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RESEARCH ARTICLE

Hypoxia and its effects on learning and memory in a zebrafish model

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ABSTRACT

Background: Zebrafish (*Danio rerio*) is the new model for human diseases. About 70% of its genome matches with that of human and 84% of diseases can be modeled in zebrafish. Zebrafish has been established as a complimentary model to the classic rodent models in the modern day's animal studies. Learning, memory, and intelligence are the most vulnerable functions due to hypoxia of brain. This study aims to model a hypoxic state in adult zebrafish and delineating its impact on cognitive functions of the brain with regard to learning and memory using a visual discrimination test. **Aim and Objective:** The objectives of this study are to create a zebrafish model for learning and memory and to study the effects of acute and chronic hypoxia on the same. **Materials and Methods:** Eighteen adult zebrafish were randomly divided into three groups (six in each): Group 1: Acute hypoxia exposed group (AHEG); Group 2: Chronic hypoxia exposed group (CHEG); and Group 3: SHAM (control group with no hypoxia exposure). All were subjected to color-cued plus maze test for 6 days for establishing memory to find the food in the red arm. Then, they were subjected to acute and chronic hypoxia and then the test is repeated again on days 9, 10, and 13 to check for short-term memory (STM) and long-term memory (LTM). The data were pooled and analyzed using SPSS by repeated measures of ANOVA. **Results:** AHEG group fishes showed significant prolongation in STM after exposure to hypoxia ($P = 0.031$), whereas LTM seems to be unaffected. CHEG group fishes showed significant changes in LTM but not in STM. STM and LTM unaffected in SHAM group. **Conclusion:** STM was affected due to acute hypoxia, whereas LTM did not have any profound effects due to acute or chronic hypoxia. Thus, this zebrafish model worked out to study the learning and memory, the results of which can be applied to humans.

KEY WORDS: Zebrafish; Hypoxia; Learning and Memory.

INTRODUCTION

Hypoxia is the imbalance between demand and supply of oxygen to tissues and organs. Brain consumes 20% of the oxygen supply to the body and brain cells can withstand hypoxia for a maximum of 5 min after which they begin to die. Hypoxia leads to the generation of reactive oxygen species or free radicals. Learning, memory, and intelligence are the most vulnerable functions due to hypoxia of brain.[1] Hypoxia to the brain due to trauma and ischemia can result in anterograde amnesia.[2] Intermittent hypoxia due to obstructive sleep apnea affects cognitive functions.[3] Individuals affected by stroke are at more risk of developing Alzheimer's disease.[4] The most complex behavioral phenomenon less studied and less explored is the learning and memory.

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Neuronal apoptosis has been demonstrated in people living in high-altitude areas where there is hypoxia and so they are more prone to decline in cognitive functions according to the years of exposure to such hypoxic conditions.[5] Glucosamine-associated metabolic pathway has been studied as a target

area for the reversal of hypoxia-induced neuroinflammation in the brain.[6] Increase in interleukin-1 levels during hypoxia which is related to cognitive functions may be the cause of the impairment of learning and memory. Three days intermittent hypoxia produced impairments in spatial memory in rat models of obstructive sleep apnea.[7] Spatial memory (water maze learning) is impaired in infant as well as adult rats exposed to hypobaric hypoxia. There was hypertrophy of hippocampus, delayed maturation of neurons, and decreased number of microglia in the cortex as well as the hippocampus.[8] Schizophrenia in adolescence and adults can be linked to exposure to hypoxia during fetal or neonatal life.[9] Hypoxia is an important cause of brain injury which causes memory loss and learning deficiencies.[10]

Zebrafish (*Danio rerio*) is the new emerging model for human diseases. About 70% of the genome of zebrafish matches with that of human genome and 84% of human diseases can be modeled in zebrafish.[11] Easy acquisition, breeding, and natural habitat are also the factors favoring the usage of zebrafish for human research.[12] There is similarity in the organogenesis and development of cardiovascular, gastrointestinal, and nervous systems between humans and zebrafish.[13] Zebrafish is an excellent organism to study the mechanisms of learning and memory. Hence, zebrafish has been established as a complimentary model to the classic rodent models in the modern day's animal experimental studies.[14]

Adult zebrafish and larvae forms are being used in neurobehavioral studies.[15-17] The mechanism of learning and memory is a complicated process involving thousands of neurons and synapses, and many of the details of the underlying mechanisms are yet to be established or proved. Incidence of Alzheimer's disease worldwide is doubling up every 20 years.[18] Many neuroprotective agents like traditional Indian herb curcumin in turmeric,[19] a traditional Chinese herb *Ginkgo biloba,*[20] have been extensively studied in rat models. The utility of elevated plus maze test has been successfully used in rodent models for neurological studies.[21] The practical simplicity of zebrafish for studying associative learning makes this an effective tool in neurobehavioral research.[22] Zebrafish can be used to study for translational research and the color-cued plus maze is a valid test to study learning and memory.[23] Thus, zebrafish has become the emerging new model for studies on neurobehavioral research, more importantly for anxiety- and stress-related states.[24]

Developing similar models in zebrafish for studying learning and memory with an intervention tool will be a preliminary step in further planning to assess the effect of many other neuroprotective agents on learning and memory. The three-chamber task is a sensitive test for studying learning and memory in zebrafish.[25] Classical conditioning is a successful model to study associative learning in zebrafish. In this study, we have adapted the associative learning using color-coded food arena for the zebrafish to find and reach the food target. The classical conditional learning could be better accomplished by zebrafish more than 6 weeks of age and thereafter,[26] and hence, in this study, we have used zebrafish above 8 weeks of age. Color preferences in cages and mazes and its neurological impact have been demonstrated in rat models.[27] As hypoxia to the brain predominantly affects the cognition, hypoxic intervention could be a better and simpler method to study the effects after establishing learning and memory in the zebrafish using a color-cued plus maze visual discrimination test. Hence, the main objective of the study was to model a hypoxic state in adult zebrafish and delineating its impact on cognitive functions of the brain with regard to learning and memory.

MATERIALS AND METHODS

The study was done after approval the Institute Animal Ethics Committee and standard guidelines for maintenance and experimentation were followed. The study was done in the zebrafish research laboratory established in the physiology department of the institution. Eighteen adult fish were randomly selected for the experiments. The fishes were maintained in a 12 h light and 12 h dark cycle. The water temperature was maintained around 28.5°C. The fish were provided with dried worms as feed.

The fishes were divided into three groups as follows [Chart 1]:

Color-cued Plus Maze

The pots were sealed by aluminum foils. Aeration was provided through a small vent made in the foil. All the fishes were starved out of food for 2–3 days. All of them were subjected to color-cued plus maze (custom-made) test. The maze contained four arms of the same length. One of the arms led to a box where food was provided, the sides of which were covered with plastic red color sheet. The ends of the other arms were covered with other colors such as yellow and green. One arm of the maze was uncovered.[25,28] A representational diagram of the color-cued plus maze was given below [Figure 1].

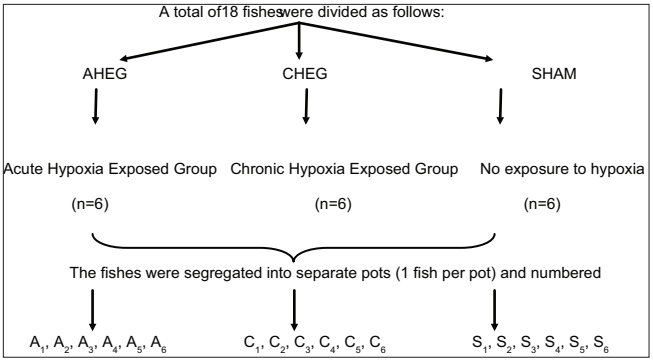

Chart 1: Grouping of Animals

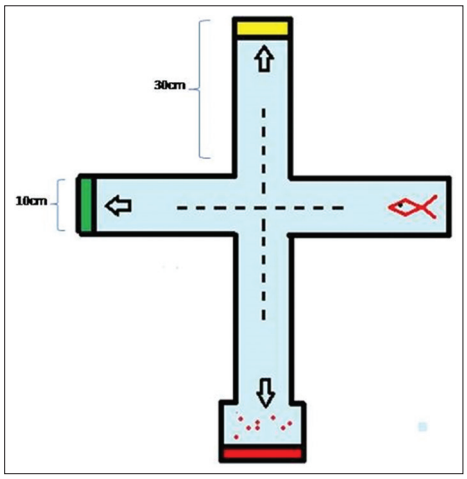

Figure 1: Color-cued plus maze, each arm measuring 30 cm × 10 cm with the red arm extended by a food arena which was 10 cm × 10 cm with an open and shut door. The fish were placed in the uncolored arm, the fish swing in different arms, when it reached the food arm, the door is shut and the fish were provided with feed

Training Period

Days 1–6

The acute hypoxia exposed group (AHEG) fish from pot 1 (A_1) were placed in the arm without any color cue. Feed was provided at the end of red-cued arm and the fish swam in various arms to reach the arm with the food. The time taken for this (working memory [WM]) was recorded using a chronometer and the fish were placed back in its pot. Next, the fish (C_1, S_1) from chronic hypoxia exposed group (CHEG) and SHAM, respectively, were subjected to the color-cued plus maze test, WM was noted. Likewise, all the fishes under each group were subjected to color-cued plus maze tests on day 1. Similar experiments were conducted on days 2–6. The fish were able to find and reach the food arm in lesser time as the trials progressed. A total number of six trials were given for all the fishes at the rate of 1 trial/day. The color cues were interchanged during each trial except in the red-cued food arm. No intermittent food was given during this trial period. Short-term memory (STM) for finding the food in the correct arm of the maze was established during the trial period. At the end of the trial period, the fish under CHEG alone were maintained in pots sealed without any aeration for 24 h to create a relative hypoxic environment. The fishes under the SHAM and AHEG groups were maintained with aeration.

Days 7 and 8

Once the STM was established, on the next day (7th day), the fishes were subjected to hypoxic stress. A fish from AHEG (A_1) were subjected to acute hypoxia by suspending in the air for 7–8 s. Then, the fish again were placed in the pot. After recovery, it was subjected to color-cued plus maze test and the time taken for finding the food STM was noted. Similarly, it was done for the other fishes (A_2–A_6) under this group. Then, the fish under CHEG (already maintained in pots without aeration) and SHAM group were also subjected to color-cued plus maze test and time taken for STM was noted. The same experiment protocol was followed on the 8th day also and readings were noted.

Days 9–12

All the fishes were maintained in their respective pots with aeration from days 9 to 12. This time period was allowed for the STM progress to long-term memory (LTM).

On 13th Day

The same color-cued plus maze experiment was repeated for all the fishes in the three groups on the 13th day to check for LTM. All these events (days 1–13) were recorded with a video camera for any future references.

Statistical Analysis

The data collected were transported to Microsoft excel. The memory curves (STM and LTM) were plotted for all the three groups (AHEG, CHEG, and SHAM) and compared. Graphical analysis was done with Igor pro software version 6 and tests of statistical significance were done using SPSS software version 23 by repeated measures of ANOVA.

RESULTS

During the Training Period

The fishes were fasted for 3 days and then subjected to color-cued plus maze test. On day 1, the fishes swing in various arms and reached the food arm where the food reward was given. All the group fishes under AHEG, CHEG and SHAM groups took longer time during the days 1 and 2. However, as the trial was continued, the fishes could swim and reach the food arm much earlier than day 1 as evident from the graphs shown below [Figure 2a-c].

The WM was slowly established in all the groups which were evident from the significant decrease in WM time and STM as the days progressed, hence, this implies that the memory for the food arm with the use of colors was very well established in the fishes during the training period of days 1–6 [Table 1a]. Similarly, when we compared the construction of STM during the training days in all the groups, there were no significant differences between the groups as it is obvious from *P* value as depicted in Table 1b.

After the Training Period

After exposure to hypoxia, fishes under CHEG group did not show much change in their STM, but the LTM was significantly affected in these fishes as they took longer time

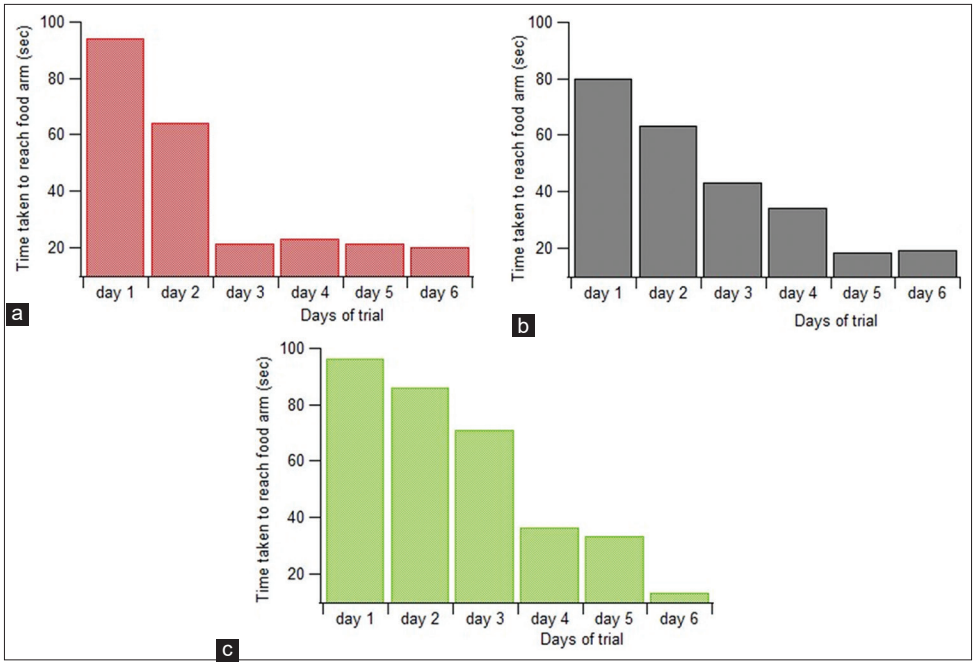

Figure 2: (a) Average time taken for memory on different days by fishes under Group 1: AHEG: Acute hypoxia exposed group; the time taken to reach the food arm significantly decreased ($P = 0.029$). (b) Average time taken for memory on different days by fishes under Group 2 – CHEG: Chronic hypoxia exposed group; in this group, there was a significant reduction in time between day 1 and day 6 ($P = 0.007$). (c) Average time taken for memory on different days by fishes under SHAM group – control group without any hypoxic exposure. There was a significant establishment of memory of food arm on day 6 ($P = 0.011$)

Table 1a: *P*-value of establishing STM during the training period of days 1–6. Significant differences were observed in all the groups. STM established

Training period (day 1 vs. day 6) before hypoxia exposure	*P*-value
SHAM	0.011*
AHEG	0.029*
CHEG	0.007*

P<0.05 is considered significant. AHEG: Acute hypoxia exposed group, CHEG: Chronic hypoxia exposed group, STM: Short-term memory

Table 1b: *P*-value of establishing STM during the training period of days 1–6. No significant differences were observed in all the groups. STM established

Training period (values of day 6 STM compared between groups)	*P*-value
SHAM versus AHEG	0.873
SHAM versus CHEG	0.962
AHEG versus CHEG	0.884

P<0.05 is considered significant. AHEG: Acute hypoxia exposed group, CHEG: Chronic hypoxia exposed group, STM: Short-term memory

to reach the food arm on the 13th day. Fishes under AHEG group showed significant difference in the both STM and LTM [Table 2]. SHAM group of fishes could reach the food arm earlier on day 8 and day 13, as they could on day 6, because they were not exposed to hypoxia, hence, the consolidation of LTM was unaffected and STM retained.

Table 2: *P*-values of the STM and LTM of the three groups (SHAM, STM, and LTM) before and after exposure to hypoxia for the test groups and the SHAM group without exposure to hypoxia

Before and after exposure to hypoxia	STM (*P*-value) (day 6 and day 8)	LTM (*P*-value) (day 6 and day 13)
SHAM	0.062	0.061
AHEG	0.031*	0.035*
CHEG	0.650	0.001*

P<0.05 is considered significant. AHEG: Acute hypoxia exposed group, CHEG: Chronic hypoxia exposed group, STM: Short-term memory, LTM: Long-term memory

DISCUSSION

In our study, learning and memory model was successfully created using the color-cued plus maze test. Conditional learning with positive rewards in terms of providing food when the zebrafish reaches the food arm helped in the establishment of STM in all the three groups of fishes (SHAM, AHEG, and CHEG). There was significant establishment of STM in all the three groups of fishes, as demonstrated by the significant *P* value between day 1 and day 6 of conditional learning in color-cued plus maze. The STM was consolidated into LTM in the SHAM group of fishes and the fishes were quickly able to reach the food arm on day 14 with significant difference when compared with day 1, which proves the establishment of LTM in this group. The fishes under test group exposed to acute hypoxia (AHEG) had effects on both the STM and

LTM, whereas the fishes under test group exposed to chronic hypoxia (CHEG) had affected the establishment of LTM only, and the STM was unaffected.

Hinz *et al*. have demonstrated zebrafish larvae exhibiting long-term associated memory by spatial preference when provided with food reward.[29] Sison *et al*. demonstrated the zebrafish acquiring significant association between the cue, reward, and the location of the reward in the plus maze.[30] Similarly, in our study also, hypoxic ischemic brain damage is a serious cause of learning and memory dysfunction. Similar results were reported in young rats exposed to hypoxic ischemic damage of brain where selective and LTM impairment were prominent in plus maze and eight-arm maze tests.[31] A study done by Kumari *et al*. has found correlation between hypobaric hypoxia and neurodegenerative changes in the limbic system.[32] It is known to cause cognitive impairment and synaptic dysfunction.[33] Connections between limbic system and the hippocampus favor the formation of associative memory.

Only consolidation of LTM was affected in test group fishes exposed to chronic hypoxia (CHEG). The mechanisms of long-term potentiation and depression which convert the STM could be affected due to the exposure to chronic hypoxia in these fishes. Pradel *et al*. have reported that antibodies against cell adhesion molecules in central nervous system have inhibited the consolidation of memory in a zebrafish learning model.[34] Prefrontal cortical structure damage and reduced neuronal density were reported in rats exposed to chronic intermittent hypoxia.[35] Similar damage could have occurred in the CHEG group of fishes as well which showed significant reduction in the LTM, but here the fishes were exposed to continuous low-grade hypoxia for three consecutive days soon after the trial period of formation of STM using the food reward conditioning method.

Few of the limitations of the study were more accurate graphical tracking software could have supplemented the data obtained for analysis. Exposure to hypoxia and its effects observed, but the oxygen saturation was not measured.

CONCLUSION

Hypoxic stress does affect cognitive functions. The LTM was predominantly affected than STM. Decline in memory due to insults to the vasculature of the brain and resultant hypoxia needs to be assessed, considering the financial, social, and psychological impact, it may produce in the patient. Preventive interventions at the primary, secondary, and tertiary levels need to be emphasized to prevent the progress of such decline in memory process. This hypoxic zebrafish model will be useful for further studying the pathophysiological mechanisms that underlie the hypoxic damage to the memory process and the reversal of the mechanisms using some neuroprotective agents.

REFERENCES

1. Sun MK. Hypoxia, ischemic stroke and memory deficits: Prospects for therapy. IUBMB Life 1999;48:373-8.
2. Chiu GS, Chatterjee D, Darmody PT, Walsh JP, Meling DD, Johnson RW, *et al*. Hypoxia/reoxygenation impairs memory formation via adenosine-dependent activation of caspase 1. J Neurosci 2012;32:13945-55.
3. Dumitrascu R, Heitmann J, Seegar W, Schulz RW. Obstructive sleep apnea, oxidative stress and cardiovascular disease: Lessons from animal studies. Oxid Med Cell Longev 2013;2013:234631.
4. Peers C, Pearson HA, Boyle JP. Hypoxia and Alzheimer's disease. Essays Biochem 2007;43:153-64.
5. Maiti P, Singh SB, Mallick B, Muthuraju S, Ilavazhagan G. High altitude memory impairment is due to neuronal apoptosis in hippocampus, cortex and striatum. J Chem Neuroanat 2008;36:227-38.
6. Lee Y, Lee S, Park JW, Hwang JS, Kim SM, Lyoo IK, *et al*. Hypoxia-induced neuroinflammation and learning-memory impairments in adult Zebrafish are suppressed by glucosamine. Mol Neurobiol 2018;55:8738-53.
7. Ward CP, McCoy JG, McKenna JT, Connolly NP, McCarley RW, Strecker RE. Spatial learning and memory deficits following exposure to 24 hours of sleep fragmentation or intermittent hypoxia in a rat model of obstructive sleep apnea. Brain Res 2009;1294:128-37.
8. Simonová Z, Sterbová K, Brozek G, Komárek V, Syková E. Postnatal hypobaric hypoxia in rats impairs water maze learning and the morphology of neurones and macroglia in cortex and hippocampus. Behav Brain Res 2003;141:195-205.
9. Manea MC, Puiu MG, Manea M, Constantin AM, Tache S. Learning and memory alterations in rats following postnatal hypoxia and effects of haloperidol; implications for schizophrenia. Indian J Appl Res 2015;5:347-52.
10. Shukitt-Hale B, Stillman MJ, Lieberman HR. Tyrosine administration prevents hypoxia-induced decrements in learning and memory. Physiol Behav 1996;59:867-71.
11. Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, *et al*. The Zebrafish reference genome sequence and its relationship to the human genome. Nature 2013;496:498-503.
12. Arunachalam M, Raja M, Vijayakumar C, Malaiammal P, Mayden RL. Natural history of Zebrafish (*Danio rerio*) in India. Zebrafish 2013;10:1-14.
13. Hsu CH, Wen ZH, Lin CS, Chakraborty C. The Zebrafish model: Use in studying cellular mechanisms for a spectrum of clinical disease entities. Curr Neurovasc Res 2007;4:111-20.
14. Levin ED, Cerutti DT. Behavioral neuroscience of Zebrafish. In: Methods of Behavioural Analysis in Neuroscience. 2nd ed., Ch. 15. Boca Raton, FL: CRC Press, Taylor and Francis; 2009.
15. Stewart AM, Kalueff AV. The developing utility of Zebrafish models for cognitive enhancers research. Curr Neuropharmacol 2012;10:263-71.
16. Kalueff AV, Stewart AM, Gerlai R. Zebrafish as an emerging model for studying complex brain disorders. Trends Pharmacol Sci 2014;35:63-75.
17. Norton W, Bally-Cuif L. Adult Zebrafish as a model organism for behavioural genetics. BMC Neurosci 2010;11:90.
18. Mayeux R, Stern Y. Epidemiology of Alzheimer disease. Cold

Spring Harb Perspect Med 2012;2:a006239.
19. Sarona JE, Ples MB, Vitor RJ. Neurotrophic effects of turmeric on the memory of the mouse using the Morris water maze test. Natl J Physiol Pharm Pharmacol 2018;8:726-31.
20. Krieglstein J, Ausmeier F, El-Abhar H, Lippert K, Welsch M, Rupalla K, *et al*. Neuroprotective effects of *Ginkgo biloba* constituents. Eur J Pharm Sci 1995;3:39-48.
21. Pellow S, Chopin P, File SE, Briley M. Validation of open: Closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Methods 1985;14:149-67.
22. Gerlai R. Associative learning in Zebrafish (*Danio rerio*). Methods Cell Biol 2011;101:249-70.
23. Sison M, Gerlai R. Associative learning in Zebrafish (*Danio rerio*) in the plus maze. Behav Brain Res 2010;207:99-104.
24. Stewart A, Gaikwad S, Kyzar E, Green J, Roth A, Kalueff AV. Modeling anxiety using adult Zebrafish: A conceptual review. Neuropharmacology 2012;62:135-43.
25. Levin ED. Zebrafish assessment of cognitive improvement and anxiolysis: Filling the gap between *in vitro* and rodent models for drug development. Rev Neurosci 2011;22:75-84.
26. Roberts AC, Bill BR, Glanzman DL. Learning and memory in Zebrafish larvae. Front Neural Circuits 2013;7:126.
27. Sherwin CM, Glen EF. Cage colour preferences and effects of home cage colour on anxiety in laboratory mice. Anim Behav 2003;66:1085-92.
28. Norton W. Learning in a Color Cued Plus Maze (Adult Fish). ZFIN Protocol, Behavioral Methods; 2012. Available from: https://www.wiki.zfin.org/pages/viewpage.action?pageid=27394156. [Last accessed on 2018 Feb 02].
29. Hinz FI, Aizenberg M, Tushev G, Schuman EM. Protein synthesis-dependent associative long-term memory in larval Zebrafish. J Neurosci 2013;33:15382-7.
30. Sison M, Gerlai R. Associative learning performance is impaired in Zebrafish (*Danio rerio*) by the NMDA-R antagonist MK-801. Neurobiol Learn Mem 2011;96:230-7.
31. Ikeda T, Mishima K, Yoshikawa T, Iwasaki K, Fujiwara M, Xia YX, *et al*. Selective and long-term learning impairment following neonatal hypoxic-ischemic brain insult in rats. Behav Brain Res 2001;118:17-25.
32. Kumari P, Kauser H, Wadhwa M, Roy K, Alam S, Sahu S, *et al*. Hypobaric hypoxia impairs cued and contextual fear memory in rats. Brain Res 2018;1692:118-33.
33. Kushwah N, Jain V, Dheer A, Kumar R, Prasad D, Khan N. Hypobaric hypoxia-induced learning and memory impairment: Elucidating the role of small conductance Ca^{2+}-activated K^{+} channels. Neuroscience 2018;388:418-29.
34. Pradel G, Schachner M, Schmidt R. Inhibition of memory consolidation by antibodies against cell adhesion molecules after active avoidance conditioning in Zebrafish. J Neurobiol 1999;39:197-206.
35. Li LQ, Cao JL, Li L, Han XQ, Wang HY, Liang XM, *et al*. The effect of chronic intermittent hypoxia on cognitive function and prefrontal cortex neurons in rats. Sichuan Da Xue Xue Bao Yi Xue Ban 2015;46:702-5, 725.

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