Research Article

Effect of Pre-Gestationally Induced Nickel Chloride in Juvenile Zebrafish

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ABSTRACT

Background: Environmental toxic ants cause an intense impact in the environment by their destructive effect affecting the human beings and they are also considered as the leading factors for infant mortality rate.

Aim: To evaluate neurotoxic effect in F1 progeny of female zebrafish subjected to heavy metal stress pre-gestationally.

Methods: Neurotoxic effect of Nickel chloride were analysed in the juvenile fish (control,30 DOE,40 DOE and 50 DOE) by behaviour analysis, gene and protein expression.

Result: In Behaviour test, Nickel chloride - 50 days induced toxicity shows more anxiety behaviour compared to control. In heat sensitization test, the increased ($p < 0.01$) latency was exhibited by juvenile fishes. The m RNA levels of BDNF, drd2a, 5-HT-1, TH and protein expression of Serotonin and dopamine receptor and transporter were also shown decreased expression in 50 days Nickel chloride induced toxicity.

Conclusion: Unexposed F1 progeny from the exposed adult female zebrafish showed severe altered behavioural patter and gene expression pattern.

Keywords: Danio rerio, Nickel Chloride, Serotonin receptor, heavy metal toxicant, Environmental disruptors, Swim motion test, Heat Sensitization test.

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INTRODUCTION

Heavy metals are widely prevalent in our environment which may occur through various processes naturally. Environmentally relevant most hazardous heavy metals and metalloids include Chromium, Nickel, Copper, Zinc, Cadmium, Lead, Mercury, and Arsenic.[1] These may cause increased risks for human health and the environment.[2] It was reported that the exposure of lead acetate to mice affects the liver and kidney.[3] It was found that mercury exposure affects the brain and kidneys. Gestational exposure to metal toxins resulted in oxidative stress and varied levels of antioxidant enzymes.[4] Heavy metals such as lead, nickel, manganese, mercury, dietary habits, pesticides, stress and other intrinsic factors exposed during the gestational period play a significant role in the brain health of an individual.[5] The heavy metal causes impairment in the blood-brain barrier (BBB) in ischaemic brain damage.[6] There is strong evidence that there exists a strong relationship between continuous metal exposure and neurological diseases including Alzheimer's disease, autism spectrum disorders, Parkinson's disease and Wilson's disease.[7] Metal exposure affects various types of nerve cells and also alters neurotransmitters. As a result, neurological symptoms such as giddiness, tiredness, lethargy develops and also cause behavioral problems.

Among the heavy metal toxicity, Nickel is the predominant one. Inhaled Nickel enters into the brain via the blood-brain barrier and also accumulates in the olfactory lobe. In a study conducted on the rats for 21 days, 10 or 20 mg/kg of $NiCl_2$ was injected via the intraperitoneal method. Behavioral and histomorphological alterations were noted in the brain of rats. It affected the neuronal morphology in the brain and also significantly reduced the percentage of intact neurons in the parts of the brain.[8] Further, it was reported that there was an alteration in the biochemical, immunohistochemical parameter, and histopathology in the rainbow trout brain exposed to 1 mg/L and 2 mg/L of Nickel chloride for 21 days.[9] Nickel exposure may lead to various neurodegenerative disorders like Parkinson's disease, Alzheimer's disease, and Schizophrenia. Behavior patterns like aggraessiveness, anxiety, depression and memory loss are also associated with nickel toxicity. In a rat model, Nickel also causes alteration in the cognitive and locomotor behaviour.[10]

Zebrafish (*Danio rerio*) is becoming a popular organism in neuroscience. *Danio rerio* (Zebrafish) are small tropical fish that are native to the Indian subcontinent.[11] The nervous systems of zebrafish share 85% genetic homology with that of humans. Zebrafish have secured prominence in neuroscience, owing to their utilities. The adult Zebrafish had well developed immune system and the translucent nature of the embryos is particularly advantageous. Zebrafish are widely employed as an animal model for neurological and toxicological studies.[12] In addition to a biological model for several human diseases.

More than 30% of women experience stress pre-gestationally or during the gestation period. It may cause an alteration in the fetal HPA axis and its functioning.[13]The maternal environment plays a key role in the development of the fetus. It also increases the risk of neuro disorders associated with the offspring. Mostly they act via neuropsychiatric pathways.[14] Maternal exposure to cigarette smoke induces oxidative stress and mitochondrial dysfunction in the parafacial respiratory group (pFRG) which are essential to the central regulation of normal breathing.[15]So, the current study focused on the neurotoxic effects of Nickel chloride in juvenile Zebrafish which was induced pre-gestationally.

MATERIALS AND METHODS

Experimental Design

After exposure to Nickel chloride in adult female fish, fishes were allowed to breed

Fish Breeding

The tanks were divided into two breeding sets of three tanks for each group. Each breeding set was bred every other day for a total of 5 spawnings per set over a 10-day period.For daily breeding set-up, fish were quickly removed using a net and transferred into breeding traps in the same tank, and eggs were collected, allowed to grow without exposure and juvenile obtained at 50th day (10-40 days also assessed but data not shown)were subjected for further investigations from the different groups Cognitive and biochemical tests were performed in juvenile (untreated F1 progeny) population (50 days post fertilisation) from the exposed mother.

Group Distribution

Group I-Control Juvenile obtained at 50thday (50 day post fertilisation) from Normal Zebrafish (n=12). (Control)

Group II –Juvenile obtained from the adult female subjected to $NiCl_2$ for 30 days at 50 th day (n=12). (J-30 DOE)

Group III- Juvenile obtained from the adultfemale subjected to $NiCl_2$ for 40 days at 50 thday (n=12). (J -40 DOE)

Group IV- Juvenile obtained from the adult female subjected to $NiCl_2$ for 50 days at 50thday (n=12) (J-50 DOE)

Swim Velocity / Swim Motion test

The swim velocity /swim motion test was performed by the method of Varga, Z. K., et al., 2018.[16] Experimental and control juvenile fishes were housed in adjacent sides during the test period. Swim Motion was then observed using a rectangle tank filled with 20 litre housing water. The tank was divided horizontally in to four equal halves indicating four quardrants. Assays were performed during the light cycle (6am-5pm). Juvenile fishes were transferred from the housing tanks to the experimental tanks using hand-nets and immediately observations are noted for consecutive 3 minutes. The control and experimental fishes were assayed alternately in identical tanks. Readings were noted for the number of quardrant changed in the given consecutive 3 minute.

Heat Sensitization test

The heat sensitisation test was performed by the method of Ahmad, F et al., 2012.[17] The experiment was carried out to understand the sensory response of the larvae to the change in water temperature. The juvenile fishes were housed at 27 degree celcius and the experimental tank was set to 32 degree celcius, the juvenile fishes was transferred from the housing tanks to the experimental tank for observation. Control Zebrafish juvenile are sensitive to the change in water temperature and were observed to respond immediately by agitated movement and moving towards the water surface, they were observed to spend very less time in the bottom of the tank.

Geno toxicity

The AO/ETBR Comet Assay was done by the method of Knopper, L. D. et al.,2005[18] to assess the DNA damage. Brain tissue was minced in 5ml of PBS using blade and a single cell suspension of brain cells equivalent to 1X10^5 cells were used. Cells were induced lysing by immersing in 0.5% NAOH for 3 minutes followed by wash in PBS. 10µl of the cell suspension were transferred to 0.8% agarose and run at 50v for 20 minutes. The slides was washed with Acridine Orange 3mg/ml followed by Ethidium Bromide 5mg/ml for 1 minute each and rinsed in PBS before analysing under fluorescent microscope. The amount of fluorescence was captured using Leica CCD camera and the fluorescence was quantified with Image J software.

Western Blot Analysis

The brain tissue was homogenised with homogenising buffer and the total protein levels were quantified using Bradford method. The homogenate containing 50µg of total protein was boiled in Laemmli buffer for five min then loaded and separated by electrophoresis at room temperature in polyacrylamide gel. After separation, the gels were electroblotted on PVDF membrane at 150mA and blocked using 5% BSA at room temperature for 3 h. The membrane was washed with TBS-T and incubated with the diluted primary antibodies in TBS-T at 4° C for 12 h: Serotonin receptor, Serotonin transporter, dopamine receptor and dopamine transporter. The membranes were

washed thrice with TBS-T and incubated with diluted horseradish peroxidase conjugated secondary antibodies (1:2000) at room temperature for 1 h and the proteins were detected using DAB reagent. The band intensity was calculated using an image J software.

Isolation of RNA for qRT PCR

For the total RNA extraction, the brain tissue was used using TRIZOL reagent (Takara, Japan). mRNA levels of BDNF, drd2A, HT-1, TH were detected with real-time PCR system (Bio-Rad Laboratories) using SYBR Green master mix (Takara, Japan). The cycle threshold (Ct) values were determined using qbase PLUS software.

The oligonucleotide used for primer synthesis:

Genes	Primer
BDNF	Forward: 5'-ATAGTAACGAACAGGATGG-3' Reverse: 3'-GCTCAGTCATGGGAGTCCA -3'
drd2a	Forward: 5'-TGTGATTGCGAATCCTGCCT-3' Reverse: 3'-CGGGATGGGTGCATTTCTTT -3'
HT-1A	Forward: 5'-CATGGTTCTCCGTTTCGAGT-3' Reverse: 3'-CAGCTCCGATGAGTTTTTCC -3'
TH	Forward: 5'-TCATCACCTGGTCACCAAGTT-3' Reverse: 3'-GGTCGCCGTGCCTGTACT-3'
GAPDH	Forward: 5'-CAACAGCCTCAAGATCATCAGC-3' Reverse: 5'-TGGCATGGACTGTGGTCATGAG -3'

Statistical analysis:

Statistical comparisons were made using GraphPad. Student's t test with a 95% confidence interval was performed using a two-way ANOVA at an alpha = 0.05 (95% confidence interval). Significance is denoted with asterisks: ns(#)- not significant, *P < 0.05, **P < 0.01, ***P < 0.001.

RESULT

Effect of maternal exposure to Nickel Chloride on the DNA damage

The DNA damage was assessed by Comet assay. The figure 1 A represents the severity of DNA damage in the control and experimental group of fishes. Figure 1 a ,1 c and 1 e represented the data of control fishes of 30,40 and 50 days of exposure whereas 1 b, 1 d and 1 f represent the DNA damage in the same 30,40,50 days of Nickel chloride exposed group. The damage was more severe as evidenced from tail length in the 50 days Nickel chloride induced group.

The figure 1 B represents the tabulation data of the length of the tail in µM in the control and experimental groups of fishes. The nuclear damage was observed in the brain of juveniles obtained from nickel chloride induced mother (exposed pre- gestationally). Group II,III,IV which was not seen in the case of control juvenile fishes (Group-I). The increased damage was noticed in the 50 days Nickel chloride induced toxicity. The Nickel chloride toxicity damages the cellular contents and further intensifies the nuclear damage as indicated in the figure 1 b,d.

Figure 1 a,c,e represented the normal image of juvenile fish without damage at different timepoints (30,40,50 days respectively).Intense orange fluorescence indicative of DNA damage observed in figure 1b,d,f from juvenile fishes from groups subjected to Nickel chloride exposure for the period of 30,40 and 50 days respectively.

Table 1: Represented the length of the tail in µM in the control and experimental groups of juvenile fish.

S.No	Juvenile day on 50th day	Length of the tail (µ)M
1	Control	0
2	J-30 DOE	6.40
3	J-40 DOE	8.40
4	J-50 DOE	9.20

Effect of maternal exposure to Nickel Chloride on the behavioural parameters in juvenile fishes

In swim motion test, the number of times quadrant change and the time spent by the fishes in both the compartment (expressed as mean duration in seconds) during the experimental period was analyzed in the control and experimental fishes. In the current study as depicted in table 2, Nickel chloride - 50 days induced toxicity spends more time ($p < 0.01$) in the bottom of the compartment indicating more anxiety behaviour compared to control juvenile fishes. In heat sensitisation test, the increased ($p < 0.01$) latency was exhibited by juvenile obtained from 50 days induced toxicity compared to control fishes as evident from table 1.2.

Table 2: Effects of Nickel chloride in assessing the swim motion and heat sensitization in fishes

S.No	Parameters	Group I	Group II	Group III	Group IV
1	Number of quadrant changed per minute	29.00±2.50	51.00±2.80*	74.00±2.70**	87.00±2.30**
2	Time spent per minute at the bottom of the tank (Sec)	35.00±1.30	45.00±1.60*	69.00±1.50**	94.00±1.80**

All data were expressed as mean ± SD (n=12). The comparisons were made with control vs J-30 DOE, J-40 DOE, J-50 DOE. Data were significant among the groups.; * $p < 0.05$; ** $p < 0.01$.

The change of quadrant and the time spent by the fishes in the bottom compartment (expressed as mean duration in seconds) during the testing period were analyzed in the control and experimental groups.

In contrast, experimental groups (Group II, III and IV) were observed to spend more time in the bottom zone of the experimental tank indicating less sensitivity to water temperature.

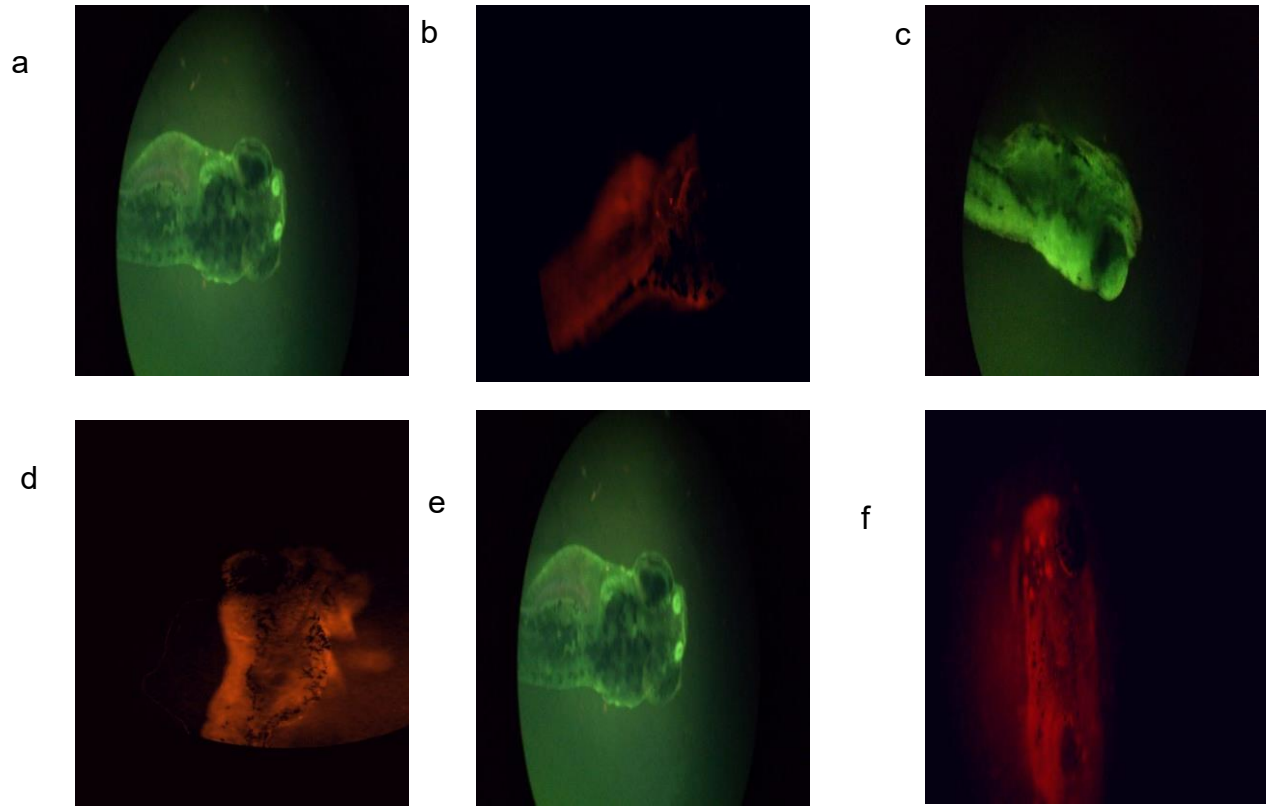

Figure 1: represented the DNA damage in control and experimental group of fishes (Figure 1 a to 1 f).

Effect of maternal exposure to Nickel Chloride on the mRNA expression of *BDNF, drd2a, 5-HT-1, TH 1:*

The m RNA levels of *BDNF, drd2a, 5-HT-1, TH 1* were depicted in figure 2. The m-RNA levels were decreased in 50 days Nickel chloride induced toxicity as compared to control fishes in figure 2.

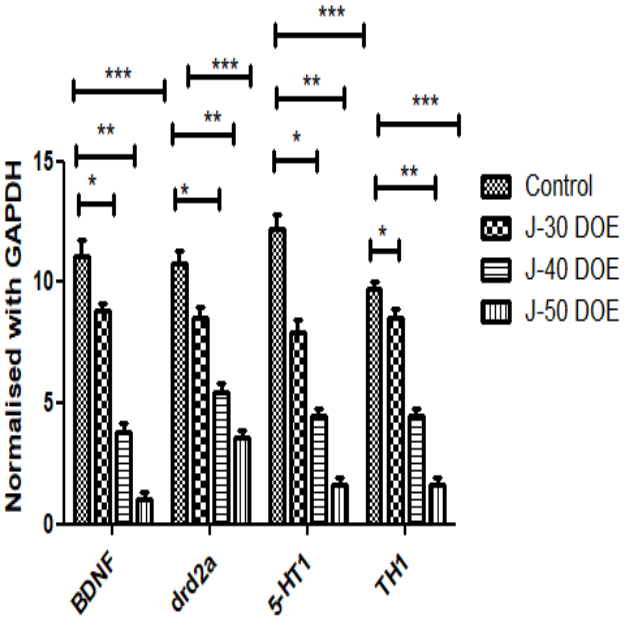

Figure 2: Expression of *BDNF, drd2a.5-HT-1, TH* 1 in the brain of the fishes in control and experimental groups of the juvenile fishes from the adult females that were exposed to different durations of Nickel chloride(1M) .Data were presented as Mean ± S.D.(n=12) and significant among the experimental group of fishes. Comparisons were made between control vs J-30 DOE, J-40 DOE, J-50 DOE. (**p*<0.05, ***p*<0.01,****p*<0.01)

Effect of maternal exposure to Nickel Chloride on the expression of Serotonin receptor, Serotonin transporter, Dopamine receptor and Dopamine transporter:

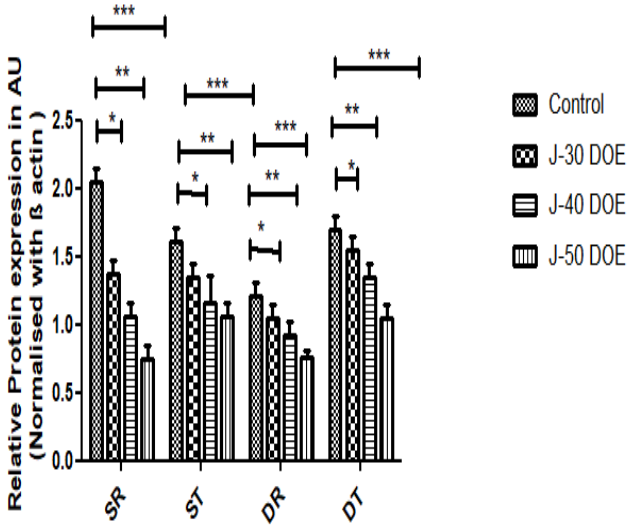

Figure 3: A represented the densiometric data on the levels of protein (expressed as relative expression in AU) were found using Image J software, the values were normalised with β actin. The results were expressed as Mean ± SEM.(n=12) and significant among the experimental group of fishes. Comparisons were made between control vs J-30 DOE, J-40 DOE,J-50 DOE. (**p*<0.05, ***p*<0.01, ****p*<0.001)

The protein expression of Serotonin receptor, Serotonin transporter, dopamine receptor and dopamine transporter were represented in the figure 4.6. The protein expression of Serotonin receptor, Serotonin transporter, dopamine receptor and dopamine transporter were also shown decreased expression in 50 days Nickel chloride induced toxicity.

DISCUSSION

Genotoxicity

It was reported that the Nickel may affect the DNA thereby inducing damage. It was observed that Nickel chloride induces single stranded DNA breakage and severe DNA damage inlymphocytes.[19]Similar to this report, current study also, showed that there was severe DNA damage in juvenile fish obtained from 50 days pre-gestationally exposed to Nickel chloride compared to that of groups. The length of the tail in µM was found to be more in Group IV fishes compared to other groups (Table 1) which might be due to production of reactive oxygen species.[20]

Behaviour changes

Behavioural responses of Zebrafish are robust, which are evolutionarily conserved, and resemble those of mammalian species. The parental exposure to EE2 (17α-ethynylestradiol) in Zebrafish has shown variations in the behaviour pattern (Novel tank test and Scototaxis test) and it was reflected in the unexposed F1 progeny.[21]The paternal exposure of herbicide (atrazine) also resulted in behavioural changes in the unexposed progeny.[22] The Zebrafish exposed to DU (depleted Uranium) parentally was reported to affect the hisotological and behavioural pattern of the progeny which was not been exposed.[23]In the current model of Nickel Chloride exposed pre-gestationally there was a decreased locomotor activity of juvenile fish as evidenced from Table 2.The results similar to previous reports [24]which had highlighted that Nickel chloride at the dose of 7.5 mg could affect locomotion ability. The oxidative stress encountered by the mother fishes (Part 1) was anticipated to affect the progeny which in turn could have altered the behaviour of juvenile fishes. Behavioural paradigms like anxiety, memory and learning capabilities was also reported by changes due to oxidative stress.[25]

Assessment of expression levels of BDNF, drd2a, 5-HT and TH 1

Danio rerio possess serotonin transporter genes similar to those of tetrapods. Serotonin receptors belong to G protein coupled receptors[26] and also (ligand gated) ion channels, located both in the central and peripheral nervous system. There are seven types of serotonin receptors available. In *Danio rerio*, a single unit of 5-HT was present and shares 62% homology with the humanbeings.[27] The increased serotonin inhibitor inhibits the seizures induced with chemoconvulscent agent. It was reported that the Nickel reduces the expression of 5 HT receptor in chlorpyrifos (pesticide) induction in Zebrafish.[28] Similar to the above research findings, in the current study also, it was observed that there was decreased level of serotonin receptor as evidenced from figure 3.

5-HT shares cent percent homology with the amino acid binding sites for dopamine receptors D1 and D3 and 85-95% homologous with D2 and D4 receptors.[29] An exposure to Nickel can cause the alteration in the stimulatory and inhibitory effect of dopamine.[30]Dopamine system has been related to locomotory behaviour and *drd1* receptor was shown to directly linked to locomotor activity. An upregulated dopamine receptor and increased locomotor activity were shown to be interrelated.[31] In another investigation, Sertaline (selective serotonin reuptake inhibitor) treatment causes increased expression of serotonin receptor and serotonin transporter in *Danio rerio*.[32]It was reported that the dopamine transporter deficiency causes Parkinson disease in infants [33]The dopamine system may also influences serotonin receptor and other receptors[34].

In *Danio rerio*, *BDNF* belongs to the neurotrophin family. The *BDNF* gene shares 91 percent homology to humans.[35] The m RNA of BDNF gene expression was similar to humans and mammals[36]. *BDNF* have been well expressed at all developmental stages. The *BDNF* synthesis had been well represented in the brain and eye[37]. The telencephalon, anterior region of the brain is involved in memory, learning, behaviour responses, receptive nature and conduction of smell. *BDNF* has been well documented in the telencephalon region. From Figure 4, it was evidenced that there was decreased expression of *BDNF*. It was supported by the study of lead exposure in rats causes altered *BDNF* expression which may be due to impaired neuronal plasticity nature and long term potentiation dysfunction[38].It was also reported that the decreased *BDNF* expression were noticed in brain and liver of Zebrafish.[39]

The current study had observed the deviated expressional data (figure 4) in F1 progeny of the 50th day $NiCl_2$ pre-gestationally exposed in adult fishes which exhibited behavioural variations. Hence, the study suggested that Nickel chloride can have influence on the neurotransmissional system of the progeny of insulted (Pregestationally) females

CONCLUSION

The heavy metal toxicant, $NiCl_2$ causes significant impact by affecting the nervous system of the adult female Zebrafishes and this detrimental effect could be reflected on the F1 progeny causing behaviour, biochemical and gene expressional variations.

Authors Contribution:

Arambakkam J Vanisree: Conceived the work, designed, analyzedthe results and wrote the major part of the manuscript.

Sudha N: Executed the bench work.

Jaya Prakash N: Involved in Manuscript Preparation

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