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Original Article

# Possible Involvement of Cellular Pathway and Cytokines in Manganese Induced Neurotoxicity in Neuroblastoma Cells via KH–Type Splicing Regulatory Protein

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## ABSTRACT

**Background:** Manganese is a toxic essential trace element and too high concentration instigates the neurodegenerative disease known as parkinsonism. Effects of manganese may lead to apoptosis. However, a detailed mechanism of manganese toxicity has not been fully elucidated. Previous published articles have highlighted the augmentation of KHSRP expression following Mn exposure. **Objectives:** In this work, the importance of KHSRP in Mn–induced toxicity was checked along with the impact of other known neurotoxicity inhibitors on KHSRP. **Materials and Methods:** KHSRP expression, pro and anti-inflammatory cytokines, chemokines, and pharmacological inhibitors (SAHA, Quercetin, and MCC950) were determined by exposing N2a cells to various $MnCl_2$ concentrations. ANOVA and Dunnett's test were used to decide on the significance. **Results:** $MnCl_2$ treatment led to the augmentation of the KHSPR mRNA expression and protein increase in N2a cell line. The $MnCl_2$ treatment of N2a cells also showed an elevated liberation of IL-6, TNF-α, MCP–1, and IL-1β. Pharmacological agents like quercetin inhibiting PI3K, MAPK, and WNT pathways, MCC950 blocking NLRP3 pathways, and SAHA showed a decrease in KHSRP expression post Mn treatment. With the inhibition of KHSRP, a decline in the release of IL-1β, IL-6, MCP–1, and TNF-α was also observed. **Conclusion:** These results suggested that $MnCl_2$ treatment of N2a cells induce the expression of KHSRP via the PI3K–or NLRP3 pathway. Furthermore, this elevated expression of KHSRP is responsible for an increment in the liberation of pro-inflammatory markers in N2a cells. More exploration is needed to throw light on the pathway driving the KHSRP.

**Keywords:** KH–type Splicing Regulatory Protein, Manganese Neurotoxicity, Neuroblastoma, Neurodegeneration, Neuroinflammation.

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## INTRODUCTION

Manganese is one of the toxic essential trace elements.[1] It is important because it has a vital role in the growth, development, and reproduction. It thus entertains the good health aspects.[2-5] However, its excess exposure is harmful and could cause parkinsonian manganism, and emotional disturbances.[6-10] The toxicity due to the manganese element is believed to be mediated by the neuroinflammation, misfolding of protein, and essentially apoptosis.[3,4,11] Mn may cross through the barrier of the blood–brain and may reach the brain by transferrin and/or DMT–1-mediated pathways, leading to the neuroinflammation, and neurodegeneration.[12-14] Thus, the search for the mechanisms/pathways involving the toxicity of Mn needs further exploration.KHSRP is a protein riches in AU residues.[15] It negatively regulates the action of chemokines and cytokines. Shi *et al.*[16] reported the induction of neurotoxicity by KHSRP following Mn exposure in PC–12 cells. In addition, a correlation of p53, caspase–3, and bax upregulation, and KHSRP expression was noticed. Furthermore, the KHSRP monitors the expression of inducible nitric oxide. The expression of iNOS is also controlled post-transcriptionally by the KHSRP through NF-κB and p38 MAPK pathways.[17-20] The regulation of WNT signalling pathway by KHSRP was also highlighted by various researchers through the β-catenin destabilisation.[21-24] Therefore,

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KHSRP could have a vital role in the inflammation, leading to the apoptosis. However, the pathway detailing the importance of KHSRP in the neuroinflammation caused by Mn exposure is not fully elucidated yet. In the present investigation, the importance of KHSRP in the induction of toxicity caused by Mn was checked along with the impact of other known neurotoxicity inhibitors on KHSRP. The possible signalling mechanisms linking the Mn toxicity, KHSRP expression, and the neuroinflammatory response, with the utilisation of various known neurotoxic inhibitors, were also proposed.

## MATERIALS AND METHODS

### Cell line

The cell line known as N2a was bought from ATCC.[25] The cell lines were cultured as per the guidelines from ATCC, in Eagle's minimal essential medium, after 10% FBS supplementation. They were incubated with 5% $CO_2$ in an incubator at 37°C. Cytotoxicity assay

The cells at the concentration of 5000 cells per well were seeded for one day in a 96–well plate. Next day, it was replaced with the fresh medium. Cells were treated with $MnCl_2$ (Sigma, M1787) group (cells treated with 0, 250, 500, and 1000 µM),[26] for one, 2, and 3 days' time period point. Celltiter glo (Promega, G7570) and Envision plate reader (from Perkin Elmer) were utilised to cheque the cell viability, and plates' luminescence, respectively. Data were analysed by calculating the viability percentage of $MnCl_2$ treated samples by normalising the data with reference to positive control as 100% viability and negative control group as 0% viability. Preparation of RNA and RT PCR

After incubation, samples were subjected to TRI Reagent® (Sigma, T9424) solution, and kept in the fridge at -80°C. The total RNA was isolated with the aid of RNeasy Kit bought from Qiagen (74004). One µg of the isolated RNA was changed in to cDNA with the aid of iScript cDNA kit from the Bio–rad (1708891). After, 100 ng of cDNA was undergone the amplification with a iTaq (Biorad, 1725121). The parameters considered were 5 min and 95°C for the pre-incubation step, 10 sec, and 95°C for the step of denaturation with 45 cycles, 10 sec, and 60°C for the annealing step, and 10 sec, and 72°C for the last step known as elongation. The expression concentration of mRNA were compared to the GAPDH and the fold variation was quantified with the delta Ct formulae. The primers utilised for the qPCR were:

KHSRP forward: 5'- TCCATCCTGCCTTAGTGGGT-3'

KHSRP reverse: 5'- TAAGCCTCTGCACCCATCG-3'

GAPDH forward:[27] 5'- CAGTGCCAGCCTCGTCCCGTAGA-3'

GAPDH reverse:[27] 5'- CTGCAAATGGCAGCCCTGGTGAC -3'[28] Western blotting

Post incubation, the phosphatase inhibitor cocktail (Sigma of USA, P2850), cell lysis buffer (9803), and proteinase inhibitor cocktail (Sigma of USA, P8340) were utilised to lyse cells. The protein levels were quantified with QuantiPro™ kit (QPBCA). The proteins (10 µg for every well) were apportioned by SDS–PAGE and then added to the membranes (Bio–Rad, 1620115). The membranes were undergone a one-day incubation with primary antibodies. They were then probed with another set of antibodies for 120 min at RT. The antibodies considered were GAPDH (14C10) rabbit mAb (2118), KHSRP antibody (NBP1–18910), HRP–linked antibody (7074), and anti-rabbit IgG. Measurement of inflammatory markers Stored media were thawed, and IL-6, IL-1β, TNF-α, and MCP–1 from mice (R&D, DuoSet ELISA) were quantified by respecting the indications highlighted by the manufacturer. The absorbance was recorded with an envision (Perkin Elmer). IL-10, an anti-inflammatory cytokine, and IL-2, a cytokine were also quantified. Cytokines and chemokines levels were deduced from standard curves, after recording the absorbance on Clariostar bought from Germany.

### Statistical analysis

After presenting the data as the means ± S.E.M, ANOVA, and Dunnett's test were utilised to check the significance at $p< 0.05$.

## RESULTS

### Induction of toxicity in N2a cell lines by $MnCl_2$

To analyse and deduce the Mn toxicity pathway and the involvement of KSHRP, N2a cells were subjected to various $MnCl_2$ concentrations of 250, 500, and 1000 µM for 24, 48, and 72 hr, and its impact on N2a cell survival, KSHRP expression, pro, and anti-inflammatory cytokines was evaluated. After 24 hr, no impact of $MnCl_2$ was observed in cell viability with either of the tested concentrations. Post 48 hr, 50, and 65% cell death were observed at 500 and 1000 µM, respectively; however, no impact on cell viability with 250 µM of $MnCl_2$ exposure. While, post 72 hr, 20 to 90% cell death was observed with increase of $MnCl_2$ exposure (Figure 1a, 1b, 1c).Induction of gene and protein expression of KHSRP in N2a by $MnCl_2$ treatment.

The KHSRP expression in N2a cells was assessed when N2a cells were exposed to $MnCl_2$ at the concentration of 250, 500, and 1000 µM for one, 2, and 3 days. 25 to 60–fold upregulation of KHSRP expression was observed in dose dependent manner after 24 hr. A minimal upregulation of KHSRP expression was observed post 48 h. However, no change in KHSRP expression was observed after 72 hr (Figure 2a, 2b, 2c). A decline in RNA and protein levels was noticed after 48 hr. KHSRP protein levels were also investigated by Western blot, and results seen were in compliance with the KHSRP gene expression results. KHSRP expression was upregulated by $MnCl_2$ treatment (Figure 3 a, b, c, d). Two to 5-folds augmentation KHSRP protein levels was observed at 24 hr; while approximately 2 folds was noticed at 48 hr. Increase of

Figure 1.

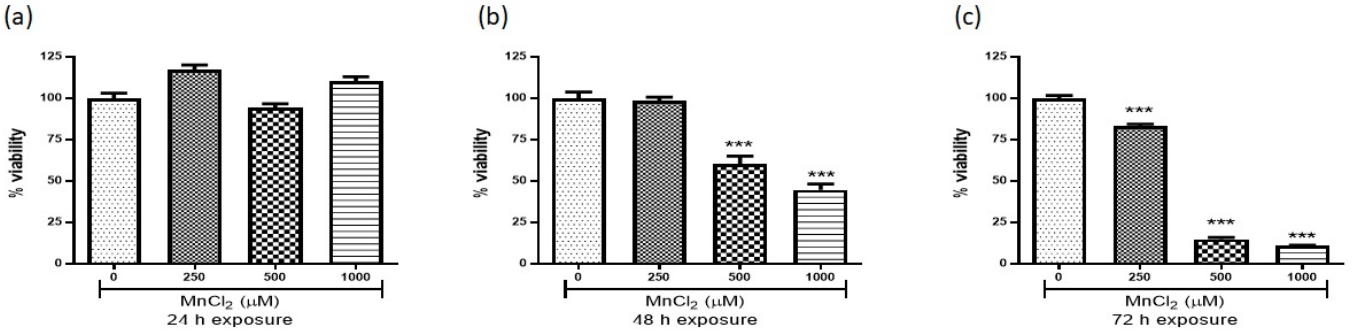


**Figure 1:** % viability of N2a cells evaluated by CTG post treatment with $MnCl_2$ at the concentration of 250, 500, 1000 μM. (a) % viability post 24 hr of $MnCl_2$ exposure, (b) % viability post 24 h of $MnCl_2$ exposure, (c) %viability post 24 hr of $MnCl_2$ exposure. Data represented as mean ± SEM, significance calculated with reference to 0 μM $MnCl_2$. *** $p$< 0.0001 vs. control group.

Figure 2.

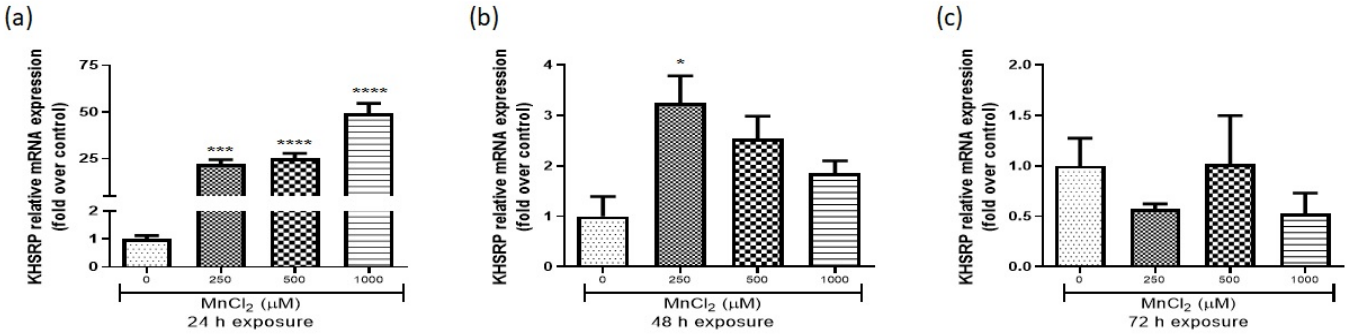


**Figure 2:** Impact of $MnCl_2$ (250, 500, 1000 μM) on relative mRNA expression levels of KHSRP noticed by qPCR, after 24 hr (a), 48 hr (b), and 72 hr (c) of Mn treatment. The significance was calculated with reference to 0 μM $MnCl_2$. *** $p$< 0.0001, **** $p$< 0.00001, vs. control group.

the expression of various pro-inflammatory markers by $MnCl_2$ treatment.

Pro and anti-inflammatory cytokines and chemokines were checked by exposing N2a cells to $MnCl_2$ at the concentration of 250, 500, and 1000 μM for 24 hr. The release of chemokine (MCP–1), pro-inflammatory cytokines (IL-1β, TNF-α, and IL6), anti-inflammatory cytokine (IL-10), and other cytokine (IL-2) was evaluated (Figure 4 a, b, c, d, e, f). An important augmentation in TNF-α, MCP–1, IL-1β, and IL6 concentrations was seen, while no change observed in IL-2, IL-10 levels.Induction of expression of KHSRP by $MnCl_2$ through mediation of PI3K–Akt and IL-1β/NLRP3/Cytokine release pathway.

Three pharmacological inhibitors (SAHA at 1μM, Quercetin at 60μM, and MCC950 1μM)[29-34] were tested to explore the activity of these inhibitors on $MnCl_2$ modulated KHSRP expression. A decline in the expression of KHSRP mRNA and protein was observed in $N_2$a cells pre-treated with MCC950 and Quercetin, while no change in KHSRP expression was observed upon treatment with SAHA (Figure 5 a, b, c). Induction of expression of pro-inflammatory markers by $MnCl_2$ through KHSRP mediation.

To investigate the impact of neurotoxicity inhibitors on $MnCl_2$ modulated cytokines, N2a cells were exposed overnight to $MnCl_2$. The release of MCP–1, TNF-α, IL6 and IL-1β was investigated. An increased level of TNF-α, MCP–1, IL-1β and IL6 was observed with $MnCl_2$ exposure (Figures 6 a, b, c, d).

## DISCUSSION

Manganese is one of the toxic essential trace element. Too high uptake leads to the health problems like neurological symptoms i.e., Parkinson's disease (PD). Central nervous system is the

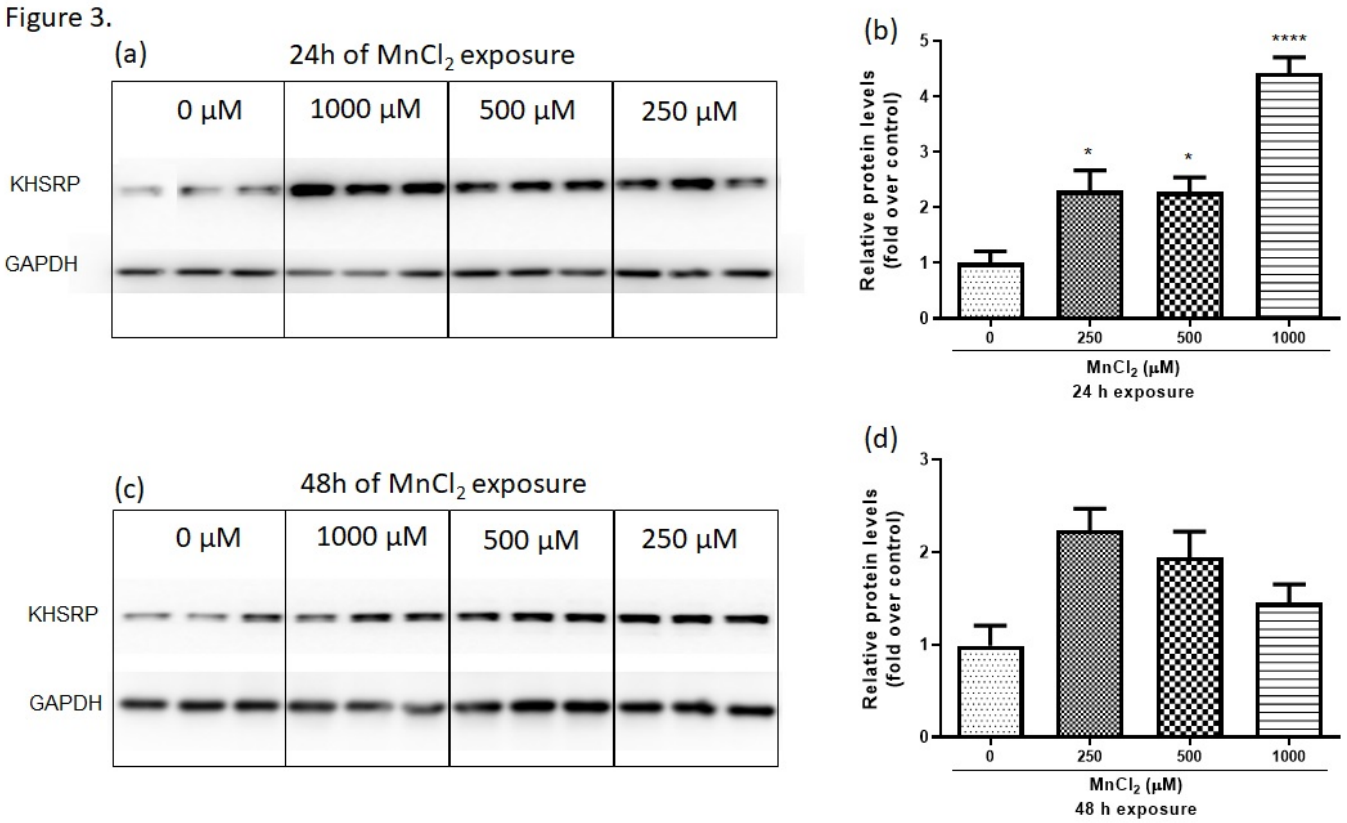


**Figure 3:** Impact of $MnCl_2$ (250, 500, 1000 µM) on protein concentrations of KHSRP, quantified by Western blot investigation, after Mn treatment. Western blot checks of KHSRP and GAPDH levels after 24 hr (a) and 48 hr (c), as well as fold variation in KHSRP protein concentration after 24 hr (b) and 48 hr exposure (d). The significance was calculated with reference to 0 µM $MnCl_2$. *$p$<0.05, ****$p$ < 0.00001 vs. control group. p.

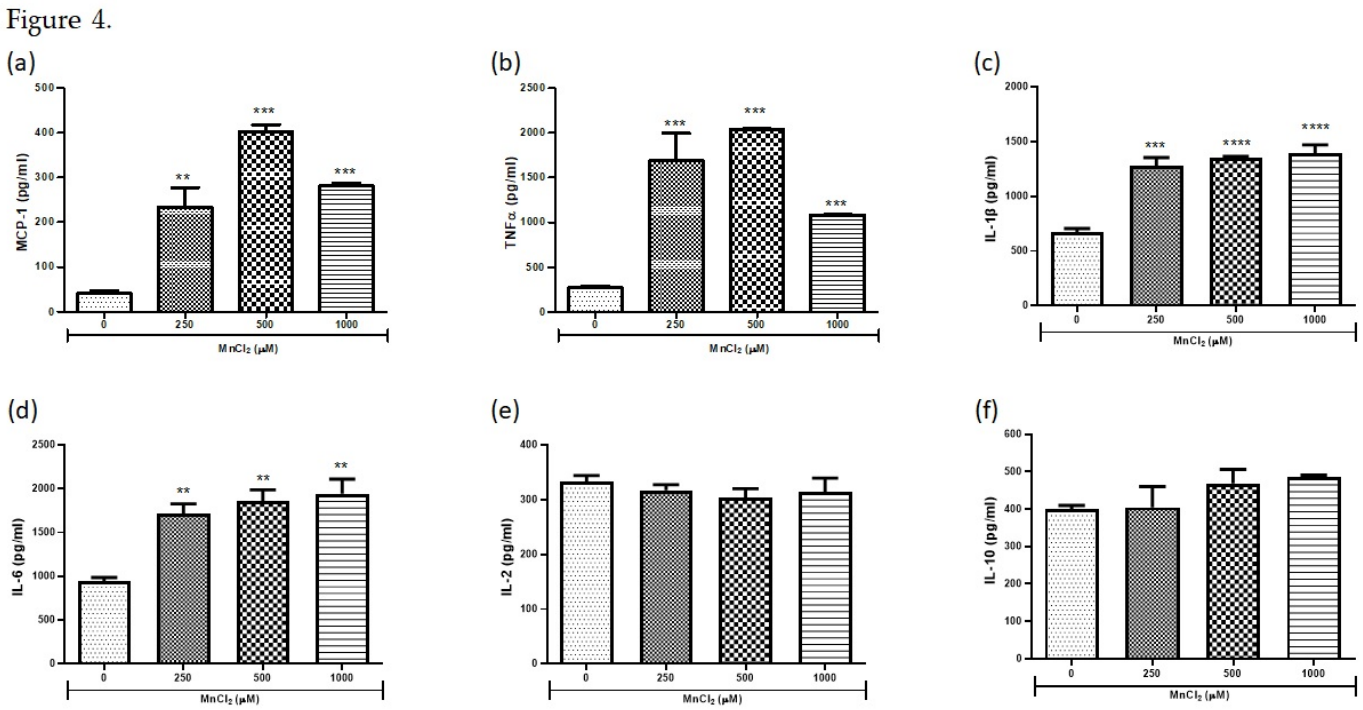


**Figure 4:** Influence of $MnCl_2$ on pro-inflammatory and anti-inflammatory cytokines, N2a cells were subjected to $MnCl_2$ (250, 500, 1000 µM) and detected by ELISA post 24 h. (a) MCP-1 (b) TNFα (c) IL-1β (d) IL-6 (e) IL-2 (f) IL-10. The significance was calculated with reference to 0 µM $MnCl_2$. **$p$<0.001, ***$p$< 0.0001, ****$p$< 0.00001 vs. control group.

Figure 5.

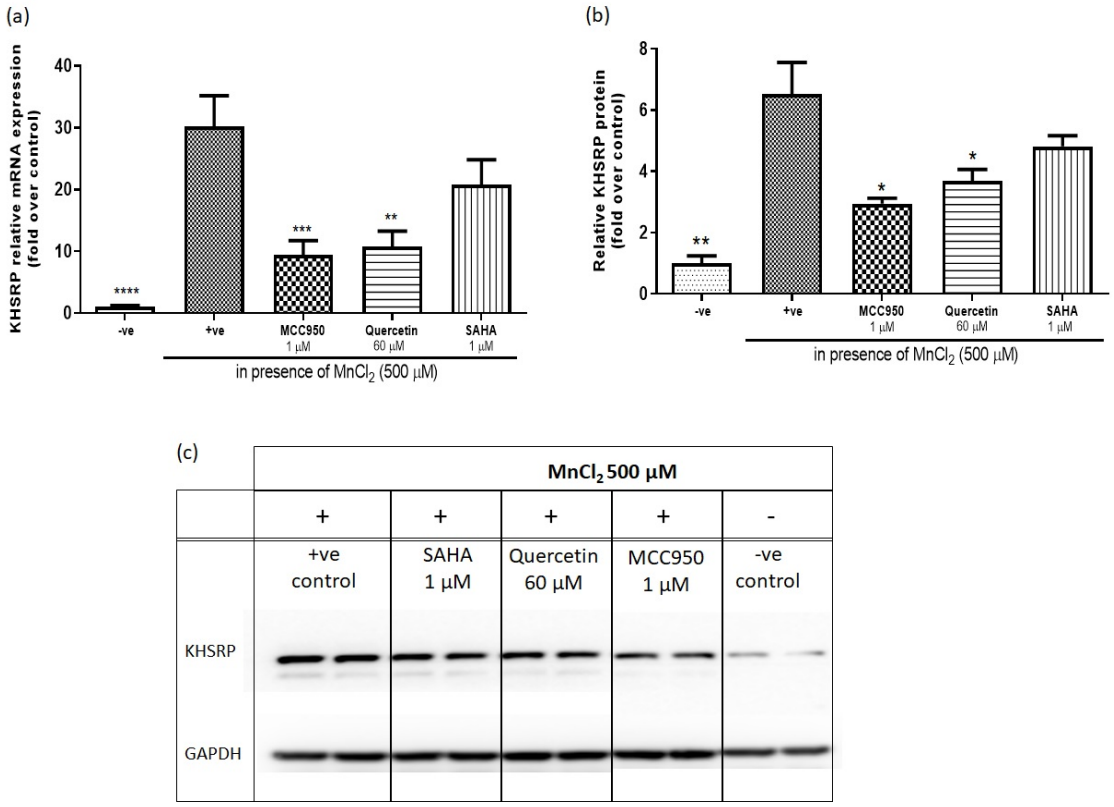


**Figure 5:** Impact of various pharmacological inhibitors (quercetin, MCC950, SAHA) on KHSRP levels post $MnCl_2$ (500 μM) exposure for 24 hr. (a) and (b) are relative mRNA expression and fold variation in KHSRP protein concentrations. (c) is the Western blot check of both KHSRP and GAPDH. The significance was quantified with reference to 0 μM $MnCl_2$. * $p$< 0.01, ** $p$< 0.001, *** $p$< 0.0001, **** $p$< 0.00001, vs. –ve control group.

Figure 6.

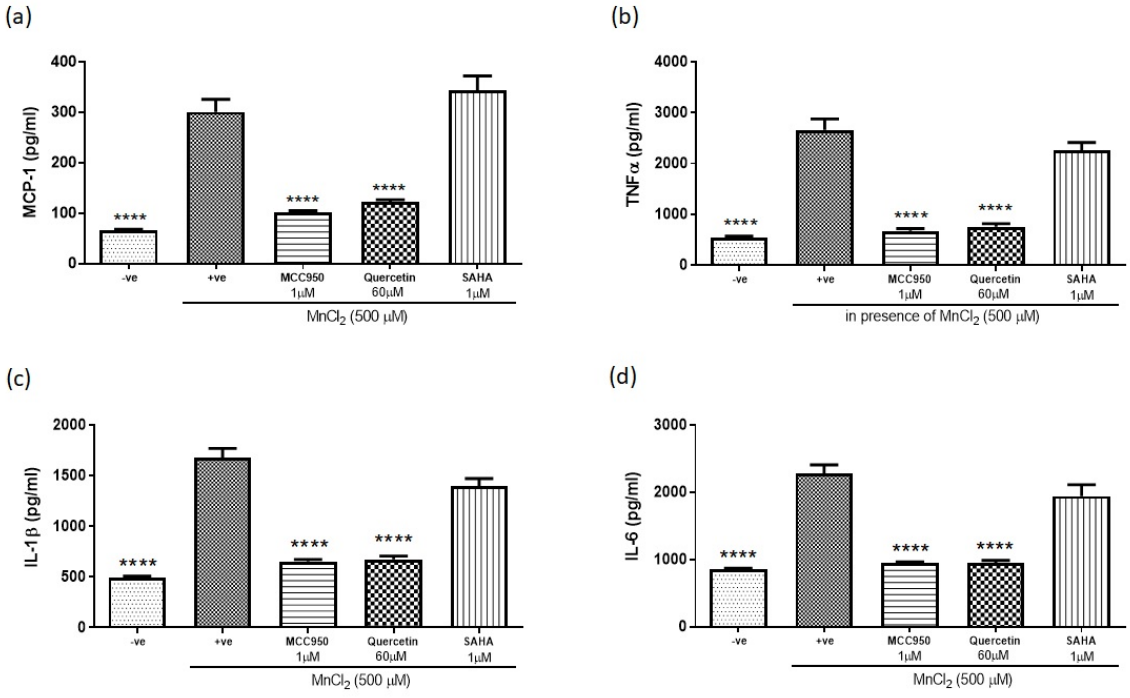


**Figure 6:** Impact of various pharmacological inhibitors (quercetin, MCC950, SAHA) on pro-inflammatory and anti-inflammatory cytokines (a) MCP-1 (b) TNFα (c) IL-1β (d) IL-6. Data represented as mean ± SEM. The significance was quantified with reference to the negative control group (without $MnCl_2$). ** $p$< 0.001, **** $p$< 0.00001.

foremost site of damage due to Mn accumulation in CNS leading to the toxicity and paralysis, and thus affecting the normal functioning of neuronal activities.[35-38] However, the mechanism of this toxicity needs to be explored more for better understanding. In the present study, $MnCl_2$ induces time–dependent toxicity;, and post 24 hr, $MnCl_2$ was observed to toxicate the N2a cells. KHSRP is reported to be playing multiple roles in the normal cell physiology.[16] In this investigation, KHSRP was found to be upregulated within 24 hr of $MnCl_2$ exposure, and this was observed to fall off by 48 hr, and back to basal point, post 72 hr. Thus, Mn treatment causes the increased expression of KHSRP gene, and protein in N2a cells. Mn is reported to be neurotoxic and KHSRP was upregulated with Mn exposure, indicating KHSRP is driven by the neurotoxicity. Longer exposure to Mn lead to complete cell death. Similarly, an important upregulation of KHSRP was noticed when a rat striatum was exposed to Mn exposure. Thus, this Mn neurotoxicity may be modulated by the p53 signalling.[16] Neurodegeneration is well known as progressive loss of basic structure and normal functioning of neurons. Multiple neurological diseases are linked with neurodegeneration. Neurodegeneration have major devastating implications on individuals. The real cases of neurodegenerative illnesses are more than what is reported worldwide. Mn was shown to cause neurotoxicity by the liberation of inflammatory cytokines and chemokines. In this analysis, cytokines estimation was performed only at 24 hr, as our previous results indicated a cell death at this time period. With the increase in KHSRP at gene and protein levels, Mn treatment also induced the stimulation of chemokines, and pro-inflammatory cytokines in these cells. However, no change was observed in the anti-inflammatory cytokines. Cytokines evaluated here were reported as the hallmarks for the neuroinflammation and neurotoxicity, indicating the role of $MnCl_2$ in inducing the neurotoxicity. Multiple cytokines are being reported to be modulated with the induction of neurotoxicity by $MnCl_2$. Mn exposure has been found to cause neurodegenerative disorders with increase in β-amyloid (Aβ1–40) and Tau production in an NLRP3–dependent manner, leading to hippocampal degeneration, and necrosis. In the process multiple cytokines are reported to be modulated as the hall mark of neurotoxicity and neurodegeneration.[34,39,40]

Many of the researchers have explored various pharmacological inhibitors in neurotoxicity studies induced via different neurotoxins. Few of these inhibitors (SAHA, Quercetin and MCC950) were tested in this study to check the influence of these inhibitors on $MnCl_2$ modulated KHSRP expression, or levels. MCC950 is a vital cytokine release inhibitor, it inhibits NLRP3.[35] Quercetin is a plant pigment (flavonoid), reported to possess strong anti-inflammatory capacities, also been shown to inhibit the PI3K pathway.[36] SAHA or Vorinostat is a histone deacetylase (HDAC) inhibitor.[37] A decline in the expression of KHSRP mRNA and protein was noticed in $N_2a$ cells pre-treated with MCC950 and Quercetin, while no change in KHSRP expression was observed upon treatment with SAHA. These results provide the evidence that activation of KHSRP via $MnCl_2$ exposure is mediated by PI3K pathway and NLRP3 inflammasome. The increase in the KHSRP expression upon $MnCl_2$ is not mediated by histone deacetylation.

Indeed, the elevated expression of KHSRP in N2a cells upon Mn exposure was demonstrated and it was mediated by PI3K/MAPK/WNT pathways, and NLRP3 inflammasome. The inhibition of the expression of KHSRP is also linked with the reduced liberation of pro-inflammatory markers indicating the role of KHSRP in neuroinflammation induced by Mn treatment. Although SAHA did not affect the KHSRP expression, it inhibited the inflammatory response by declining the expression of iNOS, and NF-Kb through STAT3 signalling pathway inhibition.[29,30] Zhang *et al.*[32] reported the prevention of diabetic nephropathy / diabetic kidney progression by MCC950 through NLRP3/caspase–1/IL-1β pathway prevention or by inhibiting NLRP3 inflammasome stimulation. The inhibition of PI3K activity by quercetin in one-hour time period was also reported in chronic lymphocytic leukaemia.[36]

The up–regulation of KHSRP was also noticed in PC–12 cells.[16] The present investigation was focused on KHSRP upregulation linked with Mn treatment and linked the upregulation of KHSRP with PI3K/MAPK/WNT and IL-1β/NLRP3/Cytokine release pathway. MAPK, viz. ERK, JNK, and p38, were activated following Mn treatment in glial cells.[24,41-44] The NLRP3 inflammasome was stimulated by Mn treatment following Mn treatment in microglial cells.[45] Thus, the Mn treatment might stimulate both NLPR3 inflammasome, and MAPK pathway, resulting/leading to the KHSRP activation.[46,47]

As mentioned, KHSRP expression in this investigation was found to be dependent on the NF–kB, PI3K–Akt, and IL-1β/NLRP3/Cytokine release pathway. Earlier studies suggested that p38/Mk2 phosphorylates the KHSRP and thereby increases its binding to ARE–containing mRNA. It therefore stabilises the transcript. Since, Mn treatment increases the p38 activation via PI3K pathway, it will inhibit the function of KHSRP. To compensate for this functional loss, the cell increases the expression of KHSRP. Hence, when the PI3K pathway is inhibited, the expression of KHSRP is decreased.[48] This decrease in the KHSRP also resulted in the reduction of pre-inflammatory cytokine release. Ba *et al.*[41] reported the regulation of iNOS expression by $Mn^{2+}$ at the transcription stage, through two signalling pathways, viz. PI3K/Akt and JNK–ERK MAPK pathways.

An increased level of MCP–1, TNF-α, IL-1β and IL6 was noticed with $MnCl_2$ exposure. This was found to be inhibited with MCC950, Quercetin, and SAHA. As there is a decrease in activation of the KHSRP upon pre-treatment of the cells with the same blockers, the release of these pro-inflammatory markers is thus mediated by KHSRP. The data presented herein showed that

Mn induced neuroinflammation in mouse neuroblastoma cell line. N2a is mediated by KHSRP, whose expression is regulated by PI3K/MAPK/WNT, and NLRP3 pathways. More exploration is needed for further understanding.

## CONCLUSION

To conclude, hints towards the co-relation between KHSRP and PI3K/MAPK/WNT, and NLRP3 pathways was shown. More detailed investigations can further throw light on the pathway driving the KHSRP.

## ACKNOWLEDGEMENT

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## ABBREVIATIONS

**KHSRP:** KH–type splicing regulatory protein; **N2a:** Mouse neuroblastoma; **NLRP3:** NOD–like receptor pyrin domain–containing protein 3; **PD:** Parkinson's disease; **IL:** Interleukin; **ARE:** AU–rich element; **iNOS:** Inducible nitric oxide synthase; **FBS:** Foetal bovine serum; **NC:** Nitrocellulose; **SAHA:** Suberoylanilide hydroxamic acid; **MAPK:** Mitogen–activated protein kinase; **NF-κB:** Nuclear factor kappa B.

## SUMMARY

In this investigation, the vital role of KHSRP in Mn–induced toxicity, and the importance of other known neurotoxicity inhibitors on KHSRP were analysed. It was discovered that $MnCl_2$ treatment of N2a cells induce the expression of KHSRP via the PI3K–or NLRP3 pathway. The increased expression of KHSRP was responsible for an augmentation in the liberation of pro-inflammatory markers in N2a cells. More exploration is needed to explain these mechanisms / pathways in details.

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