Activation of hippocampus mitochondrial Mn-SOD by a SIRT3 activator, honokiol, correlates with its deacetylation and upregulation of FoxO3a-PGC1α axis in a rat model of ammonia neurotoxicity

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Research Article

Keywords: Ammonia neurotoxicity, Hepatic encephalopathy, hippocampus, Mn-SOD, mitochondrial SIRT3, FOXO3a/PGC1a

Posted Date: April 5th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1503488/v1

Abstract

We have recently reported that honokiol (HKL), by activating mitochondrial SIRT3, restored ROS led deranged mitochondrial integrity associated with the pathogenesis of ammonia neurotoxicity induced moderate grade hepatic encephalopathy (MoHE). To delineate mechanism by which HKL does so, the present study describes activity vs acetylation level of the mitochondrial Mn-OD and its expression vs levels of its main transcription regulators; FOXO3a, PGC1α, in the hippocampus of the MoHE rat model of ammonia neurotoxicity, developed by administration of 100 mg/kg bw of thioacetamide i.p. for 10 days, and the MoHE rats treated with HKL (10 mg/Kg b.w.) for 7 days. As compared to the control, the hippocampus mitochondria from MoHE rats showed a significantly declined activity of MnSOD coinciding with the increased level of its acetylated form which however, could be restored back due the HKL treatment. Also, a significantly reduced expression of Mn-SOD in the hippocampus of those MoHE rats coincided with a similar decline in transcript level of FOXO3a and PGC1α. This was consistent with the reduced immunoreactivity of FOXO3a and PGC1α in the hippocampus DG, CA1 and CA3 regions of the MoHE rats. However, all these factors were observed to be restored back to their normal levels in the hippocampus of the MoHE rats treated with HKL. As HKL activates mitochondrial SIRT3, these findings suggest involvement of Sirt3 activation led deacetylation of MnSOD and upregulation of its transcription activators; FOXO3a and PGC1α in activating mitochondrial MnSOD in the hippocampus of the MoHE rat model of ammonia neurotoxicity.

Introduction

The persistent ammonia neurotoxicity, developed in the patients with chronic liver failure (CLF), results into development of a neuroexcitotoxic brain disorder, known as hepatic encephalopathy (HE), which is characterized mainly by the progressive loss of cognitive and motor functions (Felipo et al., 2012). The neurochemistry of HE is argued to implicate mainly glutamate-NMDAR over activation led deranged mitochondrial biochemistry at brain cells level (Jayakumar et al., 2004; Bai et al., 2001). Wherein, mitochondrial ROS load is considered one of the main precipitating events in deranging neuronal functions in most of the brain regions including cortical subregions, brain stem and cerebellum as the most affected ones in case of the HE (Bai et al., 2019; Guazzelli, et al., 2020). Thus, preventing/neutralizing mitochondrial ROS load in the cells of the susceptible brain regions could be a relevant approach to manage HE.

Mitochondria is the main oxygen consuming organelle and thus faces greater challenge of neutralizing metabolic O_2^- radical. Obviously, the mitochondrial reactive oxygen species (ROS) load is considered more critical in pathogenic mechanisms during brain disorders (Angelova & Abramov, 2018). The mitochondrial isoform of Mn-superoxide dismutase (Mn-SOD) is known to catalyze the committed step of neutralizing O_2^- radical. The literature available advocate the implication of this antioxidant enzyme in the pathogenesis of several diseases including the neurodegenerative brain disorders like; AD, PD, ALS etc. as most of them have been reported to implicate deranged mitochondrial functions (Flynn & Melov,

2013; Bresciani, et al., 2015). Obviously, Mn-SOD could be argued as a choice of therapeutic target in various pathologies, however, there was very little success. Alternatively, targeting certain up-stream regulatory steps involved in tackling ROS challenges is now emerging as a relevant approach so far preventing mitochondrial derangement during neurological disorders is concerned (Bhatti et al., 2017). This necessitates understanding the regulation of Mn-SOD in the mitochondria of the susceptible brain regions in a relevant brain disorder model.

In this respect, the development of the chronic type HE via hepatotoxin induced CLF in rats, one of the prevalent syndrome in the patients with liver cirrhosis, is neurochemically and neurobehaviorally argued as the most relevant model of neurexcitotoxicity that can be used to explore pathogenesis vs therapeutic management of HE (Singh & Trigun, 2010, 2014; Khanna et al., 2020).

So far regulation of Mn-SOD is concerned, in addition to modulating its expression, the alterations in Mn-SOD activity by modulating protein level acetylation of this mitochondrial isoform is emerging as an evolving concept in understanding ROS induced pathogenesis of the neurodegenerative brain disorders (Waddell et al., 2021). In this respect, Sirtuin3 (SIRT3), a mitochondrial isoform of SIRTUINs family deacetylase, is now emerging as a master regulator of the mitochondrial functions (Waddell et al., 2021; Meng et al., 2019) mainly by deacetylating a number of mitochondrial proteins amongst which Mn-SOD is argued to be the most important one (Meng et al., 2019). The enhanced Mn-SOD activity due to its deacetylation by SIRT3 is also on record (Santos et al., 2021). The information during recent past advocate that SIRT3 is likely to modulate Mn-SOD activity in multimodal ways. For example, SIRT3 induces transcription of Mn-SOD by increasing the level of its main transcriptional regulator Fox03a (Rangarajan et al., 2015; Ansari et al, 2017). Fox03a has been reported to protect quiescent cells from oxidative stress by increasing the expression of MnSOD (Kops et al., 2002). In response to elevated ROS, Fox03a has been demonstrated to translocate to the nucleus thereby activates transcription of MnSOD and Catalase (Olmos et al., 2009). And SIRT3 has been found to enhance FoxO3a translocation to the nucleus and augments FoxO3a-dependent antioxidant defense mechanisms through upregulating the levels of peroxisome proliferator-activated receptor-gamma coactivator 1-alpha (PGC-1α) and SOD2 (Zhang et al., 2016).

Though information is limited, it is now evident that enhanced SIRT3 activity associates with protection of neurons in culture exposed to excitotoxic and metabolic stress mainly by interacting with the mitochondrial MnSOD (Cheng et al., 2016). It has been demonstrated that Honokiol, a SIRT3 activator, is shown to improve spatial memory functions during amyloid-β (Aβ)-induced cognitive impairment in a transgenic mouse model, wherein, it involves the enhancement of PPARγ and decline of proinflammatory cytokines (Wang et al., 2018). These reports strongly suggest about a potent neuroprotective role of Sirt3 possibly by deacetylating Mn-SOD and/or by modulating transcriptional regulators of this antioxidant enzyme. Indeed, in the models of non-neuronal cells with a condition of nutritional manipulation, the SIRT3 deleted mice showed ~ 85% increase in the level of acetylated Mn-SOD (Tyagi et al., 2018). Also, SIRT3 activation in response to the increased oxidative stress could enhance the status of deacetylated Lys 122 of MnSOD leading to the enhanced Mn-SOD activity (Ozden et al, 2011). However, the study

about SIRT3 activity vs Mn-SOD acetylation/deacetylation status in the brain cells and in particular, in the animal models of a neurological disorder remains largely unexplored.

In order to map out the mechanism by which SIRT3 activation could exert its neuroprotective effects at mitochondria level in the animal models of neurological disorders, the use of honokiol, a SIRT3 specific activator is advocated as the most suitable one (Pillai et al., 2015,2017). Honokiol is a bioactive, phenolic compound, obtained from lignin of the bark of magnolia trees, which is described to maintain mitochondrial integrity via SIRT3 activation (Pillai et al., 2015). So far, its neuroprotective effects are concerned, information is limited, however, it was found to prevent age-related learning and memory impairment and neuronal deficits in senescence-accelerated mice (Woodbury, 2013). Moreover, this plant derived compound is known to cross blood brain barrier and even found to cross through the mitochondrial inner membrane as well (Wang et al., 2011). Indeed, we have recently demonstrated a correlation between the declined SIRT3 level and compromised mitochondrial function led enhanced ROS challenge in the hippocampus mitochondria of the MoHE rats (Anamika & Trigun, 2021). Also, we found that SIRT3 activation by honokiol during MoHE, resulted in recovery of pathology associated mitochondrial ROS accumulation and deranged mitochondrial function.

Therefore, in order to delineate Mn-SOD centric mechanistic aspects of HKL mediated neutralization of mitochondrial ROS load in the hippocampus of the MoHE rats, the present study investigated activity of the mitochondrial Mn-SOD vs its acetylation/deacetylation status and expression of Mn-SOD vs profiles of its transcriptional regulators (FOXO3a & PGC1α) in the hippocampus (undergoes MoHE associated neuronal atrophy; Khanna et al., 2020) of control, MoHE and the MoHE rats treated with HKL.

Material And Methods

Animals and Chemicals

The study involved adult male Charles foster for experimental studies. The rats were maintained at recommended conditions of 12h:12h light/dark cycle and were fed with the advised diet and water ad libitum at a room temperature of 25 ± 2◦C under standard hygiene conditions. Rats weighing 150–180g were grouped as per experimental plan and were kept in separate cages. All the procedures on rats were performed as approved by the institutional animal ethical committee for the care and use of laboratory animals (F.Sc/IAEC/2016-17/233).

All chemicals used were of analytical grade obtained from E-Merck and Sisco research Laboratory, Mumbai (India).

Development of model

The chronic liver failure rat model of MoHE was developed by the administration of hepatotoxin, thioacetamide (TAA) and characterized as MoHE rats on the basis of neurobehavioural parameters as reported earlier from our lab (Singh & Trigun, 2010; Khanna & Trigun, 2016). MoHE rats were further

divided into two groups (six animals each) and rats from one of the groups were administered with HKL (10 mg/kg b.w dissolved in 1:1 DMSO: PBS) i.p. once daily, as described by Anamika& Trigun, (2021), referred as MoHE + HKL group. After 24 h of the last dose given, rats were sacrificed under euthanasia. The hippocampus was dissected out and processed for biochemical and molecular studies.

Preparation of mitochondrial extract

As reported earlier (Anamika & Trigun, 2021), the mitochondrial fraction was prepared in ice cold MSH homogenization buffer (225 mM Mannitol, 75mM Sucrose, 1mM EGTA, 10mM HEPES (pH 7.2) and 1% bovine serum albumin. Briefly, the tissue was homogenized in MSH buffer in ice and cellular debris was removed by centrifugation at 1200 x g for 5min. The supernatant so obtained was again centrifuged at 10,000 x g for 10min to isolate a crude mitochondrial pellet. The pellet was further washed twice with MSH buffer without EGTA and the pellet thus obtained was re-suspended in the MSH buffer without EGTA stored in a number of aliquots at -80ºC. The mitochondrial extract was prepared by 3–4 freeze-thaw cycles. Protein content in the extract was estimated by Lowry et al., (1951) method.

Activity assay MnSOD

SOD activity was assayed using the method described by Beauchamp & Fridovich (1971). The reduced riboflavin generates O_2 radical upon oxidation in air, which in turn reduces NBT forming a blue colored formazon. Briefly, the reaction was setup by mixing 0.1M phosphate buffer (pH7.8), 12mM L methionine, 70µM NBT, 0.2mM riboflavin and 3µM EDTA. The reaction was initiated with the addition of the mitochondrial extract undergone at least three cycles of freeze-thaw releasing its matrix content. One set of tubes were illuminated under light for 30 min and parallely another set was kept in dark. A similar reaction mixture without tissue extract was run simultaneously and was used as control. O.D. was recorded at 560 nm. The difference between light and dark sets was used to calculate unit of SOD, which was defined as the amount of enzyme that produced 50% inhibition of NBT reduction/min and activity was calculated as:

SOD (U) = ([(C-E)/(C/2) X (total volume of assay mixture/ amount of enzyme extract)] Where,

C = difference in absorbance of the control tubes kept for incubation in light and dark

E = difference in absorbance of the enzyme tubes kept for incubation in light and dark

Analysis of SOD activity by non-denaturing PAGE

Non-denaturing polyacrylamide gel electrophoresis was performed as described previously (Mehrotra & Trigun, 2012). 60 µg protein of hippocampal mitochondrial extracts of control and experimental rats were loaded on non-denaturing polyacrylamide gel and electrophoresis was carried out at 4ºC at constant voltage (100V) for 2h. The in-gel activity assay was performed by incubating the gel in 20 mL activity staining mixture containing 0.25mM NBT, 28µM Riboflavin and 28mM TEMED at 37ºC for 15–20 min. Biochemically, NBT and SOD in the gel compete for O_2 radical such that the SOD activity zone appeared transparent, while rest of the region became purple blue due to reduced NBT. After development of activity

bands, the gels were photographed and intensity of bands was quantified by gel densitometry using Alphaimager 2200 gel documentation software.

Western Blotting

The western blot analysis of Mn-SOD was performed following the previously described method from our lab (Anamika & Trigun, 2021). Briefly, mitochondrial extract equivalent 80µg protein was loaded onto 12% denaturing polyacrylamide gel and electrophoresis was carried at constant voltage of 100V followed by electrotransfer of proteins on nitrocellulose membrane at 35mA for 10-12hrs at 4°C. Efficiency of Protein transfer was assessed by Ponceau S staining and then the nonspecific binding of antibody to protein was avoided by blocking of the membrane using 5% nonfat milk dissolved in 1X PBS for 90min. The membrane was then incubated with primary antibody; anti MnSOD (1:1000), anti Ac-MnSOD (1:500) dilution prepared in blocking solution and was kept at 4ºC overnight. HRP-conjugated secondary antibody was used for final immunodetection using ECL western blotting detection kit. For loading control anti hsp60 (1:1000) was used. Using normalized densitometric values of MnSOD and Ac-MnSOD vs hsp60, was recorded using gel densitometry software Alpha Imager 2200.

Real time RT-PCR

Quantification of gene expression was carried out by real time PCR. At first, total tissue RNA was isolated using TRI reagent (Sigma Aldrich) following the manufacturer's protocol. Homogenate prepared in 1ml of TRI reagent was subjected to centrifugation at 12,000g at 4°C for 10 min and in the supernatant collected, 200µl chloroform was added and vortexed. After 2 min, the tube was again centrifuged at 12,000g for 10 min, at 4°C. The colourless upper aqueous phase containing RNA isolate was collected and 0.5ml isopropanol was added to it. After 5 min, the tube was again centrifuged at 12,000g at 4°C for 10 min. The RNA precipitated as pellet at bottom of the tube was washed in 75% ethanol and air dried. RNA pellet was dissolved in 60 µL DEPC treated water and subjected to DNase treatment (DNA free-Ambion) to remove any sort of DNA contamination. The RNA samples with value of A260/A280 ratio between 1.8-2.0 were used in the cDNA synthesis reaction. cDNA synthesis was done using Revert Aid first strand cDNA synthesis kit. The reaction mixture consisting 4µg RNA, 1 µL random hexamer primer, made up to12 µL with DEPC treated water was centrifuged briefly and the following components were added; 4µL of 5X reaction buffer, 1µL of RiboLock™ Ribonuclease Inhibitor (20U/µL), 2 µL of 10 mM dNTP mix, 1µL of reverse transcriptase to make a final volume of 20 µL. The components were incubated for 5 min at 25°C followed by cDNA strand synthesis for 60 min at 42°C. The reaction was terminated by heating at 72°C for 5 min. The cDNA was stored at -80°C. Rat gene-specific primers used were: *foxO3a* (Forward primer 5'-CTCCGCTCGAAGTGGAGCTGGAC-3', Reverse primer 5'-TACAGGAGACGTGGCCGACTCTG-3') ppargc1α (Forward primer 5'AGTCCCATACACAACCGCAG 3' Reverse primer 5'-CCCTTGGGGTCATTTGGTGA-3'); mnsod (Forward primer 5'-CGGGGGCCATATCAATCACA-3' Reverse primer 5'-GCCTCCAGCAACTCTCCTTT-3') GAPDH was used for normalization as a reference gene. The real time qPCR was done with an ABI Prism 7500 Sequence Detection System (PE Applied Biosystems, CA, USA). The PCR reaction mixture of (20 µL) consisted of 1µL of sample cDNA (diluted 1:10), 1µL of 10 pmol of forward and reverse primers and 10 µL of 2X Thermo Scientific SYBR

Green/ROX qPCR Master Mix and brought to final volume with RNase free water. PCR condition was: 95°C for 30 sec, followed by 40 cycles at 95°C for 5 sec, and 60°C for 20 sec. Real-time PCR data was analyzed using the ΔΔCT method, normalizing the Ct values of the indicated gene to the Ct values of GAPDH relative to a control sample.

Immunofluroscence

Briefly, at the end of the experimental dosage, the animals were sacrificed under ether anesthesia by trans-cardiac perfusion with 4% paraformaldehyde prepared in 0.1M phosphate buffer (pH 7.4) and the brains were dissected out, immersed in the same fixative for overnight at 4ºC (post fixation); thereafter the tissue was processed with graded sucrose solutions (15%, 30%, 30%; prepared in Tris buffered saline) keeping it at 4°C. The tissue blocks were prepared by embedding the brain in O.C.T. compound submerged in isopentane at a sub-zero temperature. The blocks were stored at -20º/-80º C for long term use. The hippocampus sections of 25µm thickness were cut for the immunostaining and the section area was marked using PAP pen which provides a thin-film like hydrophobic barrier around the section. The sections were washed thrice with PBS followed by incubation in permeabilization buffer containing 0.2% Triton X-100 for 10 min. The sections were then blocked using 1% BSA, prepared in PBST and left for 1h at room temperature in humified chamber. The slides were then incubated with the Primary Antibody (FoxO3a; 1:200 dilution; PGC1α, 1;100 dilution) in 1% BSA solution prepared in PBST and was kept at 4°C overnight in the humid chamber. The slides, after primary incubation, were washed with PBS and thereafter sections were incubated with alexa fluor tagged secondary antibody suitably diluted in 1% BSA/PBST solution for 1h at room temperature in humid chamber. The sections were counter stained with DAPI (1µg/ml) for 15min at room temperature. Excess of DAPI was removed by PBS washing. Finally, the sections were mounted in DABCO and were observed and photographed under the light microscope (Olympus BX63). The image analysis was done with ImageJ software on a sample of 6 randomly selected images of hippocampus areas from 6 rat brains.

Statistical analysis

The data have been presented as mean ± SD and were analyzed using one-way Anova and Tukey's as post hoc test analysis to find out the level of significance between Control *Vs* MoHE & MoHE *Vs* MoHE + HKL. The probability of $p < 0.05$ was taken as significant difference between the two groups, where $n = 6$.

Results

HKL dependent modulation of MnSOD: activity and acetylation status

Our previous report demonstrates that HKL could normalize the MoHE pathogenesis associated enhanced ROS level in the hippocampus mitochondria mainly by activating mitochondrial SIRT3 (Anamika & Trigun, 2021). To delineate whether HKL does so by modulating Mn-SOD, the mitochondrial isoform committed to neutralize O_2^- radical, the activity and acetylation status of this enzyme was

studied in the hippocampus mitochondrial fraction from the control, the MoHE and the MoHE rats treated with HKL. The findings of Fig 1 A & B indicate that there is a significant decline in the activity ($p<0.05$) as well as in the active level (measured through native PAGE; $p<0.01$) of MnSOD in the hippocampus mitochondria of MoHE rats in comparison to the control counterparts. However, treatment of MoHE rats with honokiol, could recover the activity of MnSOD ($P<0.001$), analyzed on both the parameters. Further, to ascertain the role of HKL dependent SIRT3 activation, the level of acetylated Mn-SOD was studied in all the three sets. It is evident that there was a significant increase in the level of acetylated MnSOD ($p<0.01$) in the hippocampus mitochondria from the MoHE rats as compared to the control rats, whereas the level of acetylated MnSOD was observed to be significantly lowered ($p<0.001$) in case of the MoHE rats treated with honokiol (Fig. 1C) thereby suggesting more deacetylation of this enzyme due to the HKL treatment and thus indicating a role of SIRT3 activation in normalizing Mn-SOD activity in the HKL treated MoHE rats.

Modulation of MnSOD expression due to HKL treatment

As presented in Fig. 2A &B, the expression of MnSOD at protein and mRNA level is observed to be declined significantly ($p<0.01$) in the hippocampus of MoHE rats as compared to the control rats. However, the expression of MnSOD was found to be activated (attaining even more than normal level) ($p<0.001$) in case of the honokiol treated MoHE rats.

HKL dependent expression of FoxO3a and PGC1α

FoxO3a are winged helix structures which binds to DNA and upregulates transcription of MnSOD. FoxO3a is dependent upon PGC-1α to regulate antioxidant genes. Also, PGC1α is a known regulator of mitochondrial functions as well. Therefore, to understand the mechanism of HKL dependent over expression of MnSOD in the hippocampus of the MoHE rats, Foxo3a and PGC-1α levels were monitored in the hippocampus of the control and both the experimental groups. As presented in Fig. 3A & B, it was observed that the transcript levels of both, foxo3a and pgc1α were sharply declined in the hippocampus of MoHE rats as compared to control rats. However, the mRNA of both these factors were found to be significantly enhanced ($p<0.001$) in response to HKL treatment to MoHE rats.

In order to ascertain protein level expression pattern, both these factors were also analyzed by immunofluorescence based detection of Fox03a and PGC1α in the three main hippocampus reagions; DG, CA3 and CA1.

According to Fig. 4A &B, most of the cells in the hippocampus DG region from the control rats showed a uniform distribution and intensity of FoxO3a and PGC1α signals. However, signals for both these factors were found to be declined significantly in case of the MoHE rats. Nonetheless, after HKL treatment to the MoHE rats, immunoreactive signals for both; Foxo3a & PGC1α (Fig. 4A &B) were found to be recovered back near to the control level in the DG region. The signal intensity, analyzed through the image J

software, also matched well with the pattern of immunosignals seen for both the proteins in the hippocampus from control, the MoHE and the MoHE rats treated with HKL (Fig 4C).

Following a similar trend, the relative immunofluorescence intensity of anti FoxO3a and anti PGC1α, in CA1 region of hippocampus of MoHE rats was observed to be reduced with respect to the control rats (Fig. 5A &B). However, honokiol treatment of MoHE rats was found to restore the immunoreactivity for FoxO3a and PGC1α in CA1 hippocampus region of the MoHE rats (Fig. 5). The quantitative analysis also showed a similar pattern of signal intensities between the control, the MoHE and the HKL treated MoHE rats (Fig. 5C).

Similarly, the FoxO3a and PGC1α immunoreactivity in CA3 region, as depicted in Fig. 6A& B, we observed significantly reduced immunoreactivity of FoxO3a and PGC1α in the CA3 region of hippocampus of the MoHE rats, as compared to the control plates. However, there was a discernable increase in FoxO3a and PGC1α immune signal following HKL treatment to MoHE rats. A similar pattern was obtained when immunofluroresence intensity was recorded quantitatively (Fig. 6C). This shows that the SIRT3 activator, honokiol is able to enhance the expression level of transcriptional regulators of MnSOD.

Discussion

As mitochondria is the site of oxidative phosphorylation, it is likely to produce high amount of O_2^- radicals and thereby making this organelle the most susceptible one to undergo ROS led dysfunctions in the high energy demanding brain cells in particular. Thus, oxidative stress induced mitochondrial dysfunction is now considered the most common neurochemical aberration associated with the pathogenesis of many brain disorders. The neurotoxic effect of ammonia in CNS, symptomized mainly by the HE associated neuropsychiatric complications, is also argued to implicate enhanced mitochondrial ROS load mainly due to declined levels of antioxidant enzymes like; SOD, catalase etc. (Felipo, 2009; Heidari, 2019). The mitochondrial MnSOD is considered the committed enzyme to neutralize O_2^- radical and therefore, the declined level of this antioxidant enzyme has been reported associated with the pathogenesis of many brain disorders like AD, PD, ALS (Bresciani, et al., 2015). Consequently, by using knocked in experimental models, many workers have tried to manipulate the expression level MnSOD to prevent neurodegeneration in different neuropathology models (Flynn & Melov, 2013). However, to qualify Mn-SOD as a doable therapeutic target, there is need to define protein level reversible modification vs activity changes and the mechanism by which its expression is regulated during a neuropathogenesis.

There are reports suggesting activation of MnSOD by a number of compounds (Miriyala et al., 2012) however, the mode of action of most of them remain unexplained. In this respect, particularly in mitochondria, there is an evolving concept of activating this enzyme by a mitochondrial SIRT3 dependent deacetylation resulting into better cell survival (Santos et al., 2021). In case of the animal model of neurodegenerative brain disorders, such examples are limited. Moreover, in western diet fed $SIRT3^{-/-}$ mice model, an increased MnSOD acetylation could be correlated with the diminished activity of this enzyme (Tyagi et al., 2018). We have recently reported a cause and effect relationship between SIRT3 activation

by a natural compound honokiol (HKL) and recovery in the compromised mitochondrial functions in hippocampus of the MoHE rats (Anamika and Trigun, 2021). In this respect, the findings of Fig. 1(A-C) clearly demonstrate another cause - effect relationship between activation of Mn-SOD vs deacetylation of this enzyme due to the treatment with HKL. Though fragmentary but there are some reports on SIRT3 dependent deacetylation of certain ETS enzymes vis a vis normalizing the declined levels of those enzymes (Haigis et al, 2012). However, such reports are scanty in case of deacetylation of Mn-SOD vs recovery in ROS led mitochondrial dysfunction. Our previous report has demonstrated a correlation between the enhanced ROS level led compromised mPTP, declined ETS activity and redox ratio in the hippocampal mitochondria from the MoHE rats. However, all these parameters could be recovered back to the normal level due to SIRT3 activation by HKL (Anamika & Trigun, 2021). Herein, the finding of significantly declined level of the acetylated Mn-SOD (Fig. 1C) due to HKL treatment to the MoHE rats provides evidence to advocate Mn-SOD as another protein target of SIRT3 activation. Thus, suggesting about mechanistic aspect of how HKL could normalize ROS challenges in the hippocampal mitochondria of the MoHE rats. Additionally, this is a first report on HKL dependent alterations in the acetylation status MnSOD vs a similar pattern of its activity changes in mitochondria of a susceptible brain region of an MoHE animal model of excitotoxicity. The argument gets support from a report describing association of SIRT3 deletion with the enhanced acetylation led decreased activity of MnSOD which was found accountable for the increasing oxidative stress and decline of MPTP in an age-related loss of substantia nigra (SNc) dopaminergic neurons of the PD mouse model (Shi et al., 2017). A recent report in metal toxicity model has also shown that fluoride reduced mitochondrial antioxidant enzyme activities and elevated SOD2 acetylation by downregulating SIRT3 expression in the brain of mice and in the SH-SY5Y cells (Wang et al., 2021).

Another mechanism advocated accountable for maintaining MnSOD level in the brain cells is the regulation of its expression during pathogenesis and treatment. According to the findings from Fig. 2, a recovery in the MoHE associated declined expression of MnSOD, both at transcript and at protein levels, due to the treatment with HKL, clearly suggest about a significant role of HKL in the regulation of MnSOD expression in the hippocampus mitochondria. There are some reports, though on other neurodegenerative models, describing recovery in the Mn-SOD expression vis a vis normalization of the disease pathogenesis. In a Diabetic neuropathic pain (DNP) model in rats, upregulation of MnSOD in the spinal dorsal horn could be correlated with the pain reduction (Zhou et al., 2021). Another study suggests that a trans sodium crocetinate (TSC) could exert protection against cerebral ischemia/reperfusion (I/R) injury by increasing SOD2 protein levels and decreasing its acetylation (Chang et al., 2019). However, to our knowledge, there is a little information on the HKL dependent modulation of Mn-SOD expression in an excitotoxic brain disorder condition. Therefore, our finding of Fig. 2 necessitated investigation of how HKL could modulate expression of MnSOD expression in the hippocampus of the MoHE rats.

In most of the reports describing modulation of the expression of antioxidant enzymes, under the condition of physiological stresses, by a number of modulators, it was observed that in case of MnSOD, there appears concordant interplay of SIRT3-FoXO3a-PGC1α axis in transactivating this mitochondrial isoform of SOD. It has been suggested that FoxO3a forms a feedback loop with PGC1α to regulate

antioxidant genes and SIRT3 dependent deacetylation of these transcription factors has been argued as one of the mechanisms to regulate their expression and nuclear translocation to ultimately activate the ARE (the antioxidant response element) of the antioxidant enzymes genes (Olmos et al., 2009). Independently also, PGC1α is known to regulate mitochondrial function by stimulating antioxidant enzymes (Zhang et al., 2016). Also, the PGC1α is reported to trigger SIRT3 expression which by deacetylating MnSOD, normalizes the increased mitochondrial ROS (Wenz, 2013).

In view of our previous report, describing HKL dependent recovery in SIRT3 activity in the hippocampal mitochondria of the MoHE rats, it is argued that SIRT3 activation could be accountable for not only deacetylating Mn-SOD (Fig. 1C) but also for the modulation of FoxO3a-PGC1a axis as well. Indeed, we observed a remarkable increase in the transcript levels of both, the Foxo3a and PGC1α, in the hippocampal fraction of the MoHE rats treated with HKL (Fig. 3). Importantly, such a pattern of the enhanced expression of both these critical factors was consistent with a similar recovery in the abundance of both these proteins in all the three major regions of hippocampus; DG, CA1 & CA3, accountable for memory formation and consolidation, in the HKL treated MoHE rats (Fig. 4, 5 &6).

HKL is known to display strong anti-inflammatory and antioxidant properties in a variety of diseases including certain neuropathology as well. However, the mechanism by which it imparts its neuroprotective action remains largely unexplored. During recent past, SIRT3 is emerging as a master regulator of mitochondrial integrity and HKL is suggested as an activator of SIRT3 (Pillai et al., 2015; Anamika & Trigun, 2021) both *in vitro* and *in vivo*. Therefore, in the present context, it is argued that HKL dependent SIRT3 activation could be accountable to upregulate Mn-SOD mainly by modulating the levels of Foxo3a and PGC1α in the hippocampus of the MoHE rats. This is supported, though indirectly, by the similar findings on the multimodal modulations of SIRT3-FoxO3a-PGC1α axis including SIRT3 dependent deacetylation of both the transcription factors due to the treatment with various other compounds.

For example, Chronic fluoride exposure has been found to induce mitochondrial dysfunction through inhibition of Sirt3/FoxO3a signaling in the SH-SY5Y cell lines (Wang et al., 2021). The overexpression of spinal cord SIRT3 in DNP rat model has been demonstrated to increase the expression and deacetylation of FoxO3a to ultimately upregulate MnSOD (Zhou et al., 2021). Another study suggests that neuroprotective effect of trans sodium crocetinate (TSC) against cerebral ischemia/reperfusion (I/R) injury is mediated via increasing SIRT3 activity vs decreased acetylation of Foxo3a and SOD2 (Chang et al., 2019). SIRT3 dependent increased expression and nuclear translocation of FoxO3a have also been reported accountable to transactivate antioxidant enzymes like SOD in the activated microglia of the adult rats subjected to the traumatic brain injury (Rangarajan et al., 2015). Similarly, PGC-1α, is a multifunctional critical regulator of various cellular functions including mitochondrial quality by suppressing oxidative damage (Rius-Pérez et al., 2020). Importantly, FoxO3a has been found to protect cells from oxidative stress through direct interaction with PGC-1α in the promoter regions of the antioxidant enzymes (Olmos et al., 2009). In MPTP PD model, PGC1a-ERRα/SIRT3 pathway has been demonstrated to play critical roles in protecting DAergic neurons against oxidative damage and ATP depletion mainly by deacetylating SOD2 and ATP synthase β (Zhang et al.,2016). Also, PGC-1α

expression is reported to decline in the AD brain as a function of dementia severity (Qin et al., 2009). Taking together, SIRT3-Foxo3a-PGC1a axis act as a master regulator of maintaining the levels of the antioxidant enzymes and the mitochondrial Mn-SOD, in particular, however, evidently this axis seems to be a modifiable target in many ways and therefore, deserve special merit to be categorized as a targetable hot spot in neurological disorders.

There is no previous report on HKL dependent modulation of SIRT3-Foxo3a-PGC1a loop in modulating the antioxidant potential of the brain cells in a neurological disorder. Thus, our findings of Fig-3-6 are first of its kind to provide HKL dependent positive modulation of this axis that could be involved in normalizing ROS challenge emerged during MoHE pathogenesis.

Conclusion

ROS insult lies at the heart of mitochondrial dysfunction which serves as a central point of many neuropathology. The pathogenesis of ammonia neurotoxicity, in general and in the animal model of MoHE in particular, has also been described associated with the enhanced mitochondrial ROS load which could be demonstrated to be normalized due to SIRT3 activation by HKL (Anamika and Trigun, 2021). This article describes that HKL does so by deacetylating mitochondrial MnSOD and by recovering its expression level mainly due to HKL dependent increased expression of the constitutive type transcription factors; Foxo3a and PGC1a, of the antioxidant enzymes, in the hippocampus (the most susceptible brain regions to undergo neuroarchitectural aberrations) of the MoHE rats (Khanna et al, 2020). Thus, HKL is evident to counteract mitochondrial ROS challenge during MoHE pathogenesis by altering SIRT3 dependent acetylation status of MnSOD and by modulating Foxo3a-PGC1a axis to enhance expression of this antioxidant enzyme.

Declarations

Acknowledgment

This work was financially supported by a Govt of India DST-SERB project (EMR/2016/006501) to SKT. Anamika acknowledges the award of BHU fellowship, UGC CAS JRF and CSIR SRF. The instrumental facilities provided by BHU-DBT ISLS and DST-FIST in Department of Zoology are also acknowledged.

Declaration about the conflict of interest

The authors declare no conflict of interest

Declaration: This work was financially supported by a Govt of India DST-SERB project (EMR/2016/006501) to SKT. Anamika acknowledges the award of BHU fellowship, UGC CAS JRF and CSIR SRF.

Ethics Declaration

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figures

Fig. 1

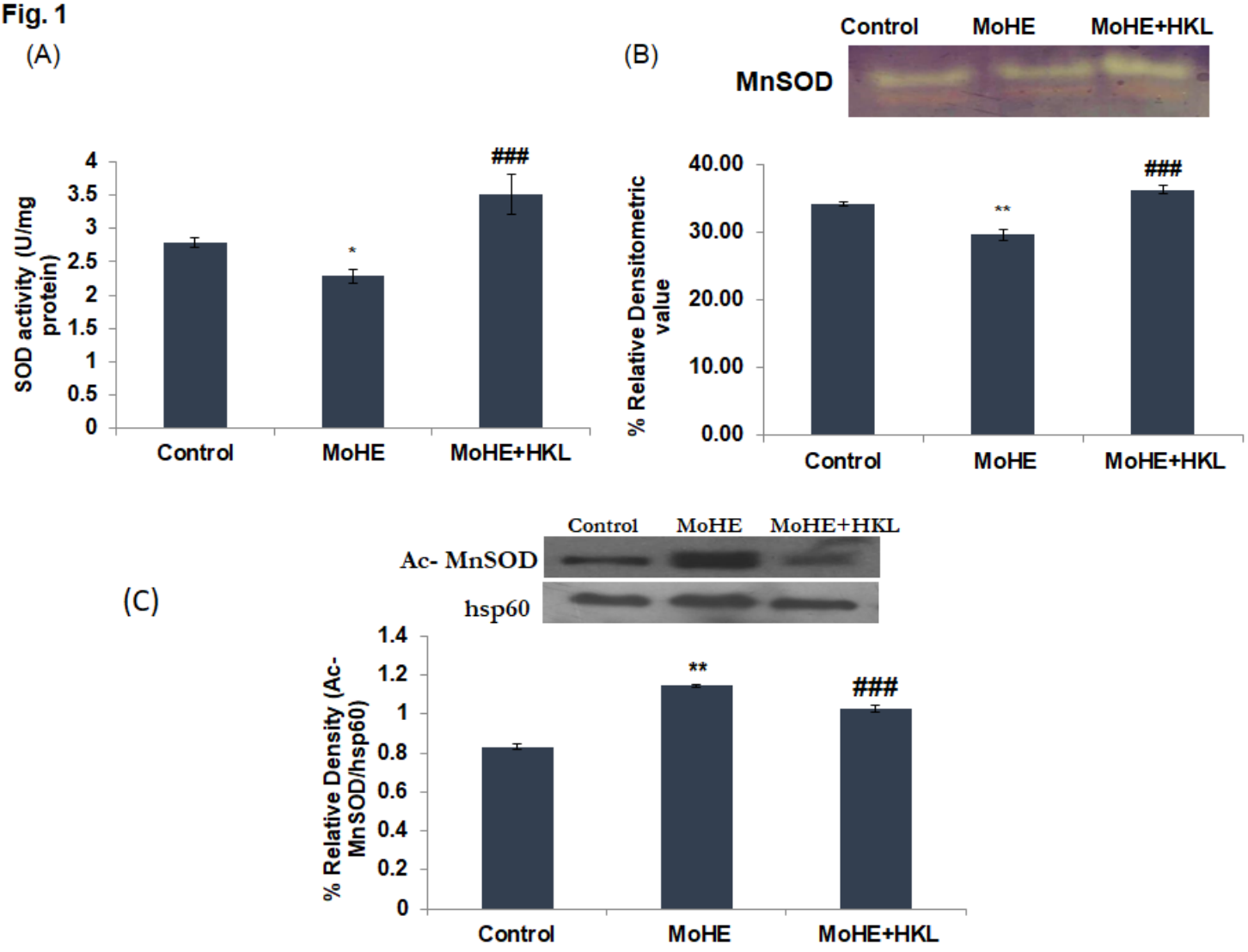


Figure 1

The effect of acetylation level on the MnSOD activity. (**A**) The spectrophotometric and **(B)** in-gel MnSOD activity profile in the hippocampus mitochondrial fraction from control, MoHE and MoHE rats treated with HKL. (**C**) Level of acetylated MnSOD in case of control, MoHE and MoHE rats treated with honokiol: Shows representative western blot photographs with 60µg protein in each lane. Lower panel shows values of MnSOD/Hsp60 as mean ± SD; n=6; ***p<0.001 (Control vs MoHE), ##p<0.001, ##p<0.001 (MoHE vs MoHE+HKL).

Fig. 2

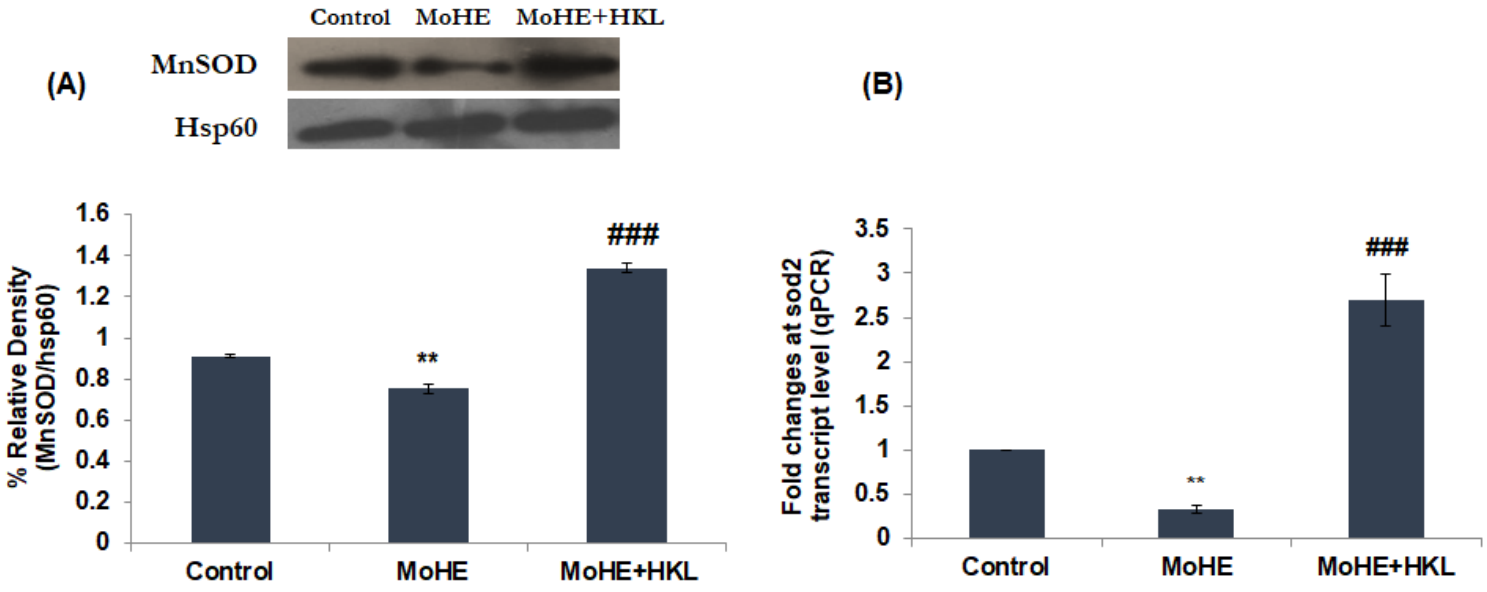


Figure 2

The expression of MnSOD at protein and mRNA levels in the hippocampus of control, MoHE and MoHE rats treated with honokiol. (A) Shows representative western blot photographs with 60µg protein in each lane. Lower panel shows values of MnSOD/Hsp60 as mean ± SEM out of the 3 repeats **(B)** Show qPCR result. The Cycle threshold value of mnsod mRNA level was normalized with the cycle threshold value of Gapdh for each sample to give a relative quantity of the mRNA expression by 2-ddCt .; Values are presented as mean ± SD n=6; ***p<0.001 (Control vs MoHE), ##p<0.001, ##p<0.001 (MoHE vs MoHE+HKL).

Fig. 3

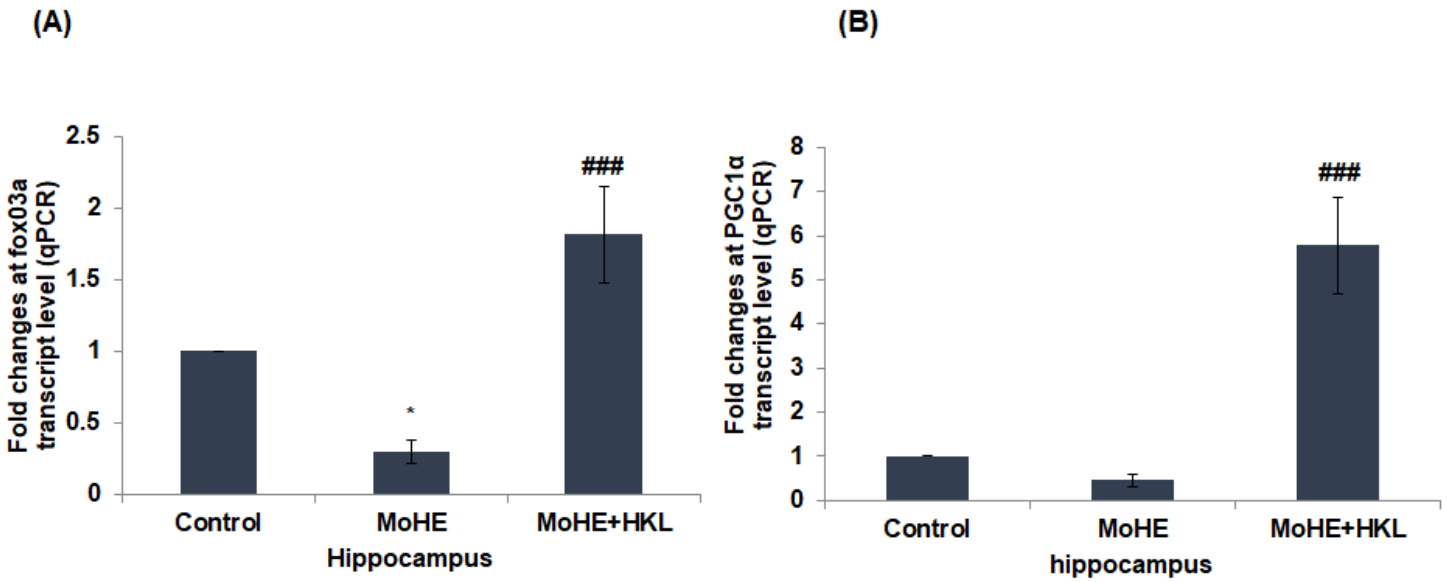
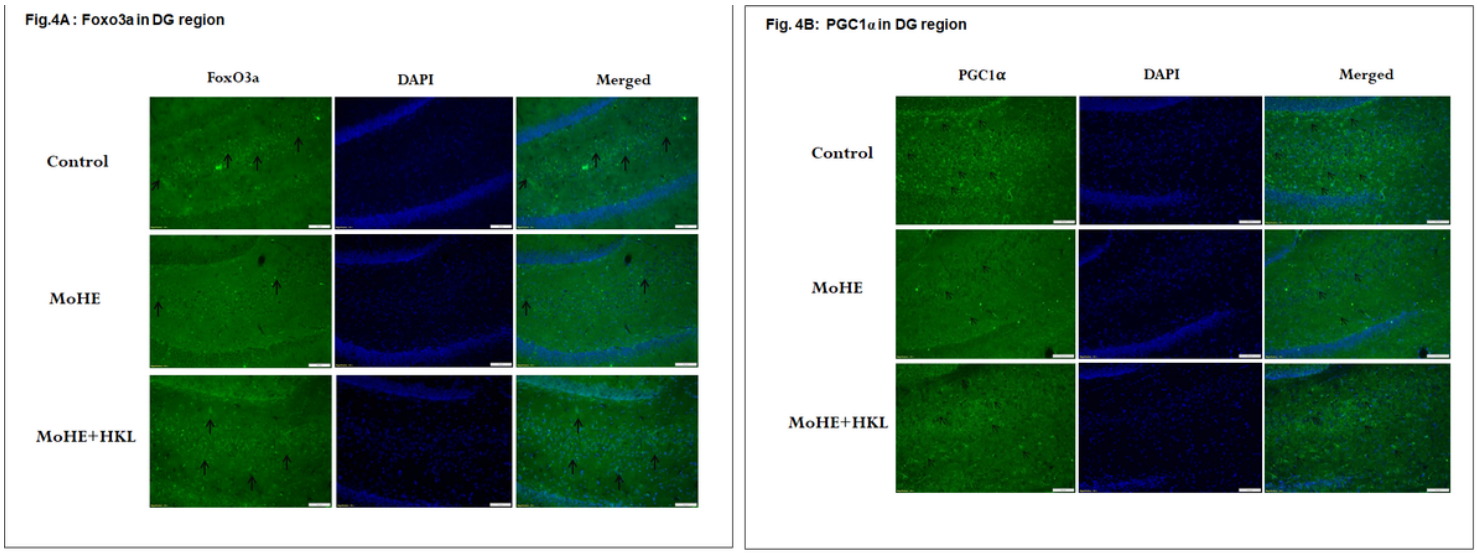


Figure 3

Level of FoxO3a and PGC1α in the hippocampus of control, MoHE and MoHE rats treated with honokiol. **(A)** & (B) show qPCR result of FoxO3a and PGC1α respectively. The Cycle threshold value of both the mRNAs level were normalized with the cycle threshold value of Gapdh for each sample to give a relative quantity of the mRNA expressed by 2-ddCt . Values are represented as mean ± SD; n=3; Gapdh was taken as the internal control. ***p<0.001, (control vs MoHE), ###p<0.001 (MoHE vs MoHE+HKL).



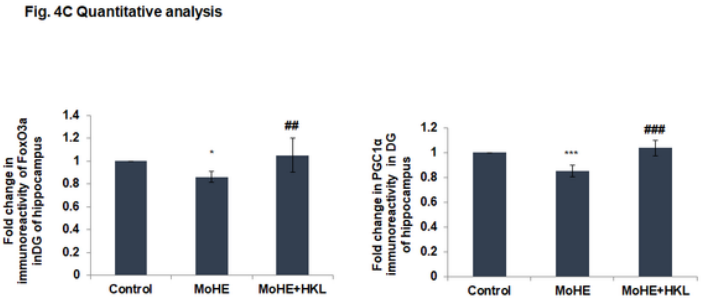
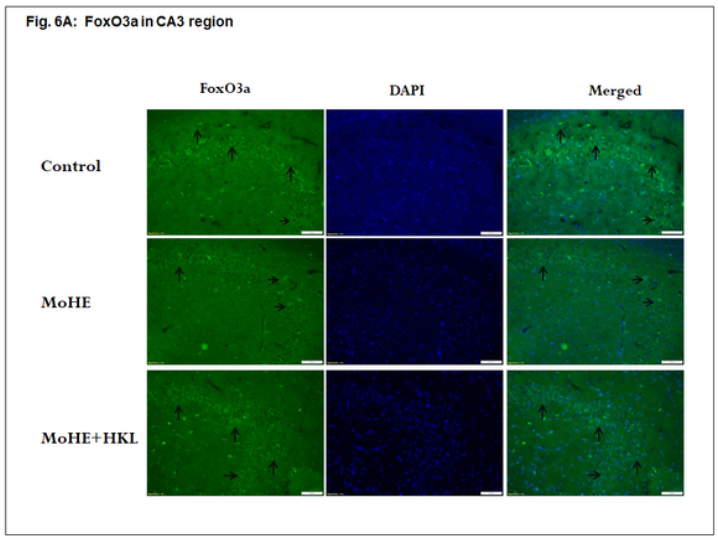


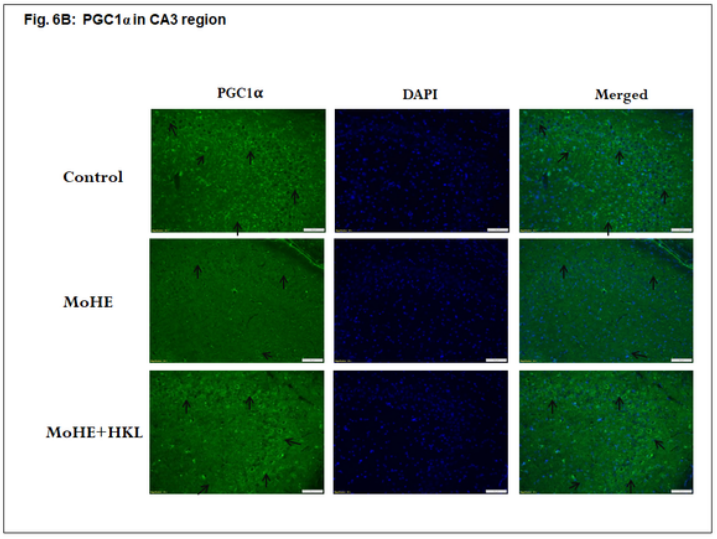
Figure 4

SIRT3 activation restores FoxO3a expression in DG region of hippocampus of the MoHE rats. Immunofluorescence of FoxO3a and PGC1α in the dentate gyrus (DG) region of hippocampus of control, MoHE and MoHE treated with Honokiol. **(A)** & (B) represent the photomicrograph at 20x magnifications. The arrows indicate FoxO3a and PGC1α immunoreactivity in neurons. **(C)** Shows the relative changes in the intensity of immunofluroscence analysed by ImageJ. Values are represented as mean ± SD, where n=6 (*p<0.05, control vs MoHE; ##p<0.01, MoHEvsMoHE + HKL groups).

Figure 5

SIRT3 activation restores FoxO3a expression in CA1 region of hippocampus of the MoHE rats. Immunofluorescence of FoxO3a and PGC1α in the Cornu amonis 1 (CA1) region of hippocampus of control, MoHE and MoHE treated with Honokiol. **(A)** & (B) represent the photomicrographs at 20x magnifications. The arrows indicate FoxO3a and PGC1α immunoreactivity in neurons. **(C)** Shows the relative changes in the intensity of immunofluroscence analysed by ImageJ. Values are represented as mean ± SD, where n=6 (*p<0.05, control vsMoHE; ##p<0.01, MoHEvsMoHE + HKL groups).





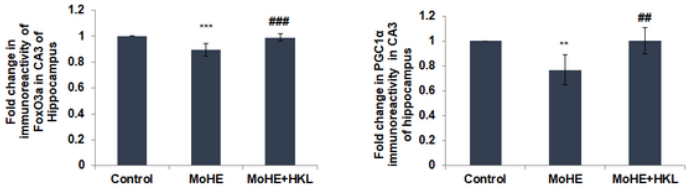


Figure 6

SIRT3 activation restores FoxO3a expression in CA3 region of hippocampus of the MoHE rats. Immunofluorescence of FoxO3a and PGC1α in the Cornu amonis 3 (CA3) region of hippocampus of control, MoHE and MoHE treated with Honokiol. **A)** & (B) represent the photomicrograph at 20x magnifications. The arrows indicate FoxO3a and PGC1α immunoreactivity in neurons. **(C)** Shows the relative changes in the intensity of immunofluroscence analysed by ImageJ. Values are represented as mean ± SD, where n=6 (*p<0.05, control vsMoHE; ##p<0.01, MoHEvsMoHE + HKL groups).