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In Vitro Cognitive Enhancement Effects of 3-Hydroxyflavone and its Solid Lipid Nanoparticles in PC12 Cells via MTT Assay and ROS Analysis

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Abstract

Oxidative stress with neuronal apoptosis exhibits a strong correlation with neurodegenerative conditions, including Parkinson's disease and Alzheimer's disease. Because of their antioxidant capabilities, flavonoids in particular 3-Hydroxyflavone (3HF), have demonstrated notable neuroprotective and cognitive-enhancing qualities. This study investigates the cognitive-enhancing effects of 3-Hydroxyflavone (3HF), a flavonoid with antioxidant properties, and its solid lipid nanoparticle formulation (3HFSLNP's), and using PC12 neuronal cell lines. Due to 3-HF's poor solubility and absorption, it was encapsulated in solid lipid nanoparticles via the solvent evaporation method to improve delivery and effectiveness. The nanoparticles were evaluated for particle dimensions, zeta potential, invitro drug release, and encapsulation efficiency.

Using MTT assays and ROS measurements, the study found that both 3HF and 3HFSLNP's amplified cell viability and reduced oxidative stress in a dose-dependent way. However, 3HFSLNP's demonstrated significantly superior results, indicating cognitive enhancing effects. The present findings imply that 3HFSLNP's may serve as an impending neuroprotective agent for neurodegenerative diseases such as PD and AD, although additional in vivo studies are required to verify thesefindings.

Keywords: 3Hydroxyflavone, 3HFSLNP's, PC12 cell lines, MTT assay, ROS analysis and cognitive enhancement

Introduction

Cognitive decline, which is a hallmark of many neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD), is primarily caused by oxidative stress, mitochondrial malfunction, and neuronal cell death .[1, 2] Oxidative stress, in particular, is caused by an imbalance between reactive oxygen species (ROS) and the body's antioxidant defences, resulting in lipid, protein, and nucleic acid damage and compromising neuronal integrity.[3,4] As a result, substances with strong antioxidant and neuroprotective properties are of great interest in looking for new treatment approaches for combating neurodegeneration.

Flavonoids, a varied group of naturally occurring polyphenolic chemicals

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found in fruits, vegetables, and medicinal plants, have been demonstrated to have considerable neuroprotective and cognitive-enhancing qualities.[5,6]Among these, 3-Hydroxyflavone (3HF), a lesser-studied monohydroxy flavone, has sparked interest due to its powerful antioxidant, anti-apoptotic, and anti-inflammatory properties.[7, 8] Previous research has shown that 3-HF can regulate neuronal survival pathways and protect against oxidative stress, making it a promising candidate for preventing or delaying cognitive decline.[9, 10]

Despite its therapeutic potential, 3HF clinical use is limited due to its poor solubility, low bioavailability, and rapid metabolism.[11]To address these constraints, nanotechnology-based delivery technologies are increasingly being investigated. Among these, Solid Lipid Nanoparticles (SLNP's) have various benefits, including regulated release, improved drug stability, increased permeability, and targeted delivery to the brain.[12, 13]Incorporating 3HF into SLNP's may boost cellular absorption and efficacy in brain cells by allowing it to penetrate biological barriers and reduce degradation.[14, 15]

In vitro models, such as the PC12 cell line generated from rat pheochromocytoma, are a reliable platform for assessing the neuroprotective and cognitive enhancing effects of pharmaceutical drugs.[16]The PC12 cells are responsive to the nerve growth factor and present some of the neuronalattributes and are therefore suitable for studying oxidative stress and the pharmacological response. MTT assays are commonly used to determine cell viability and mitochondrial activity, whereas ROS assays provide direct evidence of oxidative stress regulation.[17, 18]

Such research needs to focus on cognitive improvement of 3HF and its SLNP's preparation inPC12 cells in vitro, involving cell viability and ROS level. By comparing the effects of free 3-HF and 3HF loaded SLNP's, we expect to discover if the nano formulation provides superior neuroprotective advantages.This study's outcomesmight contribute to designing potent neuroprotective agents derived from flavonoid structure.

Materials and Methods

Chemicals and Reagents

High purity (exceeding 98%) 3-Hydroxyflavone (3HF), dimethyl sulfoxide (DMSO), Dulbecco's Modified EagleMedium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin solution and trypsin-EDTA were provided by Sigma-Aldrich. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and 2′,7′-dichlorofluorescin diacetate (DCFH-DA) were purchased from Sigma-Aldrich. All other reagents used wereof analytical grade.

Cell Culture

PC 12 cells the rat pheochromocytoma-PC12 cells were acquired from the National Centre for Cell Science (NCCS), Pune,India. Cells were cultured in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at37°C in a humidified atmosphere with 5% CO_2.[16]The culture medium was replaced every 2 to 3 days andthe cells were subcultured using 0.25% trypsin-EDTA.

Methodology

Preparation and characterization of Solid lipid nanoparticles

Solid lipid nanoparticles 3-Hydroxyflavone (3HFSLNP's) were prepared via the solvent evaporation method. The nanoparticles were evaluated for particle dimensions, zeta potential, pharmacological release, and encapsulation efficiency.

MTT Assay for Cell Viability

The MTT test is a colorimetric measureof cellular metabolism. This is based on the ability of MTT, a tetrazolium dye, to be reduced by NADPH-dependent cellular oxidoreductaseenzymes to an insoluble purple formazan product. The cell viability was measured using theMTT test reported by Mosmann.[17]PC12 cells (acquired from the National Centre for Cell Science, Pune)were

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seeded at a density of 1×10⁴ cells/well in 96-well plates and allowed to adhere for one night. The cells were then treated with different concentrations of free 3-HF and 3-HF-loaded solid lipid nanoparticles (100, 50, 25, 12.5, 6.2,and 3.125 µg/ml) for 24 h. After treatment, 50 µL MTT solution was added to each well and incubation time was 4 hours at 37°C in5% CO2. Reading at570 nm was performed with a microplate reader, and formazan crystals were dissolved with 150 µL DMSO. Viability valueswere expressed as percentage considering the control group to be 100%.

Reactive Oxygen Species (ROS) Analysis

Production of intracellular reactive oxygen species (ROS) was determined using DCFH-DA, which is a non-fluorescent probe that can be oxidized by ROS to formthe highly fluorescent compound DCF, for the measurement of intracellular ROS generation.[18] Then, PC12 cell lines were seeded in 6-well plates and treated with3-HF or 3-HF solid lipoid nanoparticles (SLNP's) for 24 h. After the treatment, the cells were treated with 10 µM DCFH-DA in serum-free media at 37°C for 30 min and then washed with PBS and the intracellular fluorescence was measured at-emission wavelength of 530 nm upon excitation wavelength of 485 nm on fluorescentmicroplate reader. Six hours post infection; experimental analyses were done on the treated cells compared with similarly treated and uninfected control cells because previous studies suggested the participation of ROS in actinobacillosis during the infection phase but notafter.

Statistical Analysis

The experimental procedures were performed independently three times,and the results are presented as the mean ± SD. Statistical significance was analyzed by one-way analysis of variance (ANOVA) and Turkey's multiple comparison tests. A p value of < 0.05 denoted significance. Statistical analysis was performed using Graph Pad Prism software (version 10.2).

Results and Discussion

The production of solid lipid nanoparticles (SLNP's) containing 3HF was successfully accomplished using the solvent evaporation technique. Various parameters, including entrapment efficiency percentage, drug loading capacity, particle size, polydispersity index (PDI), zeta potential, in vitro drug release, and drug release kinetics, were evaluated for the developed formulations.

The MTT determination was used to investigate the cytotoxic profile and cognitive enhancing potential of three treatments doxorubicin, 3Hydroxyflavone (3HF), and 3HF encapsulated in solid lipid nanoparticles (SLNP's) on PC12 cells, a commonly used neuronal model. This colorimetric assay measures mitochondrial activity, which correlates directly with cell viability while indirectly reflecting neural integrity[17]. Concentration-dependent cellular responses were tested from 100 to 3.125 µg/mL, and IC50 values were computed to determine each compound's potency. At the highest measured concentration (100 µg/mL), all treatments dramatically decreased cell viability, indicating cytotoxic stress. In contrast, as concentrations declined, cell viability gradually increased, reflecting less cellular stress and a more favorable profile for potential neuroprotective or cognitive-enhancing effects at low dosages. The negative control group (untreated) repeatedly demonstrated 100% vitality, confirming the assay parameters and the lack of spontaneous cytotoxicity. The results of Cell viability through MTT assay are shown in table 1 and graph 1.

The IC_{50} values gave vital information about the comparative efficacy of each medication. Doxorubicin, a well-known chemotherapeutic drug, has the lowest IC_{50}, indicating its high cytotoxic activity.[19] However, the neurotoxicity of such high potency makes it inappropriate for cognitive applications in general. In contrast, the flavonoid 3HF known for its antioxidant and neuroprotective effects had IC_{50} with an

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Table 1: Cell viability through MTT assay in PC12 cell lines									
Cell viability of PC12 cell									
Concentration (µg/ml)	3HF			3HFSLNP's			Doxorubicin		
100	9.43	9.20	9.58	14.30	14.07	14.22	20.15	20.18	20.16
50	36.05	35.97	36.12	41.44	41.14	41.29	31.75	31.65	31.69
25	38.02	37.95	38.10	42.43	42.21	42.28	42.03	42.13	42.11
12.5	44.26	44.33	44.18	43.95	43.73	43.88	45.53	45.35	45.45
6.2	45.63	45.48	45.70	45.48	45.32	45.55	48.03	48.13	48.09
3.125	47.07	47.22	47.00	46.54	47.07	46.69	49.12	49.15	49.13
Negative Control	100			100			100		
IC_{50} (µg/ml)	2.86			2.36			1.96		
STANDARD DEVIATION	0.08			0.04			0.02		
Surviving cell percentage (%) = Average optical density of the experimental compound / Average optical density of the negative control ×100									

Table 2: Green fluorescence intensity (%) by ROS method					
Green fluorescence intensity (%)					
SL No	Sample Code	Area	Mean	Min	Max
1	NC	3135628	12.038	0	243
2		3135628	12.862	0	243
3	3HF	3135628	18.128	0	243
4		3135628	18.462	0	243
5	3HFSLNP's	3135628	21.556	0	243
6		3135628	23.372	0	243

intermediate level, meaning an efficacy and safety profile somewhat more balanced. Notably, 3HF encapsulated in SLNP's had a substantially higher effect, as reflected by a lower IC_{50} than free 3HF.This higher activity of 3HFSLNP's is most likely due to greater cellular absorption, sustained release, and preservation of the active chemical from enzymatic degradation, all of which are benefits of nanoparticle-based delivery methods. These improvements are especially important in the context of cognitive enhancement, where persistent neuroprotection and effective blood-brain barrier penetration are important barriers.

Although the IC_{50} is traditionally used to indicate cytotoxicity, in this cognitive context, it can also reflect the therapeutic window: lower IC_{50} values for neuroprotective agents such as 3HF-SLNP's may indicate a greater ability to elicit hormetic responsesa concept in which mild cellular stress induces protective adaptation, promoting neuronal resilience and cognitive improvement[20]. Importantly, all treatments showed low inter-sample variability, indicating that the results were reliable and reproducible. Combined together, the findings suggest that 3HF, particularly in SLNP's form, has a potentially beneficial profile for cognitive enhancement. The increased efficacy of the SLNP's formulation highlights its potential as a next-generation delivery strategy for brain-targeted treatments.

To evaluate the oxidative status of PC12 neuronal cells and the antioxidant capability of 3-Hydroxyflavone (3HF) and its solid lipid nanoparticle formulation (3HFSLNP's), green fluorescence intensity—a substitute for intracellular ROS levels—was

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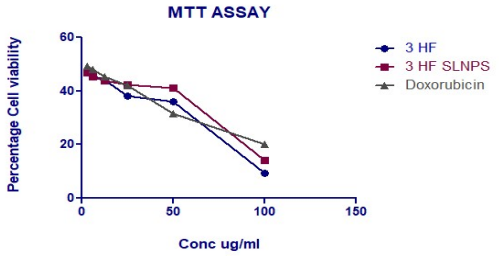

Graph 1: Percentage cell viability by MTT assay

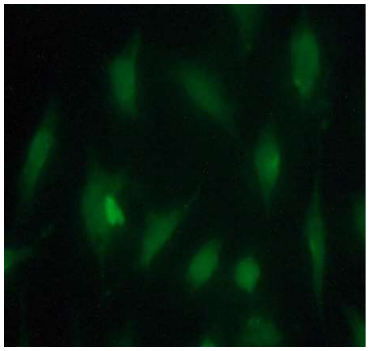

Fig.1: Normal control

measured quantitatively after treatment. PC12 cells were treated to 200 µM doses of 3HF, 3HFSLNP's, and a negative control (NC), followed by imaging and lysis for quantification. To excuse changes in cell density, the green fluorescence intensity was normalized to total protein concentrations. As shown in (Table 2 and Figs. 1,2& 3), green fluorescence intensity increased in all treatment groups as compared to the negative control (NC). The fluorescence measurements, which indicated ROS levels, were reported as follows:

The NC group had low fluorescence intensity (mean = 12.45%), indicating baseline ROS activity. Green fluorescence increased by approximately 18.3% in 3HF treated cells, whereas 3HFSLNP's treated cells showed the maximum fluorescence (around 22.5%), indicating a significant increase in ROS levels after treatment.

The increase may initially appear to contradict flavonoids' antioxidant properties. However, accumulating evidence suggests

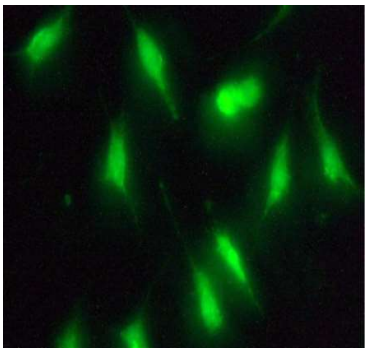

Fig. 2: 3HF

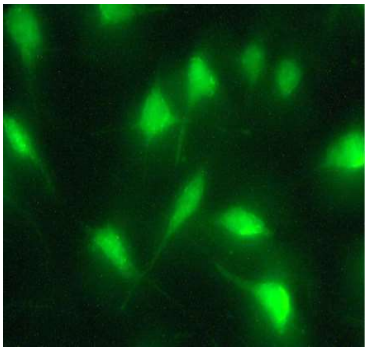

Fig. 3: HFSLNP's

that mild to moderate ROS exposure can activate endogenous antioxidant defense pathways (e.g., Nrf2/ARE signaling), improve cellular resilience, and promote synaptic plasticity an important mechanism in cognitive enhancement.[21]This condition, known as Mito hormesis, is essential for neuroprotection and cognitive function.

The observed ROS increase associated with 3HF and 3HFSLNP's treatments may thus indicate a regulated oxidative environment, which is important for neural adaptation rather than cellular harm. The hormetic effect has been shown to boost neurotrophic factors such as BDNF (Brain-Derived Neurotrophic Factor), which are required for learning and memory consolidation[22]. Furthermore, the more significant effect in the 3HFSLNP's group supports the increased intracellular transport and bioavailability of 3HF via nanocarrier systems. Thus, rather than indicating cytotoxicity, higher fluorescence intensity

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supports 3HF's pro-cognitive and neuroprotective activities, particularly in nanoparticle-encapsulated form. Such formulations could be selectively engineered to activate low-level oxidative signaling pathways that promote neurogenesis and synaptic remodeling while preventing apoptotic responses.

Conclusion

The MTT assay results show that the investigated therapies had dose-dependent effects on neuronal survival, with 3HFSLNP's dominating finish. This shows that nanoparticle formulation considerably increases 3HF neuroactive potential. Although doxorubicin had the highest cytotoxicity, its non-specific effect and concomitant neurotoxicity limit its usefulness for cognitive enhancement. In contrast, 3HFSLNP's are a potential, less toxic, and more focused option that may improve cognition by promoting neuronal health. These findings call for additional research using mechanistic investigations and in vivo behavioral models to completely establish the cognitive enhancing potential of 3HF-based nanotherapeutics.

ROS fluorescence results provide substantial proof that 3HF, specifically 3HFSLNP'sinduce positive oxidative signaling in neural cells. These findings support the importance of regulated ROS modulation in cognitive enhancement via adaptive cellular mechanisms. Future research should explore these pathways in vivo and link ROS signaling to behavioral results to validate 3HF SLNP's as a cognitive enhancing nanotherapeutic.

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Conflicts of interest

The authors report no conflicts of interest.

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