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Tjernberg peptide- A double edged sword in Alzheimer's disease

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Abstract

Alzheimer's disease is a neurodegenerative disorder affecting millions of people worldwide clinically manifested by the presence of amyloid plaques and neurofibrillary tangles. Senile plaques are composed of amyloid beta protein while neurofibrillary tangles are formed by the hyperphosphorylation of *tau* protein. A plethora of reports on the anti-oxidant and pro-oxidant properties of amyloid beta peptide are available. However the molecular candidates involved bringing about this therapeutic behaviour is not explored. To investigate this phenomenon we have used a pentapeptide sequence KLVFF, derived from the core-recognition motif of amyloid beta peptide to study the altered signaling cascade in neuronal cells. Our data showed the unique dual behaviour of KLVFF peptide as pro-oxidant and toxicant based on the dosage concentration. The peptide's inherent ability to scavenge free radicals at low concentrations <100 μM to offset oxidative stress was proved by the down-regulation of SOD1, AP-1 and FoxO3a genes. However at concentrations > 100 μM, the cytotoxic effect of the peptide dominates, leading to apoptosis through activation of p53, ERK1 and p38 in a caspase-dependent mechanism accompanied by mitochondrial membrane depolarization. The therapeutic role of KLVFF peptide stems out from the regulation of SOD1 gene by AP-1 and NF-κB and Nrf2 gene to regulate the intracellular glutathione levels. Collectively our experimental findings revealed a threshold concentration of 100 μM beyond which KLVFF peptide mimics the amyloid beta of senile plaque, which can be used as a model system to understand the pathological role of amyloid beta peptide. While concentrations below 100 μM may be actively employ for therapeutic applications to prevent the further aggregation of amyloid beta.

1. Introduction

The extracellular deposition of amyloid beta peptide 39 - 43 aminoacid residues derived from the amyloid precursor protein (APP) is a characteristic feature of amyloidosis condition during Alzheimer's disease.[1] The formation of insoluble toxic amyloid beta occurs through the amyloidogenic pathway, where APP undergoes sequential cleavage by β and γ secretase.[2] APP is an integral transmembrane protein present in neuronal cells upon cleavage by α and γ secretase yield a non-toxic soluble fragment in healthy brain through the non-amyloidogenic pathway.[2] Mutations in presenilin genes 1 and 2 result in altered proteolytic processing of APP protein through the amyloidogenic pathway.[2] The presence of beta sheet structures in amyloid beta peptide tends to aggregate with each other leading to plaque formation.

Peptide based therapeutic systems have emerged as therapeutic solution to disrupt the amyloid plaques or prevent the aggregation of plaques. The pentapeptide LPFFD (Soto peptide) and KLVFF (Tjernberg peptide) were used as beta sheet breakers to disrupt amyloid protein aggregation.[3] KLVFF peptide was derived from the amyloid beta peptide residues 16-20 and hence possesses the core recognition motif that enables it to selectively interact with the amyloid beta peptide and thereby preventing further aggregation.[3] The LPFFD peptide has been extensively used to disrupt plaques and improve cognitive deficits *in vivo*.[4] Modified KLVFF derivatives with either positively charged amino acid residues (KLVFFKKKK) or negatively charged amino acid residues (KLVFFEEE) in the C-terminal have shown to prevent protein aggregation efficiently *in vitro*.[5] *In silico* studies have suggested that KLVFF can act as a therapeutic peptide by selective binding to the core-recognition motif of amyloid beta peptide, thus preventing its further aggregation.[6] Surprisingly Hamley *et al*., have reported that KLVFF peptide and its PEGylated analogue (KLVFF-PEG) tend to self-assemble into higher order fibril structures in phosphate buffered saline.[7] The mechanism of

self-assembly followed by KLVFF peptide mimics the *in vivo* amyloid plaque formation and hence can serve as an effective model system to understand the pathogenesis of amyloid beta aggregation *in vitro*. Thus it appears that KLVFF peptide possesses a dual ability to block as well as promote aggregation of amyloid peptides under critical concentration gradient. The mechanism underlying the transition behaviour of peptides along with the molecular targets involved remains unexplored in the literature, which forms the major focus of the present study.

Literature reports suggest the amyloid beta peptide possesses a "Jekyll and Hyde" property with a neuroprotective effect at concentrations in the nanomolar range and neurotoxic effect at concentrations beyond the nanomolar range.[8] Many conflicting reports exist for the pro-oxidant and anti-oxidant properties of amyloid beta peptide.[9] However the key players involved in neuronal signaling pathway that are responsible for these complementary effects have not been elucidated thus far. In the present study, we have used KLVFF peptide derived from the core-recognition motif of amyloid beta to understand its concentration-dependent effects on neuronal cells. Since the KLVFF motif in amyloid beta peptide has been identified to be crucial for beta sheet formation, the results obtained in this study can be extrapolated to the effects caused by amyloid beta peptide. Earlier our group showed the KLVFF at low concentrations can chelate metal ions thereby overcoming metal ion-induced cytotoxicity while at high concentrations it aggravated the toxic effects of the metal ions [*unpublished work*]. However the signaling cascade involved in cell survival or death induced by the KLVFF peptide in the absence of external stress has not been explored thus far and this forms the major crux of the present study.

2. Materials and Methods

2.1. Materials

Copper chloride ($CuCl_2$), iron (III) chloride ($FeCl_3$) and zinc chloride ($ZnCl_2$) were purchased from Merck, India and were used without further purification. H_2N–KLVFF–$CONH_2$, a pentapeptide with >95% purity was purchased from Bioconcept Labs Pvt. Ltd (Gurgaon, India). 2',7'-dichlorofluoresceindiacetate (DCFDA) of >97% purity used for reactive oxygen species estimation and 5',5'-dithiobis (2-nitrobenzoic acid) also known as Elman's reagent (>98% purity) used for the measurement of intracellular glutathione, 2-thiobarbituric acid and trichloroacetic acid used for TBARS assay, 2,2-Diphenyl-1-picrylhydrazyl of >95% purity used for DPPH scavenging assay were procured from M/s. Sigma-Aldrich, USA. Fetal Bovine Serum (FBS) and Dulbecco's Modified Eagles Medium (DMEM), phosphate buffered saline (PBS), antibiotics (Penicillin/streptomycin (P/S)) were purchased from Gibco, USA.

2.2. Methods

2.2.1. Cell culture & seeding

IMR-32 human neuroblastoma cells procured from National Centre for Cell Sciences (NCCS), Pune, India were cultured in DMEM (Gibco®, USA) supplemented with 10% FBS (Gibco®, USA) and 1% penicillin/streptomycin (Gibco®, USA). The culture was maintained at 37°C in 5% CO_2 incubator.

2.2.2. Lactate dehydrogenase assay

Ten thousand cells were seeded in a 96 well plate followed by the incubation with desired concentration of peptide (50, 100, 200 and 400 μM) solution for 48 hours. The concentrations of the peptide used in this study were determined based on previous experiments from our group.[10] One hundred microliters of cell culture supernatant treated with various concentrations of peptide and untreated sample was incubated with equal amount of LDH

reagent (CytoTox-One™ Homogeneous membrane integrity assay, Promega, USA) for about 20 min at room temperature. The reaction was then terminated by addition of 25 µL of stop solution to each well. Fluorescence intensity was measured at 590 nm using a multimode reader (Infinite 200M, Tecan®, Austria) after excitation at 560 nm.

2.2.3. Reactive Oxygen Species Determination (ROS) assay

10,000 cells were seeded in a 96 well plate followed by the incubation with specific concentration of peptide (50, 100, 200 & 400 µM) for 48 hours. After incubation, the medium was removed and the cells were washed with PBS to remove any non–adherent cells. Then 10 µM DCFDA solution was added and incubated for 45 minutes. After diffusion into the cell, the DCFDA gets deacetylated by the cellular esterases to form a non-fluorescent compound, which is later oxidized by ROS into 2',7'- dichlorofluorescein, which can be detected with an excitation and emission maxima of 495 nm and 530 nm respectively.

2.2.4. Determination of intracellular Glutathione levels

100,000 cells were seeded in a 24-well plate followed by the incubation with desired concentration of peptide (50, 100, 200 & 400 µM) for 48 hours. After incubation, the medium was removed and the cells were washed with PBS to remove any non–adherent cells. Cell lysis buffer was added and incubated for 20 minutes to disrupt the cells and the lysate was collected. The proteins in the cell lysate were removed by adding 10% trichloroacetic acid and centrifuged at 4500 rpm for 15 min to precipitate the proteins. DTNB reagent was added to the supernatant and analyzed using a multimode reader (Infinite 200M, Tecan®, USA). Values were normalized with respect to the protein content present in the cells and were compared with the untreated cells.

2.2.5. Thiobarbituric Acid Reactive Substances (TBARS) assay

100,000 cells were seeded in a 24-well plate and followed by the incubation with specific concentration of peptide (50, 100, 200 & 400 μM) for 48 hours. After incubation, the medium was removed and the cells were washed with PBS to remove any non–adherent cells. Cell lysis buffer was added and incubated for 20 minutes to disrupt the cells and the lysate was collected. The proteins in the cell lysate were removed by adding 20% trichloroacetic acid and centrifuged at 4500 rpm for 15 minutes to precipitate the proteins. 0.6% thiobarbituric acid was added to the supernatant and heated to 95°C for one hour in a water bath. The absorbance at 532 nm was analyzed using a multimode reader (Infinite 200M, Tecan®, Austria). Values were normalized with respect to the protein content present in the cells and were compared with the untreated cells.

2.2.6. Imaging of cells

100,000 cells were seeded in a 96 well plate followed by the incubation with specific concentration of peptide (50, 100, 200 & 400 μM) for 48 hours. The medium was removed and the cells were washed with PBS to remove any non–adherent cells. The cells were then imaged using phase contrast microscope (Carl Zeiss Axiovert A1, Germany).

2.2.7. Gene Expression studies

Expression of genes for various transcription factors associated with the oxidative stress namely Nrf2, SOD1, p53, Akt, FoxO-3a, mTOR, GSK-3β, AP-1 NF-kB and certain MAP kinase genes namely ERK-1 and p38 were evaluated in the presence of low and high concentration of peptides (50 & 400 μM) using real time RT-PCR. The total RNA was isolated using Trizol (Invitrogen, USA) following the procedure described by the manufacturer. In brief 1 mL of Trizol was added to the samples and kept for 30 min at room

temperature. The solution was collected and RNA was extracted with 0.2 mL of chloroform (Merck, India). The solution was centrifuged at 12,000 rpm for 15 min at 4 ^{o}C. The extracted RNA was stabilized using 70% ethanol prepared with nuclease-free water (Qiagen, USA). The RNA was centrifuged using a QIA shredder spin column (Qiagen) and dissolved in RNAse-free water (Qiagen, USA). The cDNA obtained after a two-step reaction was subjected to a real-time RT-PCR (Eppendorf AG22331, Germany). The primers used in this study are shown in **Table I**. Quantitative values were determined by delta-delta method and normalized with the house keeping gene GAPDH and the control.

Table I: Primer sequences employed to determine the gene expression levels

Genes	Sequences	Base no
Nrf2	CTGCTTTCATAGCTGAGCCC	20
	CCTGAGATGGTGACAAGGGT	20
p53	CCCAAGCAATGGATGATTTGA	21
	GGCATTCTGGGAGCTTCATCT	21
ERK-1	CTGGATCAGCTCAACCACATT	21
	AGAGACTGTAGGTAGTTTCGGG	22
p38	TCGACTTGCTGGAGAAGATGCTTGT	25
	CAGGACTCCATCTCTTCTTGGTCAA	25
Akt	CTCACAGCCCTGAAGTACTCTTTCCA	26
	TCCAGCATGAGGTTCTCCAGCTTGA	25
FoxO-3a	TCTACGAGTGGATGGTGCGTT	21
	CGACTATGCAGTGACAGGTTGTG	23
mTOR	TCGCTGAAGTCACACAGACC	20

	CTTTGGCATATGCTCGGCAC	20
GSK3β	ATTTCACCTCAGGAGTGCGG	20
	AAGAGTGCAGGTGTGTCTCG	20
SOD1	ACAAAGATGGTGTGGCCGAT	20
	AACGACTTCCAGCGTTTCCT	20
NF-kB	CGCTTAGGAGGGAGAGCCC	19
	TATGGGCCATCTGTTGGCAGTG	22
AP-1	TCCTGCCCAGTGTTGTTTGT	20
	GACTTCTCAGTGGGCTGTCC	20
GAPDH	ACCACAGTCCATGCCATCAC	20
	TCCACCACCCTGTTGCTGTA	20

2.2.8. Superoxide dismutase (SOD) assay

The levels of SOD protein were estimated using standard SOD assay kit (Promega, USA). In brief the untreated cells and those treated with peptide (50 & 400 μM) were washed with PBS, scrapped and collected in lysis buffer (20mM HEPES, 1mM EDTA, 210mM mannitol and 70mM sucrose). The cell suspension was centrifuged at 1500g for 5 minutes at 4°C. The supernatant was then used for the assay. Diluted radical detector (200μL) was mixed with 10μL of sample and then 20μL of diluted xanthine oxidase was added. The plate was shaken continuously for few minutes and incubated for 20 minutes at room temperature. Absorbance was measured between 440-460 nm using multimode reader (Infinite 200M, Tecan®, Austria).

2.2.9. Caspase 3/7 activity assay

The involvement of caspases in causing apoptosis due to peptide exposure was determined by measuring caspase 3/7 activity (Apo-One® Homogeneous caspase 3/7 assay kit, Promega, USA). In brief the cells treated with peptide (50 & 400 μM) were incubated with the caspase 3/7 reagent and incubated for about 3 hours at room temperature. The fluorescence intensity was measured at 527 nm using multimode reader (Infinite 200M, Tecan®, Austria) after excitation at 485 nm.

2.2.10. MitoTracker® Red Staining

The untreated and peptide treated (50 & 400 μM) cells were washed with PBS followed by the addition of 200nM MitoTracker® Red dye (Molecular Probes, USA). After incubation for 45 minutes at 37°C in 5% CO_2 incubator, the media with dye was gently removed and rinsed with PBS. The cells were incubated with 3.7% paraformaldehyde at 37°C in 5% CO_2 incubator for 15 minutes. After gentle PBS wash, the Hoechst 33342 dye (Molecular Probes, USA) was added and incubated for 15 minutes. The excess dye was removed from the wells followed by PBS wash. Later the cells were imaged using laser scanning confocal microscopy (FV1000, Olympus, Japan).

2.2.11. JC-1 mitochondrial membrane potential assay

The measurement of mitochondrial membrane potential ($\Delta\Psi m$) in untreated and peptide treated (50 & 400 μM) cells were carried out using JC-1 assay. The JC-1 is a lipophilic cationic dye which shows two emission peaks based on the two different conformations adopted by the dye. In mitochondria of healthy cells that possess high mitochondrial potential, J-aggregates are formed which show emission maxima at 590 nm. However, in the case of dead cells or in cells undergoing apoptosis, due to decrease in mitochondrial potential, JC-1 dye exists in the monomeric form and shows a characteristic

emission at 530 nm. The cells were washed with PBS followed by the addition of staining solution (JC-1 dye at a concentration of 2 μM) and incubated for one hour in 5 % CO_2 incubator. The dye was removed and washed with PBS followed by addition of PBS with 5 % BSA. After 5 minutes incubation the BSA containing PBS was removed and fresh PBS was added before taking measurements. The fluorescence emission intensity was recorded at two wavelengths namely 530 and 590 nm after excitation at 514 nm using multimode reader (Infinite 200M, Tecan®, Austria).

2.3. Statistical analysis

The Analysis of Variance (one–way ANOVA) was performed to determine the statistical significance ($p < 0.05$) for LDH, ROS, Glutathione, TBARS assay (n=6) and RT-PCR, Caspase3/7, JC-1 mitochondrial membrane permeability assay (n=5).

3. Results and Discussion

3.1. Influence of KLVFF peptide concentration on cell viability

The results of the LDH assay shows the viability of cells remains unaltered until 100 μM concentration of the peptide, beyond which there was significant reduction in cell viability is observed (**Figure 1**). Our results suggest that KLVFF peptide at low concentrations does not exert any cytotoxic effect as it does not affect the cell viability. The drastic reduction in cell viability at high concentrations 200 and 400 μM of KLVFF peptide arises due to the tendency of the peptide to form self-assembled aggregates.[10] The molecular mechanism behind the cytotoxicity of the KLVFF peptide needs to be explored in-depth to devise possible strategies to counter its toxic effects and exploit its therapeutic potential.

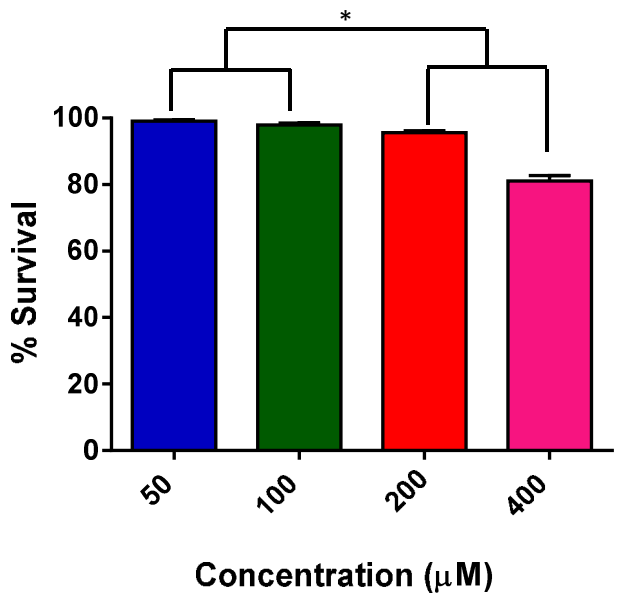

Figure 1. Viability assessment of IMR-32 human neuroblastoma cell line at 50, 100, 200 and 400 μM concentrations of KLVFF peptide. Results shown as mean ± SD (n=4; * $p < 0.05$).

3.2. Influence of KLVFF peptide concentration on reactive oxygen species (ROS) levels

As the concentration of Tjernberg peptide increases, the ability of KLVFF peptide to scavenge ROS increases as shown in **Figure 2A**. The results were in correlation with LDH assay, where the viability reduced at concentration 200 and 400 μM of KLVFF peptide. However at peptide concentration ≤100 μM, the cell viability remains unaffected while the ROS levels decline by about 35% when compared with the control cells. This indicates that the peptide exhibits an inherent radical scavenging effect until 100 μM beyond which a sudden transition to a toxic form occurs that appears to cause lethality in ROS-independent manner.

The reduction in ROS levels was observed in all peptide concentration tested from 50-400 μM, which is in contrast with recent report on $A\beta_{1-42}$ peptide toxicity in glioma and neuroblastoma cells.[11] Our results indicate that KLVFF displays contrasting properties with $A\beta_{1-42}$ depending on its concentration. Therefore, it is evident that KLVFF can serve as a $A\beta_{1-42}$ mimic only under certain conditions. The results of the ROS assay suggests that the

KLVFF peptide's ability to scavenge free radicals arises due to the nature of amino acid residues present in them. It is now recognized that the aromatic amino acids tyrosine (Y) and phenylalanine (F) have the ability to donate protons to electron deficient centres and the resultant aromatic carbanion will be stabilized through resonance.[12] The hydrophobic amino acids are known to have strong hydroxyl radical scavenging activity.[13] The protein hydrolysates containing higher concentrations of phenylalanine (F), isoleucine (I), leucine (L) and valine (V) possess strong superoxide radical scavenging activity.[14] The amino acid lysine (K) is reported to exhibit radical scavenging property by virtue of its $-NH_3^+$ group.[15] Since Tjernberg peptide possesses the amino acid sequence KLVFF, the free radical scavenging ability of the individual amino acids could have synergistically contributed to the observed results. In addition, peptides are also known to chelate metal ions via their carboxyl and amino groups.[16] which may further prevent formation of hydroxyl radicals by the metal ions.

3.3. Scavenging ability of KLVFF peptide using DPPH assay

The inherent free radical scavenging ability for KLVFF peptide in the absence of cells was quantified at various concentrations using DPPH assay and the results are shown in **Figure 2B**. A concentration-dependent increase in the free radical scavenging ability of the peptide was observed. However, maximum radical scavenging activity of the peptide was 5% indicating that the Tjernberg peptide possesses a weak anti-oxidant nature. The anti-oxidant property of the Tjernberg peptide arose from the aromatic amino acid phenylalanine that acts as a proton donor to DPPH. The Tjernberg peptide with a molecular weight of 651 Da falls in the lower molecular weight category that may also have contributed to its radical scavenging property.[17] The presence of hydrophobic amino acid leucine, valine and phenylalanine in high proportion confer the radical scavenging property by enhancing its solubility in non-polar environment thereby facilitating better interaction with the free radicals.

3.4. Influence of KLVFF concentration on glutathione levels

Alterations in the intracellular glutathione levels due to addition of various concentrations of KLVFF peptide are presented in **Figure 2C**. A marginal increase in the glutathione levels was observed at 50 and 100 μM concentrations when compared with the control cells. However, the intracellular glutathione levels were found to exhibit a concentration-dependent decrease with increasing peptide concentrations. The reduced glutathione levels at higher concentrations namely 200 and 400 μM were in correlation with reduced cell viability, owing to the toxic effects of the peptide. Small increase in glutathione levels at concentrations ≤ 100 μM suggests that the peptide recruits anti-oxidant machinery to scavenge free radicals.

3.5. Influence of KLVFF peptide concentration on lipid peroxidation

TBARS assay was performed in cells treated with various concentrations of KLVFF peptide to quantify the extent of lipid peroxidation and the results are shown in **Figure 2D**. A marginal decrease in the malondialdehyde (MDA) levels was observed in all cases when compared with the untreated control cells. Our group had earlier demonstrated the localization of KLVFF peptide in lipid bilayer.[18] Presence of hydrophobic leucine and valine in peptide sequence increases its ability to penetrate into lipid phase of membrane thereby facilitating better interaction with free radicals. It has also been reported that hydrophobic amino acids tend to protect linoleic acid from degradation by donating protons to the hydrophobic peroxyl radicals.[13] Thus from the results of ROS and TBARS assay, it may be hypothesized that KLVFF peptide can act as a lipid soluble anti-oxidant, which can localize into the lipid bilayer and scavenge the free radicals. However, beyond 100 μM, no apparent

changes in the MDA levels are observed indicating that toxicity due to the peptide does not act via lipid peroxidation.

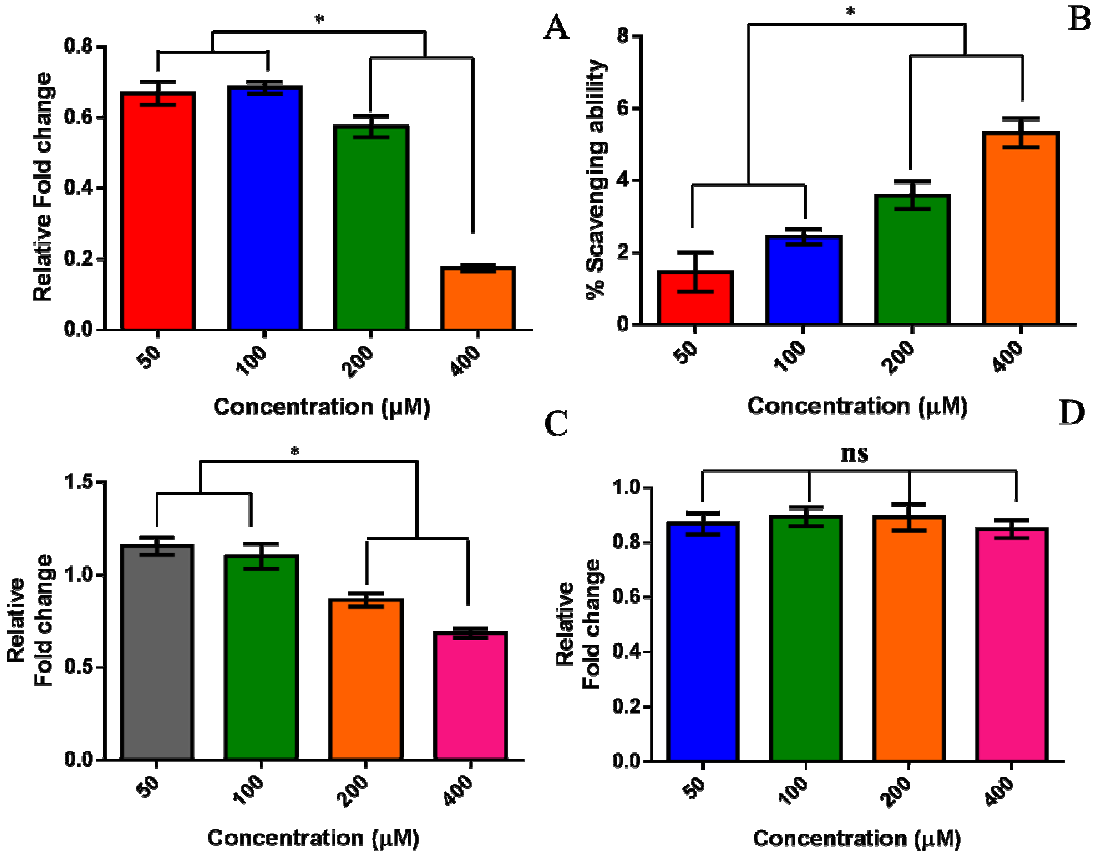

Figure 2. Biochemical analysis of KLVFF peptide at various concentrations 50, 100, 200 and 400 µM [A] ROS assay, [B] DPPH assay, [c] Glutathione assay and [D] TBARS assay. Results shown as mean ± SD (n=4; * $p < 0.05$).

3.6. Influence of KLVFF peptide on the cell morphology

The phase contrast microscopic images of IMR-32 cells treated with different concentrations of the Tjernberg peptide was shown in **Figure 3**. The results show at peptide concentration of 50 µM and 100 µM the cells exhibit their native elongated morphology with few dead cells. However at high concentrations i.e., 200 and 400µM, a significant distortion in the cell morphology is observed with more floating cells. They also lack cell-cell contacts followed by reduction in the cell number. This suggests that at high concentrations 200 and

400μM the KLVFF peptide triggers the cell death machinery by activating apoptotic pathways directly or indirectly. Therefore gene expression studies were carried out to decipher the possible pathways triggered by the peptide at low concentration (50 μM) where it exerts a non-toxic effect and cytotoxic at high concentration (400 μM).

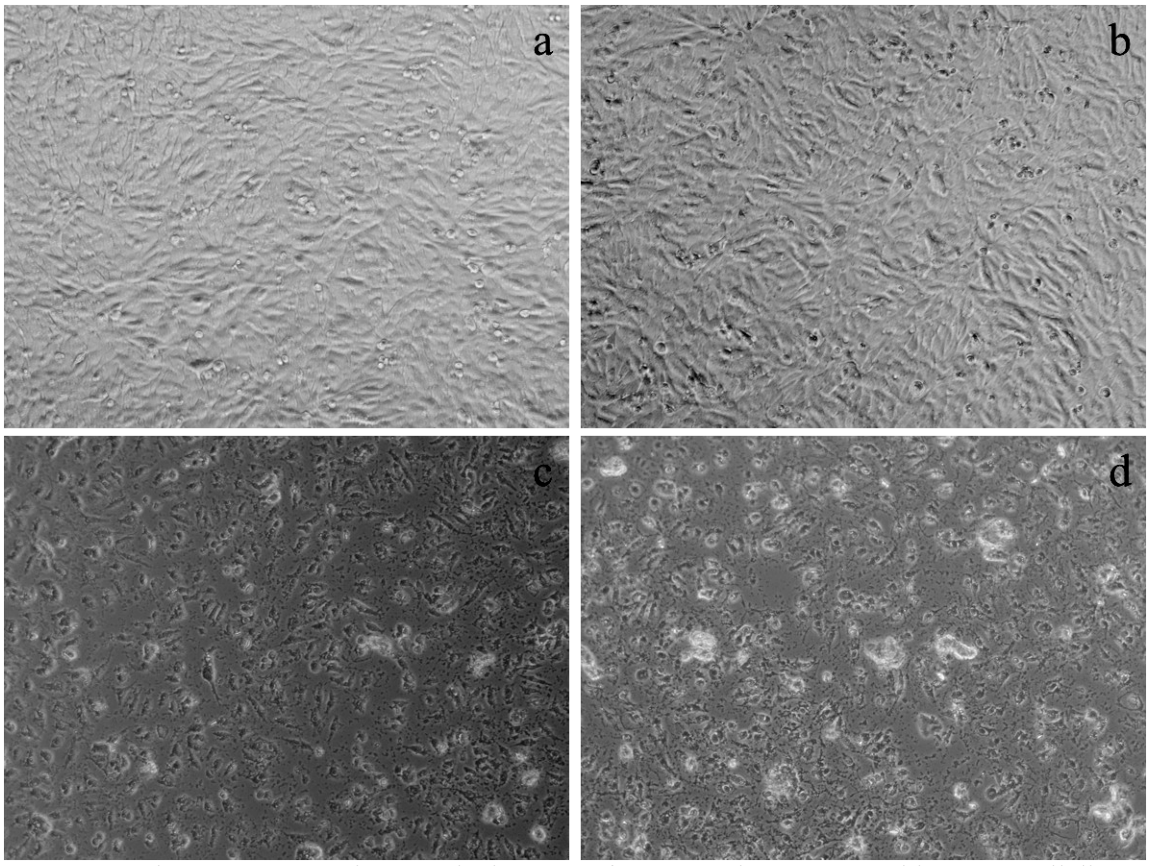

Figure 3. Phase contrast microscopic images of IMR-32 human neuroblastoma cell lines treated with various concentrations of KLVFF peptide [a] 50 μM [b] 100 μM [a] 200 μM and [b] 400 μM peptide. Magnification x 100.

3.7. Gene expression studies

The expression profiles of genes responsible for cell survival, apoptosis, autophagy and those that are induced in response to genotoxic or oxidative stress were evaluated in cells exposed to either a non-toxic concentration (50 μM) or toxic concentration (400 μM) of KLVFF. **Figure 4** summarizes the relative fold change of various genes in IMR32 cells treated with 50 or 400 μM of the peptide. A distinct difference in the expression profiles of

the genes was observed in cells treated with different peptide concentrations. Cells exposed to 50 μM of the Tjernberg peptide exhibited an up-regulation of Nrf2, Akt, mTOR and GSK3β genes while the genes SOD1, p53, ERK-1, p38, FoxO3a, NF-κB and AP-1 were down-regulated when compared with the untreated control cells (**Figure 4a**). In contrast, cells exposed to 400 μM peptide were found to over-express all genes except Nrf2 when compared with the untreated control cells (**Figure 4b**).

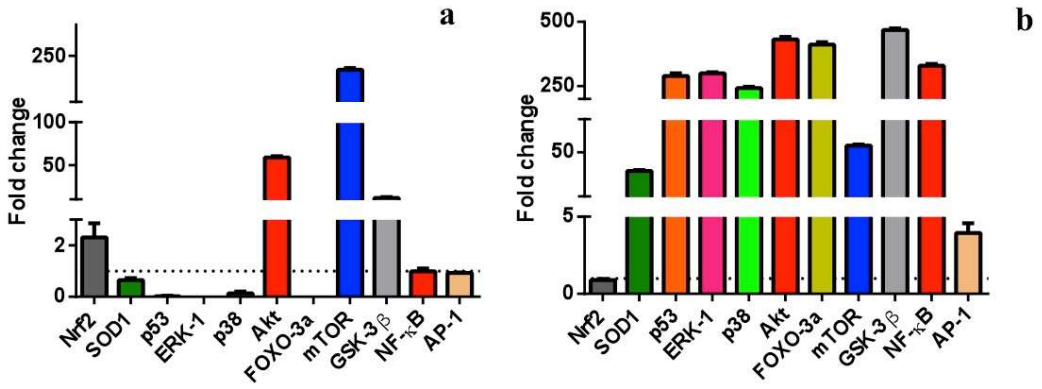

Figure 4. Gene expression analysis in IMR-32 human neuroblastoma cell lines treated with various concentrations of KLVFF peptide [a] 50 μM and [b] 400 μM peptide

Interestingly, mTOR gene expression levels were elevated in cells treated with 50 μM or 400 μM of KLVFF peptide. The mTOR exhibit multiple roles among which the sensing of nutrients and thereby enhancing cell proliferation plays a crucial role.[19] The mTOR in connection with autophagy demonstrated that inhibition of mTOR by rapamycin improved cognitive deficits *in vivo*.[20] Recent evidence demonstrate that excessive activation of autophagy pathway results in cell death due to autophagy of the cell's own cytosolic contents.[21] Elevated mTOR levels have also been found to activate the pro-apoptotic nuclear transcription factor p53.[22] It has been suggested that mTOR translocate from the cytosol to the nuclear compartment where it phosphorylates a serine residue in p53 resulting in its activation.[22] Our data reveals that cells treated with 400μM of peptide exhibit up-regulation of p53 in contrast to cells treated with 50 μM of the peptide. This indicates that high

concentrations of the peptide triggers the translocation of mTOR and the subsequent phosphorylation and activation of p53 leading to a halt in the cell cycle and initiation of apoptosis.

The upstream activator of mTOR is Akt, also known as protein kinase B.[23] The activation of Akt is seen in cells treated with the Tjernberg peptide irrespective of the concentration. The up-regulation of Akt suppresses the activation of pro-apoptotic mitochondrial proteins and inhibits caspase activation and JNK pathway.[24] This has been suggested as a potential therapeutic strategy to combat amyloid beta 1-42 induced apoptosis. Therefore our results indicate that at 50μM concentration of KLVFF peptide up-regulates Akt while causing no cytotoxicity indicating that its therapeutic activity may be due to the activation of the Akt pathway. Interestingly the Akt levels are also up-regulated in cells treated with 400μM of the Tjernberg peptide. Like many other proteins that regulate cell survival and proliferation, a dual role for Akt has been identified recently.[25] Over-expression of Akt has been implicated in inducing cell death though the exact mechanism has not been deciphered yet. It has been suggested that Akt might translocate to the nucleus where it may interact with anti-apoptotic factors like Epb1, an inhibitor of DNA fragmentation thereby altering its function.[26] Thus the treatment with high concentrations of the Tjernberg peptide is likely to promote translocation of Akt and mTOR to the nucleus thereby initiating autophagy and apoptotic signals leading to cell death.

It is reported that amyloid beta (Aβ) accumulation induces neuronal apoptosis via transcriptional activation of death-associated genes.[27] The FoxO3a is a forkhead transcriptional factor implicated in inducing apoptosis. Its transcription activity is modulated by various post-translational modifications especially phosphorylation by various kinases.[28] The phosphorylation of FoxO3a by survival kinases like Akt in the cytosol prevents its entry in to the nucleus and promotes degradation through ubiquitination.[28] In the present study, it

was found that in cells treated with low concentration (50 μM) of the Tjernberg peptide, FoxO3a gene levels are down-regulated while up-regulated in cells treated with high concentration (400 μM) of the peptide. This indicates that the presence of low concentrations of peptide promotes phosphorylation of FoxO3a by Akt leading to its subsequent degradation. However in cells treated with high concentrations of the peptide, a translocation of the FoxO3a to the nucleus might occur thereby preventing its phosphorylation by Akt and hence escapes degradation. The nuclear sequestration of FoxO3a results in activation of CDKI p27, a cell cycle inhibitor.[29] In addition the nuclear FoxO3a has been found to activate several apoptotic and autophagy factors like TRAIL (TNF-related apoptosis-inducing ligand), FasL (Fas ligand or CD95L), PUMA (p53 upregulated modulator of apoptosis), PTEN (phosphatase and tensin homolog deleted on chromosome 10) etc.[30] These events are likely to be the reason for the observed cell death in cells treated with high concentrations of the Tjernberg peptide. Recent evidence has highlighted the existence of cross-talk between FoxO3a and p53 signaling pathways as they act on common molecular targets.[31] Therefore the nuclear translocation of FoxO3a may synergistically act with p53 in inducing apoptosis of cells treated with high concentration of Tjernberg peptide.

Glycogen synthase kinase 3 beta (GSK3β) levels were found to be up-regulated in cells treated with 50 μM or 400 μM of the Tjernberg peptide. GSK3 β is involved in the phosphorylation of different protein substrates in cells. Its over-expression has been implicated in the hyperphosphorylation of tau protein leading to the formation of neurofibrillary tangles and neuronal apoptosis.[32] The GSK3β levels are regulated by the activation of Akt.[33] Our results suggest that in cells treated with high concentration (400 μM) of the Tjernberg peptide there was up-regulation of GSK3β leading to the reduction in cell viability. Interestingly both Akt and GSK3β levels are also up-regulated in cells treated with low concentration (50 μM) of the peptide. Apparently the intracellular localization of Akt and

GSK3β may be responsible for the difference in the cell viability at this concentration. Like Akt, the GSK3β found to sequester free radicals in the cytosol and nucleus though the factors promoting this sequestration and their implications on the GSK3β activity are unknown. Another aspect that may have implications on the differential effects of GSK3β expression on cell survival could be the nature of its phosphorylated site. It has been found that GSK3β exists in an inactive state, when serine residue in the 9th position was phosphorylated and transforms in to an active state if the phosphorylation occurred in tyrosine residue at 216th position.[34] It has now been demonstrated that alpha5 beta1 integrin triggers resistance to apoptosis in cells through GSK3β signaling.[35] This introduces another facet to the complex interplay of signaling factors in the cells treated with low concentration of Tjernberg peptide. An earlier report had suggested that alpha5 beta1 integrin could rescue cells from amyloid beta induced apoptosis.[35] Therefore the up-regulation of GSK3β in cells treated with low concentration of the peptide could promote cell survival and may implications in its therapeutic potential either through difference in GSK3β localization or through different phosphorylation sites or through activation of alpha5beta1 integrin or a combination of all these factors.

It is observed from Figure 7 that the ERK1 and p38 genes are down-regulated in cells treated with low concentration of Tjernberg peptide while cells exposed to high concentrations of the peptide over-express both ERK1 and p38. Both ERK1 and p38 are MAP kinases related to stress and are the downstream effectors in the anti-oxidant response of cells.[36] The up-regulation p38 gene has been implicated in early Alzheimer's disease leading to neuronal death.[37] Our results indicate that at high concentration of Tjernberg peptide p38 activation is induced leading to stimulation of pro-apoptotic factors. Low concentrations of the peptide do not activate p38. ERK1 activation has been implicated in initiation of apoptosis through activation of caspase 3.[38] The up-regulation of ERK1 in cells

treated with 400 μM of Tjernberg peptide suggests the probable activation of caspase-dependent apoptosis in these cells. The expression levels of ERK1 and p38 are also connected with the anti-oxidant levels in the cells. It has been reported that GSH depletion leads to activation of p38.[39] The glutathione assay in the present study reveals that depletion of glutathione is observed only in cells treated with high concentrations of the Tjernberg peptide and the p38 levels are up-regulated in these cells suggesting a direct correlation. GSH possesses binding sites for various transcription factors namely AP-1, AP-2 and NF-κB.[40] Intriguingly even though the GSH level is enhanced in cells treated with 50 μM peptide, neither AP-1 nor NF-κB is up-regulated. However, Nrf2 levels were up-regulated in these cells and hence it is evident that glutathione levels in cells exposed to Tjernberg peptide are regulated by Nrf2 and not through AP-1 or NF-κB. This is further substantiated from the high expression levels of AP-1 and NF-κB in cells treated with high concentration of the peptide, but the intracellular glutathione levels in these cells were depleted (**Figure 4**). These cells express low levels of Nrf2 indicating that this factor is commonly involved in the transcriptional regulation of genes encoding the antioxidant proteins and regulates the glutathione levels in cells treated with the Tjernberg peptide. The Nrf2 expression levels may be associated with Akt activation in cells treated with low concentration of the peptide. The implications of the ERK/MAP kinase pathway in Alzheimer's disease have been correlated with induction of synaptic plasticity, increased *tau* phosphorylation and development of cytoskeletal abnormalities.[41]

The phase contrast images of cells treated with high concentration of peptide reveal dystrophic neurites that may be attributed to elevated levels of ERK. Such phenotypic changes were absent in cells treated with low concentration of the peptide, which correlates well with the low levels of ERK1 expression in these cells. The gene expression level of major intracellular anti-oxidant system SOD1 was found to be up-regulated only in cells

treated with 400 μM of the peptide while it was down-regulated in cells treated with low concentration of the Tjernberg peptide. The upstream factors of the SOD1 gene are NF-κB, Nrf2, AP-1 and its downstream factors are Akt, GSK3β, mTOR, p53, FoxO3a, ERK1 and p38. In cells exposed to low concentration of the peptide, the SOD1 gene is down-regulated in spite of over-expression of its upstream transcription factor Nrf2. This indicates that the activation of SOD1 gene by the peptide requires other transcription factors such as NF-κB and AP-1 that are down-regulated in cells exposed to 50 μM of the peptide. All down-stream factors of SOD1 except Akt and mTOR are consequently under-expressed. The Akt protein, upstream factor of mTOR enhance SOD1 expression through activation of NF-κB.[42] However in cells treated with low concentration of the peptide, the NF-κB remains down-regulated and this is reflected in the low levels of expression of the SOD1 gene. In contrast, the cells exposed to high concentration of the peptide were found to over-express SOD1 gene that may have implications in increased oxidative stress and neurodegeneration. The over-expression of SOD1 can be correlated with the up-regulation of the transcription factors AP-1 and NF-κB. In addition both Akt and NF-κB are up-regulated that is also reflected in the SOD1 expression levels. The major signaling pathways activated by low (50 μM) and high (400 μM) concentration of peptide is shown in **Figure 5a and 5b**.

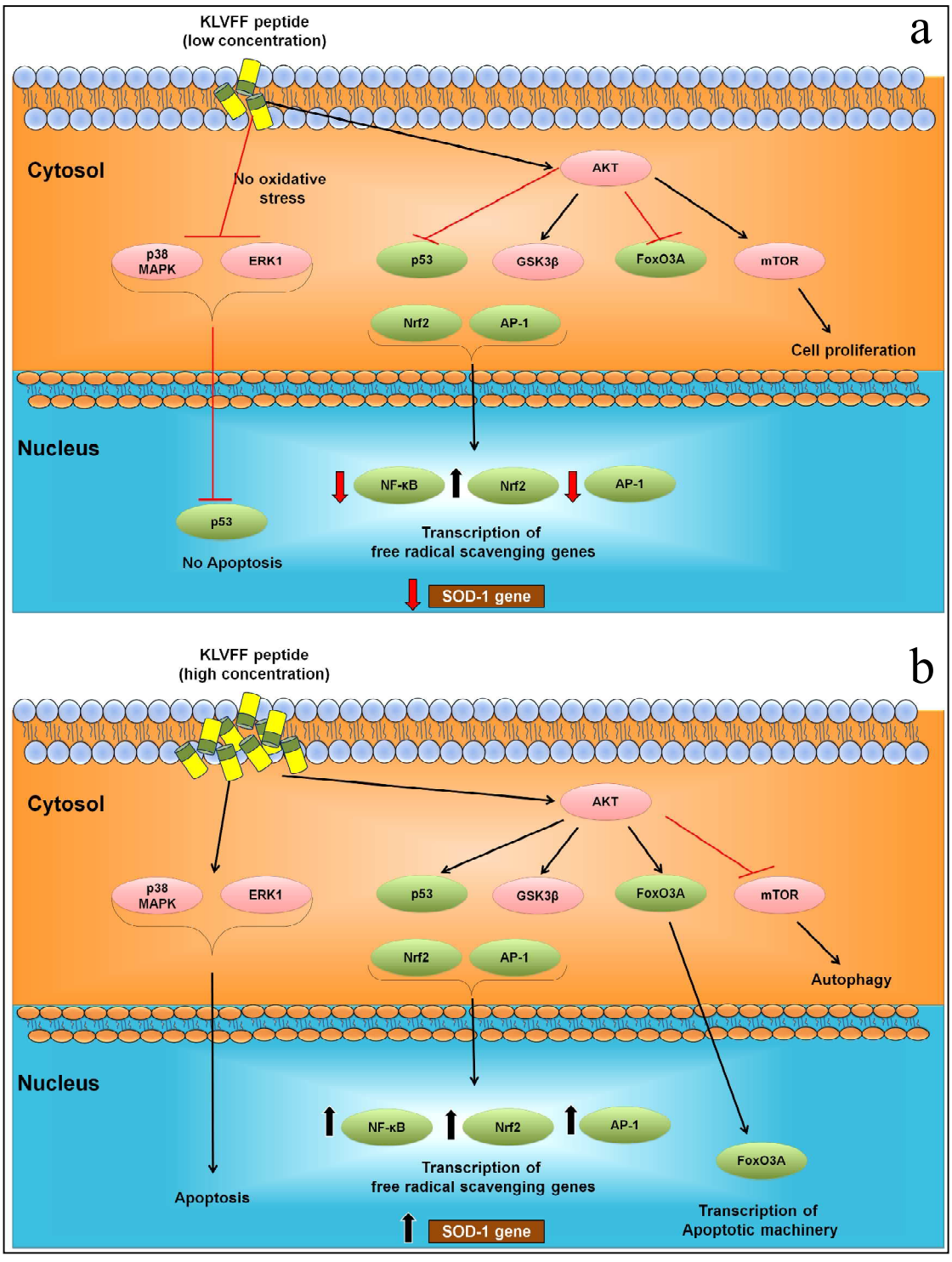

Figure 5: Major cell signaling molecules and pathways triggered in neuronal cells due to treatment with [a] 50 µM and [b] 400 µM of KLVFF peptide.

3.8. Influence of KLVFF peptide concentration on SOD activity

Figure 6A shows the SOD levels of cells treated with low and high concentrations of the Tjernberg peptide. It was observed that when cells were treated with low concentration of the peptide, no significant increase in the SOD levels are observed when compared with the untreated control cells. This is concurrent with the gene expression results where the SOD1 gene was down-regulated in cells treated with the same concentration of the peptide. The SOD levels of cells treated with high concentration of the peptide are significantly high in line with the gene expression data. The SOD has been hypothesized to promote neurodegeneration by inducing abnormal free-radical metabolism that causes production of hydroxyl radicals, toxic derivatives of peroxynitrite, metal toxicity and abnormal protein aggregation. Thus high concentrations of the Tjernberg peptide, increases SOD levels and promote protein aggregation and apoptosis.

3.9. Influence of KLVFF peptide concentration on Caspase 3/7 activity

The biochemical assays and gene expression studies have indicated that high concentration of the Tjernberg peptide induces cell death. In order to understand whether this cell death follows a caspase-dependent mechanism, caspase 3/7 activity was assessed and the results are shown in **Figure 6B**. The caspase levels were found to be significantly elevated in the cells treated with high concentration of the peptide while they were comparable to the untreated control in cells treated with low concentration of the peptide. This shows that the Tjernberg peptide at high concentration induces apoptosis through the activation of caspases. This may be due to the activation of ERK1 gene that activates the caspase 3 pathway. The activation of p53 gene in these cells may also have contributed to the apoptosis through the transcription of pro-apoptotic genes such as Bcl-2, Bax, Noxa and PUMA. This behaviour of Tjernberg peptide is akin to amyloid beta peptide, which has also been reported to cause

apoptosis through a caspase-dependent mechanism.[43] Therefore at high concentrations the Tjernberg peptide mimics amyloid beta peptide and can serve as a model system to study Alzheimer's disease.

3.10. JC-1 mitochondrial membrane potential measurements

The measurement of mitochondrial membrane potential were carried out using JC-1 probe to understand the influence of the peptide treatment on mitochondria and the results are presented in **Figure 6C**. The treatment with low concentration of peptide does not cause significant change in the mitochondrial membrane potential ($\Delta\Psi m$) when compared with the untreated control cells. However a 50% reduction in the membrane potential ($\Delta\Psi m$) is observed in cells treated with high concentration of the Tjernberg peptide. This indicates that the peptide at high concentrations can depolarize the mitochondrial membrane triggering intrinsic pathway of apoptosis. A similar observation was reported for amyloid beta protein by Moreira *et al*., who demonstrated mitochondrial dysfunction as the prime contributor to the cytotoxicity of amyloid beta and the associated alterations in energy metabolism observed in Alzheimer's disease.[44]

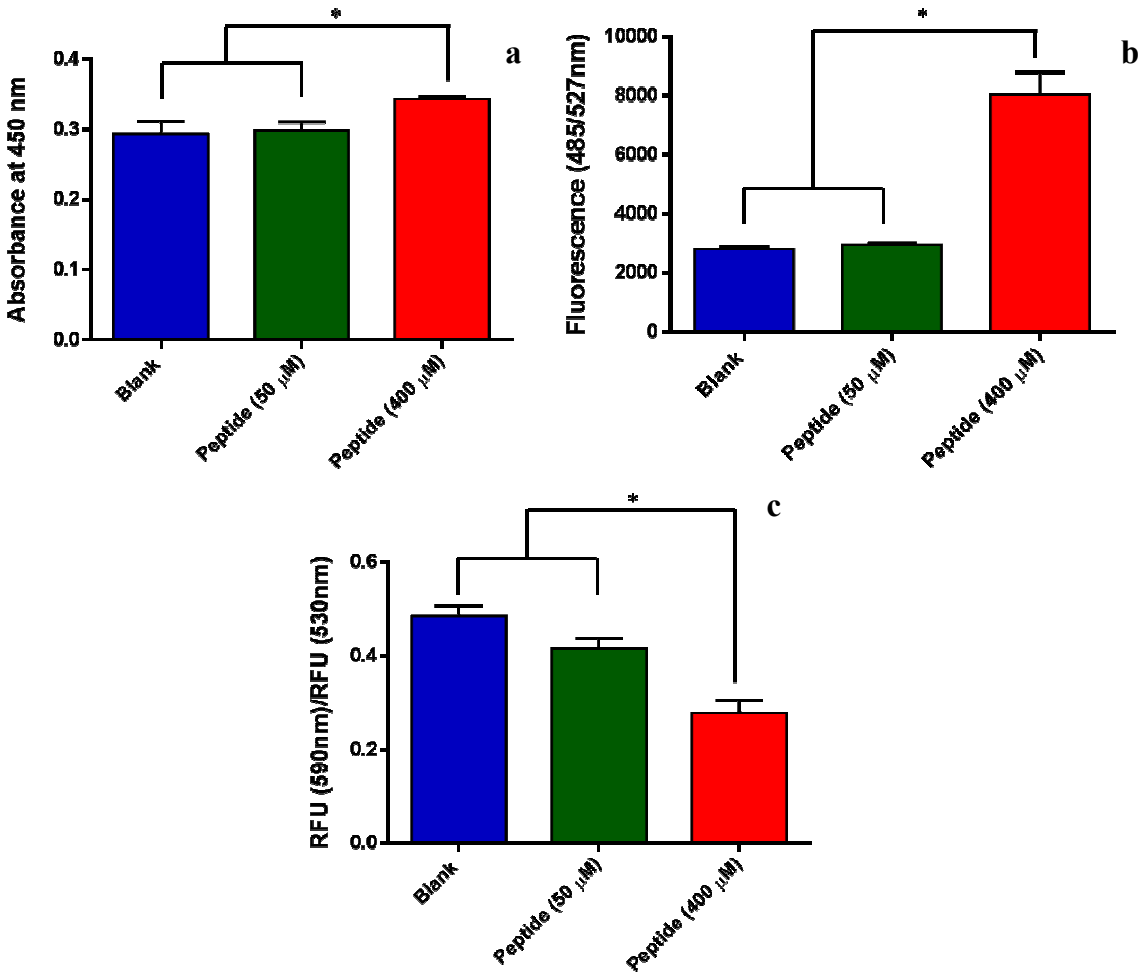

Figure 6. Intracellular alterations in the IMR32 cells due to low (50 μM) and high (400 μM) dose of KLVFF peptide [a] SOD assay, [b] Caspase 3/7 assay and [c] Mitochondrial membrane potential assay.

3.11. Mito Tracker Red® staining

To investigate the influence on the mitochondria during peptide dosing the Mito Tracker Red® staining of cells treated with different concentrations of the Tjernberg peptide was performed and the results are shown in **Figure 7**. The cells treated with the peptide exhibit a higher intensity when compared with the untreated control cells suggesting the presence of greater number of active mitochondria. However, the cells treated with higher concentration of the peptide displayed reduced viability indicating the toxic nature of the peptide at this concentration.

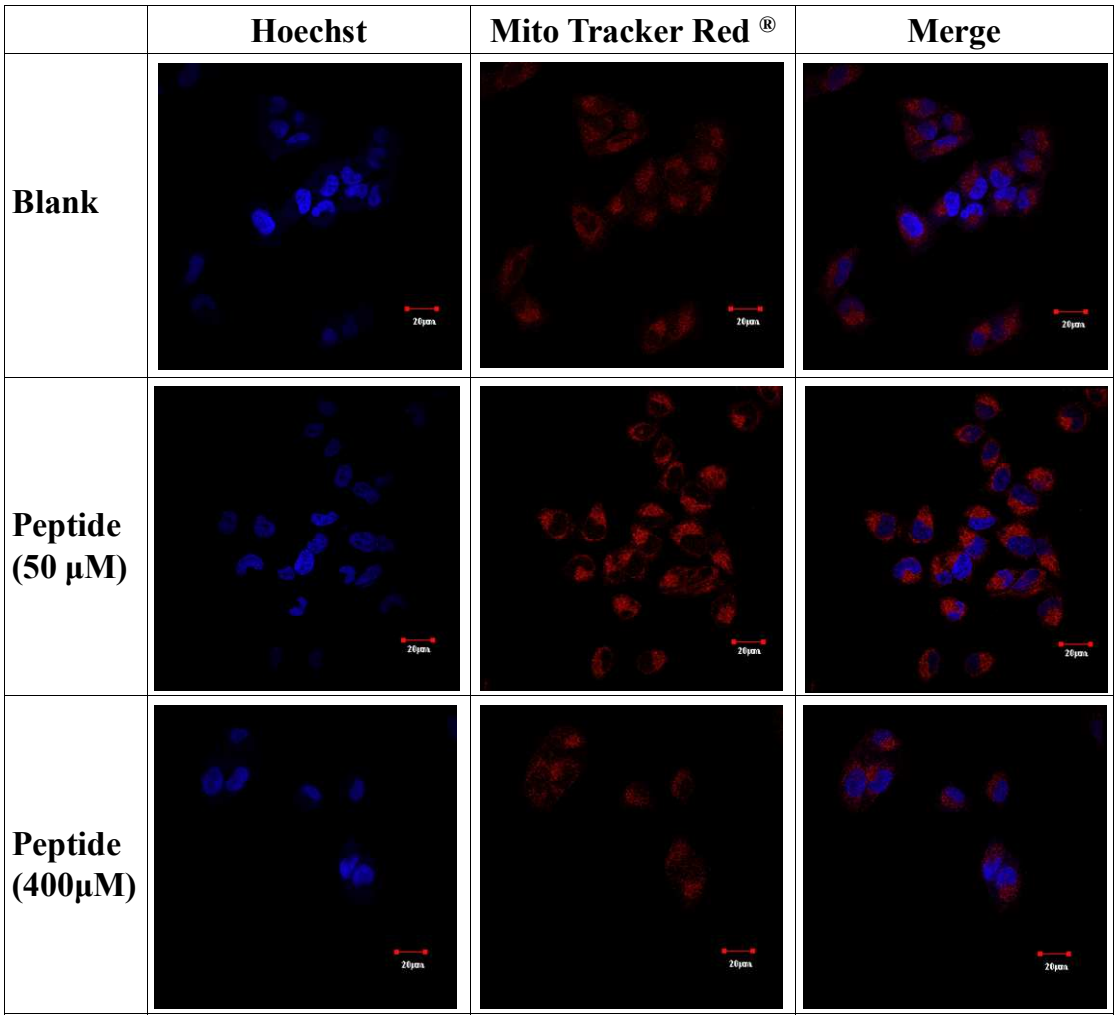

Figure 7. Mito Tracker Red ® staining of cells treated with 50 µM and 400 µM of KLVFF peptide

4. Conclusion

Our experimental results reveal the concentration dependent dual nature of KLVFF peptide. At concentrations below 100 µM, the peptide exhibits a free radical scavenging property and does not affect the cell viability. It also activates the cell proliferation genes Akt and mTOR that auger well for cell survival. However at higher concentrations, the peptide transforms into a toxic form by promoting cell death through a caspase-dependent mechanism. The peptide depolarizes the mitochondrial membrane at high concentrations and mediates the translocation of signals to the nucleus leading to activation of pro-apoptotic

genes. Activation of the MAP kinases ERK1 and p38, p53 may be postulated to be key events contributing to the cytotoxicity that strongly resembles the toxic manifestations reported for amyloid beta peptide. The present study unveils a "Jekyll & Hyde' nature of KLVFF based on its concentration. While it exhibits beneficial effects to the cells at concentrations below 100 μM, it could serve as a $A\beta_{1-42}$ mimic at concentrations beyond 200 μM. The glutathione levels in the KLVFF peptide treated cells are regulated by the expression of the transcriptional factor Nrf2 while SOD levels are regulated through the factors AP-1 and NF-κB. The molecular mechanisms involved during the transition of KLVFF peptide from its therapeutic to toxic role was elucidated in the present study, which holds immense potential in designing the KLVFF peptide based therapeutic strategies in future.

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