Neuro Protective Effect of Baicalein Against Oxaliplatin-Induced Peripheral Neuropathy: Impact on Oxidative Stress, Neuro-inflammation and WNT/β-Catenin Signaling

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Abstract

Oxaliplatin, an effective anti-cancer agent used in the treatment of colorectal cancer, associated with severe dose limiting side effect peripheral neuropathy, which currently remains a major unmet clinical need. This study was designed to investigate the possible neuroprotective potential of a bioflavonoid, Baicalein in an experimental model of oxaliplatin induced peripheral neuropathy. Rats were administered with a dose of 4 mg/kg oxaliplatin *i.p.* twice per week for four weeks and they were evaluated for behavioural and functional nerve parameters, followed by bio-chemical, immunohistochemical and western blot analysis. Results from this study show that baicalein reversed oxaliplatin-induced behavioural deficits and significantly prevented oxaliplatin-induced sensory nerve conduction deficits in rats. Molecular analysis revealed baicalein significantly strengthened the antioxidant defense system by enhancing the expression of MnSOD, HO-1 and GSH levels. Baicalein treatment neutralized the oxaliplatin-induced neuroinflammation which was evident from the significant loss of inflammatory mediators like TNF-α, IL-6 and a shunted NF-κB nuclear translocation. Additionally, Baicalein treatment resulted in a significant downregulation of active β-catenin, Wnt5b and Wnt3a proteins. In line with the *in vivo* evidences, treatment of baicalein in Neuro2a cells showed suppression of oxaliplatin induced ROS, mitochondrial superoxide levels and improved neuritogenesis against oxaliplatin-induced toxicity in Neuro2a cells. Additionally, Baicalein did not alter the cell viability of oxaliplatin in HCT-116 cell line. Collectively, these characteristics suggest that baicalein may be useful in the clinical management of peripheral neuropathy associated with oxaliplatin.

1. Introduction

Oxaliplatin, a third-generation platinum-based anti-neoplastic agent and a component of FOLFOX therapy, is used as a first-line treatment in advanced or metastatic colorectal cancer (1). However, platinum compounds are long associated with neurotoxicity, manifested as either a brief acute syndrome or a dose limiting cumulative sensory neuropathy (2). Acute neurotoxic syndrome is typically exacerbated by cold exposure and is characterized by transient paresthesia and/or dysesthesia and muscular spasms in the distal extremities. Chronic neurotoxicity mainly produces sensory dysfunction with distal paresthesia leading to sensory ataxia and functional impairment (3),(4). The incidence of peripheral neuropathy due to Oxaliplatin ranges from 81.5-98% and increases when the cumulative dose reaches 540mg/m^2 or more (5). Clinical approaches for treating peripheral neuropathy are limited to drugs that provide only symptomatic relief from neuropathic pain and none of the pharmacological interventions have found to impact the underlying pathomechanism of chemotherapy-induced peripheral neuropathy (6). Hence, it is worth exploring novel, effective therapeutic strategies that may tackle Oxaliplatin-induced neuropathic pain without interfering with its anti-cancer properties.

The major hurdle in the design of a treatment strategy targeting OIPN is that the mechanism of induction of anti-cancer activity and mechanism of induction of neurotoxicity is linked, making it difficult to reduce the neurotoxicity without compromising the anti-cancer activity (7). Though the underlying patho-mechanisms of OIPN remain elusive, a number of contributing factors have been identified which

includes ion channel dysfunction (8),(9), central glial cell activation (10), organelle failure (11),(12),(13), oxidative stress (14), neuronal apoptosis (15) and activation of various stress signaling mechanisms like NFKB, MAPK, JNK pathways (16). In addition to these, Wnt signaling which has a huge impact on the functioning of nervous system is gaining attention in the recent times (17). The involvement of Wnt signaling in pain pathology through the β-catenin-dependent pathway (Canonical Wnt signaling) in the spinal cord is well documented (18),(19),(20). Hence, a pharmacological strategy with a well-established safety profile, targeting multiple sites and without significantly altering the anti-cancer property of oxaliplatin will be a useful one in the management of OIPN.

Baicalein (5,6,7-trihydroxy-2-phenyl-4H-1-benzopyran-4-one), is a conventional Chinese herbal medicine, purified from the roots of *Scutellaria baicalensis* Georgi, widely known for its usage in the treatment of bacterial and viral infections. It exerts a wide range of properties including anti-neoplastic (21),(22), neuroprotective (23),(24),(25) cardioprotective (26),(27) and renoprotective (28),(29) effects. Baicalein has shown its potent anti-oxidant and anti-inflammatory activity in various in vitro and in vivo models(28). Fig. 9. Recent studies also suggests Baicalein's role in the negative regulation of the WNT/β-catenin signaling pathway which may be used (30). Though the efficacy of baicalein in diabetic neuropathy is established (23), the role of baicalein in oxaliplatin induced peripheral neuropathy, has never been investigated. The current study was aimed at exploring the neuroprotective mechanisms of baicalein against oxaliplatin-evoked neurotoxicity in an experimental model of OIPN.

2. Materials And Methods

2.1 Drugs and Chemicals

Oxaliplatin was a generous gift from Astron pharmaceuticals (Ahmedabad, India). All chemicals including baicalein was obtained from Sigma-Aldrich, USA unless stated otherwise. FBS was procured from Gibco (life technologies). TNF-α and IL-6 ELISA kits were purchased from BioLegend, San Diego, US. IHC detection kit was purchased from PathnSitu Biotechnologies Pvt Ltd. India.

2.2 In Vitro methods

2.2.1 Cell lines

Mouse neuroblastoma cell line, Neuro2a was procured from National Centre for Cell Science, Pune, India and HCT-116 cell line was obtained from the ATCC, Rockville, USA. These were cultured in MEM and McCoy's 5a media respectively, containing 10% FBS, 1% streptomycin/penicillin, L-glutamine (2 mM) and were maintained in a CO_2 incubator.

2.2.2 Measurement of Cell viability by MTT assay

HCT-116 cell line were seeded at an appropriate cell density in a 96 well plate and allowed to attach overnight. Later on cells were treated with different concentrations of oxaliplatin (12.5 μM to 100 μM) and also in combination of baicalein (01 to 50 μM) then again incubated for 24 h. Thereafter, MTT solution

was added and the cells were incubated for 4 h. Then the media containing MTT was discarded and the insoluble formazan crystals were dissolved using 100 µl DMSO and the absorbance was measured spectrophotometrically at 570 nm (Spectramax M4, USA) (31).

2.2.3 Measurement of intracellular ROS by DCFDA

Neuro2a cells were sub cultured in a 24 well plate and was allowed to attach overnight, the cells were then dealt with oxaliplatin (50µM) and baicalein (1 and 3 µM) drug solutions for 24 h. And were stained with DCFDA dye at a concentration of 10 µm for 30 mins to detect the levels of intracellular ROS. The fluorescent signal at an excitation wavelength of 485 nm and an emission wavelength of 535 nm was measured by multiplate reader (Spectramax M4, USA. Images were visualized using a fluorescence microscope (Nikon, Japan) (32).

2.2.4 Measurement of Mitochondrial superoxide anion (O_2^-) by MitoSox Staining

Neuro2a cells were seeded in a 24 well plate and was allowed to attach overnight, the cells were dealt with oxaliplatin and baicalein with designated concentrations for 24 h. Then cells were stained with MitoSox Red (5 µM) for 30 min. The fluorescent signal at an excitation wavelength of 530 nm and emission wavelength of 580 nm was measured by Spectramax M4, USA. Images were visualized using a fluorescence microscope (Nikon, Japan) (33).

2.2.5 Measurement of mitochondrial membrane potential by JC-1 staining

Neuro2a cells were plated in a 24 well plate and were allowed to attach overnight. Later, cells were treated with oxaliplatin and baicalein drug solutions with designated concentrations and incubated for 24 h. Later on cells were exposed to JC-1 at a concentration of 1 µg/ml for 20 min. Afterwards, images were visualised using a fluorescence microscope (Nikon, Japan) (31).

2.2.6 Evaluation of neurite outgrowth

Neuro2a cells were seeded in a 12 well plate at an appropriate density and was allowed to attach overnight. Nerve growth factor (at a concentration of 50 ng/ml) was added into cells to induce neurite outgrowth. Afterwards, cells were treated with drug solution of designated concentrations for 24 h. Images were taken using phase contrast microscope (NIKON, USA). The neurite length and the number of cells bearing neurites were recorded (34).

2.2.7 RT-PCR studies

Trizol method was used for total cellular RNA extraction, and the cDNA was generated with the help of Verso cDNA synthesis kit. Real-Time PCR was performed on SYBR Green Master Mix. Triplicate PCR reactions were performed on 1 µl of cDNA, and each reaction underwent 35 cycles of annealing at 48°C for 20 s, extension at 72°C for 25 s and denaturation at 94°C for 15 s. Relative gene expression was

calculated using the comparative ΔCt method and normalized to β-actin. RT-PCR primers were designed using Primer Express software (version 3.0.17). The following primers were used: β-actin: forward 5^1-AGACCTCTATGCCAACACAG-3^1 and reverse 5^1-ACTCATCGTACTCCTGCTTTG-3^1; β-catenin: forward 5^1-CTTGGTAGGGTGGGAATG-3^1 and reverse 5^1-GCCCTCTCAGCAACTCTA-3^1. (35)

2.2.8 Immunofluorescence studies

Neuro2a cells at a suitable cell density were cultured on coverslips in a multi-well plate and were allowed to settle. Once after the attachment of the cells, they were treated with the indicated concentrations of drug solutions for a day. Thereafter, cells were washed, fixed using 4% paraformaldehyde solution and permeabilized with 0.2% Triton-X for 15 min each at room temperature, and then blocked with 3% BSA in PBS for 30 min. Later, cells were incubated with NF-κB primary antibody at 1:400 dilution (Cell Signaling Technology, Beverly, MA) at 4°C for 12 h. After washing with PBS-T, secondary antibody conjugated with Rhodamine (Santa Cruz Biotechnology, Inc., Texas) at a dilution of 1:200 was added and incubated for 2 h at room temperature in a dark place. Thereafter cells were washed with PBS-T. Then the coverslips were mounted with antifade mounting medium with DAPI (Vector laboratories) on a glass slide. Visualization of the fluorescent images was done using confocal laser scanning microscope (Leica TCS SP8) (34).

2.3 *In Vivo* methods

2.3.1. Animals

Healthy male SD rats weighing 250-300g was employed in this study. They were fed on standard diet and water ad libitum. Optimal laboratory conditions like constant temperature of 23°C ± 1°C and relative humidity 55% ± 10%

with 12:12 h light and dark cycle was maintained during the entire study. The animal study protocols were duly approved by the Institutional Animal Ethics Committee (IAEC)-NIPER Hyderabad, and all the experiments were conducted in accordance to prevailing guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

2.3.2. Study design

SD Rats were randomized into five groups consisting of 8 animals each. The groups included normal control (NC), oxaliplatin control (OC), two baicalein treated (5 and 10mg/kg) and the perse group. Oxaliplatin at a dose of 4 mg/kg (*i.p* in 5% dextrose solution) twice every week for 4 weeks was injected. The treatment groups consist of baicalein at 5 mg/kg (OC+BA1) and 10 mg/kg (OC + BA2), *i.p.* in DMSO and baicalein alone treated group consists of normal rats treated with high dose of baicalein (BA2). At the end of the study, animals were euthanized with CO_2anesthesia, and sciatic nerves and L4-L6 region of spinal cord were collected on the spot. The collected tissue samples were stored at -80˚C to carry out biochemical and protein expression studies and in 10% NBF for immuno-histochemical studies.

2.3.3. Functional assessment

Power lab system was employed to assess motor and sensory nerve conduction velocity. The receiving electrodes were placed on the muscle of foot and by utilizing bipolar needle anode, 3V stimulus was applied at sciatic nerve (proximal to sciatic notch) and tibial nerve (proximally to ankle). The latencies of muscle action potential were recorded by Lab chart software. H-reflex latencies and negative M-Wave deflection were measured to calculate the related SNCV and MNCV, utilizing the distance between the stimulation points and latencies. The results being expressed in m/sec.

2.3.4. Behavioral assessment

Cold allodynia

Cold-allodynia was assessed via acetone spray method by spraying 100 µl of acetone onto the plantar surface of hind paw. The animals were kept under observation for the next 20s and responses were scored. Score 0 represents, no response; Score 1 represents, quick withdrawal or flicking of the paw; Score 2 represents, prolonged withdrawal or repeated flicking; and Score 3 represents, repeated flicking with licking of the paw. The minimum score obtained was 0 and the maximum score was 9.

Mechanical allodynia

Calibrated Von Frey hairs were used to explore the dynamic responses to mechanical stimulus. After acclimatizing animals in a transparent chamber on a perforated surface, the filaments were applied eventually from lower to higher pressure, perpendicular to plantar surface of rat's feet. While applying sufficient pressure to bend the filaments partially for 2–3s, responses were noted. The response was considered as positive, when there was a quick withdrawal of the paw, with its licking. The test was repeated for three times for each animal and an interval of 5 min was provided in between each reading.

Mechanical hyperalgesia

Randall-Selitto apparatus was utilized to assess mechanical hyperalgesia. Pressure was applied on the surface of the hind paws. The paw withdrawal responses to the corresponding pressure (g) were recorded. In order to avoid the damage to the paw, a cut-off pressure of 250 g was used (36).

Thermal hyperalgesia

Thermal hyperalgesia of the paw towards radiant heat (thermal stimulus) was performed by Plantar Test (Hargreaves Method) using IITC Plantar Analgesia Meter (IITC, Life Sciences, USA). The pain threshold index was taken as latency of the foremost escaping sign *i.e.*, paw licking or paw flicking, while keeping a cut-off time of 20 s (37).

2.3.5 Biochemical parameters

Estimation of MDA and GSH levels

Oxidative stress in tissues was assessed by using Lipid peroxidation assay and GSH assay (38),(39). Homogenization of the spinal tissue was carried out with phosphate buffer. The supernatant was

collected after centrifuging the spinal cord samples for 15 min (at 10,000 rpm and 4°C). The resulting supernatant was utilized to determine protein content, MDA and GSH levels. TBARS was measured to assess the level of lipid peroxidation. 100 µl of the protein supernatant was mixed with thiobarbituric acid 0.8%, SDS 8.1%, acetic acid 20% and incubated at 95˚C on water bath for 120 min. After centrifuging the samples, the absorbance of supernatant (MDA levels) was measured at 532 nm spectrophotometrically. For GSH estimation, to a 50 µl of supernatant (TCA added), 120 µl of DTNB reagent was added and allowed to incubate for 10 min at room temperature, protected from light. The absorbance of the formed yellow colour dianion was measured spectrophotometrically at 412 nm.

Estimation of inflammatory markers

ELISA estimations were performed using commercially available IL-6 and TNF-α assay kits. It was carried out according to the manufacturer's instructions.

2.3.6 Immunohistochemical analysis

Rehydrated spinal cord and sciatic nerve sections were incubated with primary antibodies WNT5b (Novus biological, USA, at a dilution of 1:200), non-phospho β-catenin, GFAP (Cell Signaling Technology, Beverly, MA, US, at a dilution of 1:200), 8-OHdG (Abcam, Cambridge, UK, at a dilution of 1:200) for overnight at 4^0c with constant shaking, followed by incubation with secondary antibody for 1 h at room temperature. Further, these micro sections after washing, were stained (using DAB) and counterstained (using hematoxylin) to observe under light OPTIKA microscope (40).

2.3.7 Western blotting analysis

Spinal tissue was homogenized using T-PER lysis buffer with1% phosphatase inhibitor and protease inhibitor each. Protein samples containing equal protein content were loaded into the well to separate them on SDS polyacrylamide gel; thereafter transferred on PVDF membrane. Membrane was blocked by using 3% BSA solution for 2 h at room temperature, and incubated overnight with primary antibodies of β-catenin, non-phospho β-catenin, WNT3a, WNT5b, NF-κB, phospho NF-κB, p38, phospho p38, SOD2, β-actin (at a dilution of 1:1000, Cell Signaling Technology, Beverly, MA, US) and COX-2, iNOS (at a dilution of 1:200, Abcam, UK) at 4^0c. After washing the membranes, they were incubated with a horseradish peroxidase-conjugated secondary antibody (at a dilution of 1:20,000), and visualized using Fusion-FX chemiluminescence imager (Vilber Lourmat, France). The relative band densities were quantified by densitometry using Image J software, NIH, US. β-actin expression were checked to confirm equal loading of proteins (33).

2.3.8 Statistical analysis

Results were demonstrated as the mean ± SEM. Behavioral results data was analyzed statistically using two-way ANOVA and end-point data was analyzed statistically using one-way analysis of variance with the help of GraphPad Prism Version-5.0 (GraphPad software, San Diego, CA). "Bonferroni's Multiple Comparison Test" was utilized for follow-up analysis. Results with probability level of p values < 0.05 reflected statistical significance.

3. Results

3.1 Baicalein did not interfere with the anti-cancer efficacy of oxaliplatin in HCT-116 colorectal carcinoma cell line

It is of critical importance to determine the interaction of oxaliplatin with baicalein, if any. Cell viability assay was performed in human colorectal carcinoma cell line, HCT-116. The IC_{50} of oxaliplatin alone and that co-treated with Baicalein at a concentration of 3 μM were found to be 12.70±0.78μm and 11.83±1.34μm respectively. This indicates that co-treatment of Baicalein with oxaliplatin did not alter its cytotoxicity.

3.2 Baicalein mitigates oxaliplatin-induced cellular and mitochondrial ROS in N2a cells.

To evaluate the redox status in N2a cells upon treatment with oxaliplatin at a concentration of 50 μM, we performed DCFDA and MitoSox staining, to measure the levels of intracellular ROS and mitochondrial superoxide (O_2^-) respectively. Augmented intracellular ROS was indicated by increased green intensity and elevated mitochondrial superoxide levels was shown by enhanced red fluorescence and both of them were increased significantly ($p < 0.001$, $p < 0.01$ respectively) in oxaliplatin treated cells when compared to the untreated control cells as shown in Fig. 1. However, Baicalein treatment at 3 μM significantly ($p < 0.001$, $p < 0.01$ respectively) reduced intracellular ROS and mitochondrial superoxide levels, suggesting a potential free radical scavenging property of Baicalein.

3.3 Baicalein prevents loss of mitochondrial membrane potential (Ψm) and improved neuritogenesis in N2a cells.

In comparison with the control group, the cells treated with oxaliplatin showed very less fluorescence intensity reflecting loss of Ψm. However, baicalein treatment significantly ($p < 0.001$ at 3 μM) prevented Ψm loss, showing its ability to maintain the integrity of Ψm as shown in Fig. 2. There was no significant alteration in baicalein alone treated cells at 3 μM when compared with untreated control cells. Further, NGF treated N2a cells, upon exposure to oxaliplatin reduced the number of cells bearing neurites (($p < 0.01$) and average neurite length significantly ($p < 0.001$) as represented in Fig. 2. Treating the cells with baicalein at 3 μM enhanced the number of cells bearing neurites ($p< 0.001$) as well as the average neurite length ($p< 0.001$) when compared to oxaliplatin alone treated cells, showing its neuritogenesis potential.

3.4 Baicalein attenuates oxaliplatin-associated behavioral and functional alterations and blocks neuropathic pain

Repeated administration of oxaliplatin resulted in the development of OIPN as evident by a marked reduction in the paw withdrawal latencies to thermal stimuli, paw withdrawal threshold to Von Frey fibers and Randall-Selitto analgesiometer and reduced mean score of allodynia to acetone spray test as

compared to normal control group. Baicalein treatment significantly restored the paw withdrawal latencies ($p < 0.001$) to Von Frey fibers, paw withdrawal threshold ($p < 0.001$) to Randall-Selitto analgesiometer (Fig. 3b and Fig. 3d respectively). The mean score of allodynia in response to acetone spray significantly ($p < 0.001$) decreased with baicalein compared to oxaliplatin rats by the 28th day as represented in Fig. 3. In oxaliplatin control group there was a significant reduction in SNCV ($p < 0.001$), while MNCV remained unchanged. However, treatment with Baicalein showed a significant ($p<0.05$) prevention of oxaliplatin-induced lowering of SNCV as shown in **Table 1**. Indeed, baicalein alone treated group did not show any significant functional and behavioral changes compared to normal control rats.

3.5 Baicalein attenuated oxaliplatin-mediated oxidative stress

MDA is an end product of lipid peroxidation and is a reliable indicator of oxidative stress. Intra peritoneal administration of Oxaliplatin significantly elevated the levels of MDA ($p < 0.001$) in comparison with normal control group. Whereas, the group treated concurrently with oxaliplatin and baicalein at 5mg/kg and 10mg/kg significantly ($p<0.05$ and $p < 0.001$ respectively) showed reduction in the MDA levels in the spinal cord in comparison with oxaliplatin control group as shown in Table 1. Marked reduction in the levels of GSH, an anti-oxidant enzyme was observed upon treatment with oxaliplatin in comparison with the normal control group ($p < 0.001$). Baicalein treatment at 5mg/kg and 10mg/kg significantly replenished the diminished GSH levels in spinal cord ($p<0.05$ and $p < 0.001$ respectively) in comparison with the oxaliplatin control group as shown in **Table 1**. The IHC analysis of dorsal part of lumbar part of spinal cord showed a significant rise ($p< 0.001$) in the expression of 8-OHdG in oxaliplatin control group compared to the normal control group. However, Baicalein treatment at higher doses significantly inhibited the elevated levels of 8-OHdG in spinal cord ($p < 0.001$) as shown in Fig. 4a. To investigate the redox profile in the spinal cord upon treatment with oxaliplatin, western blot analysis was carried out. It revealed a significant decrease in the expression of MnSOD ($p < 0.01$) and HO-1 ($p < 0.05$) in oxaliplatin control group in comparison with the normal control group. However, oxaliplatin control animals when treated with with 10 mg/kg of baicalein showed a significant increase in MnSOD ($p < 0.001$) and HO-1 ($p < 0.001$) levels as shown in Fig. 4b.

3.6 Neuro-protective activity of Baicalein on oxaliplatin induced neuro-inflammation

Oxaliplatin control group demonstrated a marked increase in the levels of TNF-α and IL-6 ($p < 0.01$ and $p < 0.001$ respectively) in the spinal cord, confirming the induction of neuroinflammation upon oxaliplatin administration. However, Baicalein treatment at 5 mg/kg ($p < 0.01$) and 10 mg/kg ($p < 0.001$) significantly halted the escalation of TNF-α and IL-6 in a dose-dependent manner in comparison with the oxaliplatin control animals as shown in Fig. 5c. Additionally, western blot analysis revealed an increase in the iNOS ($p < 0.001$), COX-2 ($p < 0.001$), NF-κB ($p < 0.01$), phospho NF-κB ($p < 0.001$), p-38 activation ($p < 0.001$) and phospho p-38 activation ($p < 0.001$) expression in oxaliplatin group when compared to normal

control group. However, oxaliplatin control group when treated with baicalein, showed a decrease in the levels of iNOS, COX-2, phospho NF-κB and phospho p-38 as shown in Fig. 6. IHC analysis showed a significant increase ($p < 0.001$) in GFAP positivity in the oxaliplatin group compared to the control group, this indicates that oxaliplatin significantly augmented the activation of astrocytes. While, treatment with Baicalein at high doses significantly inhibited this elevated level of GFAP in spinal-cord ($p<0.01$) as represented in Fig. 5a. Given the anti-inflammatory role of Baicalein in *in vivo* model, we additionally characterized the effect of baicalein on oxaliplatin-induced NF-κB nuclear translocation in N2a cells. Immunofluorescence microscopy confirmed a significant expression of NF-κB in cytoplasm in the control cells, while oxaliplatin insult resulted in nuclear translocation of NF-κB. Treatment with baicalein significantly attenuated the oxaliplatin induced nuclear translocation of NF-κB as represented in Fig. 5b.

3.7 Baicalein attenuates oxaliplatin induced activation of Wnt signaling

Wnt ligands namely Wnt5b, Wnt3a are a well-known activator of canonical β-catenin signaling. As Wnt signaling plays a critical role in the development of nervous system, we investigated the expression of its key mediators in the spinal cord by western blot analysis. It showed elevated levels of Wnt5b ($p < 0.001$) and Wnt3a ($p <0.001$) in oxaliplatin group in comparison with normal control. However, oxaliplatin control group when treated with low dose of baicalein at 5 mg/kg, showed an attenuated expression of Wnt5b ($p < 0.01$) and Wnt3a ($p< 0.05$). Significant reduction in the levels of Wnt5b ($p <0.001$) and Wnt3a ($p < 0.001$) were seen in oxaliplatin control rats treated with higher dose of baicalein at 10mg/kg as shown in Fig. 8a. The immunohistochemical analysis too disclosed a marked increase in the expression of Wnt5b characterized by brown-colored cells in the spinal cord ($p < 0.001$) and sciatic nerve ($p < 0.001$) in rats with oxaliplatin-induced neuropathy as represented in Fig. 7b. While, baicalein treatment at higher doses significantly attenuated these changes in spinal cord ($p < 0.01$) by abrogating the levels of Wnt5b. However, no prominent changes in the expression of Wnt5b were observed in sciatic nerve. As β-catenin is a substrate of the destruction complex consisting of GSK3β, Axin, APC; binding of Wnt ligands to the destruction complex frees β-catenin and the levels of active non-phosphorylated β-catenin rises in the cell. In context to this we studied the expression of active β-catenin in spinal cord and sciatic nerve. Expression levels of active β-catenin were also increased ($p < 0.001$) in oxaliplatin group in comparison with the normal control. Baicalein at 10 mg/kg dose significantly inhibited this effect in spinal cord ($p < 0.05$) and sciatic nerve ($p < 0.001$) by abrogating the levels of active β-catenin (Fig. 7a). At the mRNA level too, oxaliplatin treated group showed a significant increase in the mRNA levels of the β-catenin ($p < 0.05$) but the group given baicalein treatment showed significant downregulation of β-catenin ($p < 0.01$) in comparison with the oxaliplatin control animals as shown in Fig. 8b.

4. Discussion

OIPN is a debilitating side effect of widely used anti-cancer agents like oxaliplatin, limiting its clinical utility. The current study was designed to investigate the neuro-protective function of a Chinese herbal

medicine baicalein and its outcomes on oxaliplatin induced oxidative stress, neuro-inflammation and aberrant Wnt signaling in an experimental model of OIPN. To induce neuropathy in experimental rats, oxaliplatin was given at a dose of 4 mg/kg twice every week for 4 weeks. Behavioral examinations depicting various sensory elements of neuropathic pain were evaluated before, during and after the administration of oxaliplatin. Treatment with oxaliplatin resulted in the induction of allodynia and hyperalgesia, the hallmark symptoms of neuropathy which is consequence of hyper-responsiveness of Aβ-fibers and C-fibers. The thinly myelinated Aδ fibres mediates the touch sensation while the non-myelinated C-fibres mediates the cold sensation, the hyper-responsiveness of these fibres may contribute to the induction of neuropathic pain (41),(42). The increased pain hypersensitivity was confirmed by a marked decrease in the paw withdrawal latency (thermal hyperalgesia), decreased paw withdrawal pressure (mechanical hyperalgesia), decreased paw withdrawal threshold (mechanical allodynia) and decreased score of allodynia (cold allodynia) in oxaliplatin control group compared to the normal control group. Additionally, reduced sensory nerve conduction velocities with no prominent changes in motor nerve conduction velocities were observed upon the administration of oxaliplatin. This may be due to the fact that sensory neurons in the dorsal root ganglion had remarkably higher exposure to oxaliplatin and relatively due to the absence of its mitotoxic effect in motor neuron axons (43). Notably the treatment with baicalein at doses 5 mg/kg and 10 mg/kg significantly mitigated the oxaliplatin-induced behavioral deficits like thermal and mechanical hyperalgesia, cold and mechanical allodynia and improved SNCV, providing preliminary evidence of its possible neuroprotective role.

To shed light on the molecular mechanisms underlying neuroprotective role of baicalein, we studied the markers of oxidative stress and inflammation. Oxidative stress is a key player in the pathogenesis of OIPN (44),(45). Oxaliplatin-induced oxidative damage is likely to be mediated by increasing levels of superoxide anion, hydrogen peroxide, hydroxyl radical. It is often characterized by high amounts of free radicals and a compromised anti-oxidant defense mechanism leading to redox imbalance (46). ROS directly targets lipids and the presence of high amount of phosphospholipids in spinal cord makes them more vulnerable for oxaliplatin induced oxidative damage (45). Levels of MDA, an end product of lipid of peroxidation were markedly increased in the oxaliplatin treated rats compared to the normal control group. Compromised expression of anti-oxidant proteins like GSH, MnSOD and HO-1 resulting in inefficiency of cellular mechanisms to combat against oxidative stress induced by oxaliplatin was observed. While treatment with Baicalein not only attenuated the ROS-induced lipid peroxidation and also re-established the levels of the glutathione in spinal cord tissue of rats belonging to treatment group (Table 1). It also restored MnSOD and HO-1 levels in spinal cord (Fig. 4b). 8-OHdG is a pivotal marker in measuring endogenous damage to DNA. Free radicals generated by oxaliplatin are known to hydrolyse guanosine resulting in the formation of 8-OHdG (47). A significantly high amount of 8-OHdG was observed in the oxaliplatin control group compared to the normal control. Apparently, baicalein lessened the expression of 8-OHdG in spinal cord of rats (Fig. 4a). In agreement with the previous reports (45),(47), redox imbalance was evident in the sciatic nerves and spinal cord of oxaliplatin-treated rats. And baicalein possibly protects the neurons from ROS induced damage by alleviating the oxaliplatin-induced MDA levels and by preventing the depletion of antioxidant defenses and DNA oxidation. Consistent with

our *in vivo* findings, oxaliplatin also induces generation of ROS including mitochondrial superoxide in N2a cells. We also observed oxaliplatin mediated loss of mitochondrial potential which is a characteristic of mitochondrial dysfunction in N2a cells. However, Baicalein treatment not only suppressed the levels of ROS and superoxide, but also preserved the mitochondrial membrane potential in a dose-dependent manner (Fig. 1,2). Over and above, baicalein also enhanced neurite outgrowth and the percentage of cells bearing neurites indicating a strong neuro-protective role (Fig. 2).

The development of pathophysiological changes in the spinal cord is evidenced by neuroinflammatory processes including enhanced glial cell activation and transcriptional upregulation of inflammatory genes (45),(10). Recent findings suggest that the response of glial cells to chemotherapy induced neuropathy is different from that of trauma-induced nerve injury. Predominantly astrocyte is activated in the maintenance of OIPN with a minor role for microglial activation (48),(49). Astrocytes in addition of maintaining neuronal health, regulate the uptake and release of pro-inflammatory cytokines. The released pro-inflammatory cytokines in turn acts on the neurons increasing their excitability and triggers the glial cells creating cascading effects, which leads to long lasting neuropathic pain (50). Immunohistochemical evaluation revealed an upregulated GFAP expression in oxaliplatin control group compared to normal control group which is interpreted as an increase in the activity of astrocytes. While treatment with baicalein significantly inhibited the GFAP positive cells as shown in Fig. 5a. Additionally, as astrocyte activation co-relates with the degree of sensitivity to mechanical stimuli, attenuation of activated astrocyte by baicalein resulted in a marked reversal of neuropathic pain symptoms which is evident from restored sensitivity to mechanical hyperalgesia and allodynia as represented in Fig. 3. These proinflammatoy role of oxaliplatin are in line with the previous reports on OIPN (51),(52)(53),(54). Under physiological conditions, NF-κB exists in an inactive form in the cytoplasm bound to IκB. Upon stimulation with pro-inflammatory cytokines and/or free radicals, IκB is phosphorylated resulting in its ubiquitin mediated degradation. This leads to the translocation of free NF-κB into the nucleus and subsequent activation of inflammatory genes (10). In light of this we Oxaliplatin drastically increased the levels of inflammatory markers in the spinal cord which has been suggested cytokine mediated nociceptor sensitization in the oxaliplatin control group. Indeed, oxaliplatin treatment in N2a cells resulted in an enhanced nuclear translocation of NF-κB as observed by immunofluorescence microscopy. Treatment with baicalein ameliorated the pro-inflammatory cytokines namely TNF-α and IL-1β owing to its anti-inflammatory potential.

Next, we assessed the role of Wnt signaling in OIPN. Wnt signaling is involved in the regulation of synaptic plasticity, glial function and neuro-developmental processes (55),(17). Its function is found to be dysregulated in various neurological disorders and in cancer (56),(57),(58). Recently Wnt signaling has gained importance for its role in the development and maintenance of neuropathic pain (59),(60). Although Wnt signaling remains silent in an unstimulated cell, it gets activated upon nerve injury (17). Wnt ligands bind to N-terminal cysteine rich domain of Frizzled receptors and co-receptors leading to disruption of the destruction complex, the complex consisting of the proteins namely Axin, GSK-3β, CK-1 and APC (61),(62). The disruption of destruction complex and its subsequent escape of β-catenin from the destruction complex lead to its stabilization and build-up in the cytoplasm and its subsequent

translocation into the nucleus to activate the transcription of target genes. Recently many studies have reported a proinflammatory role of Wnt signaling and were shown to orchestrate the chemotaxis of macrophages into the injury site. Of note, β-Catenin, a multi-functional protein is found to regulate the production of pro-inflammatory cytokines (63),(64). In this study, we found that Oxaliplatin-induced nerve damage causes a rapid and an enduring activation of the Wnt signaling in the spinal cord. Repeated administration of Oxaliplatin increased the spinal expression of well-known activators of Wnt/β-Catenin signaling namely Wnt3a and Wnt5b. These elevated levels of Wnt ligands act as a trigger for the stabilization of β-Catenin, which in turn regulates the expression of pro-inflammatory genes. Upregulation of pro-inflammatory cytokines due to the dysregulation of Wnt signaling in the spinal cord leads to central sensitization and the abnormal firing of nerve impulses resulting in the persistence of neuropathic pain(65). Wnt pathway is directly linked with the induction and progression of neuropathic pain and the treatment with Wnt inhibitors has yielded promising result (59),(66). The fact that Wnt signaling contributes to neuropathic pain by regulating pro-inflammatory cytokines IL-6 and TNF-α and baicalein being a negative regulator of Wnt/β-catenin signaling, posits that spinal blockade of Wnt signaling by baicalein may abrogate neuropathic pain induced by oxaliplatin. Interestingly, accumulating evidences suggests a strong association of Wnt pathway with the tumorigenesis and aggressiveness of cancer. Wnt signaling was found to provide resistance against chemotherapy (58). Indeed, inhibition of Wnt/β-Catenin signaling by baicalein was shown to promote anti-proliferative properties (22). Hence, attenuation of activated Wnt/β-Catenin signaling has dual function; leads to sensitization to chemotherapy and further providing neuroprotection (Fig. 9). Nevertheless, Baicalein could provide neuroprotection while simultaneously preserving the anti-cancer activity of oxaliplatin.

5. Conclusion

The present study demonstrates the involvement of oxidative stress in the overactivation of Wnt/ β -Catenin and neuroinflammation in the pathogenesis of OIPN. Treatment with baicalein ameliorated the functional, behavioral and biochemical alterations associated with OIPN and reduced the expression of β -catenin and its downstream inflammatory mediators. Hence our findings suggest a potential therapeutic strategy against OIPN without altering the anti-cancer effect of Oxaliplatin.

Abbreviations

ANOVA, Analysis of variance
APC, Adenomatous polyposis coli
COX-2, Cyclo-oxygenase 2
DCFDA, 2',7'-dichlorofluorescein diacetate
DRG, dorsal root ganglia
ELISA, Enzyme-linked immune sorbent assay
FBS, Fetal Bovine Serum
GFAP, Glial fibrillary acidic protein

GSH, Glutathione
GSK3β, Glycogen synthase kinaseβ
HO-1, heme oxygenase-1
IHC, Immunohistochemistry
IL-6, interleukin-6
iNOS, Inducible Nitric Oxide Synthase
MDA, Malondialdehyde
MEM, Minimum Essential Medium
MNCV, Motor nerve conduction velocity
MnSOD, manganese-dependent superoxide dismutase
MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
NGF, Nerve Growth Factor
NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells
IκB, Inhibitor of κB
IC50,Inhibitory concentration
OIPN, Oxaliplatin-induced peripheral neuropathy
PBS, phosphate buffered saline
ROS, Reactive oxygen species
SNCV, Sensory nerve conduction velocity
TBARS, Thiobarbituric Acid Reactive Substances
TNF-α, tumor necrosis factorα
T-PER, Tissue protein extraction reagent.

Declarations

Ethics Approval

The animal study protocols were duly approved by the Institutional Animal Ethics Committee (IAEC)-NIPER Hyderabad, and all the experiments were conducted in accordance to prevailing guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data & materials

In this article, we have included all the data after doing experiments and statistical analysis, the source of majority of materials included in material and methods section. The data that support the findings of this

study are available on request.

Competing interest

Not applicable

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Conflict of Interest

The authors declare that there are no conflicts of interest.

Research involving Human Participants and/or Animals

All the procedures used for animal experimentations were approved by Institutional Animal Ethics Committee of NIPER Hyderabad.

Informed consent

Not applicable

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Simrandeep Kaur (Neuropharmacology Laboratory, Department of Pharmacology and Toxicology, National Institute of Pharmaceutical Education and Research (NIPER)-Hyderabad, Balanagar, Telangana, India), Aparna Areti (Division of Neurology, Department of Medicine, University of Alberta, 2E3.26 Walter C Mackenzie, Health Sciences Center, Edmonton, AB, T6G 2B7, Canada), Kartika N (Neuropharmacology Laboratory, Department of Pharmacology and Toxicology, National Institute of Pharmaceutical Education and Research (NIPER)-Hyderabad, Balanagar, Telangana, India). The manuscript was written by Simrandeep Kaur ((Neuropharmacology Laboratory, Department of Pharmacology and Toxicology, National Institute of Pharmaceutical Education and Research (NIPER)-Hyderabad, Balanagar, Telangana, India), Aparna Areti (Division of Neurology, Department of Medicine, University of Alberta, 2E3.26 Walter C Mackenzie, Health Sciences Center, Edmonton, AB, T6G 2B7, Canada) and Priya Saha (Department of Pharmacology and Toxicology, National Institute of Pharmaceutical Education and Research (NIPER)-Kolkata, Chunilal Bhawan, 168, Maniktala Main Road,

Kolkata-700054) and reviewed and edited by Dr Ashutosh Kumar (Department of Pharmacology and Toxicology, National Institute of Pharmaceutical Education and Research (NIPER)-Kolkata, Chunilal Bhawan, 168, Maniktala Main Road, Kolkata-700054) and Dr Ravichandiran Velayutham (Department of Pharmacology and Toxicology, National Institute of Pharmaceutical Education and Research (NIPER)-Kolkata, Chunilal Bhawan, 168, Maniktala Main Road, Kolkata-700054.) All authors commented on previous versions of the manuscript. All authors have read and approved the final manuscript.

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Tables

Table 1. Effect of Oxaliplatin and Baicalein on functional and biochemical characteristics after 28th day: *Results were expressed as mean ± SEM (n = 6 for Nerve conduction studies and n=3 for biochemical studies).^^^ p < 0.001 vs. NC, * p < 0.05, ** p < 0.01 vs. OC. NC: Normal control, OC: Oxaliplatin control (4 mg/kg, ip), OC+BA1 and OC+BA2: Oxaliplatin control rats treated with Baicalein at 5 mg/kg and 10 mg/kg, ip respectively and BA2: Normal control rats treated with Baicalein (10 mg/kg, ip).*

Parameter	NC	BA2	OC	OC+BA1	OC+BA2
MNCV (m/s)	51.05 ± 2.89	51.84 ± 3.00	45.70 ± 1.72 ^^^	46.86 ± 2.29	50.85 ± 9.07 *
SNCV (m/s)	68.60 ± 5.09	60.17 ± 3.71	42.56 ± 1.50 ^^^	46.03 ± 1.18	58.85 ± 10.46 *
MDA (μM/mg of protein)	0.95 ± 0.09	1.21 ± 0.41	4.41 ± 0.17 ^^^	2.95 ± 0.32 *	2.20 ± 0.19 **
GSH (μM/mg of protein)	33.50 ± 1.00	36.50 ± 0.93	16.11 ± 1.07 ^^^	23.09 ± 1.34 *	25.83 ± 1.52 **

Figures

Figure 1

Effect of Oxaliplatin and Baicalein on the intracellular levels of ROS and mitochondrial superoxide in Neuro2a cells. *(A) Fluorescent microscopic images of Neuro2a cells showing the intracellular ROS (upper panel) and mitochondrial superoxide (lower panel) generation. Scale shows a length of 100 μm. Photographs were captured at 200×magnification (B) Respective graphs from spectrophotometer representing their levels. Results were expressed as mean ± SEM (n = 3). ^^^ p < 0.001, ^^p<0.01 vs. NC, * p < 0.05, ** p < 0.01, *** p < 0.001 vs. OC. NC: Normal cells, OC: N2a cells exposed to Oxaliplatin (50 μM), OC+BA1 and OC+BA2: Oxaliplatin insulted N2a cells treated with Baicalein 1μM and 3μM respectively and BA2: Normal N2a cells treated with Baicalein (3 μM).*

Figure 2

Effect of Oxaliplatin and Baicalein on neuritogenesis and mitochondrial membrane potential in Neuro2a cells. *(A) Phase contrast images of Neuro2a cells with neurites (upper panel) and mitochondrial superoxide (lower panel). Scale shows a length of 100 μm. Photographs were taken at 200× magnification. (B) Respective bar graphs representing the length of neurites and % of cells bearing neurites. (C) Respective graph from spectrophotometer representing JC-1 fluorescence units. Results were expressed as mean ± SEM (n = 3). ^^ p < 0.01, ^^^ p < 0.001 vs. NC, * p < 0.05, *** p < 0.001 vs. OC. NC: Normal cells, OC: N2a cells exposed to Oxaliplatin (50 μM), OC+BA1 and OC+BA2: Oxaliplatin insulted*

N2a cells treated with baicalein 1μM and 3μM respectively and BA2: Normal N2a cells treated with Baicalein (3 μM).

Figure 3

Effect of Oxaliplatin and Baicalein on behavioural parameters. *(a) Cold chemical allodynia, (b) Mechanical allodynia, (c) Thermal hyperalgesia and (d) Mechanical hyperalgesia. Results were expressed as mean ± SEM (n = 6). ^ p <0.05, ^^^ p < 0.001 vs. NC, * p < 0.05 ** p < 0.01, *** p < 0.001 vs. OC. NC: Normal control, OC: Oxaliplatin control (4 mg/kg, ip), OC+BA1 and OC+BA2: Oxaliplatin control rats treated with Baicalein at 5 mg/kg and 10 mg/kg, ip respectively and BA2: Normal control rats treated with Baicalein (10 mg/kg, ip).*

Figure 4

Effect of Oxaliplatin and Baicalein on oxidative stress: *(a) Expression of 8-OHdG in spinal cord. Bar graph represents immunohistochemical scores of various groups. Photographs were taken at 400× magnification. Scale shows a length of 50 μm. (b) Representative western blot images of HO I, MnSOD and β-actin with corresponding graphical representations of densitometric analysis. Results were expressed as mean ± SEM (n = 3). ^ p < 0.05, ^^ p < 0.01, ^^^ p < 0.001 vs. NC, * p < 0.05, *** p < 0.001 vs. OC. NC: Normal control, OC: Oxaliplatin control (4 mg/kg, ip), OC+BA2: Oxaliplatin control rats treated with Baicalein at 10 mg/kg, ip.*

Figure 5

Effect of Oxaliplatin and Baicalein on neuro-inflammation: *(a) Expression of GFAP in spinal cord. Bar graph represents immunohistochemical scores of various groups. Photographs were taken at 400× magnification. Scale shows a length of 50 μm. (b) Pictorial representations represent NF-κB immunopositivity (Red color) in Neuro2a cells under various experimental conditions. Double positive area (Magenta color) represents nuclear translocation. (c) Bar graphs showing ELISA analysis of IL-6 and TNF-a levels. Results were expressed as mean ± SEM (n = 3). ^^ p < 0.01, ^^^ p < 0.001 vs. NC, * p < 0.05, ** p < 0.01 vs. OC. NC: Normal control, OC: Oxaliplatin control (4 mg/kg, ip), OC+BA1 and OC+BA2: Oxaliplatin control rats treated with Baicalein at 5 mg/kg and 10 mg/kg, ip respectively and BA2: Normal control rats treated with Baicalein (10 mg/kg, ip).*

Figure 6

Effect of Oxaliplatin and Baicalein on expression levels of inflammatory markers in Oxaliplatin-induced neuropathic rats*: Representative western blot images of iNOS, COX 2, p-NF-κKB, NF-κB, p-P38, P38 and β-actin with corresponding graphical representations of densitometric analysis. Results were expressed as mean ± SEM (n = 3). ^^ p < 0.01, ^^^ p < 0.001 vs. NC, * p < 0.05 ** p < 0.01, *** p < 0.001 vs. OC. NC: Normal control, OC: Oxaliplatin control (4 mg/kg, ip), OC+BA1 and OC+BA2: Oxaliplatin control rats treated with Baicalein at 5 mg/kg and 10 mg/kg, ip respectively and BA2: Normal control rats treated with Baicalein (10 mg/kg, ip).*

Figure 7

Effect of Baicalein on the levels of active β-catenin and WNT 5b in spinal cord and sciatic nerve of Oxaliplatin rats: *(a) Expression of active β-catenin in spinal cord (upper panel) and sciatic nerve (lower panel) respectively. (b) Expression of WNT 5b in spinal cord (upper panel) and sciatic nerve (lower panel) respectively. Bar graph represents immunohistochemical scores of various groups. Photographs were taken at 400× magnification. Scale shows a length of 50 µm. Results were expressed as mean ± SEM (n = 3). ^^^ p < 0.001 vs. NC, * p < 0.05, ** p < 0.01, *** p < 0.001 vs. OC. NC: Normal control, OC: Oxaliplatin control (4 mg/kg, ip), OC+BA2: Oxaliplatin control rats treated with Baicalein at 10 mg/kg, ip.*

Figure 8

Effect of Oxaliplatin and Baicalein on WNT Pathway in Oxaliplatin-induced neuropathic rats: *(a) Representative western blot images of active β-catenin, WNT5b, WNT3a and β-actin with corresponding graphical representations of densitometric analysis. (b)RT-PCR analysis of β-catenin. Results were expressed as mean ± SEM (n = 3). ^ p <0.05, ^^^ p < 0.001 vs. NC, * p < 0.05, ** p < 0.01, *** p < 0.001 vs. OC. NC: Normal control, OC: Oxaliplatin control (4 mg/kg, ip), OC+BA1 and OC+BA2: Oxaliplatin control rats treated with Baicalein at 5 mg/kg and 10 mg/kg, ip respectively and BA2: Normal control rats treated with Baicalein (10 mg/kg, ip).*

Parameter	NC	BA2	OC	OC+BA1	OC+BA2
MNCV (m/s)	51.05 ± 2.89	51.84 ± 3.00	45.70 ± 1.72	46.86 ± 2.29	50.85 ± 9.07
SNCV	68.60 ± 5.09	60.17 ± 3.71	42.56 ± 1.50	46.03 ± 1.18	58.85 ± 10.46

Figure 9

Schematic diagram representing neuroprotective mechanism of Baicalein by targeting WNT/β-catenin pathway and neuroinflammation in Oxaliplatin induced peripheral neuropathy.