

Original Research

Acetylsalicylic acid improves cognitive performance in sleep deprived adult Zebrafish (*Danio rerio*) model

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1. Abstract

Sleep deprivation (SD) is commonly associated with decreased attention, reduced responsiveness to external stimuli, and impaired locomotor and cognitive performances. Strong evidence indicates that SD disrupts neuro-

immuno-endocrine system which is also linked to cognitive function. Recently Zebrafish have emerged as a powerful model sharing organizational and functional characteristics with other vertebrates, providing great translational relevance with rapid and reliable screening results. In the current study, we examined the effects of acetylsalicylic

acid (aspirin) on cognitive and locomotor activity in sleep deprived Zebrafish model. Learning and memory were assessed by T-maze and locomotor activity was assessed by partition preference and swimming time in spinning tasks. Furthermore, brain bioavailability of aspirin was determined by high performance liquid chromatography. Following drug exposure and tasks, histopathology of the brain was performed. It was observed that three-day SD significantly reduces learning and memory and locomotion in the Zebrafish. Aspirin was found to restore SD induced cognitive decline and improve the locomotor functions. Neuroinflammation and impaired functional network connectivity is linked to cognitive defects, which implicate the possible benefits of immunotherapeutics. In the present study, aspirin decreased neutrophil infiltration, and increased spine density in dentate gyrus granular and shrinkage and basophil in the CA1 neurons of hippocampus. This hints the benefit of aspirin on neuroimmune functions in sleep deprived fish and warrants more studies to establish the clear molecular mechanism behind this protective effect.

2. Introduction

Sleep is defined as a “reversible behavioral state of perceptual disengagement from and unresponsiveness to the environment” [1] and drives an interplay between physiological and behavioral processes. Sleep helps to maintain general system homeostasis and is reported to help conserve energy, promote growth and repair, and is also involved in increasing the efficiency of metabolic processes [2]. However, in post-industrial societies, socio-cultural engagements, technological and lifestyle trends are the prime factors that have contributed to disturbances in sleep patterns [3] and have led to an imbalance between endogenous circadian rhythms and light/dark cycle that affects sleep duration and quality [4].

Normal sleeping pattern induces long term potentiation (LTP), synaptic plasticity and promotes memory formation [5]. Sleep Deprivation (SD) is reduction in sleeping time below the baseline requirement [6] which is reported to cause learning and memory deficits [7]. Although the exact pathogenetic mechanism of action of SD on memory impairment remains elusive, over activation of microglial cells and increased nuclear factor (NF- κ B) in turn levels of proinflammatory cytokines such as Interleukin 1 beta (IL-1 β), Interleukin 6 (IL6), tumor necrosis factor alpha (TNF- α) are reported as crucial factors [8]. Cyclooxygenase (COX) enzymes play a crucial role in the neuro-inflammatory cascade by converting arachidonic acid (AA) to prostaglandin G₂ (PGG₂) and then PGG₂ to prostaglandin H₂ (PGH₂). The two isoforms, COX-1 and COX-2 show >60% homology at the protein level in humans and rodents [9] and show differential sensitivities to specific inhibitors. Cyclooxygenase-2 (COX-2) or prostaglandin (PG) H synthase is the key factor in various

inflammatory processes and its up regulation in the brain leads to increase in the production of proinflammatory cytokines [10].

Acetylsalicylic acid commonly known as “Aspirin” is a non-steroidal anti-inflammatory drug that inhibits COX enzymes and prevents prostaglandin secretion. It is often hailed as the ‘wonder drug’ [11]. Aspirin is reported to down-regulate prodynorphin (PDYN) and δ opioid receptors (DOR) and helps improve learning and memory [12]. Aspirin is also reported to improve the induction of LTP and persistent strengthening of synapses and thereby contributes to increased learning and memory [13]. Hence, it is prudent to investigate the cognitive benefits of aspirin and how it may counteract the defects in learning and memory *in vivo*.

Zebrafish is an emerging real-time model system for investigating neurodegenerative diseases and the discovery of neuro-specific drugs. A study showed the marked similarity in the neuroanatomic and neurochemical pathways between Zebrafish and human brains. “Physiological, emotional and social behavioral pattern” were also reported to be similar [14, 15]. The current investigation is the first to demonstrate its beneficial effects in sleep deprivation. In the present study, we used ‘extension of light phase followed by light pulses during the dark cycle’ to induce SD as it is shown to affect humoral innate immune system in Zebrafish which leads to neuroinflammation and cognition impairment [16, 17]. We then performed a rapid and cost-effective evaluation of aspirin in terms of restoration of cognitive and locomotor functions in sleep deprived Zebrafish model. Our data shows that aspirin is neuroprotective.

3. Materials and methods

3.1 Animals

Wild type 3 months old adult male Zebrafish (*Danio rerio*) were acclimatized for a period of 9 days. Fish were maintained on 12 h light and 12 h dark cycle (250 lux intensity) with zeitgeber time (ZT) where ZT indicates the beginning of day and lights were ON at 07:00 h and turned OFF at 19:00 h in a 50 L water tank (28 \pm 2 °C) with 6 fish each. Fish were fed with commercial (Nano fish food) fish diet 3–4 times a week.

3.2 Acute toxicity study

Acute toxicity profile of aspirin was assessed in Zebrafish as per OECD test guideline 203 [18]. Fish were acclimatized for 9 days (to the laboratory conditions including water source which includes 48 h settling period + 7 days acclimatization) and were fed thrice a week until 24 h prior to test drug. The experiments were carried out at room temperature and oxygen supply was at least 60% of air saturation value. Fish (n = 7/dose) were first exposed to 1 μ g/mL dose and ascending doses of 10, 100 and 1000

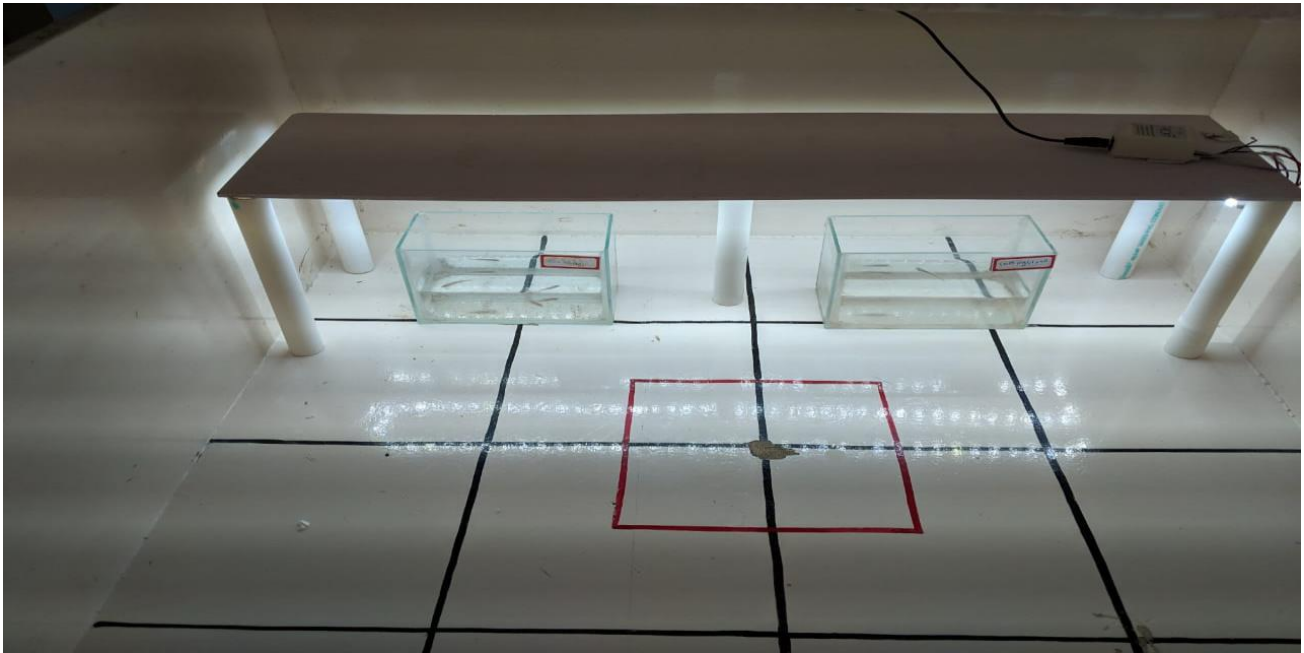


Fig. 1. Set up to induce sleep deprivation using light pulses. Two fish tanks and light source are shown.

$\mu\text{g/mL}$ were administered to separate set of animals at 96 h intervals. During the test procedure fish were observed 3 times/day at 24, 48, 72 and 96 h for mortality and/or any signs of morbidity (swimming behavior, immobility etc.). As 100% mortality was observed with 1000 $\mu\text{g/mL}$ dose within 24 h, the test with 1000 $\mu\text{g/mL}$ was repeated and confirmed for the mortality. However, there was no treatment related mortality at lower doses (1–100 $\mu\text{g/mL}$) until the completion of the experiment (96 h). Hence, further studies were carried out at safety dose range of 1–100 $\mu\text{g/mL}$.

3.3 Experimental design

Five groups of adult male Zebrafish ($n = 10$) were used in the present study. Group I: Non-sleep deprived group (without SD) was kept under regular light cycle (12 hL: 12 hD) with lights ON from 7:00 am to 7:00 pm (ZT0). Group II: Positive control (SD without any treatment) was kept at 18 h light and 6 h light pulses (which consists of 4 min white light and 1 min red light). Group III–V were subjected to SD and received aspirin treatments at three different concentrations dissolved in water tank for 3 days (1, 10 and 100 $\mu\text{g/mL}$).

3.4 Induction of sleep deprivation in Zebrafish

Light-Dark cycle is the major zeitgeber for circadian rhythm in most animals. Reports indicate that light possesses suppressive effect on sleep in Zebrafish, with no sleep rebound and consequent stress [19]. Sigurgeirsson *et al.* [20] compared SD induced by extended exposure to light and electric shock methods and found that normal sleep is altered by exposure to light and extended exposure to light causes less deviation from normal sleep wake states [20]. Hence, in the present study we have used light proto-

col to induce SD in Zebrafish and to evaluate its effect on learning and memory. SD protocol was carried out according to Pinheiro-da-Silva *et al.* [21].

In this study, SD was induced in fish by extending the light phase followed by light pulses during the dark cycle (4 min white light; 1 min red light). Non-SD group was kept in 12 L: 12 D cycles and the SD control and treatment groups were kept in light for 18 h (Extending the light phase) followed by 6 h light pulses. The apparatus used for this investigation is shown (Fig. 1).

3.5 Cognitive assessment in Zebrafish

3.5.1 T-maze

T-maze is a powerful tool for assessing learning and memory in Zebrafish. In the present study, T-maze memory test was done according to the method by Colwill *et al.* [22] with slight modification. The apparatus consists of two short arms ($20 \times 10 \times 10$ cm) wrapped with different colours-green and red. The red arm was considered as the unfavorable arm and the green arm was the favorable arm and it is followed by a wide space. The apparatus also had a long stem ($50 \times 10 \times 10$ cm). In addition, the apparatus was also equipped with Plexiglas that blocks the stem from short arms. The maze was filled with tap water to a height of 8 cm and maintained at 25 ± 2 °C.

Each fish was subjected to two training sessions per day. The fish was released from the end of long stem and allowed exploring for 2 min. Once it crosses the stem the junction is blocked with the help of the Plexiglas. If the fish enters the unfavorable arm, it was disturbed by stirring the water glass rod. Once the fish reaches the favorable arm, a reward of food pellet was given, and the fish was allowed

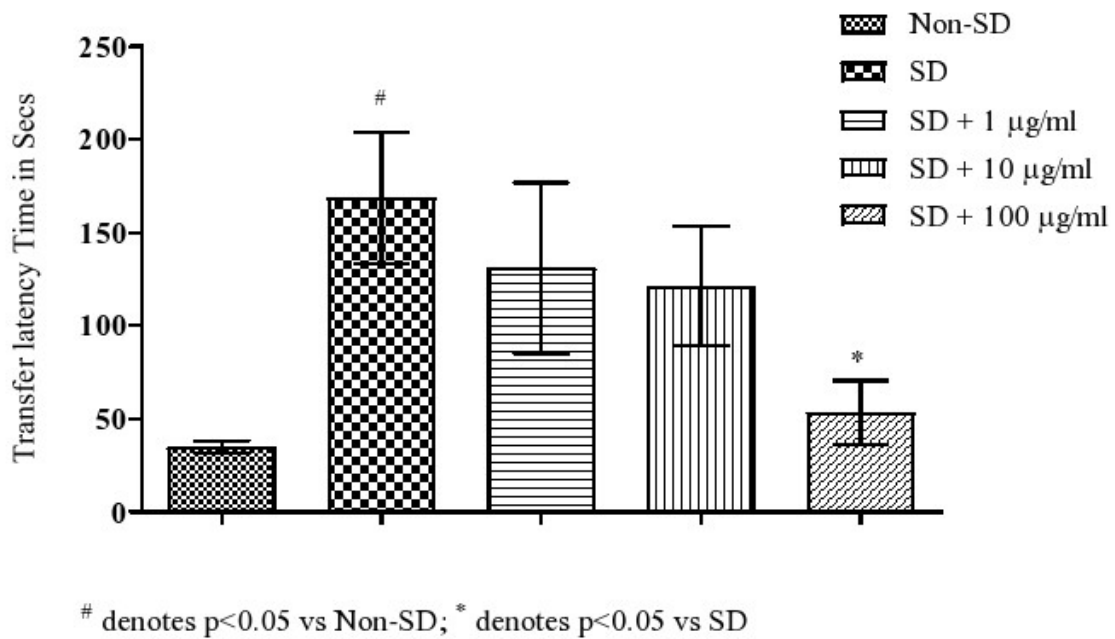


Fig. 2. Aspirin reduced the latency time to reach favorable arm in SD Zebrafish. Untreated sleep deprived Zebrafish took longer time to reach desired arm when compared to Non-SD Zebrafish (due to increased latency). Aspirin treatment was found to reduce the latency time in a concentration dependent manner when compared with untreated SD group. Data were analyzed by GraphPad Prism 5.0 (San Diego, USA software). # denotes $p < 0.05$ compared with Non-SD group, * denotes $p < 0.05$ compared with SD group. One-way ANOVA, and Tukey's multiple comparison tests was used for *post hoc* comparisons.

to swim in the wider space for 30 seconds. If the fish did not reach the favorable arm within the required time the fish was guided towards the goal. After 24 h of the second day training latency to reach the favorable arm was recorded to assess the learning and memory.

3.6 Motor function assessment

3.6.1 Partition preference test

Partition preference task assesses the locomotor activity in Zebrafish. The test was performed according to Dubey *et al.* [23]. The apparatus consists of glass chamber ($20 \times 10 \times 10$ cm) which is divided into four equal parts. The number of squares the fish crosses in 5 min gives an assessment on the locomotor activity.

3.6.2 Spinning task

Spinning task was performed to understand the motor coordination in Zebrafish. The task was performed in a 1000 mL capacity glass beaker which was filled up to 800 mL with water, along with a magnetic stir bar. The magnetic stirrer was transferred to the beaker and the beaker was covered with black walls to prevent external disturbances. Fish were kept in the beaker and allowed to explore for 2 min. Then the magnetic stirrer was turned on and the rotation speed was fixed to 400 rotation per minute (RPM). A whirlpool is formed in the beaker due to the rotation of the

stirrer. To avoid whirlpool and escape being swept, the fish tend to move towards the walls of the beaker. With the help of a stop watch the "swimming time" is determined which is defined as the time taken by the fish to be swept into the whirlpool (latency to be swept in the whirlpool) [24].

3.7 Histopathology

The brains of the fish from the treatment groups were isolated and fixed in Bouin's solution and were dehydrated in ascending grades of alcohol and cleared in xylene. The samples were embedded in paraffin blocks. Thin sections of $5-6 \mu\text{m}$ thickness were made with the help of a microtome and stained with haematoxylin-eosin stain [25] and observed for histopathological changes under microscope [26].

The pathological lesions in the various regions of brain were evaluated on ordinal scale with distinctive criteria for grading the changes and measured for its severity by counting the number of cells affected and the injury was graded as mild, moderate or severe [27].

3.8 Aspirin Bio distribution

In a separate study, a set of six Zebrafish were treated with aspirin at a concentration of $100 \mu\text{g/mL}$ for a period of 3 days. Following the treatment, the fish was euthanized by placing on ice. The brain was isolated with the help of a surgical scissor. Harvested tissues were homoge-

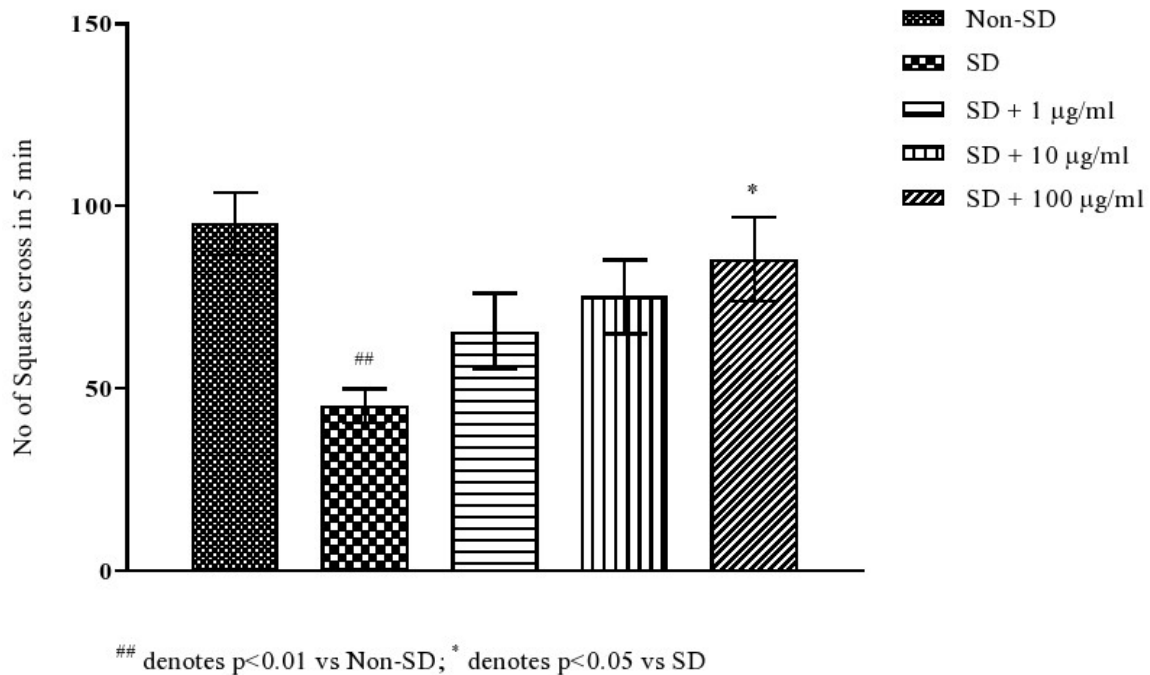


Fig. 3. Aspirin improved locomotor activity in sleep deprived Zebrafish. Sleep deprivation reduces the locomotor activity in Zebrafish when compared with Non-SD group. Treatment with aspirin (100 $\mu\text{g}/\text{mL}$) improves the locomotor activity in a concentration dependent manner significantly when compared to SD group. Data were analyzed by GraphPad Prism 5.0 (San Diego, USA software). ## denotes $p < 0.01$ compared with Non-SD group, * denotes $p < 0.05$ compared with SD group. One-way ANOVA, and Tukey's multiple comparison tests was used for *post hoc* comparisons.

nized in ice cold methanol. Homogenates were centrifuged and the supernatant was collected and subjected to High Performance Liquid Chromatography (HPLC) estimation. Aspirin was dissolved in methanol at standard concentrations of 2, 4, 6, 8, 10 $\mu\text{g}/\text{mL}$. Estimation was carried out by LC-20 AD, Shimadzu with C18 phenomenex column after 10 $\mu\text{g}/\text{mL}$ samples were injected into the column. Methanol : acetonitrile (65 : 35) was used as the mobile phase and the flow rate was adjusted to 1 mL/min. The samples were detected with photo diode array detector and the concentrations of aspirin were estimated using a spectrophotometer at 240 nm.

3.9 Data analysis

Data analyses were performed using GraphPad Prism 5.0 (San Diego, USA) software. Multiple comparisons were conducted using one-way ANOVA, and Tukey's multiple comparison tests was used for *post hoc* comparisons. p value ≤ 0.05 was considered as significant. Data were expressed as mean \pm SEM.

4. Results

4.1 Acute toxicity studies

Within 6 h of aspirin exposure at 1000 $\mu\text{g}/\text{mL}$, 100% mortality was observed in the Zebrafish. However, no mortality was observed at 1, 10 and 100 $\mu\text{g}/\text{mL}$ concentrations throughout the experimental period (96 h). Thus, we chose 1, 10 and 100 $\mu\text{g}/\text{mL}$ as treatment concentrations for evaluating efficacy.

4.2 Aspirin alleviated SD induced memory deficits in T-maze

SD fish took significantly ($p < 0.05$) longer latency in reaching the favourable arm when compared to the Non-SD group. Treatment with aspirin produced a reduction in the time taken to reach the favourable arm in a dose dependent manner. Though a decrease in time to reach the favourable arm was observed in aspirin exposure at all the concentrations only 100 $\mu\text{g}/\text{mL}$ showed a significant ($p < 0.05$) decrease in latency time (Fig. 2).

4.3 Aspirin reversed SD induced locomotor dysfunction in Zebrafish

Following SD Zebrafish were seen to have reduced locomotor activity. The number of squares crossed

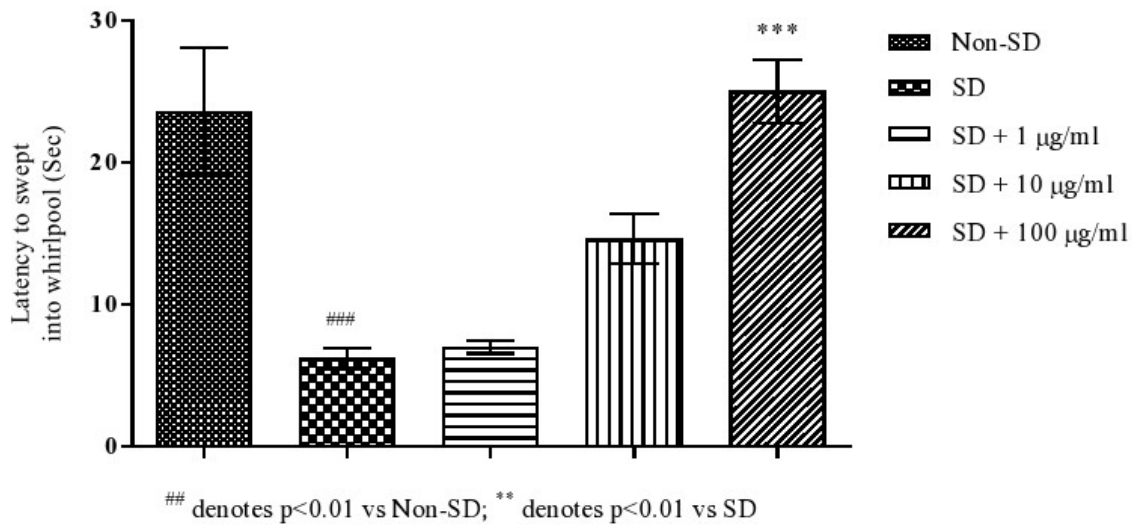


Fig. 4. Effect of aspirin on spinning task in sleep deprived Zebrafish. Sleep deprivation reduces the swimming time significantly when assessed using spinning task when compared to Non-SD group. Treatment with aspirin (100 $\mu\text{g}/\text{mL}$) improves the swimming time when compared to SD group. Aspirin increases the swimming time in SD group. Data were analyzed by GraphPad Prism 5.0 (San Diego, USA software). ### denotes $p < 0.001$ compared with Non-SD group, *** denotes $p < 0.001$ compared with SD group. One-way ANOVA, and Tukey's multiple comparison tests was used for *post hoc* comparisons. SD, Sleep Deprived.

in glass chamber in 5 min was used to assess the locomotor activity. SD significantly ($p < 0.01$) decreased the locomotion of Zebrafish when compared to Non-SD fish. Treatment with aspirin causes improvement in the locomotion and exploration in the SD Zebrafish. 100 $\mu\text{g}/\text{mL}$ concentration significantly increases ($p < 0.05$) the locomotion when compared to untreated SD fish (Fig. 3).

4.4 Aspirin improves the swimming time in spinning task

Spinning task experiment is used to assess the locomotor activity in Zebrafish. SD fish showed significant ($p < 0.01$) decrease in swimming time and decrease in the ability to cope up with whirlpool swept as compared to the Non-SD group. Treatment with aspirin produces a dose dependant increase in swimming time in SD fish. A significant increase in swimming time was observed at 100 $\mu\text{g}/\text{mL}$ dose (Fig. 4).

4.5 Aspirin bioavailability

Serial dilutions of aspirin were prepared at concentrations ranging from 2–10 $\mu\text{g}/\text{mL}$. The standard calibration curve is shown (Fig. 5A). A separate set of Zebrafish ($n = 6$) were exposed to aspirin (100 $\mu\text{g}/\text{mL}$) like SD study as above and brain tissues were isolated, homogenised and subjected to HPLC estimation. The aspirin content was found to be 12.35 $\mu\text{g}/\text{mg}$ in wet tissue (Fig. 5B,C).

4.6 Histopathology

The histopathological examination of brain tissues in Non-SD Zebrafish revealed normal neuronal and neu-

rophil architecture. The SD group showed neutrophil infiltration, reduced spine density of basal dendrites in dentate gyrus granular cells, shrinkage and basophilia of CA1 neurons of hippocampus. SD fish treated with aspirin at 100 $\mu\text{g}/\text{mL}$ showed increased spine density of dentate gyrus granular cells and hyperplastic CA1 neurons of hippocampal region. Also, SD fish treated with aspirin at 1 and 10 $\mu\text{g}/\text{mL}$ showed moderate improvement in neuronal cell degeneration (Fig. 6).

5. Discussion

The present study is the first scientific evidence that demonstrates the beneficial effects of aspirin in sleep deprivation and associated cognitive dysfunction. SD is one of the major risk factors that impair learning and memory. Adverse changes in cognitive performance have been associated with total and partial SD [6]. In the present study, fish exposed to extended light phase and light pulses showed decreased locomotor strength in partition preference and spinning task and increased working errors in T-maze tests, signifying the induction of SD. Learning a new task leads to increase in intracellular calcium (Ca^{2+}) and adenylyl cyclase leading to the formation of cyclic AMP (cAMP) [28]. cAMP activates cAMP response element binding protein (CREB) that upregulates expression of proteins involved in memory consolidation and long-term potentiation [29]. SD is reported to impair these signalling pathways and inhibit memory formation [30].

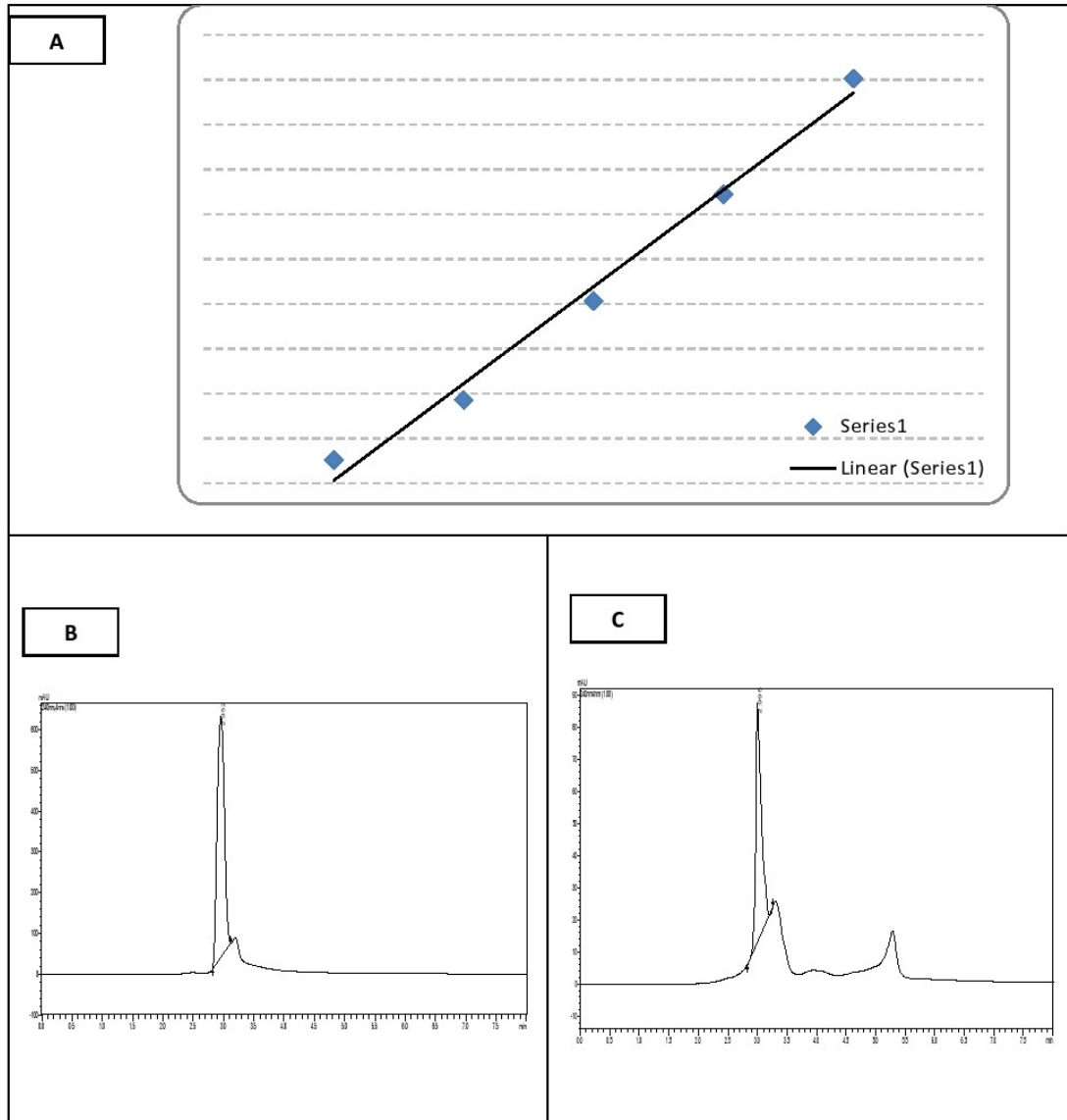


Fig. 5. Aspirin HPLC chromatogram. (A) Standard calibration curve for aspirin. The R^2 value was calculated using linear regression analysis. (B) Representative HPLC chromatograms of standard aspirin. (C) Representative chromatogram of aspirin detected in Zebrafish brain.

Reports indicate that sleep loss causes “a systemic low-grade inflammation” which is characterized by the release of cytokines, chemokines, and acute-phase proteins [31]. Both acute and chronic SD causes bursts of pro-inflammatory cytokines like hsCRP and TNF- α , and IL6 even in the absence of an infection or injury [32, 33]. Release of these cytokines and chemokines is reported to be associated with changes in the cellular components and disruption of Blood Brain Barrier (BBB) [34]. This causes the activation of microglial cells and impairs phagocytic activity ultimately leading to the accumulation of toxic metabolites in brain [35]. Investigations in the *in vivo* models have also confirmed that microglial overactivation leads to changes in synaptic maturation and plasticity [36, 37]. In addition, SD increases the deposition of amyloid- β ($A\beta$)

and tau proteins which over-activates microglial cells in the brain [38–40]. Accumulation of $A\beta$ increases the levels of pro-inflammatory cytokines such as TNF- α , IL6, IL-1 β , and granulocyte-macrophage colony stimulating factor (GM-CSF) [41] and promotes neuroinflammation.

Substantial evidences indicate that microglial activation causes cognitive dysfunction and loss of functional synapses in SD [42]. Treatment with minocycline, a microglial activation inhibitor, reverses the cognitive impairment [43]. Aspirin, the most used anti-inflammatory drug, acetylates COX1 and COX2 and decreases prostaglandin and thromboxane synthesis [44]. In the present study, we established the non-toxic concentrations of aspirin through acute toxicity assay and treated the Zebrafish with three different safe concentrations (1, 10 and 100 $\mu\text{g}/\text{mL}$) to assess

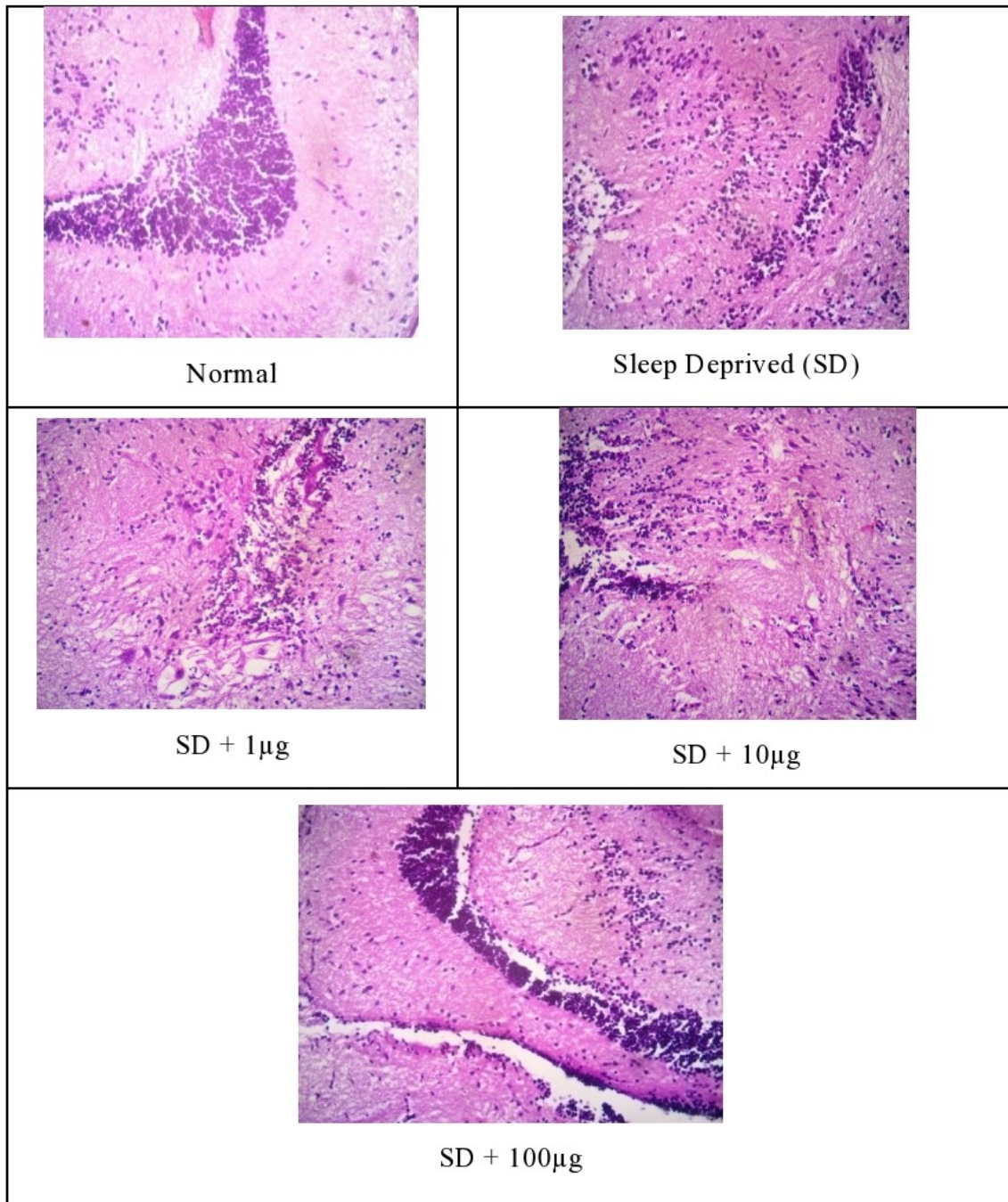


Fig. 6. Effect of aspirin on histopathological features of brain tissues in SD. The SD group showed neutrophil infiltration, reduced spine density of basal dendrites in dentate gyrus granular cells, shrinkage and basophilia of CA1 neurons of hippocampus. Aspirin (100 $\mu\text{g}/\text{mL}$) treatment increased spine density of dentate gyrus granular cells and hyperplastic CA1 neurons of hippocampal region. SD, Sleep Deprived.

if aspirin treatment can alleviate the cognitive and locomotor impairment caused by SD. Aspirin improved cognitive performance in T-maze and locomotor functions in partition preference and spinning task tests, revealing its beneficial role in sleep deprived state. These protective effects of aspirin correlate to its bio distribution in the brain of SD Zebrafish. Several mechanisms were reported on the neuro-

protective properties of aspirin [45]. Recent study showed that aspirin could inhibit microglial activation and reduce $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ release in LPS challenged microglial cells [46]. It is also said to activate the lipoxin A4, an endogenous lipoxygenase-derived anti-inflammatory mediator, through inhibition of $\text{NF-}\kappa\text{B}$ in BV2 microglial cells [47]. Microglial activation in the form of exacerbated

phagocytosis have been earlier shown to decrease synaptic protein levels in sleep deprived rats indicating on the role of microglial cells in cognition [42]. Thus, microglial inhibition is found to be viable target in sleep disorders and is associated with cognitive dysfunction. In alignment with earlier scientific reports, the improved cognition with aspirin treatment in the present study may be correlated to microglial inhibition in sleep deprived fish. Improvement in locomotor function with aspirin may be correlated to its effect on tyrosine hydroxylase and dopaminergic function [48].

Architecturally and functionally hippocampus consists of distinctive neuronal subfields such as cornu ammonis sectors (CA1-4), dentate gyrus (DG), and subiculum. Sleep disturbances are report to cause high degree of hippocampal CA1 atrophy. Interestingly, atrophy of CA2-4-DG region is linked to cognitive impairment [49]. Neutrophil invasion contributes to neurodegeneration possibly via the release of IL17 or neutrophil extracellular traps (composed of chromatin and proteases) or through microgliosis [50]. Furthermore, activation of neutrophils by microglial cells contributes to neuronal death [51]. We have recently reviewed the role body fluids in sleep [52]. In the current study, histopathological examination of brains of SD fish revealed increased neutrophils infiltration, reduced spine density and neurodegeneration in the dentate gyrus and CA1 regions of hippocampus. This was effectively reversed by aspirin and can be correlated to the improved cognitive performance. These data indicate the potential involvement of aspirin in mitigating the invasion by neuroimmune cells such as microglia, neutrophils, etc. in sleep deprived state. In line with this, we propose that investigations at the molecular level in SD Zebrafish and higher models may help extrapolate data to clinical trials and facilitate the repositioning of aspirin as a possible therapeutic option for SD and associated cognitive dysfunctions.

6. Conclusions

In summary, the present study demonstrates the beneficial effects of aspirin in improving learning and memory and locomotor activity induced by sleep deprivation in a Zebrafish model. Histopathological reports support the behavioural observations. These effects were proposed to be correlated to aspirin's role on neuro-immune and dopaminergic system. Further studies are warranted to establish the molecular mechanism in higher phylogenetic orders which may help to extrapolate data to clinical trials and facilitate the repositioning of aspirin as a possible therapeutic option for SD and associated cognitive dysfunctions.

7. Author contributions

MB and MA performed the study. MB analysed the data, and drafted the manuscript. AB, BR helped during

experiment. PE, JPR, LR, JY, SLC, MME helped in drafting manuscript. MKS and SBC designed the study, corrected and finalised the manuscript. All authors have given their final approval for the manuscript.

8. Ethics approval and consent to participate

Not applicable.

9. Acknowledgment

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11. Conflict of interest

Authors declare no conflicts of interest.

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Abbreviations: $A\beta$, Amyloid- β ; AA, Arachidonic Acid; BBB, Blood Brain Barrier; cAMP, cyclic COX, Cyclooxygenase; CREB, cAMP response element binding protein; GM-CSF, Granulocyte-macrophage colony stimulating factor; HPLC, High Performance Liquid Chromatogra-

phy; IL-6, Interleukin 6; LTP, Long Term Potentiation; NF- κ B, Nuclear Factor κ B; PDYN, Prodynorphin; PGG₂, Prostaglandin G₂; PGH₂, Prostaglandin H₂; RPM, Rotation Per Minute; SD, Sleep Deprivation; TL, Transfer Latency; ZT, Zeitgeber Time.

Keywords: Sleep deprivation; Acetylsalicylic acid; T-maze; Spinning task; Cognition; Neuro-immune; Zebrafish

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