidating the Role of Extracellular Vesicles Released by *Toxoplasma gondii*: A Review

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ABSTRACT

Toxoplasma gondii is one of the most successful pathogens worldwide. Its high secretory capability is related to its dynamics for motility, invasion and tissue dissemination within the infected host. *Toxoplasma* is a highly secretory parasite of proteins released from secretory organelles such as micronemes, rhoptries and dense granules, but it also secretes/excretes a huge variety of extracellular vesicles with unknown role. Herein, it is discussed some characteristics of the extracellular vesicles released by *Toxoplasma*, such as their different sizes, different identification names, and their possible biological functions

Keywords: *Toxoplasma gondii*; Micro Vesicles; Exosomes; Ectosomes.

OVERVIEW

Toxoplasmosis is an infection caused by the intracellular parasite *Toxoplasma gondii (T. gondii).* This cosmopolitan parasite can invade almost all the cell types in warm-blooded organisms, including humans. To date, the infection with *Toxoplasma* has been considered as a neglected disease [1]. In developing countries, at least two-thirds of the human population is considered seropositive to this pathogen; while in developed countries, such as the United States, less than one-third of the population is seropositive to *Toxoplasma* [2,3].

T. gondii is an opportunistic pathogen in immunocompromised patients (cancer patients, HIV/AIDS, and transplant recipients), in whom produces encephalitis and death [4-7]. In pregnant women, the infection may be highly severe resulting in congenital malformations and abortion [8,9]. Toxoplasmosis also affects immunocompetent patients, although the infection is slight or with minor symptoms similar to a flu; in addition, it has been related to schizophrenia and suicide attempts [10].

The most common route of infection of *Toxoplasma* is by the ingestion of under cooked meat contaminated with tissue cysts (which are the infective structures containing bradyzoites) [11], or by the ingestion of poorly washed vegetables or water contaminated with infective oocysts (structures containing sporozoites that are excreted in feces of infected cat) [12]. After the ingestion of any of those structures, the parasites are released directly on the gut lumen and from there they can infect the epithelium of small intestine [13]. Once inside in a parasitophorous vacuole (PV) within the host cell cytoplasm, bradyzoites and sporozoites differentiate into the most replicative, virulent, and disseminative stage of the parasite, the tachyzoite [11]. The tachyzoite is responsible of the acute toxoplasmosis and of a fast tissue dissemination throughout the host, reaching immune-privileged organs, such as brain, eye and in pregnant women, the placenta [14]. The success of this parasite is based on its high secretory property of proteins, a process that continues through the entire life cycle including invasion, replication inside the PV, egress from the host cell, tissue dissemination throughout the body, evasion of the host immune response and differentiation to form tissue cysts [15-18].

During host cell adhesion, invasion, and replication of *Toxoplasma*, occur a sequential secretion process of proteins from micronemes (MIC proteins), rhoptries (ROP proteins), and dense granules (GRA proteins) [17]. However, in the last years, the secretion of molecules anchored to the membrane of released vesicles or located in the vesicle lumen as soluble proteins, has acquired a great importance in the understanding of the biology of several pathogens including *Toxoplasma* [19, 20].

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In a recently published paper by our group, it was described the excretion/secretion of extracellular vesicles (EVs) by freshly mouse-isolated extracellular tachyzoites of the highly virulent RH strain of *T. gondii*. The EVs were morphologically characterized by transmission electron microscopy (TEM) and classified as exosomes and ectosomes [21]. The analyses of both types of nanovesicles by mass spectrometry, identified 210 proteins from ectosomes and 285 proteins from exosomes [21]. Although the function of these EVs continues to be unknown, it was demonstrated that such mixture of exosomes and ectosomes can auto-fuse to form vesicle-tubular structures, which resemble the nanotubular network organized inside the PV [21, 22]. Apparently the nanovesicles were released from posterior and anterior ends of the parasite [21, 23].

In the present review, we focus our attention on the most relevant topics in the field of the exosomes and ectosomes in the parasite *Toxoplasma gondii* in order to understand the possible role of the EVs in different processes in the biology of the parasite.

EXTRACELLULAR VESICLES

In mammalian cells, EVs have been proposed as powerful tools that cells use to communicate themselves [24]. One of the most studied EVs are exosomes, which are apparently related to broad range of diseases including neurodegenerative conditions and cancer [25,26]; however, other EVs are also being studied nowadays. The EVs are found in almost all biological fluids and cell types, and in diverse organisms including parasites [19-21,23,27-31]. Composition of EVs includes proteins, lipids, a huge variety of RNA such as messenger RNA (mRNA), microRNA (miRNA), circular RNAs (circRNA), and others, which are enveloped by a lipid bilayer that protects the cargo from enzymatic digestion [24]. Although in mammals these kinds of vesicles are related with a variety of processes such as cell-cell communication, cell differentiation, tissue homeostasis, organ remodeling, and development [32-34], in parasite field, they have been poorly studied.

Toxoplasma has the ability to secrete a large number of proteins including soluble proteins as well as proteins anchored to the membrane of EVs and soluble proteins located in the lumen of the EVs. According to the most recent reports published in the field, the tachyzoites of *Toxoplasma* can secrete at least two types of EVs: exosomes and ectosomes [21]. Exosomes are nanovesicles of about 50-100 nm in diameter, while ectosomes can reach more than 100 nm [21]. Of note, the mixture of exosomes and ectosomes are known as EVs.

EXOSOMES

Generalities

In most cell types, exosomes are defined as membrane nanovesicles secreted through the fusion of multivesicular bodies (MVB) with the plasma membrane. The invagination of the Golgi membrane forms early endosomes which can contain a wide variety of cargo molecules. Such process generates the so-called, intraluminal vesicles (ILV). Maturation of early endosomes is characterized by the presence of abundant acidified ILV´s, which progress to form the MVB. The fusion of MVB with the plasma membrane triggers the release of the vesicles, now named exosomes [35,36]. In the '80s, it was thought that exosomes were cellular waste [37]. However, in the last decades, it has been shown that exosomes are structures formed through active processes and they are related to cell communication during health and disease conditions in mammalian cells; particularly exosomes have been studied in cancer [35,38]. They usually contain a high amount of proteins, lipids, and nucleic acids. According to the latest update in the exosomes database ExoCarta (www.exocarta.org), the statistics include 9769 proteins, 3408 mRNAs, 2838 mRNAs, and 1116 lipids.

Exosomes have been studied in the field of *Toxoplasma* by few laboratories. However, it is important to distinguish between the studies realized by the isolation of exosomes from *Toxoplasma* directly [21,23,39-42], and from those studies where exosomes were isolated from *Toxoplasma-* infected cells (exosomes from host cells) [43,44]. Herein, we will only discuss the role of exosomes isolated directly from *Toxoplasma gondii* since EVs released by infected cells would also involve components from the host cell not necessarily related with the presence of the pathogen.

EXOSOMES AND *T. gondii*

To our knowledge, Wowk and collaborators in 2017 published the first report in where exosomes of about 200 nm isolated from *T. gondii* were analyzed using mass spectrometry, given the first proteomic profile [41]. In this work, it was compared the proteomic profiling of exosomes isolated from *T. gondii* with exosomes isolated from infected human foreskin fibroblasts (HFF cells). As a brief report, they showed the classical content of proteins previously reported for exosomes in mammalian cells, such as calcium-binding proteins, HSP proteins, annexin family members, between others [41].

Recently, our group published a quantitative proteomic characterization of exosomes in secretion/excretion fraction from tachyzoites that were isolated from infected mice and then purified [21]. Reporting for the very first time the quantification of proteins in these EVs, Their possible secretion source and a structural relationship [21]. In addition, the existence of another population of EVs, the ectosomes, was also demonstrated after enrichment by differential centrifugation and characterization by TEM, scanning electron microscopy (SEM), and mass spectrometry [21,23,39,42].

MORPHOLOGY OF THE EXOSOMES SECRETED BY *TOXOPLASMA*

The use of TEM and SEM has contributed in the characterization of exosomes morphology. There are two approaches mostly used to perform the morphological characterization of the exosomes (or EVs):

By an enrichment of the exosomes isolated from culture supernatants following several steps of high-speed centrifugation and then observation under the electron microscope, or

By direct observation of the parasites under the process of secretion by TEM or SEM [21,23]. Also, it has been very useful the nanoparticle tracking analysis (NAT) and posterior confirmation by TEM [39,41,45].

The NAT analysis combines the laser light scattering microscope and the Brownian motion to obtain a size distribution of the sample in suspension [46]. The NAT analysis in *T. gondii* could provide not only the size of the EVs but also the concentration of the particles. In most reports of exosomes secreted by *Toxoplasma*, the morphology analysis by TEM shows vesicle sizes lower that 100 nm [21,23,40-42]. However, recently an extensively morphological characterization of the vesicles by SEM and by TEM was reported by our group [21]. In this report, not only the thin sections of the parasites and the sample of exosomes were visualized directly by TEM, but also the parasites were analyzed during secretion conditions by SEM. This feature allowed to show how the parasites are releasing the vesicles to the media and to characterize the morphology of at least two populations of vesicles (exosomes and ectosomes) [21]. Besides, the negative staining was quite clear to observe the morphology of exosomes, it was possible to identify rounded shape vesicles as well as the presence in the lumen of electron-dense material [21]. A recent report in mast cell line (HMC-1) showed nine different morphological categories for vesicles [47], organized as:

Single vesicles

Double vesicles

Triple vesicles or more

Small double vesicles

Oval vesicles

Small tubules

Large tubules

Incomplete vesicles

Pleomorphic vesicles

In *Toxoplasma*, it was found singles vesicles, oval vesicles, small tubules, and large tubules. Interestingly, the most common morphology in exosomes was visible by TEM after negative staining showing single vesicles with a donut shape, specifically, rounded and with a depression similar to a concave side [21]. Interestingly, vesicle-tubular structures were detected after the EVs fraction was incubated at 37°C for different period of time. These structures were apparently the result of the fusion of individual exosomes [21]. Nevertheless, it is still unclear the precise function of every type of vesicle. Altogether, these finding indicate that the heterogenous morphology of exosomes described in mammalian cells, occurs also in pathogens such as *Toxoplasma*.

THE ROLE OF EXOSOMES IN THE BIOLOGY OF *Toxoplasma gondii*

The role of exosomes in *Toxoplasma* is still unknown, however, it has been reported their participation during the host immune response by the induction of IL-10, TNF-α, and iNOS in murine macrophages, apparently by the effect of miRNA [39].

In the same way, it was reported the stimulation of secretion of inflammatory cytokines in macrophages through the JNK pathway, generating a production of IL-12, IFN- γ, and TNF-α [42]. Besides the increased production of the above-mentioned cytokines, BALB/c mice inoculated with exosomes, showed humoral and cellular immune responses as well as a prolonged survival time [40].

The function of most proteins contained within exosomes or proteins anchored to the membrane of the vesicles is still unknown in the biology of *Toxoplasma*. Of note, in the last proteomic profile of exosomes obtained from mouse-isolated tachyzoites, there were detected 210 proteins [21], including cytoskeleton and Rab proteins which have been related with the process of exosome transport and fusion in mammalian cells [48]. Proteins GRA, ROP, and MIC were also found in exosomes, interestingly these kinds of proteins are exclusively released from secretory organelles during invasion or intracellular proliferation of the parasite. Therefore, their presence in the secretion/excretion fraction would imply that the parasites are also releasing them by following a secretion constitutive process [49]. As GRA, ROP, and MIC proteins are highly immunogenic, they could be considered as great candidates in the quest of vaccines [50].

THE ORIGIN OF EXOSOMES IN *TOXOPLASMA GONDII*

Although the source of the vesicles in *Toxoplasma* is still unknown, recent studies have proposed that they come from the dense granules and rhoptries [21,51]. It has been shown by TEM, the attachment of these vesicles on the plasma membrane of the host cell in the first steps of invasion [21,23,39]. But also, it was possible to detect the release of these vesicles from the apical and posterior end from extracellular parasites in absence of host cells [23]. By immunoelectron microscopy (IEM) using a polyclonal antibody against a vesicle enriched fraction it was demonstrated their localization in dense granules, suggesting this secretory organelle as the possible storage site. The proposal of dense granules as the reservoir of exosomes is supported in a previous report about the cytosolic transportation of dense granules in the parasite in a process dependent on actin and TgMyoF [52]. Also, it has been reported the role of the GTPase Rab11A in promoting the dense granules transport along the cytoskeleton, to reach the plasma membrane followed by membranes fusion and release of their content to the extracellular medium [53]. But also, by the fact that there are different population of dense granules in Toxoplasma with uncharacterized roles to date [54].

Other functions related to exosomes in *T. gondii*

It is worth to mentioning that, it was recently described a structural role of the extracellular vesicles secreted by *Toxoplasma gondii*. Vesicles that were secreted/excreted from extracellular tachyzoites and then were maintained under incubation a 37°C, aggregated themselves and then exhibited an auto-fusion event resulting in the formation of vesicle-tubular structures which sizes increased as time passed. The vesicle-tubular structures structures showed morphological similarities with the intravacuolar network generated by parasites located inside the PV [21,55,56].

Although further studies must be done to corroborate this proposal, would be of great interest the knowledge of the proteome of the IVN or the PV of *T. gondii* which to date only has been partially determined because the difficulty to isolate enriched fractions of such structures. Besides, some studies have demonstrated the translocation of GRA16 and GRA24 proteins across the membrane of the PV, although the precise mechanism is poorly understood, but could be related to the presence of some proteins named MYR2 and MTR3 [57]. Due to the high content of proteins in the tubules formed by the auto-fusion of EVs in *Toxoplasma*, it is possible that those structures could work as a bridge between the host and the parasite [21]. Another possibility is that these vesicle-tubular structures could contribute in the anchorage of the parasites between them and to the inner face of the PV during their replication process, as was previously suggested [56].

ECTOSOMES

Generalities

Although is common to be confused between ectosomes and exosomes properties, the last ones are quite different and are also known as micro vesicles [58]. The confusion relies on the similarity of morphology, or even protein content; however, those characteristics have been clarified in the last decade [59]. While exosomes are small vesicles (50-100 nm) that come from MVB, ectosomes have larger sizes (100-1000 nm) [59]. The ILV are not formed directly from the plasma membrane; in contrast, ectosomes are formed by budding of the plasma membrane with the carrying of some membrane proteins such as certain proteases [60,61]. The lipid composition of the exosomes in multicellular organisms is quite similar to the composition of the cell membrane, being the biggest difference that the phosphatidylserine and phosphatidylethanolamine are not exclusively sequestered in the inner layer of the membrane, but are also distributed along the membrane [62].

ECTOSOMES AND *Toxoplasma*

Information about morphological and the proteomic characterization of ectosomes is very limited and much less is known about their role [21, 23]. One group of the well-characterized proteins in EVs are proteases, which have been described as virulence factors in different pathogens including the metalloprotease GP63 secreted in EVs by *Leishmania* [19,20]. Presence of proteases has been reported in ectosomes secreted by *Toxoplasma* [21]. The knowledge of the participation of proteases in the pathogenesis of *Toxoplasma* has just been recently reported [23]. However, it has been discarded the participation of EVs during the modification of intercellular junctions in MDCK cells treated with excretion/secretion products of *Toxoplasma gondii*, where the participation of ectosomes and exosomes was discarded [23]. A comparative proteome of EVs obtained from cancer cells is available, reporting a higher amount of proteins in micro vesicles than in exosomes (910 vs. 785, respectively) [63].

MORPHOLOGY

Micro vesicles above 100 nm have been detected during the NAT assay in previous reports of EVs of *Toxoplasm gondii*. However, ectosomes were not reported in such studies [39,41]. About morphology, it is important to emphasize that most reports have stated that micro vesicles are a synonym of ectosomes; however, for some researchers in the field, exosomes could be defined as micro vesicles with a diameter around 100-350 nm and this has been also accepted [59]. In addition, other structures with sizes of 1000 nm or more, which correspond to apoptotic blebs, also known as apoptotic bodies, can also be detected [64]. In order to differentiate the apoptotic blebbing in the samples, several viability controls have been included during the isolation and secretion of the parasites when obtaining the EVs [21,23]. After that, it is possible to detect vesicles of no more than 200 nm of diameter, giving the population of ectosomes [21,23]. The morphology of ectosomes found in *Toxoplasma*, has rounded shapes with similarities to exosomes but bigger in size [21]. In mammalian cells, the micro vesicles have been described as oval or pleomorphic vesicles [47]. In *Toxoplasma,* these structures appeared as tubular structures, apparently formed by the fusion between the different types of EVs [21]. From the EVs in *Toxoplasma*, ectosomes are the less studied vesicles, their study in futures approaches could result in a better understanding of their composition and functional roles.

CONCLUSION

In this review, we have summarized the features of EVs released by *Toxoplasma gondii* and their possible roles in the biology of this parasite. It was discussed the recent studies and a comparison was done with data in mammalian cells in order to understand the role of EVs in *Toxoplasma*. This information could help in futures directions in the field of EVs not only in *Toxoplasma gondii* field, but also in other pathogens

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