Aim & Objectives
3.1 Aim of the study

Hyperalgesia or pain is an unpleasant feeling which needs immediate remedy. Use of conventional medicines poses limitations due to their side effects, which has attracted scientists for search of alternative medicines. Limited literature available in the past decade indicates that reactive oxygen species play an important role in inflammatory hyperalgesia. Complementary and alternative medicines with natural herbal products or extracts are acquiring importance in treatment of hyperalgesia owing to their lesser side effects. Most of the herbal products possess antioxidant property. Therefore, it is hypothesized that antioxidant property of these herbal products may be involved in their anti-hyperalgesic property.

To validate the hypothesis, the present study was aimed to investigate the regulation of hyperalgesia by ROS as well as mechanism of anti-hyperalgesic action by antioxidants curcumin and resveratrol.

3.2 Objectives

Hyperalgesia was developed by intraplantar administration of CFA or H\textsubscript{2}O\textsubscript{2} in hind paw of rats and the following objectives were adapted to achieve the aim of the study-

1. To study the role of curcumin in CFA induced hyperalgesic rats
   a. Effect on thermal hyperalgesia
   b. Effect on ROS, antioxidant enzymes and pro-inflammatory cytokines

2. To study the role of resveratrol in CFA induced hyperalgesic rats
   a. Effect on thermal hyperalgesia
   b. Effect on ROS and antioxidant enzymes
   c. Effect on pro-inflammatory cytokines, enzymes and ERK signaling

3. To study the molecular mechanism of ROS mediated hyperalgesic signaling
   a. Modulation of ERK signaling in H\textsubscript{2}O\textsubscript{2} induced hyperalgesia
   b. Validation of H\textsubscript{2}O\textsubscript{2} induced modulation of ERK signaling in CFA induced hyperalgesia
Following experimental approach was adopted to fulfill the objectives

1. Inflammatory hyperalgesia was developed by standard inflammatory stimulant ‘complete Freund’s adjuvant’ (CFA) in rats. H$_2$O$_2$ was used to develop hyperalgesia for study of ROS signaling.

2. Paw withdrawal latency (PWL) test was performed to measure thermal hyperalgesia.

3. Expression of neuronal marker c-Fos was evaluated by IHC.

4. Spectrofluorometric method was used for measurement of total ROS level using ROS sensitive dye DCFH$_2$DA.

5. Lipid peroxidation was measured by spectrophotometric method to study oxidative stress.

6. Enzyme activity was assayed by spectrophotometric method and activity gel assay was used to measure the isozymes pattern of antioxidant enzymes catalase, SOD, GPx and GR.

7. Proteins level of different inflammatory mediators IL-1β, TNF-α, IL-6, iNOS and COX2 was determined by ELISA/Western blot analysis.

8. In order to evaluate ERK activation, pERK level was measured by Western blot analysis/IF.

9. Co-localization studies were performed using cell specific markers NF-200 and NeuN to identify pERK expressing cells.

10. SFK, PTP and MEK inhibitors were used to delineate the signaling pathway in H$_2$O$_2$ and CFA induced hyperalgesic rats.
Materials and Methods
5.1 Materials

5.1.1 Chemicals

All the chemicals used for experiments were analytical and molecular biology grade and used without further purification. Chemicals were purchased from Sigma–Aldrich, Fermentas Life Science, Ambion, Sisco Research Laboratories, Merck and Himedia. CFA and curcumin (purity ≥99%) were purchased from Sigma-Aldrich (Saint Louis, USA). Resveratrol (purity ≥98%) was purchased from Cayman Chemicals, (Ann Arbor, USA). ELISA kits IL-1β from KOMA Biochem (Seoul, Korea), and IL-6 and TNF-α from Qiagen (Hilden, Germany). Hydrogen peroxide was purchased from Merk (Kenilworth, New Jersey, USA). NBT (Nitro blue tetrazolium), ferric chloride, potassium ferricyanide, riboflavin, PMS (Phenazine methosulphate) and GSH (Reduced glutathione) were purchased from SRL (Mumbai, India). Polyclonal anti-TNFR1, anti-ERK-1/2 and anti-pERK-1/2 rabbit antibodies were purchased from BioVision (Milpitas, CA, USA), anti-pERK-1/2 mouse antibody from Santa Cruz Technology (Texas, USA), Polyclonal anti-COX-2 rabbit antibody from Cayman Chemicals (Ann Arbor, USA), anti-iNOS polyclonal rabbit antibody from Enzo Life Sciences (New York, USA), anti-NeuN monoclonal mouse antibody from Novus Biologicals (Littleton, Colorado, USA), monoclonal anti NF-200, anti-c-Fos polyclonal rabbit antibody from Abcam (Cambridge, UK) and HRP conjugated anti-β-actin monoclonal antibody from Sigma-Aldrich (Saint Louis, USA). FITC conjugated anti rabbit, TRITC conjugated anti mouse and HRP conjugated anti-rabbit secondary antibodies were purchased from Merk-Genei (Bangalore, India). Src inhibitor (PP1) was purchased from BioVision (Milpitas, CA, USA), MEK inhibitor (PD98059) from Cayman chemicals (Ann Arbor, USA), and PTP inhibitor (Sodium orthovanadate) from Sigma-Aldrich (Saint Louis, USA).

5.1.2 Animals

Rats of Charles-Foster strain were used for the experiments. Rats were bred and maintained with the approval of CPCSEA (Licence:1802/GO/Re/S/15/CPCSEA) to Department of Zoology, BHU under standard laboratory conditions; at 25±2°C with 12h light/dark schedule with ad libitum supply of standard animal feed and drinking water. Normal adult (12-14W old) male rats were used for the experiments. All experiments
were performed with the approval of Central Animal Ethical Committee, Banaras Hindu University.

5.2 Methods

5.2.1 Induction of hyperalgesia and drug administration

Two different hyperalgesia models were used in the present study namely CFA induced rats as a classical model, and H$_2$O$_2$ induced rats representing ROS induced hyperalgesia model.

5.2.1.1 CFA induced hyperalgesia

Animals were randomly distributed into four groups. 100µl CFA was injected via the intraplantar (i.pl.) route in three groups for induction of hyperalgesia. Out of the three CFA induced groups, one group received 50µl saline (C), the second group received 50µl DMSO as vehicle (CD) and the third group received resveratrol dissolved in 50µl DMSO (CR) via i.p. route. Control (N) rats received 100µl saline via i.pl. route and 50µl saline via i.p. route. Resveratrol was injected immediately after CFA administration. A dose of 20mg/kg body weight/day of resveratrol or 100mg/kg body weight/day of curcumin was selected on the basis of earlier reports and used after standardization (Pham-Marcou et al., 2008; Torres-Lopez et al., 2002; Mittal et al., 2009). 8-12 animals were included in each group for molecular studies, out of which 4-6 were sacrificed after 6h and remaining animals were sacrificed after 48h of CFA administration. For behavioral studies involving the paw withdrawal latency test, each group included 6-9 rats.

5.2.1.2 H$_2$O$_2$ induced hyperalgesia

Animals were divided into four groups. First group was injected with 100µl saline (i.pl.) and served as control group (N). For induction of hyperalgesia, remaining three groups received 100µl of 5, 10 and 20µmole H$_2$O$_2$ respectively via i.pl. route. The doses were standardized based on the dose prescribed for mice (Keeble et al., 2009). Further experiments were performed with the best suitable dose of H$_2$O$_2$.

5.2.2 Assessment of thermal hyperalgesia

Animals were acclimatized for 1 week prior to experimentations by handling and keeping them in hyperalgesiameter at RT for 5min daily. Thermal hyperalgesia was measured by paw withdrawal latency (PWL) from a hot plate. All assessments were performed 3 times during baseline conditions and after different treatments. Animals were individually
placed on hot plate of hyperalgesiameter (Eddy’s Hot-Plate, Orchid Scientific, India) with the temperature adjusted to 50±0.5°C. Hyperalgesia was assessed by measuring the latency period of paw licking or jump response as index of pain threshold. Each withdrawal value was taken as an average of three consecutive latencies, measured at 10min intervals. The cut-off time was 15s in order to avoid tissue damage. Experiments were performed at different time intervals after induction of hyperalgesia. The observer was blinded to all treatments until analysis of results.

![Figure 1. Hot plate hyperalgesiameter](image)

5.2.3 Measurement of paw edema

H$_2$O$_2$ induced inflammation was analyzed by measurement of paw edema. The dorso-ventral thickness of hind paw was measured by using Vernier caliper, placed at the border of phalanges and metatarsals. Measurement was done at different time intervals after H$_2$O$_2$ administration. Each measurement was repeated three times.

5.2.4 Preparation of inhibitors

Sodium orthovanadate (Sigma) was activated following the procedure of Gordon JA (1991). A stock solution of orthovanadate (100mM, pH 10) was prepared in distilled water and boiled until it turned colorless. After cooling down at RT, the solution was re-adjusted to pH 10 with NaOH and boiled again to be colorless. After three to four cycles, the solution was stabilized at colorless state and then stored at -20°C. Just before use, the stock solution was diluted to the desired working concentration.

Src inhibitor 1-(1,1-Dimethylethyl)-1-(4-methylphenyl)-1H-pyrazolo[3,4-d] pyrimidin-4-amine (PP1; Biovision) and MEK inhibitor 2-(2-Amino-3-methoxyphenyl)-4H-1-
benzopyran-4-one (PD98059; Cayman Chemicals) were dissolved in dimethyl sulfoxide (DMSO), and diluted with saline before use. The final concentration of DMSO was not more than 2%.

5.2.4.1 Intrathecal administration of inhibitors

All the inhibitors were intrathecally (i.t.) injected 30min prior to induction of hyperalgesia by H₂O₂ or CFA. Doses of orthovanadate (0.4µg), PD98059 (1µg) and PP1 (10nmol) were selected on the basis of earlier published reports (Azkona et al., 2016; Kawasaki et al., 2004; Zhang et al., 2010).

For intrathecal injection, rats were anesthetized with diethyl ether and were placed in the prone position. Hair of the caudal back was shaved, and the injection was given with a 30 gauge needle connected to a 50µl Hamilton syringe following the method of Mestre et al. (1994). The needle was inserted into the inter-vertebral space between the fifth and sixth lumbar vertebrae at 45° angle with respect to the vertebral column, facing the cranial direction. Penetration of the needle tip into the inter-vertebral space was signified by a sudden advancement of needle and lateral movement of the tail. 10µl of the inhibitors or saline were slowly injected for 5 seconds. The syringe was held in place for 10 more seconds before removal in order to avoid spillage. Any chance of nerve damage was monitored by gait analysis. Foot prints of fore paw and hind paw overlap each other in case of normal as well as intrathecally injected rats which suggested no nerve injury after intrathecal injection (Appendix - Fig. 1).

5.2.5 Immuno-histochemical staining of c-Fos

Rats were deeply anaesthetized by sodium pentobarbital (65mg/kg i.p.) at different time intervals after H₂O₂ or CFA administration and were transcardially perfused with 0.6M phosphate-buffered saline (PBS), followed by ice-cold fixative (4% paraformaldehyde in 0.6M phosphate buffer). The lumbar region (L4-L6) of each spinal cord was dissected out and fixed for 8h in 4% formalin and cryoprotected overnight in 20% sucrose at 4°C. Sections of 12μm thickness were cut using a cryo-microtome (Microm HM525, Thermo Scientific) and collected on poly L-lysine coated slides. Endogenous peroxidase activity was quenched with 2% H₂O₂ for 30min. Non-specific binding was blocked with 2% normal goat serum. Sections were incubated overnight in rabbit anti-c-Fos antibody (1:200) at 4°C, washed in PBS for 5-10min and incubated with horseradish peroxidase
(HRP) conjugated goat anti-rabbit secondary antibodies (1:500) for 2h at room temperature (RT). c-Fos positive cells were detected by DAB (di-amino benzidine) staining. Stained sections were observed under a light microscope (Leitz “laburlux S” microscope, Earnst Leitz GmbH, Wetzlar, Germany) and images were taken with Leica DCF290 camera (Leica Microsystems Ltd., Germany). The number of c-Fos positive cells was considered as marker of pain intensity, irrespective of intensity of stain.

5.2.6 Preparation of Tissue Homogenates

Tissues were homogenized in 50mM potassium phosphate buffer, pH 7.0, containing 1mM PMSF, 0.1% Triton X-100 with a polytrone homogenizer in a cold-room maintained at 4±2°C and centrifuged at 14,000g for 20min at 4°C. The supernatant was collected, total protein content in each sample was determined using the method of Bradford by using bovine serum albumin (BSA) as standard. Protein samples were diluted to 100X in autoclaved triple distilled water. Diluted protein sample (100µl) and Bradford regent (900µl) were mixed well and incubated for 1min at RT. Absorbance was measured at 595nm (Bradford M, 1976). Protein samples were stored in aliquots at -70°C for further use or used directly.

5.2.7 Lipid peroxidation assay

Malondialdehyde (MDA) level, an indicator of lipid peroxidation was measured in paw skin and spinal cord using the method described by Ohkawa et al. (1979). Reaction mixture containing 0.2ml tissue extract, 0.2ml of 8.1% sodium dodecyl sulphate (SDS), 1.5ml of 20% acetic acid (pH 3.5), 1.5ml of 0.8% aqueous solution of thiobarbituric acid (TBA) and distilled water up to a final volume of 4.0 ml was heated at 95°C for 60min in a water bath. After cooling 1.0ml of distilled water and 5.0ml of mixture of n-butanol and pyridine (15:1; v/v) was added, the reaction mixture was vortexed and centrifuged at 3,000g for 15min. The absorbance of upper organic layer was measures at 532nm using appropriate control in a spectrophotometer (GE Health Care). The concentration of MDA was determined using the standard curve, generated by taking known quantities of 1,1,3,3-tetramethoxypropane and expressed as nmols of MDA/mg of protein.

5.2.8 Measurement of ROS

Total ROS level was determined by the oxidative conversion of nonfluorescent 2′,7′-dichlorofluorescein diacetate (H2DCFDA) to highly fluorescent 2′,7′-dichlorofluorescein
(DCF). Skin or spinal cord extracts were incubated at 37°C for 60 min with equal volume of 2 mM H$_2$DCFDA (Invitrogen) in PBS. Fluorescence was recorded at 485 nm (excitation) and 527 nm (emission) with a Victor3 1420 Multilabel Counter (Perkin Elmer, MA, USA). The levels of ROS were presented in arbitrary units in terms of fluorescence intensity/mg protein.

5.2.9 Immunofluorescence staining
Spinal cord, DRG and sciatic nerves were dissected out and processed as described for IHC. Sections were rinsed in 0.01 M PBS (pH 7.4) for three times (10 min each), blocked with 2% goat serum in 0.01 M PBS for 2 h at the RT and then used for immunofluorescence staining. The sections were incubated overnight at 4°C with the primary antibodies: Rabbit anti-pERK (1:200) only or mixed with mouse anti-NF-200 (1:500) or mouse anti-NeuN (1:500), rabbit anti-TNFR1 (1:200) only or mixed with mouse anti-pERK (1:500) for co-localization. The sections were then washed for three times in 0.01 M PBS (5 min each) and incubated for 2 h at RT with the corresponding secondary antibody: FITC conjugated goat anti-rabbit antibody (1:500) and TRITC conjugated goat anti-mouse antibody (1:500). Images were obtained using a fluorescence microscope (Motic BA410, Japan) and images were captured with Moticam-5 (Motic, Japan) camera.

5.2.10 Western blotting
Tissues were homogenized in 50 mM potassium phosphate buffer (pH 7.4) containing 1 mM PMSF (Phenylnmethylsulfonyl fluoride), protease inhibitor cocktail and 0.1% Triton X-100 using polytron homogenizer, and centrifuged at 14,000 g for 20 min at 4°C. The supernatant was used for Western blot analysis. Equal amount of total protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with non-fat milk for 2 h to prevent non-specific binding. Blots were incubated overnight with primary antibodies (1:1000), washed in PBS for 5-10 min and incubated with HRP conjugated secondary antibodies (1:2500) for 2 h. Bands were detected by enhanced chemiluminescence (ECL) on X-ray film. Alpha Imager 2200 software (Alpha Innotech) was used for densitometric analysis. β-actin served as a loading control.
5.2.11 Assay of antioxidant enzymes

5.2.11.1 Catalase activity assay
Activity of catalase was measured in terms of H₂O₂ decomposition per unit time by the enzyme according to method of Aebi (1984). For final volume of 1ml reaction mixture 20mM H₂O₂, 50mM phosphate buffer (pH 7.0) and 15μg protein was added. Decomposition of H₂O₂ was monitored by recording absorbance at 240nm. Activity of catalase was calculated by using molar extinction coefficient of 43.6 M⁻¹cm⁻¹ for H₂O₂. Specific activity was defined as U/mg of protein.

5.2.11.2 Superoxide dismutase (SOD) activity assay
The enzymatic activity of superoxide dismutase was measured spectrophotometrically according to method of Winterbourn et al. (1975). The reaction mixture in final volume of one ml containing 67mM potassium phosphate buffer pH 7.8, 100mM EDTA, 0.12 mM riboflavin, 1.5mM NBT and 15μg of protein. The reaction mixture was incubated under fluorescent light for 10 min at RT and absorbance was measured at 560nm at time intervals. One unit was defined as the amount of enzyme causing one half the maximum inhibition of NBT reduction.

5.2.11.3 Glutathione peroxidase activity assay
Glutathione peroxidase (GPx) activity was measured according to Paglia and Valentine (1967) and Ho et al. (1997). The reaction mixture was prepared in final volume of one ml containing 50mM phosphate buffer (pH-7.0), 1mM EDTA, 1mM sodium azide, 0.5mM NADPH, 0.2mM GSH and 1U glutathione reductase. Equal amount of protein was added in reaction mixture and allowed to equilibrate for 1min at RT. The reaction was initiated by addition of 0.1mM of H₂O₂. Oxidation of NADPH was monitored by recording absorbance at 340nm. The activity of GPx was calculated by using extinction coefficient for NADPH of 6.22 mM⁻¹cm⁻¹. Specific activity was defined as U/mg protein.

5.2.11.4 Glutathione reductase (GR) activity assay
Glutathione reductase activity was measured spectrophotometrically by following the rate of oxidation of NADPH by GSSG according to method of Horn (1965) with minor modifications. The reaction mixture was prepared in final volume of one ml containing 50mM phosphate buffer pH 7.0, 12mM NaHCO3, 0.5mM EDTA, 0.5mM 2-mercaptoethanol, 1.5mM GSSG and 0.2mM NADPH. 15μg protein was added in
reaction mixture and decreasing absorbance was recorded at 340nm. The activity of GR was calculated by using extinction coefficient for NADPH of 6.22 mM⁻¹ cm⁻¹. Specific activity was defined as U/mg protein.

5.2.12 In-gel activity assay of antioxidant enzymes

5.2.12.1 Catalase
The activity of catalase was analyzed by native poly-acrylamide gel electrophoresis followed by ferricyanide method of Woodbury et al. (1971). 30μg of protein was mixed with equal amount of native loading buffer and loaded on the wells of 4.5% stacking gels and resolved on 8% resolving gel. The electrophoresis was carried out at 4°C. After completion of electrophoresis, the gel was washed with two changes of distilled water and soaked in H₂O₂ (0.03%) for 5 min and rinsed with distilled water to remove excess H₂O₂. The gel was stained in a solution containing 1% ferric chloride and 1% Potassium ferricyanide for 4-5 min at room temperature.

5.2.12.2 Superoxide dismutase (SOD)
The activity gel assay of SOD was performed according to modified procedure of Beauchamp and Fridovich (1971). 30μg of total protein from each sample was loaded and separated on 10% resolving gel by non-denaturing PAGE at 4°C. After completion of electrophoresis, the gels were soaked in 1.23mM NBT solution for 20 min at RT under dark. Gels were washed with distilled water and incubated in 100mM phosphate buffer (pH 7.0) containing 28mM TEMED and 0.28mM riboflavin for 15-20 min at RT in dark. Thereafter, the gels were exposed to fluorescent light until achromatic bands appeared with purple-blue background.

5.2.12.3 Glutathione peroxidase (GPx)
The activity gel assay of GPx was performed according to the method of Lin et al. (2002). 100μg of protein from each sample was loaded and separated on 10% resolving gel by non-denaturing PAGE at 4°C. After completion of electrophoresis, the gel was soaked in 50mM tris-HCl (pH 7.9) containing 13mM GSH and 0.004% H₂O₂ for 20 min in dark. Thereafter, the gels were washed and developed in darkness at 30°C containing 1.2mM NBT and 1.6mM PMS for 10 min and exposed to light until appearance of clear zone of GPx band on purple background.
5.2.13 Estimation of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6)
Level of cytokines was measured by respective kits based on sandwich ELISA method following manufacturer’s instruction. Briefly, 100μl of antigen standard solutions and tissues samples were added to capture antibody coated ELISA plate wells. Captured antigens were allowed to bind with biotin conjugated detection antibodies. Detection was done by Avidin-HRP conjugate. TMB was used as color development reagent. Reaction was stopped by stop solution (H₂SO₄). Color intensity was measured by recording absorbance at 450nm in ELISA reader (micro scan, ECIL, India). Concentration of cytokines was calculated by standard curve generated by serial dilutions of antigen standard.

5.2.14 Statistical analysis
Behavioral data were analyzed by repeated measure ANOVA. ROS level was compared in two tissues during two phases by two-way ANOVA. Other results were analyzed by one-way ANOVA. These tests were followed by Tukey post hoc test using SPSS software (version 23). Values were expressed as mean ± S.E.M. obtained from three different sets of experiments; p<0.05 was taken as statistically significant.