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Combination treatment of celecoxib and ciprofloxacin attenuates live *S. aureus* induced oxidative damage and inflammation in murine microglia via regulation of cytokine balance

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ABSTRACT

Microglial activation is the most common phenomenon in *S. aureus* induced brain abscesses as well as other common neurodegenerative diseases. The main objective of this study is to reduce the microglial inflammation with effective bacterial elimination. Ciprofloxacin and celecoxib were used in combination to regulate *S. aureus* induced oxidative stress and inflammation in primary murine microglial cells. Our results showed that combination treatment effectively killed viable *S. aureus* and reduced the inflammatory consequences. It can be concluded that lower production of pro-inflammatory cytokines and higher anti-inflammatory IL-10 level may be responsible for microglial polarization switching from pro-inflammatory M1 to anti-inflammatory M2.

1. Introduction

Brain abscesses are known to arise from the localized parenchymal infection with different pyogenic bacteria such as *Streptococcal* strains and *Staphylococcus aureus* (*S. aureus*). Those etiologic agents can penetrate the brain tissue through paranasal sinuses, middle ear and upper molars which are considered as the sites of chronic infection (Mathisen and Johnson, 1997; Townsend and Scheld, 1998). When a considerable portion of brain parenchyma is destroyed the typical signs of brain abscess begin to arise including seizure, hemiparesis or cognitive decline (Esen and Kielian, 2009). Therefore, if fruitful treatment is not available or diagnosis is delayed it can be a life threatening issue for human health. Brain abscess remains a significant medical problem with an increased prevalence in developing countries mainly due to the development of multidrug resistant strains of *S. aureus* (Fournier and Philpott, 2005).

Staphylococcus aureus (*S. aureus*) is a Gram positive, opportunistic microorganism causing localized infections to bacteremia in human (Archer, 1998). Already exsisting studies revealed that *S. aureus* is a potential candidate to cause brain abscess. Primary microglia produces significant amount of proinflammatory mediators in response to intact *S. aureus* as well as its cell wall product like peptidoglycan (PGN) (Kielian et al., 2002). Proinflammatory cytokines namely TNF-α, IL-1β, IL-6, IL-12 and different chemokines like MIP-2, MCP-1 mediate the host response to infection in CNS usually, within 1 to 3 h after acute *S. aureus* infection (Kielian and Hickey, 2000). Exposure of microglia to *S. aureus* leads to release of other bactericidal mediators including reactive oxygen species (ROS), reactive nitrogen species (RNS), lysozyme as well as enhanced expression of surface receptors that play a pivotal role in bacterial recognition and antigen presentation (Kielian et al., 2002; Kielian et al., 2005). Among them the toll like receptor-2 (TLR-2) is critically expressed by activated microglia (Syed et al., 2007). Some recent in vitro research illustrates that TLR-2 plays a pivotal role in recognition of peptidoglycan (PGN) from *S. aureus* in microglial cells and it is responsible for a maximal cytokine response of primary astrocytes (Esen et al., 2004; Jack et al., 2005). Therefore, targeting of the TLR-2 signalling pathway may provide a new avenue for developing drugs to treat infectious diseases (Carpenter and O'Neill, 2007).

Microglial activation is considered as the hallmark of brain abscess. Actually, microglias are commonly known as brain macrophages. Their main physiological functions are antigen presentation, cytokine production and the secretion of neurogenesis promoting factors (Nayak et al., 2014; Zhan et al., 2014). It is evident that microglia robustly respond to *S. aureus* infection and provide an initial line of defence against invading microorganisms into the CNS prior to leukocyte infiltration (Kielian et al., 2001). Microglias rapidly become activated and mobile by retracting their processes and efficiently phagocytose causative microorganisms during CNS infection (Davalos et al., 2005). In

Abbreviations: CNS, Central nervous system; CPCSEA, Committee for the purpose of control and supervision of experiments on animal; DMEM, Dulbecco's Modified Eagle's medium; HBSS, Hank's balanced salt solution; ELISA, Enzyme linked immunosorbant assay; FBS, Foetal bovine serum; MRSA, Methicillin resistant *S. aureus*; NF-κB, Nuclear factor kappa B; RPMI-1640, Roswell Park Memorial Institute-1640; SOD, Superoxide dismutase; TSST-1, Toxic shock syndrome toxin-1

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addition, microglia also helps in damaged tissue repair by secreting growth factors and anti-inflammatory molecules but sometimes microglial immune responses become detrimental to host and promote neurodegenerative diseases (Perry et al., 2010).

Classically, microglia possesses two different phenotypes i.e., proinflammatory or M1 type and anti-inflammatory or M2 type. Previous in vitro studies suggest that differentiation of classically activated M1 microglia dependents upon LPS (lipopolysaccharide) and IFN-γ stimulation where as the neuroprotective M2 phenotype is induced by IL-4 and IL-10 (Durafourt et al., 2012). M2 microglia shows its pro-healing activity via down regulation of NF-κB (Mosser and Edwards, 2008). At early stage of infection the M1 microglia is predominantly expressed to control pathogen spread by increased production of ROS and cytokines including TNF-α, IFN-γ, IL-1β, IL-6 (Colton, 2009). On the other hand persistent M1 programming is believed to be detrimental to the host. Inducible nitric oxide synthase (iNOS) is considered as one of the well known M1 polarization markers seen in microglia (Hu et al., 2012). M1 to M2 transformation has a protective role against severe inflammatory diseases by attenuating the recruitment of immune cells at the site of infection. Several animal models demonstrated that M2 phenotype switching plays beneficial role in cerebral damage (Xu et al., 2017). So, targeting of this switching mechanism may be helpful to promote the resolution of inflammation and tissue repair after pathogenic insult (Benoit et al., 2008).

Already existing studies show the effects of various virulent components of *S. aureus* including peptidoglycan (PGN) and lipoteichoic acid (LTA) on microglial TLR-2 activation in terms of infection but simultaneous administration of both of them causes fourfold higher NO production than LTA alone (De Kimpe et al., 1995). Therefore, we use live *S. aureus* to stimulate murine microglia for evaluating the cumulative effects of all the virulent factors (i.e. TSST-1, hemolysins, adhesins, α-toxin, protein A) together over primary microglia. On the other side chronic microglial activation is directly correlated with excessive cellular stress and tissue damage in infected brain. In the CNS, levels of prostaglandins are low under normal condition but increase with stimulation of IL-1β, TNF-α during inflammatory processes (Zarghi and Arfaei, 2011). It was reported that some prostaglandin may control TLR-2 mediated inflammatory events in brain immune cells (Yoon et al., 2008). Normally, vasoactive prostaglandins are synthesized from arachidonic acid and molecular oxygen by the action of a key enzyme cyclooxygenase-2 (COX-2) with the production of reactive oxygen free radicals as by-products. So, COX-2 inhibitors may play an anti-inflammatory role in brain by attenuating prostanoid and free radical generation (Strauss, 2008). Therefore, an understanding of mechanisms that govern TLR-2 signalling in microglial cells, with respect to cyclooxygenase-2 (COX-2) expression, will undoubtedly facilitate the design of therapeutic regimens for CNS infections that would be capable of pathogen elimination without destruction of surrounding brain parenchyma (Konat et al., 2006).

So, it is evident that control of excessive microglial activation is the main gateway to the treatment of brain abscess and its inflammatory consequences. After accurate diagnosis of infection, proper choice of antimicrobial agents with adequate penetration power through blood-brain-barrier is recommended (Brook, 2004). Beside successful bacterial elimination, anti-inflammatory agents cease further chances of neuroinflammation related to microglial activation (Zhuang et al., 2011). Non steroidal anti-inflammatory drugs (NSAIDs) such as selective COX-2 inhibitors have effective role on microglial function by controlling inflammation and pain (Ajmone-Cat et al., 2010). Previous studies suggest that it also efficiently maintains pro-inflammatory cytokine release, NO production and phagocytic uptake in microglia (Cho, 2007). Therefore, a suitable antibiotic with a selective COX-2 inhibitor can efficiently manage the inflammatory consequences of *S. aureus* induced microglial activation. Ciprofloxacin, a fluoroquinolone is a potent antimicrobial agent against different bacterial species in-vitro. The enhanced accumulation of this antibiotic within the infected phagocytes provides more killing of microbes (Dey and Bishayi, 2016). Here, we tried to treat multidrug resistant *S. aureus* infected microglia with ciprofloxacin and a selective COX-2 inhibitor namely celecoxib. Being an anti-inflammatory agent, celecoxib shows broad-spectrum antimicrobial activity against a variety of Gram-positive pathogens including *Staphylococcus* (Thangamani et al., 2015). In addition it also helps to minimize the therapeutic dose of antibiotics to treat drug-resistant bacteria (Annamanedi et al., 2017). Hence, there are ample of reasons to choose celecoxib over others as an anti-inflammatory agent for the combinatorial treatment along with ciprofloxacin to treat infected microglia. Currently, no other study was done on this combination to treat *S. aureus* induced microglial inflammation.

In this in-vitro study we want to induce microglial inflammatory responses against acute live *S. aureus* infection in terms of ROS, NO and other inflammatory cytokine production. It is also very crucial to judge infection mediated cellular stress to the host. Our main objective is to evaluate whether this combination treatment can effectively ameliorate inflammation in microglia by means of effective bacterial neutralization along with less oxidative cellular damage. This study will also explore the relationship between TLR-2, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and pro-inflammatory cytokines profile in microglial cells both before and after combinatorial treatment with ciprofloxacin and celecoxib. Moreover this study will also demonstrate the cytokine mediated M1/M2 polarization switching in murine primary microglia which will facilitate better understanding of microglial polarization with respect to this treatment strategy.

2. Materials and methods

2.1. Maintenance of animals

All experimental animals were maintained and experiments were performed according to the protocols that had been approved by the Institutional Animal Ethics Committee (IAEC), Department of Physiology, University of Calcutta, under the guidance of CPCSEA [Proposal submitted on 27.12.2016 to IAEC, proposal number: IAEC/IV/Proposal/BB-01/2017 and approved on 20.03.2017], Ministry of Environment and Forest, Govt. of India. Male Swiss albino mice were used throughout this study. Upon arrival, mice 6–8 weeks of age with body weight 20 ± 4 g were randomized into plastic cages followed by one-week of quarantine period. Mice were fed a normal rodent diet. To minimize the feeling of hypoxia or discomfort during animal dissection and brain tissue collection mice were anesthetized with ketamine hydrochloride (Sigma, Life Sciences) at a dose of 1 mg/kg body weight through the tail vein followed by cervical dislocation.

2.2. Isolation of murine primary microglial cells

After sacrifice, brains from 6 to 8 weeks old male Swiss albino mice (4 mice/group) were collected in HBSS, dispersed with scissors, re-suspended in HBSS containing 0.3% collagenase D and 10 mM HEPES buffer and incubated for 30 min at 37 °C. Then homogenates from brain tissue were prepared and filtered in 70 μm pore cell strainers (Becton Dickinson), centrifuged at 1500 rpm for 7 min, washed, and re-suspended in 70% isotonic Percoll (Sigma-Aldrich). 3.5 ml of cell suspension was transferred to 15 ml polypropylene conical tubes with 5 ml of 25% isotonic Percoll, which were sequentially layered on top with 3 ml of PBS. After centrifugation (30 min, 800 *g*, 4 °C), the 70%: 25% Percoll interphase layers were collected, and the cells were washed. Finally, the adherent cells, which contained CD11b + cells (as determined by FACS), were cultured in DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml sodium pyruvate, and 10 mM HEPES buffer. Microglial cells were washed with PBS and resuspended in medium containing 1% heat-inactivated FBS, and then cultured for the required times at 37 °C (Arroyo et al., 2013). Then smear was prepared from the