European Medicinal Leeches

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Before the advent of modern medicine, natural resources were widely used by indigenous populations for the prevention and treatment of diseases. The associated knowledge, collectively described as folk medicine or traditional medicine, was largely based on trial-and-error testing of plant extracts (herbal remedies) and the use of invertebrates, particularly medicinal maggots of the blowfly Lucilia sericata and blood-sucking leeches. The widespread use of traditional medicine in the West declined as scientific advances allowed reproducible testing under controlled conditions and gave rise to the modern fields of biomedical research and pharmacology.

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1. The Biology of Medicinal Leeches

European medicinal leeches of the genus *Hirudo* are blood-feeding annelids. The most relevant species are *H. orientalis* (Asian leech), *H. medicinalis* (European leech) and *H. verbana* (Hungarian leech). All three species are ectoparasites that live in freshwater ponds and slowly flowing streams, where they locate their vertebrate hosts by sensing heat, chemicals or movement [1][2]. Leeches attach to the host body surface and cut the skin using hundreds of calcified teeth [3]. They can then draw blood for up to one hour while secreting saliva into the wound. The secreted salivary proteins and peptides reach the vascular system of the host via thousands of tiny salivary gland cell ducts [4]. After ingestion by the leech, the host blood is compressed in the crop by the excretion of water and salts [5][6]. The remaining highly viscous blood comprises plasma proteins and blood cells and can be stored in the crop for up to one year [7]. It is thought that the morphology of the concentrated erythrocytes remains stable during storage [8], which means that proteolysis induced by host proteases released from leukocytes is inhibited [5]. Furthermore, leeches inevitably make contact with (and thus ingest) some bacteria on the surface of the host's skin during feeding, but the stored blood does not become overrun with pathogens. Indeed, foremost symbiotic core bacteria such as *Aeromonas veronii*, *A. hydrophila* and *Rikinella*-like species survive in the alimentary tract of the leech [9][10][11]. It is supposed that symbionts like *A. veronii* support the digestion of host blood by facilitating hemolysis [10][12][13] and may also help to suppress the growth of other bacteria in the crop of the leech [9]. In most parasitic leeches the host blood is stored in the crop, while food digestion and the absorption of nutrients occur predominantly in the intestine. It can be assumed that medicinal leech enzymes (e.g., endopeptidases, aminopeptidases, phosphatases) promote digestion processes [14].

2. The Pharmacological Potential of Medicinal Leeches

Medicinal leeches were used by Egyptian, Indian, Greek and Arab physicians thousands of years ago. The main application was bloodletting, but leeches were also recommended for the treatment of systemic ailments such as inflammation, skin diseases, rheumatic pain or problems with the reproductive system [15]. As an advocate of leech therapy, the Greek physician Galen of Pergamon (130–201 AD) described leeches as an effective treatment for numerous diseases. Later, in the Middle Ages, leech therapy was popular because it was less painful than conventional treatments and was recommended even for diseases of the nervous system and eyes. The use of leeches declined in the age of modern medicine, but medical interest was rekindled when one of the strongest natural anticoagulants—hirudin—was discovered in leech saliva by John Berry Haycraft in 1884, further characterized by Fritz Markwardt in the 1950s [15].

In the 1960s, physicians rediscovered the pharmacological potential of leech saliva. For example, medicinal leeches were used to prevent vascular disorders after reconstructive surgery [16], to re-establish disrupted blood vessel networks and as an alternative to anti-inflammatory drugs. Most reports concerning medicinal leech therapy focus on cosmetic and reconstructive surgery. However, leech therapy has been tested for many conditions over the past two decades, including migraine [17][18], knee osteoarthritis [19][20][21][22][23][24][25], cardiovascular disease [26][27][28], skin disorders [29], diabetic foot ulcers [30][31][32], priapism [33], macroglossia [34][35], cancer [36][37] and skin wounds [38][39]. For most of these conditions only individual case studies were published [39], but migraine and knee osteoarthritis are exceptions. Migraine is a primary neurological disorder and, for most patients, a lifelong illness associated with headaches, vomiting, nausea, photophobia and phonophobia. In a case series of seven patients who were unresponsive to conventional drugs, post-auricular leech therapy was shown to significantly reduce the frequency of migraine headaches, which the authors attributed to the presence of potent anesthetic, anti-inflammatory and vasodilator substances in the leech saliva [17]. Osteoarthritis is a disorder of the joints that is prevalent in older people (>65 years) and causes pain after activity and stiffness after rest [40]. A meta-analysis of seven articles published between 2000 and 2017 showed that leech therapy could improve the symptoms of knee osteoarthritis and reduce pain [39]. Importantly, leeches placed on the knee often achieved comparable or even better pain relief than conventional drugs, and patients reported that mobility was restored and the benefits of leech therapy were sometimes still evident after six months [41]. Although the benefits of leech therapy were evident from these studies, the salivary compounds responsible for these effects and the underlying molecular mechanisms were not characterized in detail.

3. Salivary Proteins: Natural Drugs from Medicinal Leeches

Antagonistic interactions between parasites and their hosts have led to an evolutionary “arms race”, during which ectoparasites adapted to feed on host body fluids [42][43]. To ingest and digest host blood, medicinal leeches synthesize more than 100 salivary proteins and peptides [44][45][46][47]. The molecules are secreted during feeding and target physiological pathways involved in host defense, working as analgesics (kininases), anticoagulants (hirudin, calin, saratin and apyrase), anti-inflammatories (eglins, bdellins and tryptase inhibitor), cell matrix-degrading proteins (hyaluronidase) or antimicrobials [7][45][47][48][49][50][51][52][53][54][55][56][57][58]. Salivary transcriptome data from *Macrobdella decora* [59] and *Hirudo nipponia* [60], as well as expressed sequence tag libraries constructed

from the salivary glands of *H. verbana*, *M. decora* and *Aliolimnatis fenestrata* [61], provided insight into the spectrum proteins found in leech saliva. For European medicinal leeches, the combined transcriptomic analysis of salivary gland cells and proteomic analysis of saliva in *H. medicinalis*, *H. orientalis* and *H. verbana* revealed a much wider repertoire of components than previously known [44], indicating that only ~15% of the salivary proteins in these species were identified and characterized (Table 1).

Analysis of the salivary transcriptomes of *H. medicinalis*, *H. orientalis* and *H. verbana* revealed the presence of transcripts representing 189, 86 and 344 salivary proteins, respectively [44]. The three closely related species were found to share 39 orthologous clusters, whereas 50 orthologous clusters were shared by any two of the three species [44]. Many of these newly discovered leech salivary proteins are either associated with blood feeding or related to proteins found in animal venoms [44]. The salivary proteins predicted from transcriptomic and proteomic data can be assigned to various functional groups based on their structural similarities, including metalloproteases representing the M12, M13 and M28 families, hyaluronidases, apyrases, adenosine deaminases, antistasins, cysteine-rich secretory proteins (CRISPs), eglins, cystatins, PAN/apple domain proteins, α2-macroglobulins, low-density lipoprotein receptors, R-type lectins, and salivary proteins containing a von Willebrand factor type A (vWA) domain. These proteins are likely to be involved in the regulation of blood coagulation, the temporary adjustment of blood pressure, the regulation of inflammation, the suppression of microbial growth or the digestion of blood in the crop [44]. Interestingly, differential gene expression analysis indicated that genes encoding salivary proteins, such as hirudin, eglins, saratins and destabilases, were also expressed in other leech tissues, showing that at least some leech “salivary proteins” are not restricted to the saliva and may have additional physiological functions [44]. Some leech-specific anticoagulants were also found in leeches that do not feed on blood, such as *Whitmania pigra* [62]. Interestingly, these anticoagulants were upregulated after feeding [62] just as they are in blood-feeding leeches [63].

The identified metalloprotease families in leech salivary encompass astacins (M12), neprilysins (M13) and aminopeptidase S (M28). Members of these metalloprotease families were also determined in the salivary secretion of medicinal maggots of *Lucilia sericata* [64]. Astacin-like metalloproteases are endopeptidases, which were originally identified in the crayfish *Astacus astacus*, which contribute to digestion. A homologues were found in the venom of the brown spiders *Loxosceles,* with the recombinant form able to induce morphological changes, such as loss of adhesion of muscular aorta cells in vitro and hydrolyzed purified fibrinogen and fibronectin [65]. Mammalian neprilysin is involved in reproduction and the modulation of neuronal activity and blood pressure [66]. Interestingly, the transcriptomic analysis of the salivary glands from medicinal maggots *L. sericata* elucidated a diversification of proteolytic enzymes [64], whereas the most diverse groups of molecules in the saliva of leeches represented protease inhibitors.

Table 1. Leech salivary proteins from *H. medicinalis, H. verbana* or *H. orientalis.* Isoforms of individual proteins are not shown.

Many leech salivary proteins, including antistasin-like inhibitors, hirudins, hirudin-like factors and Kunitz-type proteinase inhibitors, show remarkable diversity [44][67], possibly reflecting target-oriented evolution [68] promoted by

gene duplication events [69]. Gene duplication events are likely to have promoted the acquisition of two major salivary protein families—salivary blood coagulation inhibitors and platelet aggregation inhibitors—in blood-feeding ticks [70]. Gene recruitment also supports the diversification of salivary protein isoforms, based on the hypothesis that regulatory evolution is fundamental for adaptive evolution [71]. Accordingly, at least some venom and salivary proteins were recruited from other tissues, where they fulfilled distinct biological functions. The recruitment of alternative splice variants and 5′ exon evolution might explain the adaptation of vampire bats to hematophagy and may be a more common source of genomic complexity in sanguivorous animals than the evolution of new genes [71]. This led to the identification of novel and convergently recruited venom proteins in blood-feeding leeches and vampire bats [71].

Evolutionary models explaining the adaptation of leech salivary proteins to specific hosts are still a matter of debate. Current challenges include the lack of well-characterized proteins in terms of mode of action and target. The isoproteins in leech saliva may have more than one target in the host, or their activity may be dependent on pH, temperature, the season or the developmental phase. Both the redundancy of salivary proteins (multiple proteins directed against the same target) and the potential cooperative interactions among multiple salivary proteins should be considered. The interplay of several salivary proteins can be seen in the bloodsucking arthropod *Rhodnius prolixus*, which produces four isoforms of salivary nitrophorin. All of them are vasodilators (working in cooperation) and histamine suppressors, but one is a strong inhibitor of factor IXa, another is a weaker anticoagulant and the remaining two isoforms appear to have lost their anticoagulant activity [72].

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