Review Article

Sand fly saliva: toward a vaccine against leishmaniases

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ABSTRACT

Leishmaniases are a group of sand fly-borne diseases caused by protozoan parasites from species of *Leishmania* genus. These diseases are reported in about 100 countries with a prevalence of 12 million people infected and incidence of 2 million people per year, putting approximately 350 million people at risk of the infections. Leishmaniases are endemic and are considered as important public health problems in many provinces of Iran. The infection is transmitted through the bite of phlebotomine sand flies. The sand fly salivates while biting the vertebrate host. The saliva of phlebotomines consists of different molecules that are necessary for a sand fly to successfully take a blood meal. Additionally, previous exposures to sand fly saliva indirectly affect the establishment of *Leishmania* in the vertebrate host. Moreover, mice previously exposed to the saliva by injection or by uninfected sand fly bites have shown both humoral and cellular immune responses against the salivary antigens that protects them against *Leishmania* infection. Importantly, the immunization of mice with defined molecules from the saliva of the vector species has also conferred a strong protection against *Leishmania* infection. This suggests that such salivary components may be considered as candidates for a cocktail vaccine against leishmaniases. The current article briefly explains the potential of salivary components of sand fly vectors as immunological items to prevent leishmaniasis. So far, there is no efficient vaccine against these infections and efforts are required to be focused on developing effective and applicable vaccines against leishmaniases.

KEYWORDS: Sand fly saliva, vaccine, leishmaniasis.

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1. Epidemiology of leishmaniasis

Leishmaniases are a group of neglected tropical diseases caused by protozoan parasites of *Leishmania* genus. The inflicted disease is transmitted through the bite of sand flies. Leishmaniases are reported from about 100 countries with a prevalence of 12 million infected people and an incidence of 2 million people per year while approximately 350 million people are at risk of the infection [1, 2]. The estimate of disease burden is 2357000 DALY (disability adjusted life years) [3]. These complex diseases have different clinical forms, namely cutaneous leishmaniasis (CL), visceral leishmaniasis (VL), post kala-azar dermal leishmaniasis (PKDL) and mucocutaneous leishmaniasis (MCL). Approximately 20 species of *Leishmania* parasites are the causative agents of the infections, among them, VL caused by *Leishmania donovani* is the most serious form which is fatal if left untreated. CL is a public health problem; however, it is not fatal and is caused by a number of various *Leishmania* species. CL is endemic in approximately 82 countries with 1-1.5 million new cases per year. About 90% of global CL cases are reported from Afghanistan, Algeria, Iran, Iraq, Saudi Arabia, Syria, Brazil and Peru [4-7]. CL is categorized to four clinical forms; namely, localized, recidivans, diffuse and mucosal. In localized form, the parasite is limited to the skin. After the incubation period, lesions (0.5 to 3 cm in diameter) appear on some exposed body parts like the face, the legs and the arms. Self-healing is seen in most lesions after months or years while they may become everlasting scars [8]. In recidivans form, the lesions relapse at the edge of the previous scars and it occurs in approximately 5% of CL patients infected with *Leishmania tropica* who have deficiency in cell-mediated immune responses [4, 9]. Diffuse leishmaniasis leads to diffused lesions on the skin which occurs mainly in Africa and is transmitted by *Leishmania aethiopica* [10]. PKDL is a form of diffuse leishmaniasis that occurs after up to 20 years in affected individuals with incomplete treatments [11]. MCL causes disfiguring lesions and extensive damages in nasal, oral and pharyngeal cavities which occurs mainly in South America. In the Old World, it is caused by *L. tropica*, *Leishmania major* and *Leishmania infantum* [9].

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2. Sand fly vector biology

The vectors of leishmaniases are a member of *Phlebotominae* subfamily (*Diptera*, *Psychodidae*) [12, 13]. Approximately 800 species of phlebotomine sand flies have been described from

which about 10% have been considered as vectors of leishmaniases. Around 70 out of 800 species of phlebotomine sand fly are proven or suspected vectors of leishmaniases [14]. So far, 44 species of the sand flies (26 *Phlebotomus* species and 18 *Sergentomia* species) have been reported in Iran [15, 13]. Phlebotomine sand flies are small and hairy, with slender legs and with a body length of seldom more than 3 mm. While resting, they hold their wings in V shape above their abdomen [12]. For their blood meal, they usually hop around on the host before coming down to bite. Phlebotomine sand flies, unlike mosquitoes, have silent attack. Because of their hopping behavior, they are supposed not to disperse far from their breeding sites. The dispersal distance varies with the species and the habitat. The maximum dispersal distance is rarely more than 1 km, except for one species (*Phlebotomus ariasi*) which has a maximum dispersal distance of more than 2 km [16-21].

Phlebotomines are nocturnal or crepuscular insects, despite a few species which are diurnal. Diurnal resting sites are rather cool and humid. Their resting places include rodent borrows, birds and termites nests, stables, caves, house basements, toilets, cracks in walls, rocks or soils and forest vegetation as well as tree holes. In most species, the females are mainly exophagic and exophilic which bite outside and during the gonotrophic cycle. Since they rest outside, they cannot be efficiently controlled using inside residual spraying with insecticides [12, 16].

Both male and female sand flies have sugar feeding from natural sources such as juices of plants [22] and honeydews of aphids [23-26]. The females have also blood feeding because they need the blood to produce the nutrition required for their egg production. Some species have autogeny, meaning that they produce the first batch of eggs without the blood feeding [27]. The saliva of a sand fly has a composition which helps it to have a successful blood meal and it also helps the parasite to establish in its vertebrate host [28-31]. The saliva components are not constant in sand flies with different species, sex, age, generation and physiological stages [32-34]. Environmental factors and geographical locations seem to affect the saliva composition [35, 36].

3. Life cycle of Leishmania in sand fly and vertebrate host

The life cycle of *Leishmania* has two parts; one part in the sand fly vector and the second part in the vertebrate host. The development of the parasite inside the vector initiates when the female sand fly bites on its mammalian host and ingests the blood containing macrophages infected with the amastigote form of the parasites. Sand flies cut the skin with their mouthparts and create wounds into which skin macrophages or freed amastigotes are released and are then taken-up into the sand fly gut. The environmental conditions change when the parasites move from their mammalian host to the sand fly gut. These changes include a decrease in the temperature and an increase in the pH. These changes activate the morphological transformation of the parasite into the procyclic promastigotes which are weakly motile organisms with a short flagellum at the anterior end of the cell. In this phase, the amastigotes transform into procyclic promastigotes which are located at the posterior end of the midgut. The parasites maturation period takes 1-2 weeks, resulting in infective metacyclic promastigotes, located in the anterior of the gut [37]. During biting, the metacyclic promastigotes are delivered into the skin of a new mammalian host during the next blood meal, leading to the transmission of the disease. When the sand fly bites a new mammalian host, the infective metacyclic promastigotes released during the blood feeding are transmitted into the upper dermis of the skin. The promastigotes are then phagocytosed by the macrophages and differentiate into obligate intracellular amastigotes. The amastigotes will eventually proliferate within the macrophages and are finally released to the tissue to infect more cells. This life cycle is completed when the sand fly takes up the parasite during a blood meal at the infected skin [38].

4. Sand fly saliva and induction of immune responses and protection

A Sand fly salivates while biting the skin of a vertebrate host. The saliva of sand fly consists of different molecules which are necessary for the insect to take its blood meal successfully and to establish the parasite in the vertebrate hosts [39, 40]. The salivary glands have a unicellular epithelial layer surrounding a container for the saliva which consists of a repertoire of proteins that vary upon parameters such as the physiological state of the adult insect as well as its sex, age, generation, species and geographical location [41, 32]. Moreover, the saliva composition has been shown to change upon the environmental conditions of the sand fly habitats [36]. After emerging, the number of protein components gradually increase with the age of the insect, reaching to the full amount in 3 to 5-day-old sand flies. The amounts and components of the salivary proteins in the adult females is more than the males [32].

The sand fly saliva has immunomodulatory characteristics and induces specific immune responses including antibody production and cellular immune responses. All examined vector species in the Old and New World have shown to produce immune responses in their vertebrate hosts [42]. The saliva of sand fly is known to enhance *Leishmania* infection. Belkaid and colleagues [43] have demonstrated that the injection of a low number of *L. major* plus saliva of *Phlebotomus papatasi* could enhance the infection in the ear dermis of naive mice. Moreover, AMP and adenosine as immunomodulatory components of *P. papatasi* have been shown to induce IL-10 production, to suppress TNF-α and IL-12 in a mouse model and to decrease the expression of nitric oxide synthase gene in the activated macrophages in order to prevent the generation of nitric oxide [44-46]. In another study, AMP and adenosine treatment in an experimental murine model of arthritis, have affected the dendritic cells (DC) function to decrease Th-17 immune responses and to suppress the autoimmune responses as well [47]. Another study has shown that *P. papatasi* saliva could induce IL-4 response at injection site in mice [43]. Taking together, these works emphasize on the Th2 potential of the sand fly saliva and its exacerbating properties in leishmaniases. The work by Titus and Ribeiro [39] has shown that the infection with *L. major* is highly exacerbated by the presence of *Lutzomyia longipalpis* saliva. This saliva contains Maxadilan, a 6.5 KDa peptide which is an effective vasodilator and has the potential of inhibiting or modulating the inflammatory and immune responses in mice, suggesting the disease-exacerbating qualities of *Lu. longipalpis* saliva [48, 49]. Furthermore, upon addition of Maxadilan to mouse macrophages *in vitro*, cytokines associated with Th2 responses including IL-6, IL-10, TGF-β are upregulated; however Th1 cytokines such as IL-12p70 and TNF as well as nitric oxide are downregulated [50]. Maxadilan affects the cells which are important for controlling of *Leishmania* infection. DCs incubated with Maxadilan have been shown to exhibit lower expression of co-stimulatory molecules (i.e. CD80 and CD86)

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and chemokine (CCR7) while inducing secretion of type 2 cytokines [51].

In a recent study with mouse macrophages, *Lu. longipalpis* saliva has been shown to stimulate lipid body creation, leading to production of prostaglandin E2, a molecule which could have effects on the parasite dissemination [52]. Moreover in human DC, macrophages and monocytes, *Lu. longipalpis* saliva is reported to induce apoptosis of neutrophils, resulting in a higher parasite load while it could change the expression of co-stimulatory molecules and decrease the creation of TNF and IL-12p40 in LPS-stimulated monocytes [53, 54]. The saliva samples of *P. papatasi*, *Phlebotomus sergenti* or *Lu. longipalpis* have been used to treat murine macrophages and monocytes which have resulted in decreased multiplication of the mitogen-activated murine splenocytes and inhibition of the production of the Th1 cytokine IFN-γ [55].

In addition to *in vitro* incubation of the saliva to induce immune responses, sand fly bites have also been used to induce immunity in vertebrate hosts, in order to mimic the natural route of the transmission. Repeated exposure to sand fly bites can induce antibody production and cellular immune responses. In this regard, a recent study in Esfahan province (a hyper endemic area for CL in central Iran) has been published where *P. papatasi* and *Rhombomys opimus* (commonly known as great gerbil) are the main vector and reservoir hosts, respectively. In this area, the main leishmanial agent detected from *P. papatasi* was *L. major* [56]. This study has shown the presence of antibody response in *R. opimus* against the sand fly salivary antigens. *R. opimus* serum has been shown to strongly react with a salivary antigen of *P. papatasi* collected in the study area with a molecular mass of approximately 28 kDa [35] . This protein may be PpSP32, a protein that is highly recognized by humans bitten by *P. papatasi* [57]. More studies are recommended to confirm the immunogenicity of this protein in *R. opimus* to assess its potential as a marker of exposure to *P. papatasi*. Another study which contributes to our knowledge of the differential expression of the salivary genes among different groups within a *P. papatasi* population has been conducted under natural field conditions in Iran. This study has reported the expression pattern of two *P. papatasi* salivary transcripts of PpSP15 and PpSP44 to be regulated by sand fly blood feeding, activity season, accessory gland status and leishmanial infection [58, 59].

In laboratory examinations, immune responses to sand fly saliva have been detected in mice, hamsters, dogs and humans after multiple exposures to the bites or inoculation of dissected salivary glands from female *P. papatasi*, *Phlebotomus argentipes*, *P. ariasi*, *P. sergenti*, *Lu. longipalpis and Lutzomyia intermedia* [60-62, 49, 63-71, 33, 72-75]. Anti-saliva antibodies have been associated with increased risk of CL caused by *L. major*, *L. tropica* and *Leishmania braziliensis* in Tunisia, Turkey and Brazil, accordingly [76, 68, 77]. In CL cases, anti-saliva antibodies have been shown to induce inflammation and vasculitis resulting in a greater numbers of harboring cells, especially the local neutrophils of the skin, leading to the exacerbation of the disease outcome [42]. Conversely, the presence of antibodies against the salivary proteins of VL sand fly vector has resulted in protection in humans and dogs [78, 79, 75]. Antibodies are believed to neutralize the salivary proteins which can have an effect on hemostasis, therefore preventing the migration of the infected cells to the peripheral circulation and then to liver, spleen and bone marrow [42].

The protection against leishmaniasis has been acquired when experimental hosts were immunized by salivary gland homogenate (SGH) or were repeatedly bitten by the sand fly and then were challenged with SGH of the same sand fly species and *Leishmania* parasites. However there is some level of antigenic cross-reactivity among the salivary proteins of some species. In a recent study, hamsters immunized with *Lu. longipalpis* SGH, have shown protection when were challenged with *L. braziliensis* plus SGH prepared from *Lu. intermedia* or *Lu. Longipalpis* [80]. A summary of studies on the sand fly saliva protective potentials is shown in Table 1. Although to mimic *Leishmania* transmission in nature, it would be better to use the *Leishmania*-infected sand fly challenge, this kind of experiments are scarce and most studies are conducted with the needle injections of SGH plus *Leishmania* parasites [41].

Table1. Protective potential of sand fly saliva against leishmaniasis.

Salivary Proteins	Sand fly	Treatment with	Protective immunity
PpSP15	*P. papatasi*	*L. major*	+ (Yes)
PpSP44	*P. papatasi*	*L. major*	- (No)
Maxadilan	*L. longipalpis*	*L. major*	+
LJM19	*L. longipalpis*	*L. infantum*	+
LJM19	*L. longipalpis*	*L. braziliensis*	+
LJM11	*L. longipalpis*	*L. major*	+
LJM11	*L. longipalpis*	*L. infantum*	-
LJL11	*L. longipalpis*	*L. infantum*	-
LJM17	*L. longipalpis*	*L. infantum*	-
LJL143	*L. longipalpis*	*L. major*	-

Previous studies have shown that anti-saliva antibodies are not required for the protection in rodents [63]. In fact, a protective anti-saliva immunity is associated with a delayed type hypersensitivity (DTH) response distinguished by cellular recruitment of macrophages and monocytes to the bite site and the production of Th1 cytokines such as IFN-γ and IL-12 which make the bite site environment unaccommodating for *Leishmania* parasites and results in a less successful establishment of the parasite in the host [61, 67]. A significant fact in anti-saliva mediated protection is that at the moment of

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sand fly biting, there is a close proximity between the parasite and the salivary proteins , in the microenvironment of the host skin where anti-saliva DTH responses will interfere with the parasite establishment [42]. After identification of the protection, it is important to know which salivary proteins are responsible for this protective immunity. The low number and low complication of approximately 30 salivary proteins have made it possible to screen for the salivary proteins accountable for a DTH-Th1 response in several sand fly species [42].

The first sand fly species for which the protective salivary proteins were identified was *P. papatasi* and the protein was called PpSP15. Mice pre-exposed to PpSP15 exhibited a strong DTH response against *L. major* co-injected with SGH of *P. papatasi* [63]. Interestingly, after immunization with PpSP44 (a different protein of *P. papatasi* saliva), *L. major* infection was enhanced. This result emphasized the different induced immunity responses from distinct molecules of the same sand fly species which had led to different outcomes of the disease [67]. In spite of the fact that PpSP44 could produce a DTH response and cellular recruitment to the skin bite site, the cells did not produce IFN-γ but they produced IL-4, instead. This result suggests that anti-saliva DTH immune responses along with IFN-γ production is able to provide protection against *L. major* infection [42]. Similar studies have been done to identify salivary proteins from *Lu. longipalpis* which could induce protective immunity against Leishmania infection. Following immunization of a hamster model of VL with LJM19 salivary protein from *Lu. longipalpis*, the parasite burden was shown to be decreased in the liver for 5 months after the infection and a strong DTH response with IFN-γ production was induced, 48 hours after the exposure to the sand fly bites [81].

5. New approaches to the vaccine development

Previous studies have shown that the immune responses to salivary proteins provide protection in rodent models of leishmaniases. To be converted to a commercial vaccine, the salivary proteins should overcome a few barriers such as the variations among the sand fly populations, the differences between the wild and the colonized sand flies and the possibility of human desensitization [42]. Recently it was shown that the colonization of *P. papatasi* can provide a saliva associated protection. Mice immunized with SGH of F29 lab-bred female *P. papatasi* could produce protection against *L. major* co-inoculated with the same type of SGH while the mice immunized with SGH of the wild-caught sand flies did not produce any protection [33, 72, 82]. The reason for this may be associated with the different amounts of the salivary proteins in the colonized versus the wild sand flies rather than a genetic variability [42]. The protection provided by PpSP15 against *Leishmania* has been confirmed in mice and this protective immune response has not been observed in Rhesus monkeys [83]. Therefore, in different vertebrate hosts, different molecules of the saliva are responsible to provide protection against *Leishmania* infection [81, 70]. So far, no salivary molecule from *P. papatasi* has been identified to confer protection in humans [83]. Elnaiem et al. [84] have examined the variability of PpSP15 between the colonized and the wild-caught *P. papatasi*. The results have shown that the genetic variation of PpSP15 was higher in the wild compared to the lab-bred sand flies.

Currently, the majority of studies on the saliva rely on the long term laboratory-reared sand flies. By using transcriptomic analysis, the saliva of the wild versus the colonized sand flies, collected from different geographical localities, have been compared. The obtained results indicate high levels of homology between the saliva transcripts from different sand fly groups [85]. Further proteomics analyses are required to illuminate such differences.

Another barrier is possibly the desensitization of humans living in the endemic areas. This concern should be tested in humans from areas with frequent sand flies. In a recent study, this theory has been examined where experimental mice were bitten by 30 *P. duboscqi* every week for 15 weeks. The mice which were repeatedly exposed to the sand fly bites were unable to produce a protective anti-saliva immune response [74]. In the endemic areas, humans are exposed to multiple sand fly bites every day. The effect of such multiple exposures may lead to human desensitization over time [42].

Another barrier may be the genetic variations among different populations of the sand flies. The salivary protein Maxadilan, has been reported to show a high degree of variation among sand fly populations from different geographical locations [86], while PpSP15 salivary protein was more conserved at amino acid level among populations from Sudan, Egypt, Jordan and Saudi Arabia [84]. It can be concluded that a conserved salivary protein which successfully works across different geographical locations would be a better vaccine candidate.

A vaccine candidate against leishmaniases should also be examined by a challenge with *Leishmania*-infected sand fly. A previous study has shown that the challenge with infected sand flies is more powerful to disable the protection provided by a vaccine than a challenge with parasites injected by the needle [87]. A challenge with infected sand flies which mimics the natural route of transmission, combines several unique parameters including the sand fly saliva, the promastigote secretory gel [88, 89], the infective metacyclic *Leishmania* parasite and the sand fly probing and injury of the skin during the host biting. It has been suggested that mimicking all of the abovementioned parameters in a natural route of parasite transmission would lead to the development of an efficient human vaccine against leishmaniasis.

This review provides a summary of the studies on different aspects of the sand fly saliva. The salivary proteins of sand flies have been extensively studied and their functions have been investigated. Vaccination with the components of the sand flies saliva could bring protection against leishmaniases. In spite of new findings in the vaccine industry, an effective vaccine against leishmaniases has not yet been developed. In this regard, the sand fly salivary proteins could be considered as a vector-based vaccine against *leishmania* infections.

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