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Research Article

POSSIBLE INVOLVEMENT OF HEME OXYGENASE-1 IN PATHOPHYSIOLOGICAL MECHANISMS OF OPIOID TOLERANCE IN DIABETIC NEUROPATHIC PAIN

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ABSTRACT

Diabetic neuropathic pain (DNP) is immedicable and progressive neuronal damage seen in 30% patients with diabetes mellitus. It is a painful condition characterized by hypersalgesia, allodynia and dysthesia. Opioids like morphine and oxycodone are used for treatment of DNP but major drawback of opioids are that tolerance develops after short span of treatment. Hemeoxygenese-1 (HO-1) is an enzyme which catalyzes heme degradation and produces iron, biliverdin and carbon monoxide and has a potential role in DNP. In this study, we plan to investigate the possible role of HO-1 in tolerance due to use of opioids. A single dose of STZ 50 mg/kg, i.p., instigated diabetes within 7 days and DNP develops within 4 weeks. Our investigative drugs; hemin (HO-1 inducer) 4 mg/kg, i.p., ZnPP (HO-1 inhibitor), 50µg/kg, i.p., and morphine, 5 mg/kg s.c., were administered from day 28th to 35th day. This study unveils that with single time administration of STZ showed reduction in body weight, symptoms of DNP like thermal hyperalgesia, thermal allodynia, mechanical allodynia etc. appeared from 3rd week after STZ administration, increased levels of TBARS, nitrite whereas decreased antioxidant defense enzymes (reduced glutathione and catalase) and levels of TNF-α and IL-6 had also increased. It was seen that treatment with hemin recedes thermal hyperalgesia, mechanical, thermal and cold allodynia. With treatment with hemin, TBARS and nitrite levels attenuate whereas reduced glutathione levels and catalase activity increases, so hemin upsurge anti-nociceptive effect of morphine which has reduced due tolerance.

Keywords: Hyperalgesia, Allodynia, Hemin, Neuropathic Pain, Opoid Tolerance, Diabetes.

INTRODUCTION

Diabetes mellitus is a metabolic disorder which is characterized by hyperglycemia, polyphagia, polydipsia and polyuria. Persistent hyperglycemia for long coon's age fountains secondary complications like neuropathy, cardiomyopathy, nephropathy and retinopathy. Neuropathic pain is a devitalizing pain which is characterized by the sensory abnormalities such as dysesthesia (unpleasant), hyperalgesia (noxious stimuli) and allodynia (innocuous)[1,2]. For inciting diabetes in experimental animals, streprozotocin (STZ) is used as diabetogenic agent that is specifically toxic to β-cells of the pancreas and leads to destruction of β-cells. Streptozotocin (STZ, 2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose) is acceded naturally from soil microbe species *Streptomycetes achromogenes* and instigate both insulin-dependent and non-insulin-dependent diabetes mellitus[3].

Pharmacological intervention available for treatment of DNP includes tricyclic antidepresent, selective serotonin reuptake inhibitors, anticonvulsants, opioids, COX-2 inhibitors[4]. Opioid like morphine, oxycodone are centrally acting analgesic which comport its antinociceptive action through μ, κ, δ receptors (central and peripheral nervous system) and recedes the neuronal firing. Morphine is μ- opioid used for the treatment of DNP but after few days tolerance develops due to receptor desensitization or down-regulation[5,6] and anti-nociceptive effect fades off. Morphine exerts its anti-nociceptive through activation of nitric oxide-cyclic guanosine monophosphate-protein kinase G-adenosine trihosphate-sensitive potassium channel signaling[7]. Long time use of opioids like morphine that we used in our study escalate the release of pro-inflammatory cytokines which further releases iNOS that produces nitric oxide (NO) and then peroxynitrite that augments analgesic tolerance. Persistant hyperglycemia, prompt downregulation of μ-opioid receptor expresion leads to opioid tolerance[8]. Opioid tolerance restricts the patients from its beneficial effects.

Heme oxygenase is a rate limiting enzyme that fastens the degradation of heme into biliverdin, carbonmonoxide and

Fe^{2+}. Ho-1 has been reported to have inverse relation with iNOS[9]. HO-1 possesses many beneficial effects like anti-nociceptive, anti-inflammatory, anti-apoptotic, anti-athereoscleortic and prevention of ischemia reperfusion injury myocardial arrhythmia[10]. HO- upsurge the effects and expression of μ-opioid receptor[7].

Therefore, the present study aims to investigate the possible role and mechanism of HO-1 in opioid tolerance in diabetic rats.

MATERIALS AND METHODS

1. Experimental animals

Wistar rats (200-250g) of either sex of 8-10 weeks in age are used. They were housed in central animal house facility of ISF College of Pharmacy, Moga, Punjab, India and were kept under the normal animal house provided with a12-h light/dark cycle and were given standard chow and ad libitum access to water. The experimental protocol was approved by the Institutional ethical committee of ISF college of pharmacy, Moga, Punjab and was carried out in accordance with the guidelines of committee for the purpose of control and supervision of experiments on animals (CPCSEA) for the use and care of experimental animals.

2. Drugs and chemicals

STZ was purchased from Spectrochem Pvt. Ltd (Mumbai, India) and was dissolved in 0.1M citrate buffer at pH 4.5. Hemin and ZnPP were purchased from Himedia (Mumbai, India). Morphine was obtained as ex-gratia sample from Guru Gobind Singh Hospital, Faridkot. All other chemical were of analytical grade. All the solutions were freshly prepared before the experiment. A constant volume of 0.5 ml per 100 g body weight of animal was injected.

3. Induction and assessment of diabetes mellitus in rats

STZ is used to induce diabetes mellitus because of its diabetogenic property is due to selective cytotoxic action upon pancreatic beta-cells[11]. Animals were weighed and injected with STZ, a diabetogenic agent, 50 mg/kg, i.p., once (Taliyan et al., 2010). Serum glucose levels are measured by GOD-POD method[12] (Trinder, 1969) using commercially available kit in blood sample obtained from tail vein or capillary retro-sinus before and after STZ injection. Serum glucose ≥ 200mg/dl were considered diabetic animals.

4. Experimental protocol and treatment schedule

The animals were divided into following groups and each group consists of 6 animals. Group-1 served as normal control and received no treatment; Group-2 served as STZ control (50mg/kg, i.p., once); Group-3 served as hemin treated group (4mg/kg, i.p.) with treatment starting from day 28 to 35 to diabetic animals. Group 4 served as ZnPP treated group (50μg/kg, i.p.); Group 5 received morphine treated group (5mg/kg, s.c.) for 7 days; Group 6 served as hemin (4 mg/kg, i.p.) and morphine treated group (5mg/kg, s.c.). Group 7 served as morphine (5mg/kg, s.c.) and ZnPP treated group (50μg/kg, i.p.);

5. Body weight

Body weight of animals was recorded on the first (BW_F) and last day (BW_L) of the study. Percent change in body weight (BW) was calculated as per following formula:-

$$\% BW = [(BW_F - BW_L) / BW_L] \times 100$$

6. Behavioral parameters

6.1 Hot Plate Test

Pain responses to thermal stimuli were measured by Eddy and Leimbach hot plate method[13]. In brief, rats were placed on the hot plate (25.4 cm x 25.4 cm), maintained at constant temperature of 52ºC which was surrounded by a clear acrylic cage, and the start/stop button on the timer was activated. Cut-off time was 30 sec; animals were removed immediately from hot plate to avoid tissue damage.

6.2 Thermal Hyperalgesia

The threshold level of pain to thermal heat was evaluated using the plantar test (Ugo Basile, Varese, Italy)[14] (Hargreaves et al., 1988) before and after diabetes instigation. The paw withdrawal latency to heat was taken as an index of pain threshold. The cut-off time of the test was 20 s to avoid tissue damage. In short, each animal was placed in a clear plexiglas box and hind paw was exposed to a constant beam of radiant heat through a plexiglas surface. Time taken to withdraw its paw is noted.

6.3 Thermal allodynia

In brief, each rat tail was immersed in a water bath maintained at 42°C (a temperature that is normally innocuous in normal rats[15] (Courteix et al., 1993) until tail withdrawal or signs of struggle were seen (cut-off time- 20 sec).

6.4. Mechanical Allodynia

The nociceptive threshold was defined as the force at which the animals withdrew its paw. The mechanical threshold was measured using dynamic plantar anesthesiometer (Ugo, Basile, Varese, Italy). In brief, each animal was placed in a test cage with a wire mesh floor, and the tip of a von Frey-type filament was pricked to the middle of the plantar surface of the hind paw. Paw-withdrawal threshold was expressed as threshold level in g. The maximum force applied by a von Frey-type filament was set 50 g.

6.5. Cold Allodynia

Cold allodynia was assessed using the acetone drop method[16] (Choi et al., 1994). Cold allodynia was observed as number of foot withdrawal after application of acetone to the paw. In short, acetone (0.1 mL) was applied to each hind paw with the 1-mL insulin syringe. Acetone was applied five times to each paw at intervals of 5-10 min. The time period of withdrawal response was recorded. (Cut-off- 20s)

7. Biochemical tests

Biochemical tests were carried out immediately after behavioral observations on day 36. Animals were sacrificed and sciatic nerves were removed quickly and tissue homogenate was prepared as under:

7.1. Tissue preparation

Animals were killed by decapitation and sciatic nerves were quickly removed and washed with ice-cold isotonic saline. Sciatic nerve samples were then homogenized with ice-cold 0.1M phosphate buffer (pH 7.4) 10 times (w/v). The homogenate was centrifuged at 10,000 g for 15 min and aliquots of supernatant were separated and used for biochemical estimation.

7.2. Measurement of TBARS

The quantitative measurement of lipid peroxidation in the sciatic nerve was performed according to the method of Wills

(1966). The amount of malondialdehyde was measured by reaction with thiobarbituric acid at 532 nm by using the UV-1700 spectrophotometer, Shimadzu, Japan were calculated by using molar extinction coefficient of chromophore (1.56 x 105/mol/l/cm) and expressed as mg pr.

7.3. Estimation of reduced glutathione

Reduced glutathione in the sciatic nerve was estimated according to the method described by Ellman (1959). Results were calculated using molar extinction coefficient of chromophore (1.36x 104/ mol/l/cm) and expressed as mg pr.

7.4. Estimation of nitrite

The accumulation of nitrite (serum and tissue) in the supernatant, an indicator of the production of nitric oxide, was determined with a colorimetric assay with Greiss reagent [0.1% N-(1-naphthyl) ethylenediame dihydrochloride, 1% sulfanilamide and 2.5% phosphoric acid] as described by (Green et al., 1982). The concentration of nitrite in the supernatant was determined from a sodium nitrite standard curve and expressed as mg pr.

7.5. Estimation of catalase

Catalase activity was measured by the method of Luck (1971), wherein the degradation of hydrogen peroxides was measured at 240 nm. Mixture consisted of 3 ml of hydrogen peroxides phosphate buffer and 0.05 ml of supernatant of tissue homogenate (10%), and change in absorbance was recorded at 240 nm with UV-1700 Spectrophotometer, Shimadzu, Japan. The results were expressed as micromole H_2O_2 decomposed per mg of protein/min.

7.6. Estimation of protein

Protein estimation was done by Biuret method[17] (Gornall et al., 1949) using Bovine Serum Albumin as standard.

7.7. Estimation of tumor necrosis factor-alpha (TNF-α) levels

TNF-α level was estimated by using rat TNF-α kit (Ray Bio, Rat TNF-alpha ELISA kit protocol). It is a solid phase sandwich enzyme linked immunosorbent assay (ELISA), which uses a microtiter plate reader read at 450 nm. Concentrations of TNF-α were calculated from plotted standard curve.

7.8. Estimation of interleukin (IL-6) levels

IL-6 level was estimated by using rat IL-6 kit (Ray Bio, Rat IL-6 ELISA kit protocol). It is a solid phase sandwich enzyme linked immunosorbent assay (ELISA), which uses a microtitre plate reader read at 450 nm. Concentrations of IL-6 were calculated from plotted standard curve.

2.8. Statistical analysis

The data were analyzed by using analysis of variance (ANOVA). One way ANOVA followed by Tukey's multiple comparison test and two way ANOVA followed by Bonferroni post-hoc test. All the values are expressed as mean± S.D. In all tests, $p \leq 0.05$ was considered as statistically significant. All the data were analyzed using software GraphPad prism 5.0 (Graphpad software, California, USA).

RESULTS

1. Effect of pharmacological interventions on the % change in body weight

On 35th day STZ treated group showed highly significant decrease in body weight as compared to normal control (Fig 2). Hemin treated rats gained weight as compared to STZ control whereas treatment with morphine did not showed any significant change in body weight due to development of tolerance as compared to STZ treated group. The group in which hemin is given prior to morphine administration, in that group body weight of animals improved as compared with STZ control and STZ+ morphine treated group. ZnPP showed completely opposite effects than that produced by hemin both alone and in combination.

2. Effect of pharmacological interventions on serum glucose levels

STZ treated rat had elevated high serum glucose levels on day 7 and these levels remained high till last day of study as compared with normal control (Fig. 3). Morphine and hemin treatment had no effect on serum glucose levels; it remained high throughout the study period.

3. Effect of pharmacological interventions on hot plate test

Jumping and licking response of animals on a hot plate significantly augmented on day 21 in STZ administered rats as compared to normal control (Fig. 4). Treatment with hemin alone and in combination with morphine from day 28 showed marked rise in pain threshold as compared to STZ control. ZnPP showed completely opposite effects than that produced by hemin both alone and in combination.

4. Effect of pharmacological interventions on thermal hyperalgesia

Withdrawal latency significantly decreased in STZ treated rats as compared to normal rats (Fig. 5). Treatment with hemin from 28 to 35 days showed reduced withdrawal latency as compared to STZ control group and same results were seen in morphine and hemin combination group in which withdrawal latency increased significantly as compared to STZ control and STZ+ morphine group. ZnPP showed completely opposite effects than that produced by hemin both alone and in combination.

5. Effect of pharmacological interventions on thermal allodynia

Paw withdrawal latency (PWL) in sec is defined as time taken by animals to withdraw its paw to hot stimuli (Fig. 6). PWL receded in diabetic rats that were given STZ as compared to normal group. Treatment with hemin from 28 to 35 days showed increased PWL as compared to STZ control group and same results were seen in morphine and hemin combination group in which significant result were seen both as compared with STZ control and STZ+ morphine treated group. ZnPP showed completely opposite effects than that produced by hemin both alone and in combination.

6. Effect of pharmacological interventions on mechanical allodynia

Paw withdrawal latency (PWL) in g is defined as force applied by rats to withdraw its paw to the painful stimuli. PWL is receded in diabetic rats that were given STZ as compared to normal group (Fig. 7). Treatment with hemin from 28 to 35 days showed reduced PWL as compared to STZ control group but best result were seen in morphine and hemin combination group in which significant result were seen both as compared with STZ control and STZ+ morphine treated group. ZnPP showed completely opposite effects than that produced by hemin both alone and in combination.

7. Effect of pharmacological interventions on cold allodynia

Paw withdrawal latency (PWL) in sec is defined as time taken by animals to withdraw its paw to the cold stimuli. PWL increased in diabetic rats that were given STZ, 50 mg/kg, i.p., as compared to normal group (Fig. 8). Treatment with hemin showed increased PWL as compared to STZ control group but best result were seen in morphine and hemin combination group in which significant result were seen both as compared with STZ control and STZ+ morphine treated group. ZnPP showed completely reversed effects than that produced by hemin both alone and in combination.

8. Effect of pharmacological interventions on TBARS, total protein and nitrite level

STZ treated rats showed augmented levels of TBARS, total protein and nitrite level as compared to normal group (Fig. 9, 10). Treatment with hemin lowered the TBARS, total proteins and nitrite levels as compared to STZ control group. Pre-treatment with hemin in combination group showed marked decrease in TBARS, total protein and nitrite levels as compared to STZ control as well as STZ+ morphine group. . ZnPP showed completely opposite effects than that produced by hemin both alone and in combination.

9. Effect of pharmacological interventions on catalase activity and reduced glutathione level

STZ treated rats showed attenuated catalase activity and reduced glutathione levels as compared to normal group (Fig. 11, 12). Treatment with hemin increased catalase activity and reduced glutathione levels as compared to STZ control group. Pre-treatment with hemin showed marked improvement in catalase activity and reduced glutathione level as compared to STZ control as well as STZ+ morphine group. ZnPP completely reversed these levels than that produced by hemin both alone and in combination.

10. Effect of pharmacological interventions on tumor necrosis factor-alpha (TNF-α) and interleukin (IL-6) level

STZ treated group showed significant increased levels of tumor necrosis factor-alpha (TNF-α) and interleukin (IL-6) as compare to normal group. Treatment with hemin alone and in combination with morphine significantly attenuated the increased levels of TNF-α and IL-6 as compared to the STZ group (Fig.13, 14). ZnPP showed completely opposite effects than that produced by hemin both alone and in combination.

DISCUSSION

In the present study, STZ (50 mg/kg, i.p.) was administered to induce diabetes mellitus in rats, which was confirmed by assessment of chronic hyperglycemia by God-Pod method using spectrophotometer. Neuropathic pain is subsidiary complication of diabetes mellitus which is characterized by slow nerve conduction, sensory loss and nerve degeneration[18] (Weifeng et al., 2014). Streptozotocin has cytotoxic and diabetogenic action which lead to development of this model. STZ is selectively toxic to β-cells of pancreas, the reason underlying is that its structure is similar to that of glucose so it competively inhibits glucose binding to GLUT-2 transporter and is taken up by β-cells and ultimately leads to its destruction. STZ first inhibits the release of insulin from pancreas that lead to transient hyperglycemia and after the destruction of β-cells it release all the stored insulin, causing hypoglycemia and lastly when no β-cells survived it cause persistant hyperglycemia[19] (Skhudelksi, 2001). The normoglycemic rats were selected for the study. Serum glucose levels before and after diabetes inductions were measured. On day 7, rats with serum glucose levels more than 200 mg/dl were considered diabetic. Serum glucose levels remained high in all the groups except the normal group throughout the time period of study after induction by STZ.

In this present study, diabetes was induced by single injection of STZ, 50 mg/kg i.p., and then diabetes was induced within 7 days after STZ injection whereas symptoms of DNP can be seen from 21st day and complete neuropathic pain was developed after 28 days. So, we started our treatment from 28th day and continued till 35th day. Hemin, HO-1 inducer, 4mg/kg was administer to group 3 and ZnPP, HO-1 inhibitor 50 µg/kg, i.p., was given to 4th group. To group 5 morphine, 5mg/kg, s.c. was given while in group 6, hemin was given along with morphine and to group 7, ZnPP was co-administered with morphine.

Body weight was measured on 1st day and on the last day of the study. On 35th day, the STZ treated group showed huge loss in the body weight as compared to normal control which may be due to dysphagia. Treatment of hemin (4 mg/kg, i.p.); alone in group 3 and in combination with morphine in group 6 was started from 28th day which gradually improved the weight of rats

The behavioral parameters were assessed on 1st, 7th, 14th, 21st, 28th and 35th day. The behavioral parameters include hot plate test, thermal hyperalgesia, thermal allodynia, mechanical allodynia and cold allodynia. It was observed that the symptoms of DNP were visible from 3rd week and pain threshold of STZ treated rat's decreases as compared to normal group. In hot plate test the paw licking or jumping behavioral signs appeared earlier as compared with normal group. In hemin treated group pain threshold upsurged as compared to STZ treated group while ZnPP (50 µg/kg, i.p.) administered group 4 produced completely reversed effects as compared to hemin treated group 3. Thermal hyperalgesia and thermal allodynia were observed from 3rd week in group 2 which is STZ administered rats as compared to normal group 1 while these symptoms improved in hemin treated group 3 as compared to STZ administered group 2. Cold allodynia was noticed after 4th week after STZ injection and result were quite similar to that produced in all other tests.

Hemin treatment in group 2 receded the levels of TBARS and nitrite as compared to STZ treated group suggesting that it is a negative regulator of oxidative stress. Also it has been earlier reported that that HO-1 has inverse relationship with iNOS expression (Kitamura et al., 1999). As discussed above hemin plays a significant role in anti-oxidant defense system, in this present study, results support this point as the levels of reduced glutathione and catalase activity significantly increased in hemin treated group as compared to STZ treated group. Hemin treated group attenuated the upsurged levels of neuroinflammatory markers such as TNF-α and IL-6 which may be due to its anti-inflammatory action.

In this present study, DNP have completely developed till 4th week of STZ injection. Opioids like morphine and oxycodone which are classic analgesics are used for the treatment of DNP but are not used for the long time management of DNP because of the tolerance. So, in the present study, it was observed that in morphine treated group 5, tolerance develops after 7 day administration of morphine. Hemin treatment reverses the morpnine tolerance and ameliorates the anti-nociceptive effect of morphine. In STZ+ morphine treated group 5 results of all the pain parameters were not significant as compared to STZ control group 2, this suggests that morphine after short time causes tolerance which could be due to release of cytokines and increase iNOS release whereas in STZ+ morphine+ hemin treated group 6 results were significant as compared to STZ+ morphine treated group. Pretreatment with hemin improved the pain threshold and ameliorate the anti-nociceptive effect of morphine and this group shows the maximum increment in pain threshold which supports the previous studies that CO produced by HO-1 has antinociceptive effect in neuropathic pain. Hemin treatment in combination with morphine receded the levels of TBARS and nitrite level. Glutathione level and catalase activity in STZ+ morphine+ hemin treated group 6 have improved tremendously as compared with STZ control and STZ+ morphine treated group. Morphine+ hemin treated group 6 attenuated the upsurged levels of neuroinflammatory markers such as TNF-α and IL-6 which may be due to its anti-inflammatory action. ZnPP in combination with morphine produced completely opposite effects as compared to morphine+ hemin treated group 6.

CONCLUSION

From above discussion, it can be concluded that heme oxygenase-1 (HO-1) has potential role in diabetic neuropathic pain and analgesic tolerance due to chronic use of opioids. So, the study warrants the future prospective of HO-1 in neuropathic pain for clinical practice.

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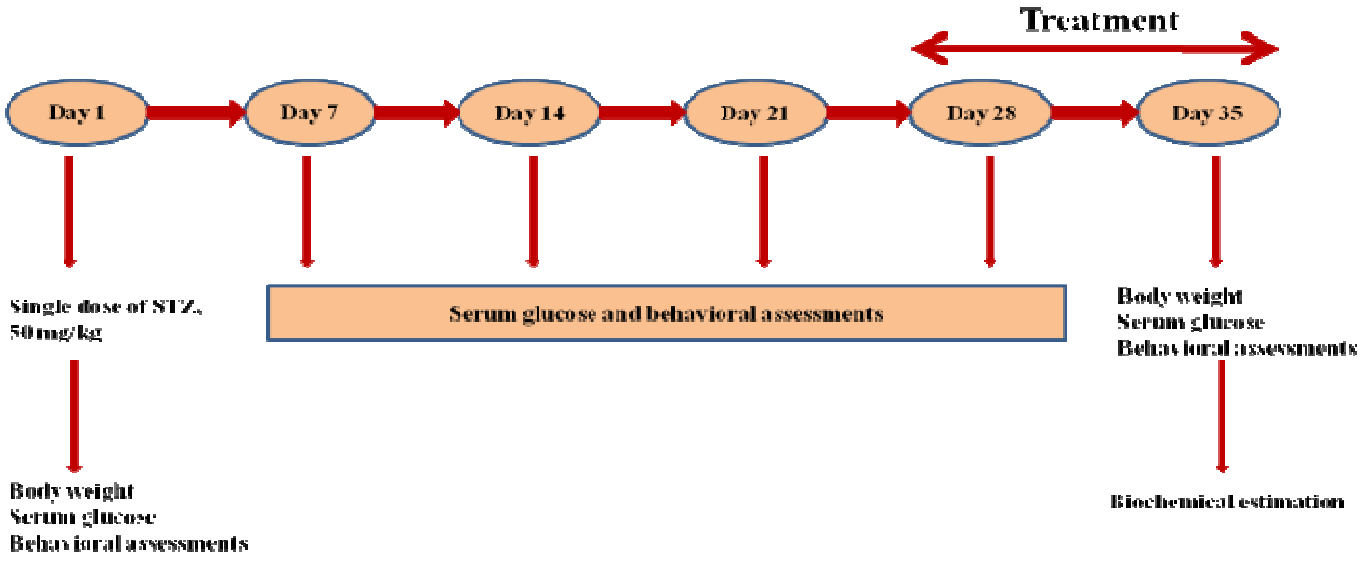

Figure 1: Experimental design

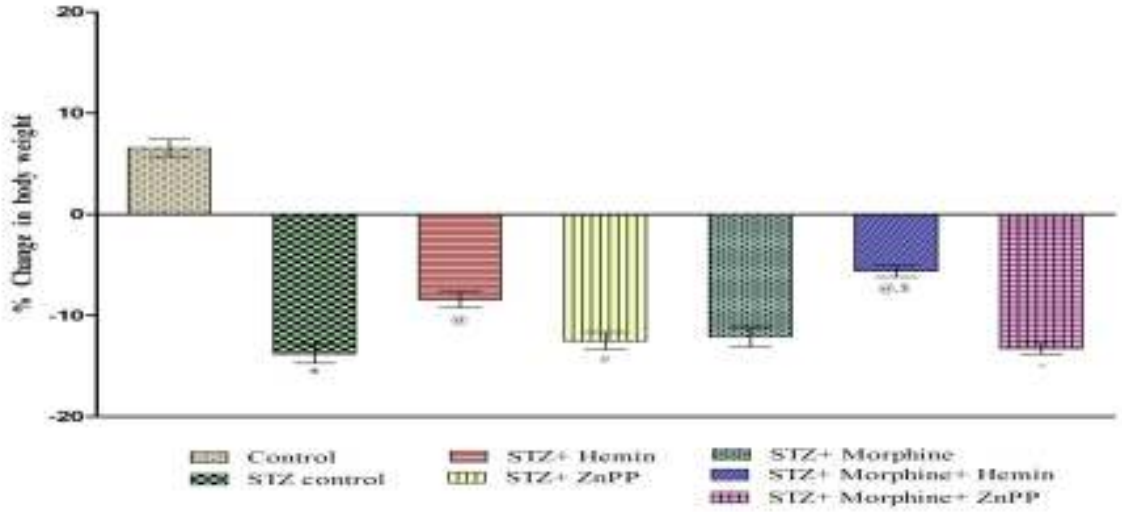

Figure 2: Effect of pharmacological interventions on % change in body weight
Values are expressed as Mean ± S.D.* p<0.05 vs. normal group, @ p<0.05 vs. STZ group, #p<0.05 vs. STZ+ Hemin group, $p<0.05 vs. STZ + Morphine (5 mg/kg), ^ p<0.05 vs. STZ+ Morphine+ Hemin

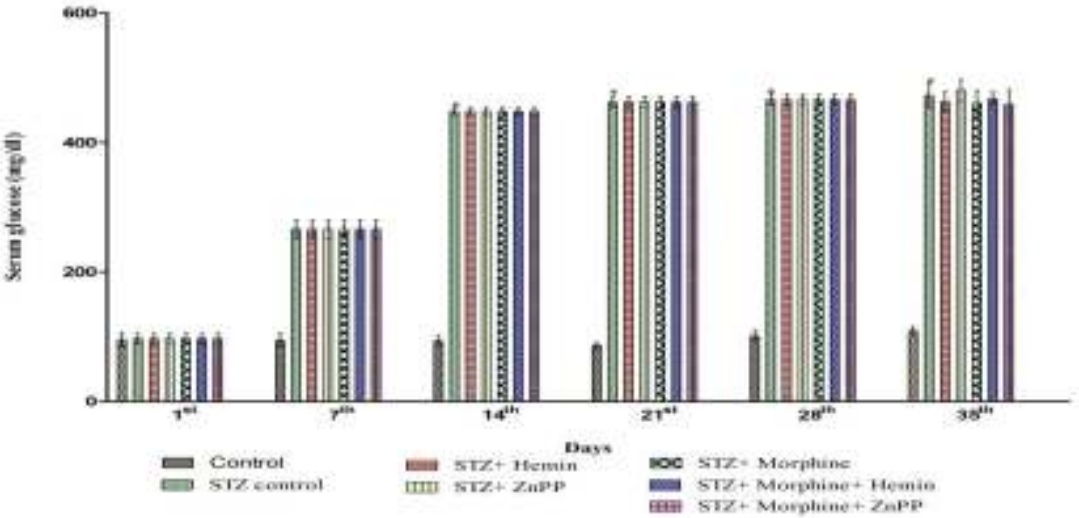

Figure 3: Effect of pharmacological interventions on blood glucose levels.
Values are expressed as Mean ± S.D.* p<0.05 vs. normal group

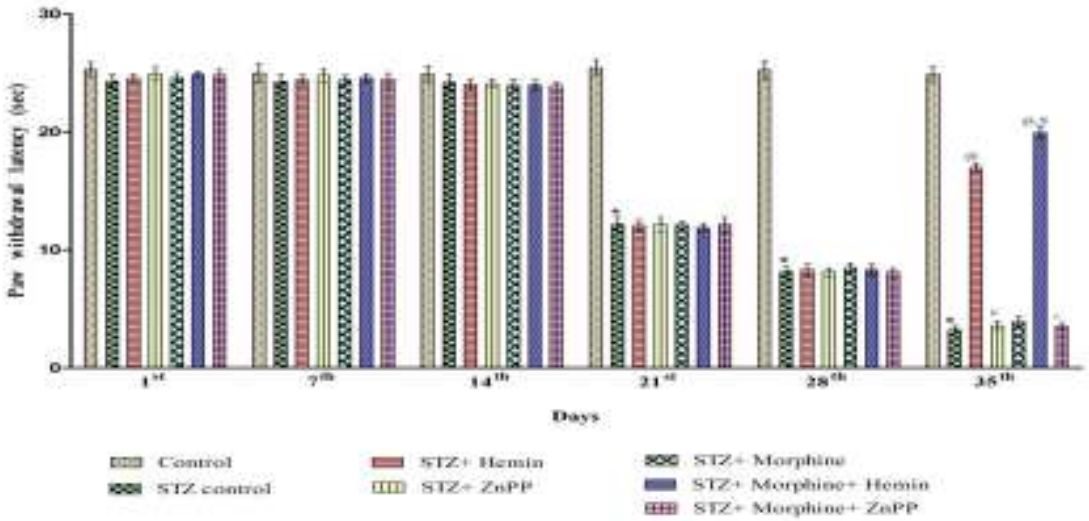

Figure 4: Effect of pharmacological interventions on hot plate test.
Values are expressed as Mean ± S.D.* p<0.05 vs. normal group, @ p<0.05 vs. STZ group, #p<0.05 vs. STZ+ Hemin group, $p<0.05 vs. STZ + Morphine (5 mg/kg), ^ p<0.05 vs. STZ+ Morphine+ Hemin

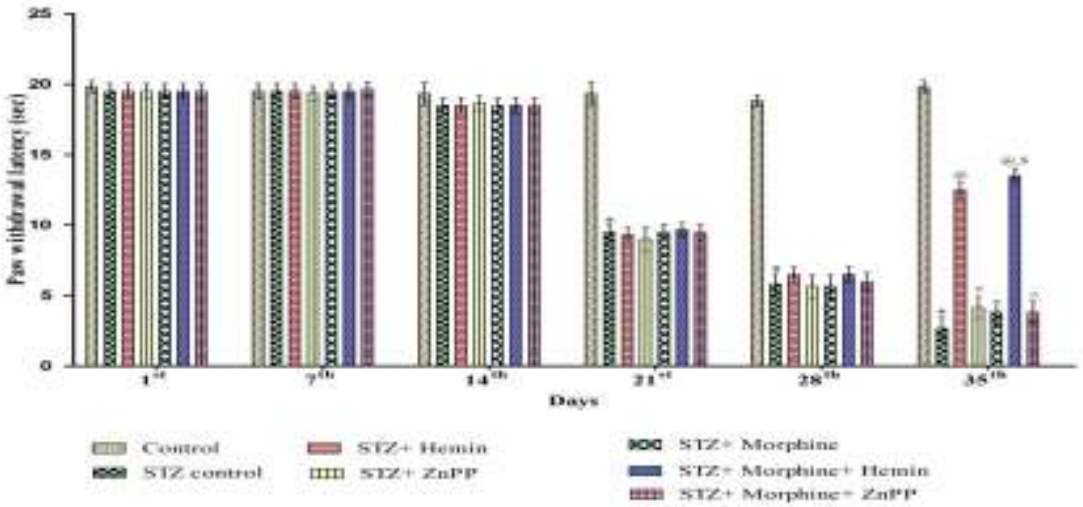

Figure 5: Effect of pharmacological interventions on thermal hyperalgesia
Values are expressed as Mean ± S.D.* p<0.05 vs. normal group, @ p<0.05 vs. STZ group, #p<0.05 vs. STZ+ Hemin group, $p<0.05 vs. STZ + Morphine (5 mg/kg), ^p<0.05 vs. STZ+ Morphine+ Hemin

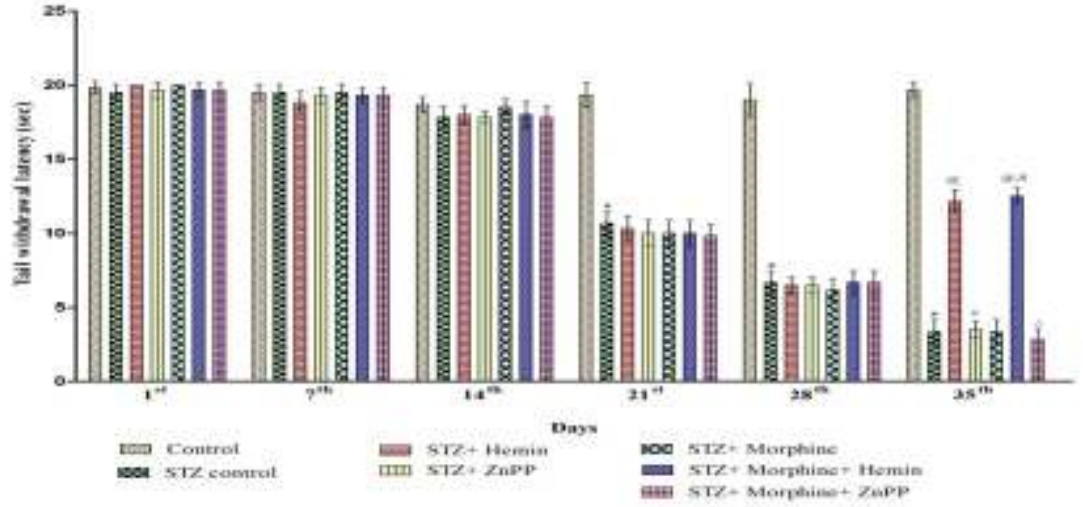

Figure 6: Effect of pharmacological interventions on thermal allodynia
Values are expressed as Mean ± S.D.* p<0.05 vs. normal group, @ p<0.05 vs. STZ group, #p<0.05 vs. STZ+ Hemin group, $p<0.05 vs. STZ + Morphine (5 mg/kg), ^p<0.05 vs. STZ+ Morphine+ Hemin.

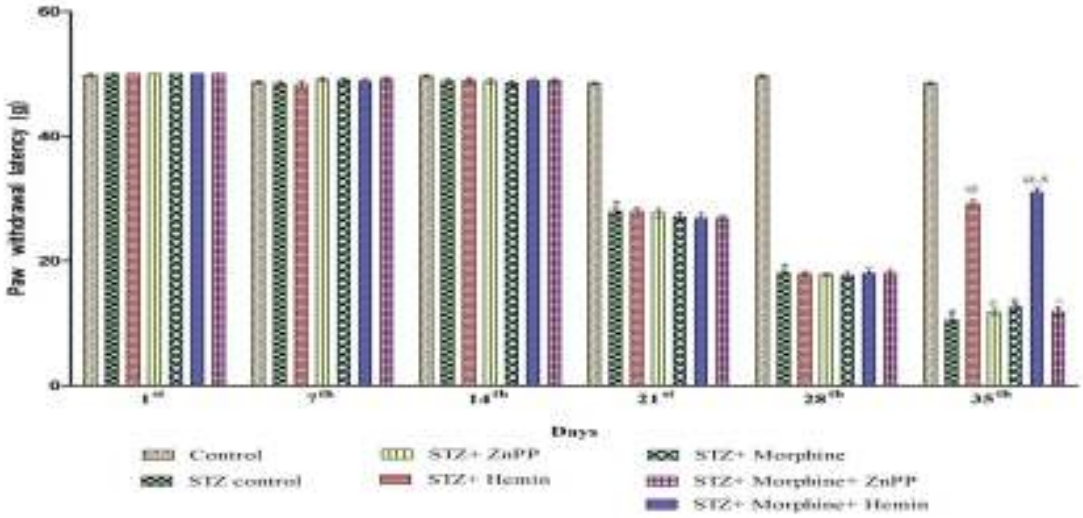

Figure 7: Effect of pharmacological interventions on mechanical allodynia
Values are expressed as Mean ± S.D.* p<0.05 vs. normal group, @ p<0.05 vs. STZ group, #p<0.05 vs. STZ+ Hemin group, $p<0.05 vs. STZ + Morphine (5 mg/kg), ^p<0.05 vs. STZ+ Morphine+ Hemin.

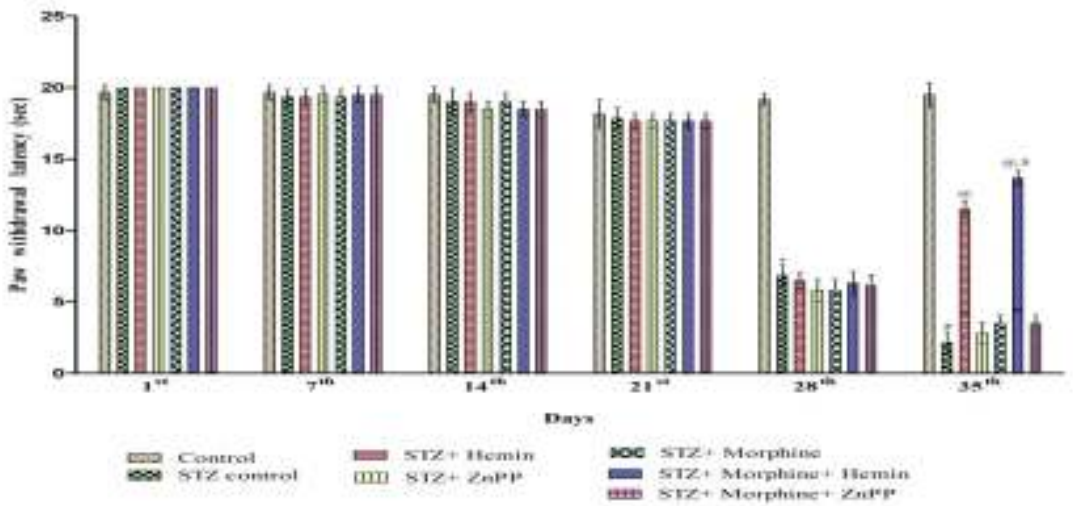

Figure 8: Effect of pharmacological interventions on cold allodynia
Values are expressed as Mean ± S.D.* p<0.05 vs. normal group, @ p<0.05 vs. STZ group, #p<0.05 vs. STZ+ Hemin group, $p<0.05 vs. STZ + Morphine (5 mg/kg), ^p<0.05 vs. STZ+ Morphine+ Hemin.

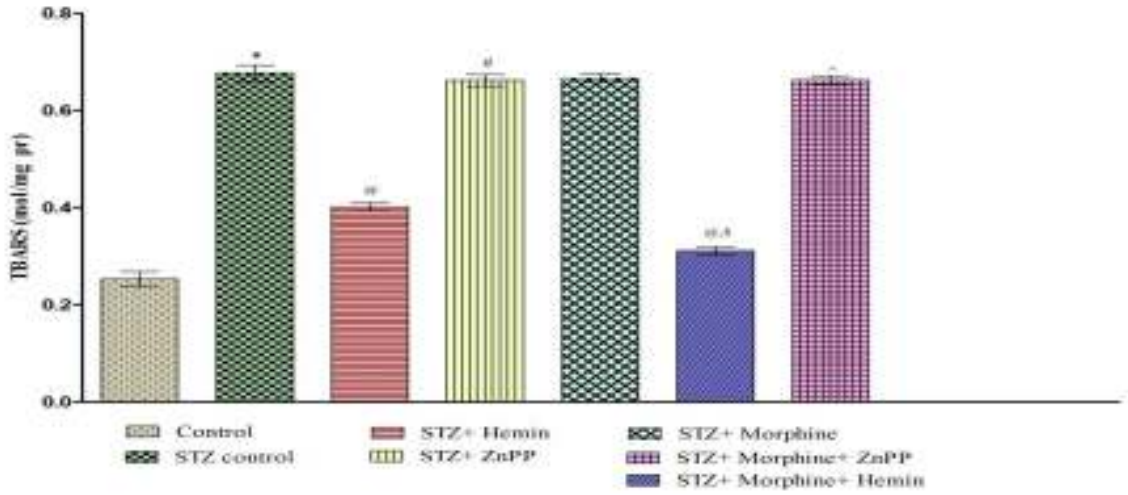

Figure 9: Effect of pharmacological interventions on TBARS levels
Values are expressed as Mean ± S.D.* p<0.05 vs. normal group, @ p<0.05 vs. STZ group, #p<0.05 vs. STZ+ Hemin group, $p<0.05 vs. STZ + Morphine (5 mg/kg), ^p<0.05 vs. STZ+ Morphine+ Hemin.

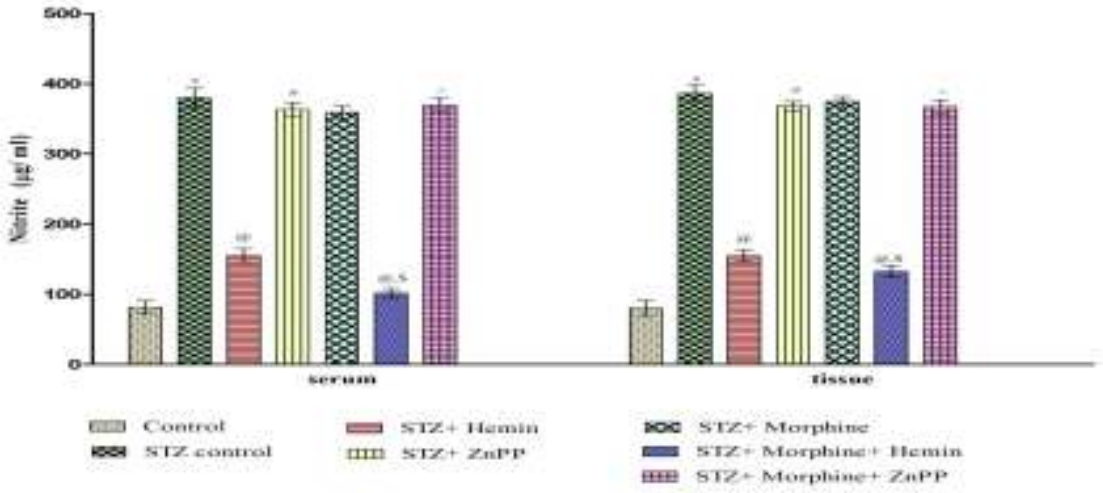

Figure 10: Effect of pharmacological interventions on serum and tissue nitrite levels.
Values are expressed as Mean ± S.D.* p<0.05 vs. normal group, @ p<0.05 vs. STZ group, #p<0.05 vs. STZ+ Hemin group, $p<0.05 vs. STZ + Morphine (5 mg/kg), ^p<0.05 vs. STZ+ Morphine+ Hemin.

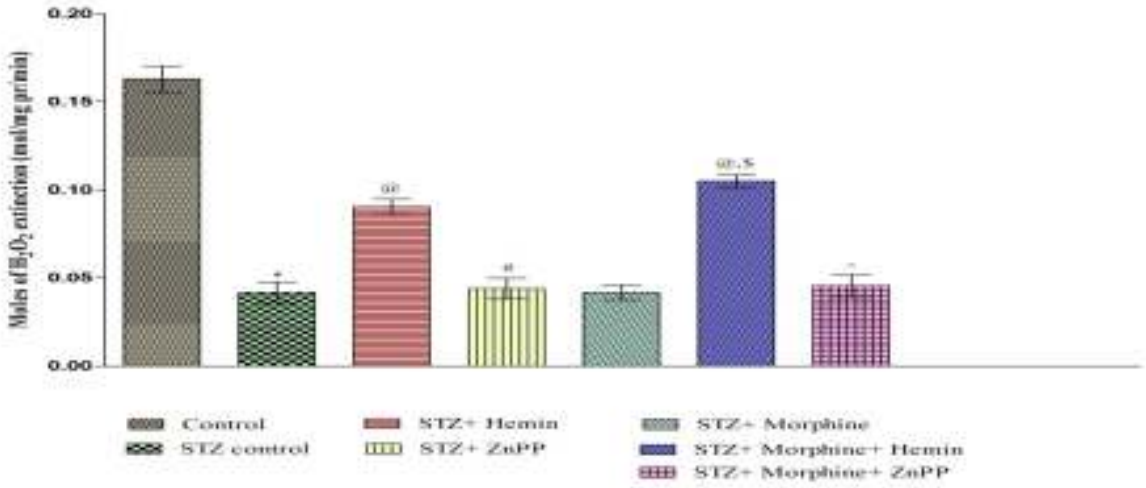

Figure 11: Effect of pharmacological interventions on catalase activity
Values are expressed as Mean ± S.D.* p<0.05 vs. normal group, @ p<0.05 vs. STZ group, #p<0.05 vs. STZ+ Hemin group, $p<0.05 vs. STZ + Morphine (5 mg/kg), ^p<0.05 vs. STZ+ Morphine+ Hemin.

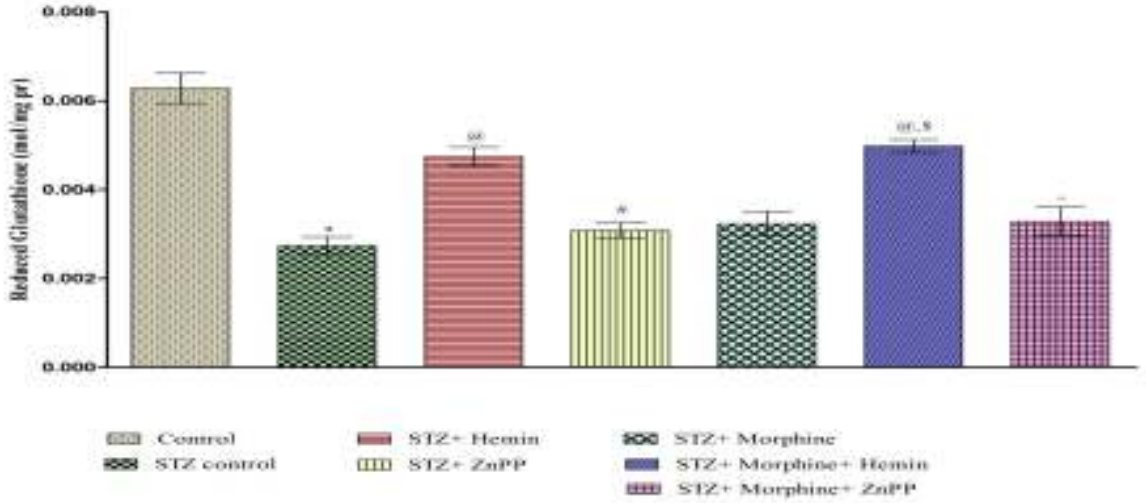

Figure 12: Effect of pharmacological interventions on reduced glutathione levels
Effect of pharmacological interventions on Values are expressed as Mean±S.D.* p<0.05 vs. normal group, @ p<0.05 vs. STZ group, #p<0.05 vs. STZ+ Hemin group, $p<0.05 vs. STZ + Morphine (5 mg/kg), ^p<0.05 vs. STZ+ Morphine+ Hemin.

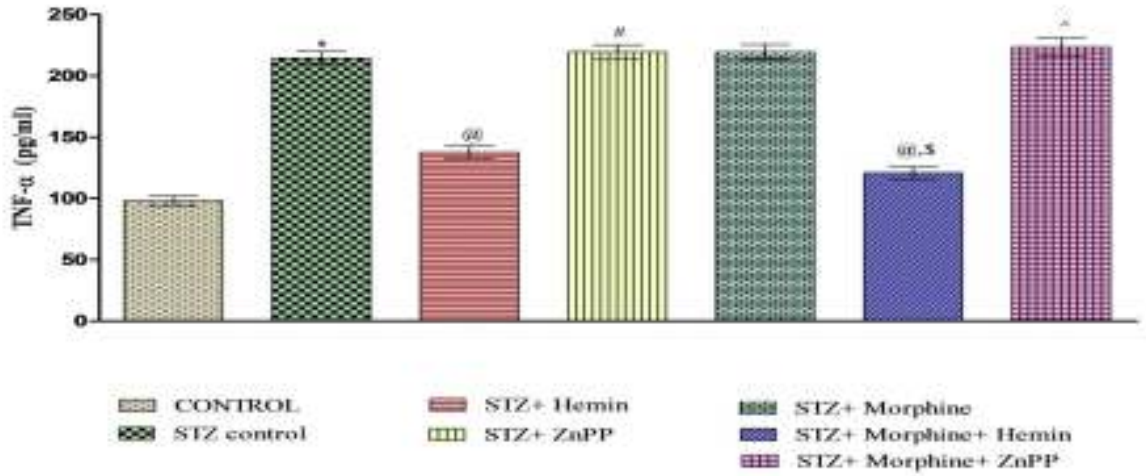

Figure 13: Effect of pharmacological interventions on TNF-α level
Values are expressed as Mean ± S.D.* p<0.05 vs. normal group, @ p<0.05 vs. STZ group, #p<0.05 vs. STZ+ Hemin group, $p<0.05 vs. STZ + Morphine (5 mg/kg), ^p<0.05 vs. STZ+ Morphine+ Hemin.

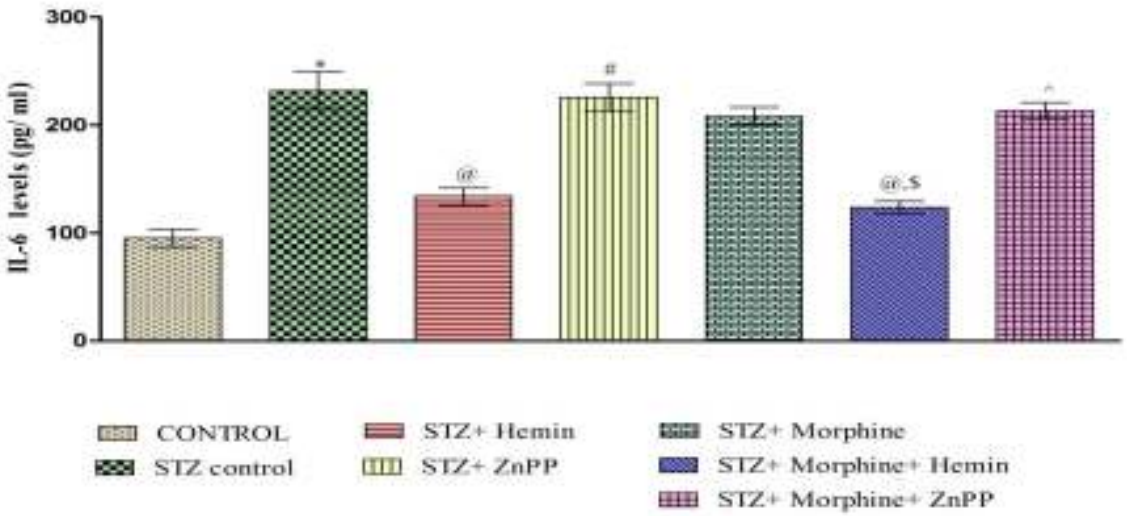

Figure 14: Effect of pharmacological interventions on IL-6 levels
Values are expressed as Mean ± S.D.* p<0.05 vs. normal group, [@] p<0.05 vs. STZ group, [#]p<0.05 vs. STZ+ Hemin group, [$]p<0.05 vs. STZ + Morphine (5 mg/kg), [^]p<0.05 vs. STZ+ Morphine+ Hemin.

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