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Bullfrog Oil and its Potential Anti-inflammatory Effects as Neuroprotective Therapy in Sepsis-associated Encephalopathy

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Abstract

Sepsis is a life-threatening multiple organ dysfunction due to secondary infection and systemic inflammatory responses, which evolves to encephalopathy in 8% to 70% of the cases. Sepsis-associated encephalopathy is characterized by a potential inflammation of the nervous system that can result in cognitive damage, short and long term, in surviving individuals. To reduce these effects, scientists investigate the use of natural resources as more biocompatible and lower-cost therapeutic alternatives. Here, we investigated the rationale behind the role of the bullfrog oil (BFO) in neuroinflammation and neuroprotective potential. Results of the analyses of the articles demonstrate the anti-inflammatory aspects of BFO in different tissues. Additionally, the analyses establish microemulsions as a safe delivery system capable of securing medicinal effects and preventing bullfrog oil-induced hepatotoxicity. Considering the importance of inflammation in sepsis-associated encephalopathy and cognitive impairment, BFO emerges an excellent pharmacological tool to decrease inflammation and prevent the post-sepsis cognition impairment. Due to the excellent therapeutic potential of using bullfrog oil on the treatment of sepsis-associated encephalopathy, future studies should further investigate its therapeutic properties.

Keywords: Septic Encephalopathy, Inflammation, Central Nervous System, Microemulsion

Introduction

Sepsis can be defined as an acute multiple-organ dysfunction due to the responses of an infection [1]. It is characterized by the development of associated biochemical, hematological, and immunological changes that lead to the excessive production of free radicals, enzymes, and pro-inflammatory mediators by the host, initiating a cascade of immunological reactions [2]. The "cytokine storm" induced by the systemic infection is responsible for large-scale tissue damage and multiple organ failure, an important cause of morbidity and mortality worldwide [3, 4]. Sepsis affects 1.5 million individuals in the U.S. each year and is responsible for approximately 30% of hospital deaths [5].

Septic encephalopathy (SSc) is a complication that affects 8 to 70% of septic patients. As a result, the brain becomes highly affected by the extensive inflammatory cascade, aggravated by the systemic activation of toll like receptors (TLR) [6, 7]. In addition to being associated with a high index of morbidity and mortality, this reaction can lead to a wide spectrum of brain dysfunctions, and long-term cognitive and functional limitations [8-10]. Young et al. (1990) reports that the most recurrent behavioral changes cause by sepsis are cognitive deficits, psychiatric disorders, reduced levels of consciousness, agitation, and concentration deficit. The main concern regarding SAE is the long-term neurological sequelae among survivors that range from mild confusion and disorientation to convulsions and deep coma [9].

The standard treatment for sepsis is the use of broad-spectrum antibiotics within the first hours of diagnosis, fluid resuscitation, vasopressors, such as norepinephrine, and, in some cases, corticosteroids [11]. However, this treatment does not offer a therapy to reduce or minimize brain damage, emphasizing the need for developing novel treatment strategies. The search for techniques that rely on the use of natural resources is gaining more and more space in science and in the pharmaceutical industry due to their low cost and the possibility of reducing iatrogenic factors, pointed out as one of the aggravating factors for the occurrence of SAE [2]. In this context, the American Bullfrog Oil, extracted from an animal of the species Rana catesbeiana shaw, also called Lithobates catesbeianus, has been used - by the Brazilian population, specifically the people of the state of Rio Grande do Norte - in the treatment of allergic processes and asthma [12]. Its therapeutic potential is due to the presence of 62 compounds of polyunsaturated fatty acids such as oleic acid (omega 9), linoleic acid (omega 6), alpha-linolenic acid (omega 3), stearic acid, palmitic acid, and muriatic acid [12, 13].

In this work, we review the main findings regarding the role of BFO in reducing cognitive damage induced by the inflammatory processes that affect the central nervous system after sepsis. We focus on functional responses, such as memory loss prevention.

Methods

The present work carries out a methodological analysis of the studies about the therapeutic potential that BFO can present when associated with the treatment of pathologies that affect the central nervous system. It focuses on the processes of brain dysfunction caused by sepsis, through an integrative review that accommodates data from both empirical and theoretical literature. It aims at defining parameters and identifying gaps in that field of study. The data used in this analysis were gathered from the largest national and international databases PubMed (National Library of Medicine), SciELO (Scientic Eletronic Library OnLine), and MEDLINE (Medical Literature Analysis and Retrieval System online). The goal of this search was to answer the proposition "What evidence-based literature can indicate anti-inflammatory/neuroprotective properties of BFO that can help reduce the sequelae of brain damage induced by systemic inflammation?", A quantitative review survey was conducted between 1990 and 2020. The studies referenced in this review were selected from the search “bullfrog oil” in the database described above. Only articles addressing the cytotoxic aspects of the oil and its role in inflammatory modulation were selected. Seven studies were discarded because their approaches did not address the inflammatory properties of the central nervous system.

Results

The search from databases came back with a total of 15 articles. After a careful analysis of these articles, which demonstrate the scarcity of studies about the therapeutically use of the BFO, 7 of them were discarded because they did not evaluate inflammatory-related aspects of BFO. Hence, the remaining 8 articles were selected for containing the most relevant information about the BFO properties regarding inflammatory and neuroprotective role, and its relationship with the behavioral response (Table 1).

Table 1: Synthesis of the results found that support this review

Reference	Type of cell/animal model	Concentration	System	Results
Oliveira et al., 2022	Human melanoma cells (A2058)	50 ug/mL	Pure	↓ MTT
		100 ug/mL		↓ MTT
		50 ug/mL	Microemulsion	No diffence in MTT
		100 ug/mL		↓ MTT, ↑ ROS
		50 ug/mL	Nanoemulsion	↓↓ MTT
		100 ug/mL		↓↓ MTT, ↑ ROS
		50 ug/mL	Nanocapsule	↓ MTT
		100 ug/mL		↓ MTT, ↑ ROS
Amaral-Machado et al., 2021	Murine macrophage cells (Raw 264.7)	100 ug/mL	Pure	No difference in MTT
		100 ug/mL	Nanocapsules	No difference in MTT
	Murine macrophage cells (Raw 264.7) treated with LPS	50 ug/mL	Pure	No difference in ROS, NO and cytokines
		100 ug/mL		No difference in ROS, NO and cytokines
		500 ug/mL		No difference in ROS, NO and cytokines
		50 ug/mL	Nanocapsules	↓ ROS, ↓ IL-6
		100 ug/mL		↓ ROS, ↓ IL-6
		500 ug/mL		transient ↑ ROS, transient ↑ NO, ↓ IL-6
	Acute carrageenan-induced paw edema in mice	50 ug/mL	Pure	↓ Paw edema ↓ myeloperoxidase
		100 ug/mL		↓ Paw edema ↓ myeloperoxidase
		500 ug/mL		↓ Paw edema ↓ myeloperoxidase
		50 ug/mL	Nanocapsules	↓ Paw edema ↓ myeloperoxidase
		100 ug/mL		↓ Paw edema ↓ myeloperoxidase
		500 ug/mL		↓ Paw edema ↓ myeloperoxidase

Barbosa et al., 2020	Acute carrageenan-induced paw edema in rats	50 ug/mL	Pure	↓ paw edema ↓ leukocyte infiltration ↓ vasodilatation
		100 ug/mL	Pure	↓ paw edema ↓ leukocyte infiltration ↓ vasodilatation
		200 ug/mL	Pure	↓ paw edema ↓ leukocyte infiltration ↓ vasodilatation
	Murine macrophage cells (Raw 264.7)	50 ug/mL	Pure	No difference in MTT, ↑ NO, ↑ IL-6, ↓ TNF-α
		100 ug/mL	Pure	No difference in MTT, ↓ NO, ↓ IL-6, ↓ TNF-α
		200 ug/mL	Pure	No difference in MTT, ↓ NO, ↓ IL-6, ↓ TNF-α
Amaral-Machado et al., 2018	Human melanoma cells (A2058)	50 ug/mL	Pure	↓ ROS
		100 ug/mL	Pure	↑ ROS ↓ MTT
		200 ug/mL	Pure	↑ ROS ↓ MTT
Davim et al., 2018	Sepsis experimental model in mice	100 ug/mL	Microemulsion	↓ leukocytes migration to the lungs ↓ pulmonary parenchyma injury
		100 ug/mL	Pure	↓ leukocytes migration to the lungs
Davim et al., 2017	Acute formalin-induced muscle injury in mice	100 ug/mL	Microemulsion	↓ muscle edema and ausence of hepatoxicity
		100 ug/mL	Pure	↓ muscle edema and discrete signs of hepatoxicity
Bonatto et al., 2015	Murine melanoma cells (B16F10)	NR	Microemulsion	↓ ↓ cell viability
		NR	Pure	↓ cell viability

The table 1 shows the contains the indicators that led the authors to the conclusions that corroborate to the main hypothesis of this work, that is, the probable anti-inflammatory properties of BFO, which can be used to help the recovery of patients with brain damage caused by inflammation.

Discussion

Understanding the Inflammatory Aspects of Sepsis-associated Encephalopathy

After the installation of the systemic infection, the innate and adaptive immune system reacts by initiating an excessive inflammatory cascade with the release of pro-inflammatory mediators, such as cytokines, reactive oxygen species (ROS), and nitric oxide (NO) [14]. This exacerbated immune response may reach the central nervous system (CNS) by different pathways such as neuronal tracts as the vagus nerve or by infiltration of cells and mediators in a disrupted blood-brain barrier (BBB) [15]. After sepsis, there is an increase of brain endothelial permeability, and modulation of astrocyte end feet which is a known consequence of BBB breakdown [16, 17]. In post-mortem investigation, the brains of individuals with severe sepsis presented lower expressions of occluding, a tight junction protein highly expressed by astrocytes [18]. Pro-inflammatory cytokines in the circulating blood increases the expression of adhesion molecules in the endothelial and immune cells inducing endothelial barrier dysfunction, which allows the entrance of activated lymphocytes into CNS tissue [19, 20]. Both microglia and astrocytes are considered, not only the immune cells in the CNS, active participants in the synaptic environment, preventing excitotoxicity and regulating oxidative stress and neuronal damage [21-24]. Additionally, astrocytes and microglia express different pattern recognition receptors (PRRs) and are able to release an array of pro-inflammatory cytokines, contributing to the inflammatory cascade within the CNS and attracting immune peripheral cells to the injury site by NF-kB signaling [25]. Similar to macrophages, microglia activation releases pro-inflammatory cytokines, enzymes, ROS, and excessive glutamate, which promotes tissue inflammation, neurotoxicity, axonal damage, and neuronal dysfunction or death [26-28]. Once activated, microglia also induce the classical activation of astrocytes which is also responsible for the release of pro-inflammatory mediators [29]. However, once activated, microglia and astrocytes lose their protective functions contributing to the deleterious consequences of inflammation [30, 31].

Sepsis-associated Encephalopathy and Cognitive Impairment

Sepsis and its neuropathological complications are challenging worldwide. The high incidence and severity associated with this phenomenon alone would be enough to justify deepening the knowledge of its pathophysiology and the search for new therapeutic possibilities. One of the main consequences related to sepsis is the prevalence of moderate to severe cognitive impairment, which is recurrent in 11% of recovered individuals [32, 33]. Molecular alterations caused by sepsis, such as increase of TNF-a in the cerebrospinal fluid and a decrease in thiobarbituric acid-reactive species and mitochondrial activity in the prefrontal cortex, may be appear after 30 to 60 days of the original infection and are highly related to cognitive impairment [34-36]. Additionally, sepsis is a risk factor for dementia development since systemic inflammation can contribute to cognitive decline [37, 38]. Several factors have been pointed out in studies as aggravating factors for the occurrence of SAE, such as pre-existing conditions of the patient (such as age, liver and kidney diseases, and depression); acute conditions that led the patient to that clinical condition (i.e. drug overdose and fever); iatrogenic and environmental factors (use of sedatives, enteral feeding, and central venous catheter) [39].

As described in a previous section, the exacerbated activation of a pro-inflammatory cascade relates intrinsically to the function-

al characteristics of sepsis, which, despite being complex, has been partially elucidated and is known to involve many organic resources, including the release of endotoxins and cytokines by defective bacteria and cells. The immediate results of this process at molecular levels include changes in the BBB, generation of pro and anti-inflammatory cytokines, interruption of amino acid metabolism, cerebral ischemia, and imbalance of neurotransmitters [40]. In this sense, sepsis decreases the level of neurotransmitters, such as acetylcholine, noradrenaline, dopamine, and serotonin while increases sharply glutamate concentration [6, 40-45]. The exacerbated glutamate activates NMDA receptors leading to neuronal over-stimulation and injury, characterizing excitotoxicity [46]. In long-term sepsis, it is observed neuronal dysfunctions due to high excitotoxicity and oxidative stress (40), which are strongly related to cognitive impairment [34-36].

Some of the most recurring behavioral changes in sepsis patients were cognitive deficits, psychiatric disorders, reduced levels of consciousness, agitation, and deficit in concentration [7]. In fatal cases, the proliferation of astrocytes and microglia in the cortex, cerebral infarctions, cerebral purpura, multiple hemorrhages in white matter, and dissemination of micro abscesses have been observed [47]. To further investigate cognitive impairment associated with encephalopathy, many pre-clinical models have been used. These animals show discrepancies in memory evaluated by novel object recognition test, radial maze test, and Morris water maze social behavior by social exploration and social interaction between mice and even sleep evaluated by “clock” proteins related to circadian rhythm [48-52]. These cognitive impairment behaviors also accompanied neuronal loss in the hippocampus and sub-regions of the prefrontal cortex and reduced cholinergic innervation of cortical areas changes that result in consequences such as memory loss [10, 32, 53, 75]. As previous described, astrocytes, which are supposed to maintain the glutamatergic homeostasis, are unable to perform their tasks because of the inflammatory classical phenotype [31]. Beyond the hemodynamic dysfunctions induced by sepsis, such as hypovolemic and cardiogenic shock induced by increased capillary leak and decreased venous return to the heart cerebral perfusion pressure, observed by cerebral blood flow, is also low in these individuals regardless of cardiac or blood pressure changes, Indeed, Maekawa at al. found a lower cerebral blood flow in individuals with sepsis-associated delirium than in awake controls [54-56]. Taken together, these data suggest that exacerbate inflammation leads to neuronal damage and disrupted cerebral oxygenation of individuals affected by sepsis which contributes to the cognitive impairment.

Anti-inflammatory Aspects of the Bullfrog Oil

Considering the importance of inflammation in the sepsis-associated encephalopathy and its role in cognitive impairment, it is pivotal to investigate biocompatible therapeutic alternatives, which can cause minor side effects, and can be more accessible and less costly. In this sense, our group is currently investigating the therapeutic potentials of the oil extracted from bullfrog (Rana catesbeiana Shaw) as well as searching for the most appropriate delivery systems aiming to improve their biopharmaceutical performance in a sepsis pre-clinical model. Different authors have shown interesting results regarding the role of BFO in controlling inflammation, which may be relevant in the sepsis context.

As described in table 1, the role of BFO in inflammation may depend on the type of the tissue investigated. In melanoma cells, BFO decreases cell viability and decreases inflammatory markers, such as nitric oxide, TNF-a and IL-6 in high concentration, but have the opposite response in lower concentrations [57, 58]. However, Amaral-Machado at al. (2019) and Oliveira et al. (2019) showed decreased ROS in lower concentrations, and increased ROS but lower cell metabolic activity in higher concentrations in melanoma cells. These conflicting results may be related to the components of the BFO [59].

Because of these components, BFO in its pure formulation may lead to hepatotoxicity by increasing ROS and liver injury biomarkers in vivo [47]. Hence, Davim et al. (2017) investigated the role of BFO in an acute inflammatory pain model considering two delivery systems: pure and in microemulsion regarding the anti-inflammatory properties and the in vivo safety of these delivery systems. Both formulations were able to decrease muscle edema, suggesting an improvement in muscle inflammation. However, the pure system showed high levels of hepatotoxicity biomarkers, while microemulsion system prevented this phenomenon. The formulation used in the microemulsion system was composed of an oily phase of 90%, an aqueous phase of 5% and a tensile phase of 5% in order to preserve the medicinal properties [60]. Additionally, microemulsion of BFO decreased leukocyte migrating and cellular infiltration to the lung [61].

The BFO contains polyunsaturated fatty acids with approximately 13% omega-3, which decreases arachidonic acid-derived eicosanoids such as prostaglandins, an important inflammatory mediator [62, 63]. Interestingly, eicosanoids are related highly to vascular permeability, leukocyte recruitment, and BBB disruption [64, 65]. Omega-3 can also decrease leukotrienes-induced bronchoconstriction effect and COX-2 expression while increasing the expression of resolving lipids, which inhibits peripheral immune cells response [63]. Additionally, omega-3 inhibits PRRs and NF-kB activation [66, 67, 68], and interacts with epigenetic microRNA regulation of the immune system [69]. In this sense, BFO can be considering an extraordinary tool to prevent and control systemic and brain inflammation. Large doses of omega-3 improved the executive functions of healthy older adults suggesting a beneficial effect of cognition accompanied by an increase of brain derived neurotrophic factor (BDNF) [70]. In sepsis individuals, omega-3 nutritional supplementation reduced time in the intensive care unit and duration of mechanical ventilation [71]. Regarding cognitive impairment, omega-3 decreased decline in neurogenesis and contributed to improve cognitive performance probably by increasing BDNF and transporters related to glutamatergic clearance [71].

Perspectives

Understanding the main pro and anti-inflammatory events that induce tissue damage is undoubtedly the first step to improve the prognosis of the inflammatory diseases and establish an appropriate therapy. More recent studies point out to bidirectional communication between the CNS and the immune system through the "anti-inflammatory cholinergic pathway." Stimulation has shown effectiveness in improving inflammatory diseases such as endotoxemia and sepsis [2].

Literature reports that the presence of unsaturated and saturated fatty acids (linoleic, linolenic, arachidonic, eicosapentaenoic, and decosapentaenoic) in natural oils may be related to apoptotic effects on tumor cells, suggesting that the protective capacity of BFO can be attributed to its composition [57, 72]. In studies with melanoma cells, the BFO was able to increase the production of ROS by 51%, being associated directly with mitochondrial damage that compromises cellular metabolism and induces intrinsic apoptosis [73].

Other studies also evaluated the possible cytotoxicity of the BFO against non-tumor cells, such as a lineage of fibroblasts (3T3) and human erythrocytes. These studies demonstrated, through in vitro tests, that the oil did not promote damage to these cell lineages, suggesting, thus, that its cytotoxic activity may be related to specific characteristics of tumor cells [35, 59, 74]. An overview of the main therapeutic properties of the BFO is shown in Figure 1.

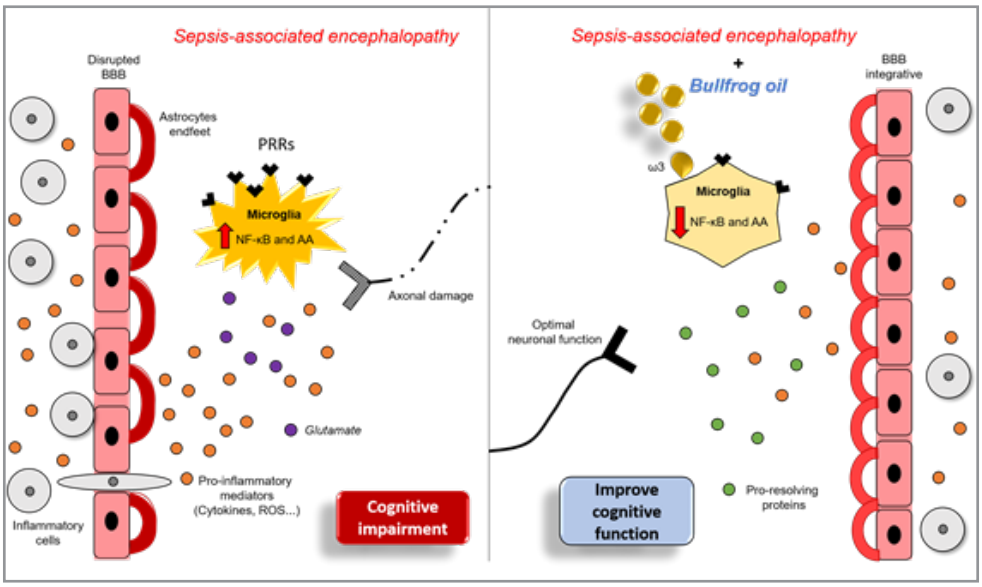

Figure 1: Representative schematic of anti-inflammatory mechanisms of bullfrog oil.

After the consolidation of the infection, peripheral immune cells pass through a breakdown blood-brain barrier (BBB) releasing pro-inflammatory mediators into the central nervous system characterizing the sepsis-associated encephalopathy. These mediators are recognized by microglia pattern recognition receptors leading to the classic activation of microglia cells by NF-kB and arachnoid acid. The classic activated microglia release pro-inflammatory mediators and glutamate deteriorating the synaptic cleft modulation leading to axonal damage and cognitive impairment. The saturated fatty acids present in the bullfrog oil control the peripheral inflammation preventing the BBB breakdown and decreasing the central inflammation. Additionally, these fatty acids also inhibit microglia activation, and promotes the release of pro-resolving mediators, preventing axonal damage and leading to an improved executive function.

The knowledge, already presented in the literature, about the biocompatibility of BFO, together with the data obtained on its anti-inflammatory effect and cytotoxic and apoptotic activities, make the BFO an interesting therapeutic tool, but this can only be ascertained with further experimentation. More recently, different preparation of BFO have been purposed in the literature in the attempt to increase its effects and attenuate possible side effects. In this sense, it has been shown that BFO in nanoemulsion has an increased potential of cell injury in human melanoma cells when compared to microemulsion or nanocapsule [76].

On another hand, BFO delivered in nanocapsules were able to attenuate LPS-induced oxidative stress and inflammation in murine macrophages when compared to pure BFO [77]. That would highlight the positive effect of the nanocapsule formulation into the beneficial effects of BFO. However, the same authors showed that both pure and nanocapsule BFO had similar effects regarding decreased paw edema and myeloperoxidase (MPO) in a carrageenan-induced paw edema preclinical model. These articles shed light into the importance of further studying the effects of BFO in different preparations. However, the role of these different preparations in sepsis-induced encephalopathy are still in need of investigation.

We consider that a growing number of natural products have been explored for pharmacological purposes, and the proof of their effects by scientific institutions contributes to the development of innovative possibilities in biotechnology for application in therapeutic resources. However, it is important to encourage the development of more research to address brain damage in SAE.

Conclusion

In view of the results obtained, we can suggest that the BFO is promising as therapeutic tool to sepsis and other inflammatory diseases that impair brain tissue, due to its anti-inflammatory properties and its composition with a high content of polyunsat-

urated fatty acids that should be applied in slow release systems such as microemulsion, to prevent liver damage. BFO has been proven to be a bioactive input capable of acting against inflammatory processes, thus, expanding knowledge about the physiological mechanisms antagonistic to inflammatory processes that result in neurological damage.

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