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***In vitro* alternative test system development for ocular Irritation**

(Project No. 5/20/4(Bio)/10-NCD-1)

(16th August 2011 to 27th March 2014)

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Submitted to:

Indian Council of Medical Research

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Objectives

The objective of the present project is, to develop an *in vitro* test system for acute and sub acute ocular irritation and will be suitable for evaluating the biomaterials, medical devices, pharmaceuticals and chemicals. The main objective of the project include,

1. Development of primary corneal cells from rabbit and human tissues
2. *In vitro* cell viability assay of rabbit and human corneal cells using MTT assay
3. Development of rabbit and human corneal tissue constructs (3D), followed by acute and sub acute treatment with 10 chemicals and evaluation of ;
    - Viability of cells using MTT assay
    - Inflammatory markers such as IL 1α, IL 1β, IL 8, TNF
    - Corneal opacity measurement
    - Histopathological analysis (sub acute)
4. *Iv vivo* acute and sub acute ocular irritation tests with 5 chemicals
5. Evaluation of inflammatory markers such as IL 1α, IL 1β, IL 8, TNF and comparison of the scoring with *in vitro* system
6. Validation of assay

Methodology

I. Acute Studies (single exposure)

1. *In vitro* assay using corneal cells

2. *In vitro* studies using tissue constructs

Using this tissue construct 10 known chemicals (already classified as irritant in in vivo system) will be evaluated with appropriate positive and negative controls. Here the tissue construct will be treated (single exposure) with different concentrations of the irritant chemicals and incubated at 37ºC. The inflammation is the end mark of all the tissue reactions and there by following inflammatory markers will be evaluated using ELISA method

1. Interleukin 1 alpha (IL 1α)
2. Interleukin 1 beta (IL 1β)
3. Interleukin 8 (IL 8)
4. Tumor necrosis factor (TNF)
5. Oedema measurement (if possible)
6. Corneal opacity (spectrophotometer)

3. *In vivo* Method: Acute ocular irritation studies

The acute ocular irritation tests will be carried out as per ISO 10993/OECD methods using 5 chemicals in rabbit model. The scoring will be carried out as per international standard. After the last scoring the corneal tissue will be collected and subjected to analyze the inflammatory

markers such as IL 1α, IL 1β, IL 8 and TNF. Subsequently the *in vivo* acute score will be compared with the inflammatory markers. Appropriate negative and positive controls will be carried out simultaneously.

II. Sub acute studies

1. *In vitro* sub acute ocular irritation studies using tissue construct

Using above tissue construct 10 known chemicals (already classified as irritant in in vivo system) will be evaluated with appropriate positive and negative controls. Here the tissue construct will be treated for 7 days with different concentrations of the irritant chemicals and incubated at 37°C. The following inflammatory markers will be evaluated using ELISA method

1. Interleukin 1 alpha (IL 1α)
2. Interleukin 1 beta (IL 1β)
3. Interleukin 8 (IL 8)
4. Tumor necrosis factor (TNF)
5. Oedema measurement (if possible)
6. Corneal opacity (spectrophotometer)
7. The same tissue construct (7 days treatment) will be subjected to histopathological analysis

2. *In vivo* Method: Sub acute ocular irritation studies (repeated exposures)*

The corneal tissue of same rabbits (7 days treatment) will be subjected to histopathological analysis. Later, the *in vivo* sub acute score, inflammatory response, histopathological scores will be compared with the *in vitro* scores, inflammatory response and hispathological results

III. Validation

On successful completion of the tissue construct development, the following studies as given in the table will be carried out for final validation.

In vitro method: 10 chemicals each for acute and sub acute studies

In vivo methods: 05 chemicals each for acute and sub acute studies

Results

- **Development of Primary corneal cells from rabbit tissues**
  Almost all explants showed cellular outgrowth (approximately 95%) with epithelial morphology (Figure 1).
- ***In vitro* cell viability assay of rabbit corneal cells using MTT**
  The cells obtained from explants showed optical density of 0.5 ± 0.1(n=4) indicating good viability (Figure 2). Figure 2 shows formazan crystals indicating viability of rabbit corneal cells.
  The data showed that corneal cells isolated from primary explants culture were viable, varying from 40-90 % of the maximum value (Figure 3). The variation may be due to the non uniformity of cell outgrowth from different explants.
- **Development of Rabbit Corneal tissue construct**
  The goat corneal explant which was taken from the limbal area was used to develop a 3D tissue construct. On the 10^{th} day, the multilayerd tissue construct was detached from the NGMA surface by lowering the temperature below 20◦C (Figure 4 and 5). Rabbit corneal construct was also prepared as described above (Figure 6)
- ***In vitro* acute ocular irritation test based on Viability of cells in corneal construct using MTT assay**
  Results showed variations in cellular activity not respective to the concentration. In Figure 7, MTT assay of primary corneal construct treated with SS. MTT assay did not show the expected trend with respect to various doses of chemicals. This may be due to variation in the number of cells in the construct. To confirm this, the cytotoxicity of SS was studied using uniform number of L-929 fibroblast cells. Similar doses of SS used for explants culture system

was also used for L-929 cells. In addition to SS, serial dilutions of CPC ranging from 15.6 ug/ml to 0.015 ug/ml were also prepared. Briefly, 2 x 104 cells were seeded in 96 well plates and allowed to form monolayer in MEM containing serum. After 24h treatment, MTT assay was performed. The result showed the dose responses of SS and CPC to L-929 cells (Figure 8). The results of MTT assay on SS treated L-929 cells confirmed that difference in the trend of dose response on primary corneal cells could be due to the non-uniformity of cell number obtained from explants culture.

- **Neutral Red uptake**

  The staining showed variations in neutral red uptake by primary corneal cells corresponding to the concentrations (Figure 9, 10 and 11). From Image Pro plus software, the results showed variable uptake of neutral red relative to the concentration of chemicals used (Figure 12).

- **Alamar Blue assay using SIRC (Rabbit corneal Cell line)**

  Graph was plotted to observe the dose response of SIRC cells towards lower dilutions of SS (Figure 13). From the graph it was concluded that as the concentration of SS increases, the percentage reduction of alamar blue decreases and thereby reducing the viability of cells.

- **MTT and Alamar Blue assay (Rabbit corneal tissue construct)**

  Both MTT assay and alamar blue assay was performed on each plate (Figure 14 - 17).
  Results showed that the selected concentrations did not elicit cytotoxic response when analyzed by alamar blue assay. In all three cases, the cells maintained similar metabolic activity (Figure 18, 19 and 20).

- **Acute studies**
  - ***In vitro* acute ocular irritation studies**
    - ***In vitro* cytokine analysis:**

      It is clear from Figure 21(a) that IL-1α production in CPC treated rabbit corneal cells were highest in the higher doses (5 and 2.5mg/ml) when compared to the control. In the lower doses (1.25, 0.625 and 0.3125mg/ml) IL-1α production was comparable to that of control. However, statistically significant increase in IL-1α was seen at 5mg/ml dose only. Similarly, IL-1β production in CPC treated cells increased at high doses (5 and 2.5mg/ml) while the lower doses (1.25, 0.625 and 0.3125mg/ml) were similar to control (Figure 21(b)). In Figure 21(c), it was seen that the IL-8 production was comparable to that of control in the lower doses (2.5, 1.25, 0.625 and 0.3125 mg/ml). However there was an increase in IL-8 production at the highest dose (5mg/ml) when compared to control, which was statistically significant. The production of TNF-α in rabbit corneal cells treated with CPC is mentioned in Figure 21(d). This shows that TNF-α production was similar to that of control at the lower doses (2.5, 1.25, 0.625 and 0.3125 mg/ml) whereas at 5mg/ml dose (high dose), it showed increase TNF-α production, that was statistically significant.

      The result of the study shown in Figure 22a suggests that IL-1α production in SS treated rabbit corneal cells (media) increased slightly, when compared to that of the control, with increase in the concentration of SS. Figure 22b shows that IL-1β production also shows an increase in production in a dose dependent manner when compared to that of the control. IL-8 production in rabbit corneal cells treated with SS is shown in Figure 22c. This suggests that there is a slight increase in production of IL-8, when compared to that of control, at higher concentrations (10 and 5 mg/ml). Whereas IL-8 production at the lower concentrations (2.5, .125 and 0.625mg/ml) was almost similar to control. TNF-α production also show an increase in production in a dose dependent manner in comparison to that of control (Figure 22(d)).

      Interestingly, IMI, a known eye irritant, does not induce cytokine (IL-1α, IL-1β, IL-8 and TNF-α) production at any doses, rather it was seen to inhibit cytokine production when compared to that of control (Figure 23a, b, c, d). The decreased production of TNF-α, at higher concentrations (5 and 10 mg/ml) was found to be statistically significant when compared to control.

Illustrated in Figures 24 a, b, c, d is cytokine production of ACT treated corneal construct. There was an increase in IL-1α production in rabbit corneal cells treated with ACT in a dose dependent manner when compared to control. However, this increase was statistically insignificant. IL-1β production in rabbit cornea treated with ACT also showed an increase in level with increase in concentration (when compared to control) but it remains statistically insignificant, even at the highest dose (10mg/ml). It was found that IL-8 production showed an elevation in production, when compared to control. At concentrations 2.5, 5 and 10 mg/ml, there was a statistically significant increase in IL-8 levels with respect to control. ACT treated rabbit corneal cells also showed an enhanced production of TNF-α, which was statistically significant at 5 and 10 mg/ml doses, in comparison to control.

NIC treated rabbit corneal cells showed a slight increase in the level of IL-1α at higher concentration (2.5, 5 and 10 mg/ml), whereas remained comparable with control at lower concentrations (0.625 and 1.23 mg/ml). At 5 and 10mg/ml, the level of IL-1α was statistically significant. Stimulation of IL-1β in NIC exposed rabbit corneal cells showed a dose dependent increase in the cytokine level with regards to control. Only at the highest dose, i.e. 10mg/ml, was it found to be statistically significant. IL-8 production was comparable to control at lower doses (0.625 and 1.25 mg/ml) but at higher doses (2.5, 5 and 10 mg/ml), there was an increase in the levels, when compared to control. Nonetheless, it remained insignificant statistically. The level of TNF-α in supernatant of rabbit cornea exposed to NIC showed a slight increase at the higher doses (2.5, 5 and 10 mg/ml) when compared to control. However, upon statistical analysis, it was found to be insignificant. These are depicted in Figure 25(a-d).

- **Corneal opacity measurement**

  Cells showed toxicity relative to the concentration of CPC (Figure 26) and IMI (Figure 27). The construct in normal culture medium was considered as control (Figure 28). Control is shown in Figure 29.

- **Histopathology of corneal construct**

  Normal goat corneal epithelium is shown in Figure 30. Sections were so much mutilated that satisfactory assessment was not feasible (Figure 31). This may be due to the thin nature and difficulty in embedding. Better substrates which are transparent and will allow histological processing are being looked into. Using rabbit tissue construct five chemicals were used for acute toxicity and three for sub acute toxicity out of the ten chemicals selected for *in vitro* studies based on *in vivo* data. Other chemicals were not suitable for cell culture assays due to insolubility, precipitation, severity of reactions etc.

- ***In vivo* acute ocular irritation studies**

- **Scoring**

  The right eye (treated) and the left eye (control) was given a score from 0-4 (Table 1). All the treated samples showed typical eye irritation. There was redness, discharge from eye and chemosis in the conjunctiva. Cornea did not show opacity, however there was visible swelling.

- **Cytokine analysis**

  IL-1α, IL-1β, IL-8 and TNF-α analysis in the supernatant of the corneal homogenate of rabbits treated with CPC, SS, IMI, ACT and NIC for a period of 24h is shown in Figure 32-35. IL-1α production is illustrated in Figure 32. It shows a statistically significant increase in CPC, SS, ACT and NIC (CPC: 0.157±0.007, SS: 0.173±0.014, ACT: 0.162±0.012 and NIC: 0.156±0.012 pg/ml) treated animals in comparison with control (0.130± 0.006 pg/ml). However, in IMI treated animals, the IL-1α production remained comparable with that of control (IMI: 0.133±0.009 pg/ml).

  The cornea of ocular irritant chemicals treated rabbits, showed increase in production of IL-1β (CPC: 25.9±0.002, SS: 31.78±0.014, IMI: 23.9±0.002, ACT: 35.73±0.005 and NIC: 32.56±0.007 pg/ml) when compared to control (20.06±0.003 pg/ml) which is statistically significant (Figure 33).

  From Figure 34, increased production of IL-8 is evident in cornea of rabbits treated with CPC (1.59±0.02 pg/ml), SS (1.56±0.03 pg/ml), ACT (1.68±0.01 pg/ml) and NIC (1.18±0.003

pg/ml) but not statistically significant in IMI (1.01±0.003 pg/ml) treated group when compared with control (1.01±0.009 pg/ml).

Production of TNF-α in cornea of rabbits were significantly elevated in all groups except IMI treated animals, when compared to control (CPC: 1.083±0.006, SS: 1.397±0.011, IMI: 1.016±0.009, ACT: 1.193±0.009, NIC: 1.206±0.014 and control (0.891±0.009 pg/ml). This is demonstrated in Figure 35.

- **Histopathology**

Histopathological analysis of eyes of CPC treated animals showed that the morphology or cornea remained normal (Figure 36 B). However, there was mild acute inflammation in the ciliary body and blood vessel congestion in the choroid of CPC treated animals.

Animals treated with SS, showed corneal morphology similar to control, as evident from Figure 36 C, whereas, chronic inflammation was visible in the ciliary body. Choroid and retina showed mild inflammation as well.

From Figure 36 D, it is observed that IMI treated animals, when compared to control, had normal corneal morphology. There were no evident signs of inflammation, except slightly congested blood vessels in the ciliary body.

In comparison to control, cornea was normal in ACT treated animals (Figure 36 E). Sign of mild inflammation was detected in the ciliary body due to macrophage infiltration. It was observed that the blood vessels in the choroids region were also congested.

In cornea of ACT treated animals, it was seen that there was corneal thickening (oedema) when compared to control, as seen in Figure 36 F. Mild inflammation of the ciliary body was also noticed in ACT treated animals.

- **Hematological and Biochemical parameters**

Blood from animals treated with ocular irritant chemicals were collected and subjected to hematological analysis (HB, WBC, RBC, PLT, MCV, MCH and MCHC). Table 2 indicates that all the groups of treated animals showed slight alterations. However, they were comparable with results of control and were statistically insignificant.

Biochemical parameters such as GPT, GOT, ALP, GGT, uric acid, bilirubin, calcium, phosphorous, chlorides, creatinine were analyzed in the blood of the treated animals. Even though there were slight variations, it was found to be statistically insignificant when compared to control (Table 3).

- **Spleenocyte proliferation assay**

Figure 37 depicts the proliferation of spleenocyte in the rabbits treated with CPC, SS, IMI, ACT and NIC. There is a slight decrease in the proliferation of spleenocyte (CPC: 830±73.53, SS: 725.33±135.79, IMI: 762.25±116.28, ACT: 763.33±121.41 and NIC: 742.5±195.22 CPM), when compared with control (933.67±96.01 CPM). Nevertheless, it is statistically insignificant.

- **Antioxidant enzyme assay**

Lipid peroxidation (LPO)

The liver and brain homogenates of treated animals were assessed for the presence of malonedialdehyde (MDA). From Figure 38, it is seen that there is a slight increase in LPO in the liver of the treated rabbits (CPC: 16.78±2.48, SS: 15.10±1.27, IMI-15.56±2.01, ACT: 13.46±0.37 and NIC: 13.65±0.91 nmol/mg protein) when compared with control (12.98±0.85 nmol/mg protein). Yet, it is not significant.

LPO in the brain of treated rabbit (Figure 39) shows similar pattern as that of liver (Control: 8.89±0.84, CPC: 8.08±1.61, SS: 14.01±0.07, IMI: 11.27±1.03, ACT: 11.85±1.82 and NIC: 10.63±1.39 nmol/mg protein). However, it is also statistically insignificant.

Reduced Glutathione (GSH)

The concentration of GSH in the liver of rabbits exposed to ocular irritant chemicals is shown in Figure 40. There is a slight decrease in the concentration in IMI, ACT and NIC (IMI: 1.40±0.19, ACT: 0.98±0.29, and NIC: 1.22±0.43 nmol/mg protein) treated groups, when compared to control (1.61±0.15 nmol/mg protein), but it was found to be insignificant. GSH

levels in CPC (1.53±0.44 nmol/mg protein) and SS (1.57±0.66nmol/mg protein) treated groups were almost similar to that of control.

From Figure 41 it can be seen that brain of the rabbits showed decreased concentration of GSH (CPC-: 0.44±0.05, SS: 0.47±0.13, IMI: 0.57±0.11, ACT: 0.58±0.18 and NIC: 0.69±0.22 nmol/mg protein), when compared to the control (0.74±0.14 nmol/mg protein). Still, upon statistical analysis it was found to be insignificant.

Glutathione Reductase (GR)

Liver homogenate of rabbits which were exposed to chemical ocular irritants showed slight changes (CPC: 0.40±0.07, SS: 0.37±0.4, IMI: 0.33±0.15 ACT: 0.27±0.004 and NIC: 0.32±0.09 units/mg protein) when compared to control (0.26±0.11units/mg protein), but it is not significant. This is depicted in Figure 42.

GR activity in brain of treated animals (CPC: 0.46±0.07, SS: 0.49±0.03, IMI: 0.52±0.08, ACT: 0.46±0.04 and NIC: 0.41±0.01 units/mg protein) was almost similar to that of control (0.39±0.05 units.mg protein) and did not show any statistical significance (Figure 43).

Glutathione Peroxidase (GPx)

Activity of GPx showed slight increase, when compared to control, in the liver of rabbits exposed to the chemicals (Control: 0.12±0.02, CPC: 0.18±0.02, SS: 0.16±0.04, IMI: 0.15±0.005, ACT: 0.11±0.01 and NIC: 0.12±0.05 units/ mg protein). This is shown in Figure 44. Statistical analysis, when compared with control, indicates that it is not significant.

GPx activity in the brain of treated rabbits showed a similar trend as liver as seen in Figure 45. There was a slight variation in the when compared to control but it is statistically insignificant (Control: 0.03±0.01, CPC: 0.04±0.004, SS: 0.04±0.007, IMI: 0.04±0.01, ACT: 0.05±0.002 and NIC: 0.03±0.01 units/mg protein).

Super Oxide Dismutase (SOD)

SOD activity in liver of exposed animals is illustrated in Figure 46. The activity of the enzyme in the treated animal's liver, (CPC: 0.13±0.02, SS: 0.14±0.02, IMI: 0.11±0.02, ACT: 0.09±0.004 and NIC: 0.10±0.01 units/mg protein) is almost comparable with that control (0.10±0.008 units/mg protein) and not significant.

In brain as well, the slight alteration in treated animals in comparison with control is statistically insignificant as seen in Figure 47. The values are – Control: 0.12±0.01, CPC: 0.16±0.001, SS: 0.16±0.006, IMI: 0.13±0.001, ACT: 0.14±0.002 and NIC: 0.12±0.01 units/mg protein.

- **Sub Acute studies**
  - ***In vitro* sub-acute test on corneal construct by MTT assay**

    The results showed that all the chemicals expressed a reduction in cellular activity (Figure 48).
  - ***In vitro* sub acute ocular irritation studies**
  - ***In vitro* cytokine analysis**

    In vitro cytokine analysis of CPC, SS and IMI treated rabbit corneal construct were carried out using the supernatant.

    Rabbit corneal cells exposed was exposed to CPC at a dose of 0.0012 and 0.0015 mg/ml/day, over a period of 7 days, showed a statistically insignificant increase in IL-1α production, when compared to control (Figure 49 a). IL-1β also showed a slight increase in levels when compared to control. However, this remained insignificant (Figure 49 b). From Figure 49 c, it is clear that IL-8 production showed slight alterations when compared to control, which was not statistically significant. Figure 49 d illustrates the TNF-α level when treated with CPC. It was seen that TNF-α showed a statistically insignificant increase in concentration, when compared to control.

    SS (0.3125 and 0.781mg/ml/day) was exposed to rabbit corneal cells for 7 consecutive days and cytokine levels were analyzed. IL-1α production was comparable to that of control at 0.3125 mg/ml and slightly increased at 0.781 mg/ml. However, it was statistically insignificant (Figure 50 a). Figure 50 b shows IL-1β production in rabbit corneal cells. It can be seen that there is an increased production of the cytokine, with respect to control, though it is

insignificant. IL-8 shows an elevated production in SS treated group, but it is insignificant when compared to control as shown in Figure 50 c. TNF-α showed increased levels in a dose dependent manner, which was insignificant (Figure 50 d).
A dose of 0.3125 and 1.5625 mg/ml/day of IMI were exposed to rabbit corneal cells. IL-1α production is depicted in Figure 51a. There is a statistically insignificant alteration in the cytokine production. In Figure 51b, IL-1β shows a decrease in level, when compared to control. This was found to be statistically insignificant. IL-8 concentration in the supernatant of treated cells showed a minute increase in its level, which was statistically insignificant (Figure 51c). Figure 51d illustrates TNF-α production in rabbit cornea treated with IMI. There is a little increase in its level, though it is insignificant.

- ***In vivo* sub acute ocular irritation studies**
- **Scoring**

The treated right eye and the control left eye were scored based on severity from 0-4, where 4 was assigned most severe. It can be seen that prolonged treatment caused swelling in the cornea. Redness, chemosis and discharge from the conjunctiva. This is shown in Table 4.

- **Cytokine analysis**

The production of IL-1α, IL-1β, IL-8 and TNF-α, in rabbit cornea, after treatment for 7 days with CPC, SS, IMI, ACT and NIC are shown in Figure 52-55.

In Figure 52, it can be clearly seen that CPC (0.166±0.01 pg/ml), SS (0.156±0.01 pg/ml), ACT (0.163±0.007 pg/ml) and NIC (0.149±0.01 pg/ml) induced a significant increase in the production of IL-1α in comparison with control (0.130±0.01 pg/ml). IMI (0.143±0.01 pg/ml) also showed a minor increase in IL-1α when compared to control, but it was statistically insignificant.

It was seen that IL-1β production was significantly stimulated in the cornea of rabbits treated with ocular irritant chemicals, when evaluated with control (Figure 53). CPC (34.77±0.02 pg/ml) showed the most increased production with respect to control (15.02±0.002 pg/ml). IMI (20.73±0.007 pg/ml) treated corneas showed slight alteration with that of control but was found to be statistically insignificant.

There was a marked increase in IL-8, a chemotactic factor, in the cornea of rabbits treated with the different ocular irritant chemicals, with respect to control. This was found to be statistically significant and is illustrated in Figure 54.

TNF- α, showed a noticeable increase in production, as evident in Figure 55, in the rabbit cornea that were treated with CPC (1.88±0.02 pg/ml), SS (1.96±0.01 pg/ml), NIC (1.70±0.01 pg/ml) and ACT (1.80±0.02 pg/ml) with regards to control (1.31±0.01 pg/ml). However, IMI (1.57±0.01 pg/ml) treated animals showed statistically insignificant variation in comparison to control.

- **Histopathology**

Histopathological analysis of eyes of rabbits treated with CPC showed an oedematous cornea and macrophage and neutrophil infiltration (Figure 56 B). There was also acute inflammation and congestion of blood vessels in the ciliary body of the eye, with respect to control. From Figure 56 C, it was seen that the cornea had increased in size in comparison to control, due to oedema, in SS treated animals. Mild acute inflammation of the ciliary body was also noticed.
The eyes of rabbits treated with IMI did not show any significant signs of inflammation in the cornea (Figure 56 D. But ciliary body showed congested blood vessels, which can be an indication of inflammation.

Rabbits treated with ACT showed an odematous cornea when compared with control, as evident from Figure 56 E. However, mild acute inflammation was noticed in the ciliary body of the exposed animals.

NIC treated rabbits showed oedema in the cornea, when compared to control, as seen in Figure 56 F. There was acute inflammation in the ciliary body, which was clear from the infiltration of macrophages and neutrophils.

- **Hematological and Biochemical parameter**

  Blood collected from the rabbits after sub acute exposure to ocular irritant chemicals was subjected to hematological and biochemical parameters. From the hematological parameters (HB, WBC, RBC, PLT, MCV, MCH and MCHC) shown in Table 5, it can be seen that there are slight variations in the values when compared with control. But statistically, they are insignificant.

  Biochemical parameters (GPT, GOT, ALP, GGT, uric acid, bilirubin, calcium, phosphorous, chlorides and creatinine) are shown in Table 6. The alterations seen in the treated group, when compared to control are statistically not significant.

- **Spleenocyte proliferation assay**

  It is seen from Figure 57, that the spleenocyte proliferation has some variation in the animals that were treated with the ocular irritant chemicals, when compared to control. However, since it is statistically in significant, it is safe to assume that the ocular irritant chemicals did not affect the spleenocyte proliferation capacity.

- **Antioxidant enzyme assay**

  Lipid Peroxidation (LPO)

  The liver of rabbits treated for 7 days with ocular irritant chemicals did not show any significant increase in LPO in comparison to control, as seen in Figure 58. MDA concentration in control was 2.98±0.84, nmol/mg protein, whereas values of CPC, SS, IMI, ACT and NIC were as follows – 3.77 ±0.38, 3.96 ±0.51, 4.03 ±0.12, 4.55 ±0.14 and 4.01 ±0.65 nmol/mg protein respectively.

  Rabbit exposed to ocular irritant chemicals in the eyes showed slight increase in LPO(CPC: 10.22±2.02, SS: 15.53±5.10, IMI: 8.98±2.22, ACT: 10.10±0.71 and NIC: 12.61±2.74 nmol/mg protein), which was insignificant, when compared to control(8.89±0.45 nmol/ mg protein). This is depicted in Figure 59.

  Reduced Glutathione (GSH)

  GSH concentration in the liver of rabbits treated with CPC (1.27±0.37 nmol/mg protein), SS (1.13±0.04 nmo/mg protein), IMI (1.44±0.21 nmol/mg protein), ACT (1.23±0.10 nmol/mg protein) and NIC (1.33±0.20 nmol/mg protein) were statistically insignificant, when compared with control (1.61±0.15 nmol/mg protein). This is shown in Figure 60.

  Concentration of GSH in the brain homogenate of treated rabbits is shown in Figure 61. Though there is a slight decrease in GSH levels with respect to control (Control: 0.61±0.23, CPC: 0.47±0.22, SS: 0.41±0.03, IMI: 0.03±0.10, ACT: 0.35±0.12 and NIC: 0.33±0.08 nmol/mg protein) it still remains statistically insignificant.

  Glutathione Reductase (GR)

  The activity of GR in the liver (Figure 62) and brain (Figure 63) of animals exposed to ocular irritant chemicals showed slight variations with regards to control. However, both in liver and brain, it was statistically insignificant. Control (liver: 0.26±0.01; brain: 0.42±0.03), CPC

(liver: 0.21±0.01; brain: 0.50±0.01 units/mg protein), SS (liver: 0.17±0.03; brain: 0.52±0.07 units/mg protein), IMI (liver: 0.50±0.004 units/mg protein), ACT (liver: 0.43±0.09; brain: 0.41±0.8 units/mg protein) and NIC (liver: 0.41±0.11; brain: 0.41±0.05 units/mg protein).

Glutathione Peroxidase (GPx)

Liver homogenates treated with CPC (0.15±0.04), SS (0.11±0.002), IMI (0.13±0.02), ACT (0.15±0.01) and NIC (0.12±0.01) was almost similar to that of control (0.14±0.002) value. This is shown in Figure 64. All the values are expressed in units/mg protein.

It is seen in Figure 65 that in brain homogenates as well, the GPx activity was not significant in the treated group (CPC: 0.02±0.01, SS: 0.06±0.02, IMI: 0.03±0.01, ACT: 0.03±0.02 and NIC: 0.08±0.04 units/mg protein) even though there were slight alteration when compared to control (0.02±0.002 units/mg protein).

Super Oxide Dismutase (SOD)

From Figure 66, it can be seen that the SOD activity in the liver homogenate of ocular irritant exposed animals (CPC: 0.08±0.01, SS: 0.09±0.004, IMI: 0.06±0.003, ACT: 0.09±0.008 and NIC: 0.08±0.01 units/mg protein) were similar to that of control (0.12±0.01 units/mg protein).

SOD activity in the brain of treated animals (CPC: 0.19±0.01, SS: 0.17±0.02, IMI: 0.14±0.07, ACT: 0.21±0.001 and NIC: 0.19±0.01 units/mg protein) was comparable with that of control (0.21±0.01 units/mg protein) as shown in Figure 67.

III. Validation

Acute

Acute treatment (24h) with ocular irritants was done in rabbits (*in vivo*) and in corneal tissue construct (*in vitro*). The pro-inflammatory cytokine release is shown below. Mean value of cytokine production in rabbit cornea treated with the highest dose of the known chemical irritants was taken and compared with that of mean value of control.

IL-1 α

Chemicals	*In vitro* IL-1α production (pg/ml)		*In vivo* IL-1α production (pg/ml)	
	Test	Control	Test	Control
Cetyl pyridinium chloride	585.00	123.21	157.38	130.17
Sodium Salicylate	124.64	112.85	173.75	130.17
Imidazole	102.50	122.50	132.85	130.17
Acetaminophen	157.85	119.28	162.38	130.17
Nicotinamide	156.42	112.85	156.90	130.17
Mean	**202.88**	**118.07**	**156.65**	**130.17**

In vivo, a cytokine level above 130.17 indicates irritation. *In vitro*, it can be seen that any chemical which gives a cytokine release above 118.07, can be considered as an irritant. That is, nearly a 100pg/ml increase in IL-1α production when compared to control *in vitro*, suggests that the chemical is an eye irritant and there is no need to proceed further to animal testing. However, IL-1α below this level should be subject to further analysis and comparison with release of other cytokines as well before marking it as safe to the eye.

IL-1 β

Chemicals	*In vitro* IL-1 β production (pg/ml)		*In vivo* IL-1 β production (pg/ml)	
	Test	Control	Test	Control
Cetyl pyridinium chloride	93.15	74.90	25.90	20.06
Sodium Salicylate	76.65	64.15	31.77	20.06
Imidazole	52.90	55.90	23.90	20.06
Acetaminophen	47.15	28.90	35.73	20.06
Nicotinamide	68.15	48.65	32.56	20.06
Mean	**67.57**	**54.50**	**29.97**	**20.06**

IL-1β production both *in vivo* and *in vitro* shows approximately a 0.5 fold increase when compare with that of control. Any chemical, which shows a value above 54.5 or a 0.5 fold increase in IL-1β production, can be ascribed as a potential eye irritant and *in vivo* testing can be avoided.

IL-8

Chemicals	*In vitro* IL- 8 production (pg/ml)		*In vivo* IL-8 production (pg/ml)	
	Test	Control	Test	Control
Cetyl pyridinium chloride	7672.22	2605.55	1588.88	1007.41
Sodium Salicylate	3044.44	2894.44	1563.88	1007.41
Imidazole	2505.55	2422.22	1014.81	1007.41
Acetaminophen	3044.44	2505.55	1681.48	1007.41
Nicotinamide	2994.44	2627.77	1188.88	1007.41
Mean	**3852.22**	**2138.11**	**1407.59**	**1007.41**

Rabbit cornea treated with eye irritant chemicals (*in vivo*), shows IL-8 levels which is 400 times more than that of control. *In vitro*, IL-8 levels showed 2136.11 as mean, suggesting that any chemical above this value can be a potential eye irritant.

TNF-α

Chemicals	*In vitro* TNF-α production (pg/ml)		*In vivo* TNF-α production (pg/ml)	
	Test	Control	Test	Control
Cetyl pyridinium chloride	2473	1398	1083.00	890.5
Sodium Salicylate	1628	1198	1396.33	890.5
Imidazole	968	1323	1016.33	890.5
Acetaminophen	1655	1330	1193	890.5
Nicotinamide	1590	1330	1206.33	890.5
Mean	**1662.80**	**1315.80**	**1178.91**	**890.50**

TNF-α shows approximately 300 times more release in test when compared to control, both *in vivo* and *in vitro*. Hence corneal tissue construct treated with chemicals, if they show TNF-α more than 1315.80 *in vitro*, they can be potential eye irritants.

Sub acute

Sub acute treatment (7 days) with known ocular irritants was carried out in rabbit cornea and rabbit corneal tissue construct. The pro-inflammatory cytokine release is shown below. Mean value of cytokine production in rabbit cornea treated with the highest dose of the known chemical irritants was taken and compared with that of mean value of control.

IL-1α

Chemicals	*In vitro* IL-1α production (pg/ml)		*In vivo* IL-1α production (pg/ml)	
	Test	Control	Test	Control
Cetyl pyridinium chloride	199	162	166.19	130.17
Sodium Salicylate	201	144	156.19	130.17
Imidazole	176	162	143.57	130.17
Mean	**192**	**156**	**155.31**	**130.17**

In vitro and *in vivo*, the IL-1α release was approximately 30 pg/ml more than control. So, any chemical showing IL-1α level more than 156 *in vitro*, can potentially cause harm to eyes and *in vivo* animal experiment can be eliminated.

IL-1β

Chemicals	*In vitro* IL-1β production (pg/ml)		*In vivo* IL-1β production (pg/ml)	
	Test	Control	Test	Control
Cetyl pyridinium chloride	28.75	19.83	34.77	15.02
Sodium Salicylate	30.00	19.83	23.52	15.02
Imidazole	18.75	19.83	20.73	15.02
Mean	**25.83**	**19.83**	**26.34**	**15.02**

IL-1β production *in vitro* and *in vivo* showed a slight increase when compared to control. It can be said that, chemicals are potential eye irritants if they show IL-1β more than 19.83 when compared to control.

IL-8

Chemicals	*In vitro* IL-8 production (pg/ml)		*In vivo* IL-8 production (pg/ml)	
	Test	Control	Test	Control
Cetyl pyridinium chloride	2794.44	2538.88	1055.55	1007.41
Sodium Salicylate	2944.44	2538.88	1119.44	1007.41
Imidazole	2511.11	2472.22	1086.11	1007.41
Mean	**2749.99**	**2516.66**	**1087.03**	**1007.41**

IL-8 production *in vivo* shows a marked increase when compared to control. In the rabbit corneal tissue construct (*in vitro*), chemicals which stimulate IL-8 beyond 2516.66 are potential ocular irritants.

TNF-α

Chemicals	*In vitro* TNF-α production (pg/ml)		*In vivo* TNF-α production (pg/ml)	
	Test	Control	Test	Control
Cetyl pyridinium chloride	1335	1220	1883	1310.5
Sodium Salicylate	1305	1220	1963	1310.5
Imidazole	1270	1220	1575.5	1310.5
Mean	**1303.33**	**1220**	**1340.5**	**1310.5**

TNF-α shows 40 and 80 pg/ml increase, when compared to control, in *in vivo* and *in vitro* respectively. It can be said that any chemical that stimulates TNF- α more than 1220 pg/ml, then that chemical can be classified as an ocular irritant.

Conclusions

In conclusion, the corneal construct was successfully developed from primary rabbit tissue could serve as a model for *in vitro* toxicity screening. The construct was exposed to different chemicals on actue and sub-acute mode. This was evaluated using different assay methods of qualitative and quantitative nature. Evaluation with vital staining (neutral red) technique and image analysis can bring quantitative representation of microscopic data. The feasibility of Alamar blue assay as an *in vitro* toxicity screening technique is a scientific knowledge that can be communicated. The *in vitro* results corroborated well with the *in vivo* results as well. This study was also able to confirm that systemic toxicity through the ocular route was in significant. The results of this study is promising in the fact that primary corneal construct can be used for ocular toxicity screening against currently practiced systems that use cell lines. Cytokines production using tissue construct may be used as a biomarkers for irritation potential. This culture system is important in developing indigenous commercially viable *in vitro* screening system. Hence, the present study can concluded that the corneal tissue construct can be used as an indigenous potential alternatives (using novel cell culturing, tissue construct techniques and with inflammatory markers) for acute and sub acute ocular irritation tests, which will be very cost effective, readily available to the various segments of health care industry and a total replacement of animal experimentation.

FIGURES AND TABLES

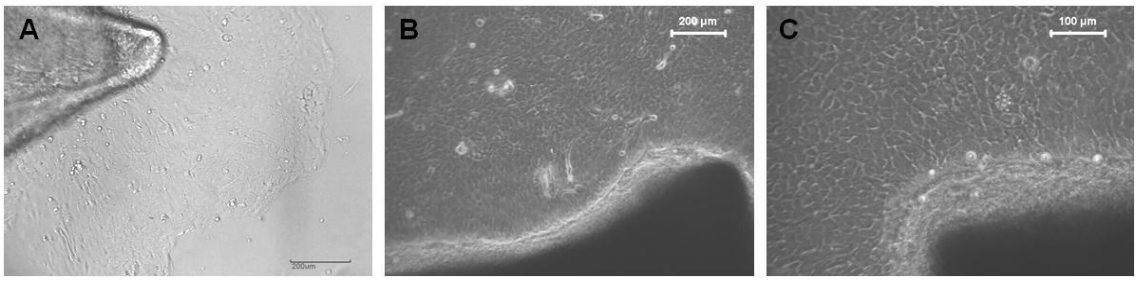


Figure 1. (A,B) Primary corneal cells from rabbit on 3 and 6 days after isolation. (C) Explant culture showing corneal epithelial morphology(6th day)

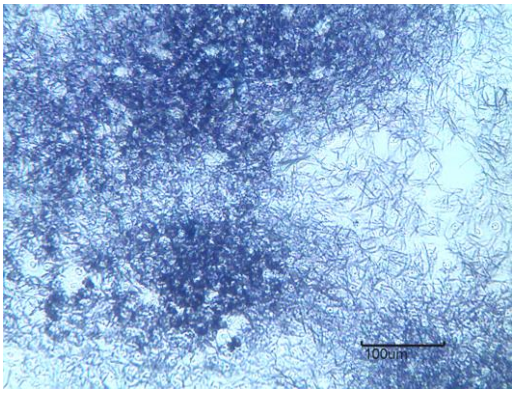


Figure 2. Formazan crystals showing the viability of rabbit corneal cells

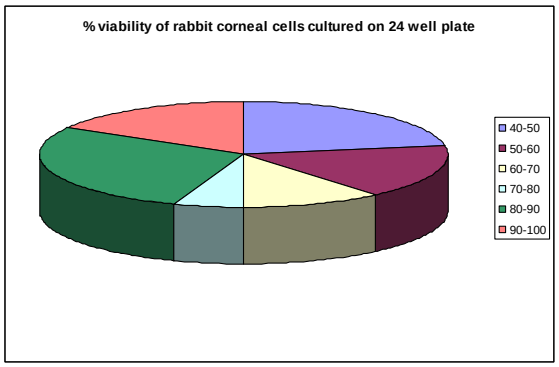


Figure 3. MTT assay on rabbit corneal cells on 7th day of isolation

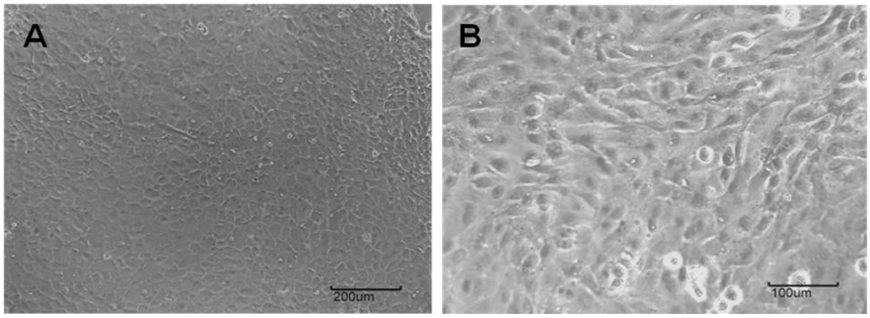


Figure 4 : (A and B) Goat corneal tissue construct on thermoresponsive NGMA surface on the 10th day

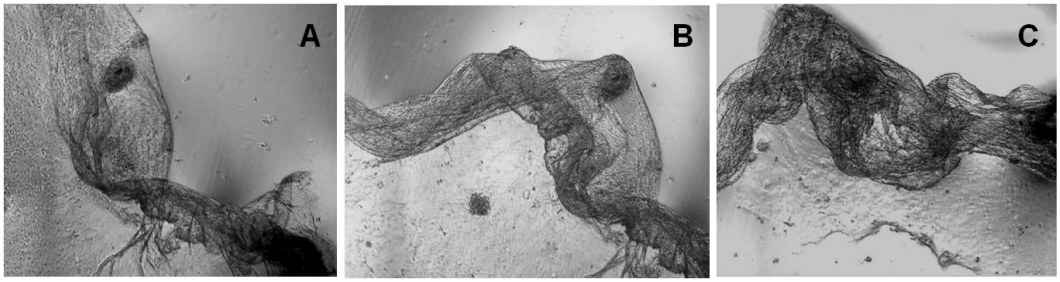


Figure 5: Phase contrast image of goat corneal tissue construct detached from the NGMA

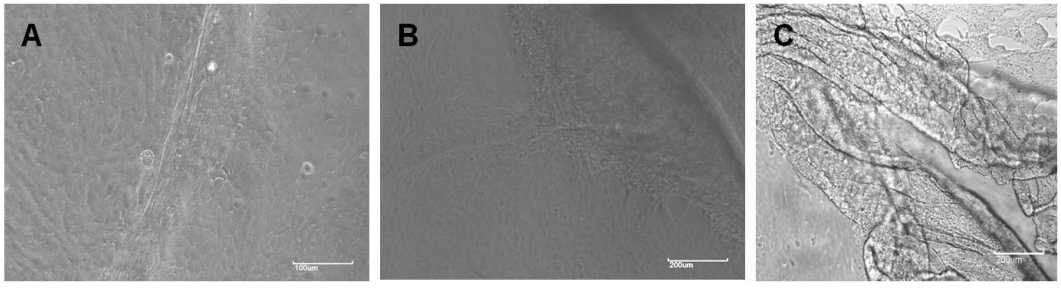


Figure 6: Phase contrast image of rabbit corneal tissue construct detached from the NGMA

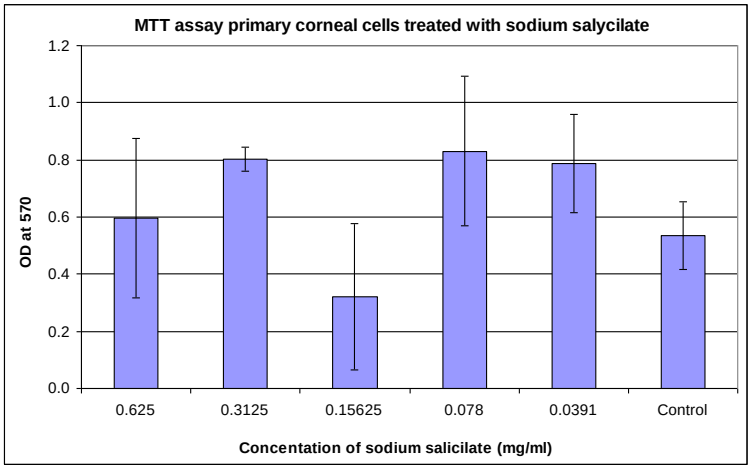


Figure 7 MTT assay of primary corneal construct treated with sodium salycilate.

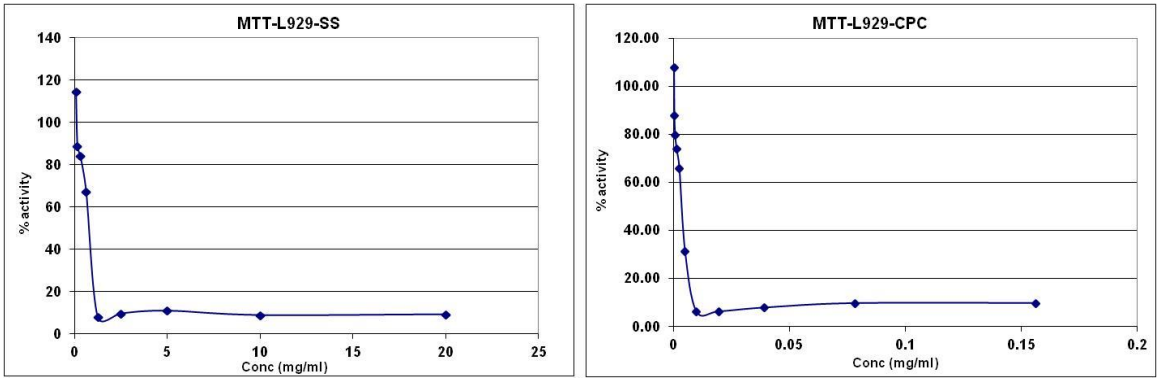
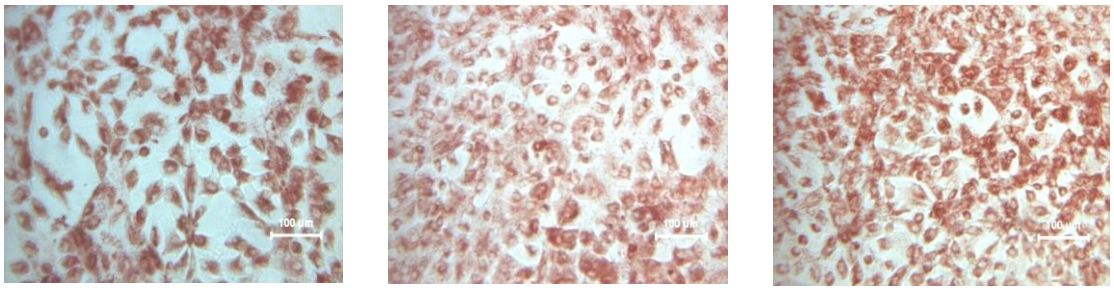
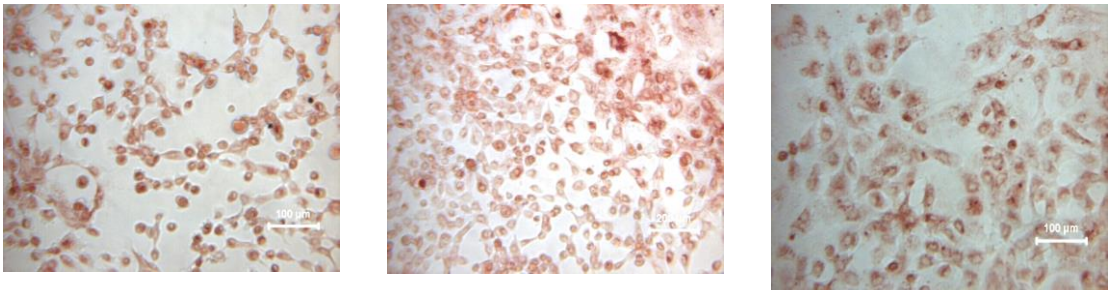


Figure 8: MTT assay of L929 cells treated with SS and CPC.



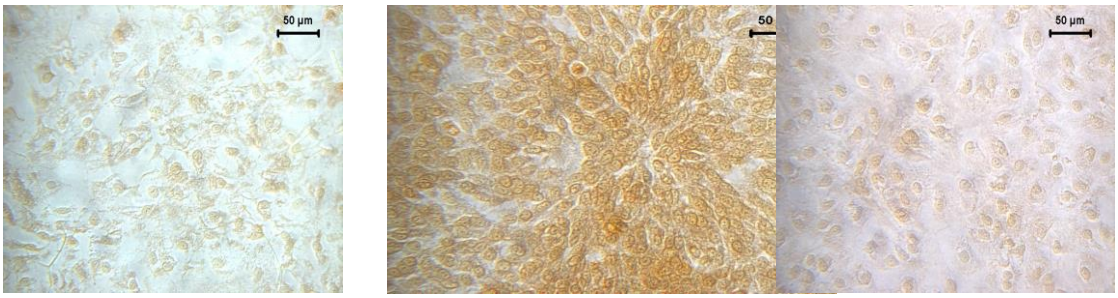
A B C

Figure 9. Neutral red stained primary corneal cells treated with Imidazol. A) 50 mg/ml B) 25 mg/ml and C) 12.5 mg/ml.



A B C

Figure 10. Neutral red stained primary corneal cells treated with Sodium Salycilate. A) 50 mg/ml B) 25 mg/ml and C) 12.5 mg/ml



A B C

Figure 11. Neutral red stained primary corneal cells treated with Cetyl Pyridinium chloride. A) 50 mg/ml B) 25 mg/ml and C) 12.5 mg/ml

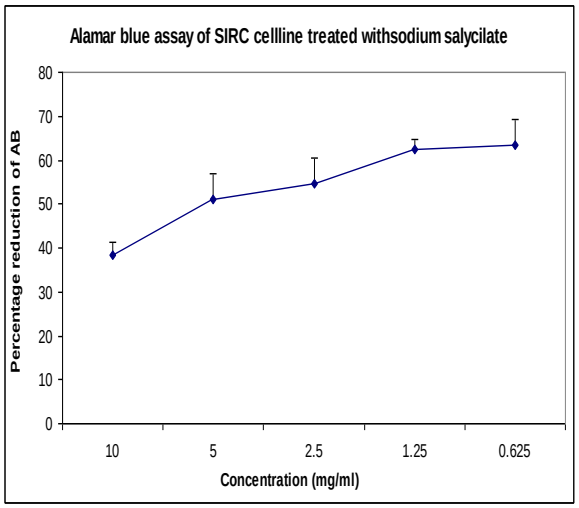


Figure 13. Alamar blue assay of SIRC cells treated with Sodium Salylcilate

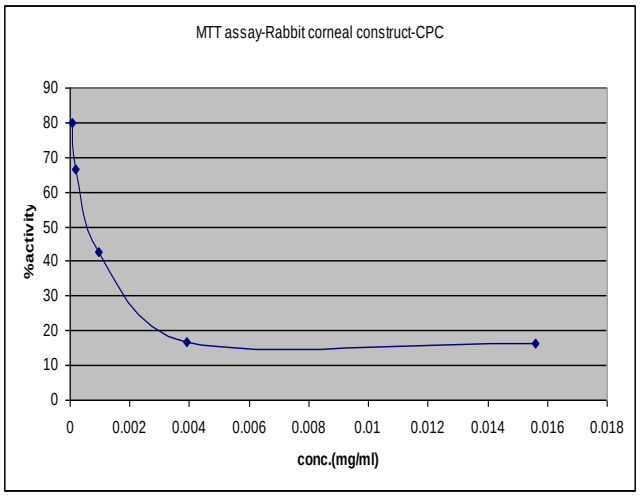


Figure14. Percentage activity of Rabbit corneal construct after treatment with Cetyl pyridinium chloride.

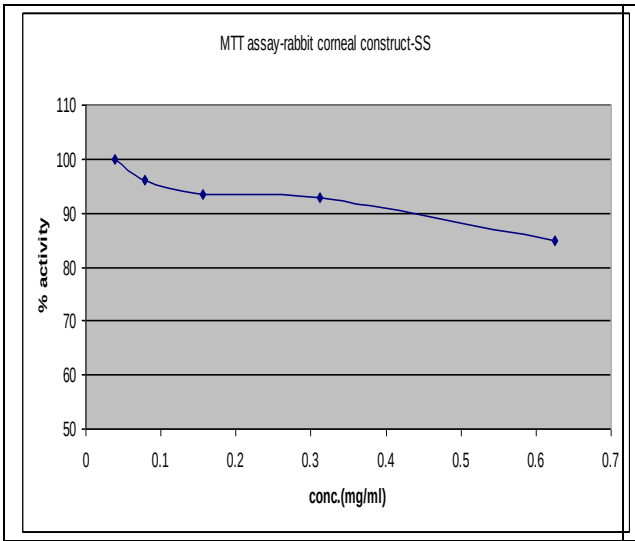


Figure 15. Percentage activity of Rabbit corneal construct after treatment with Sodium salycilate

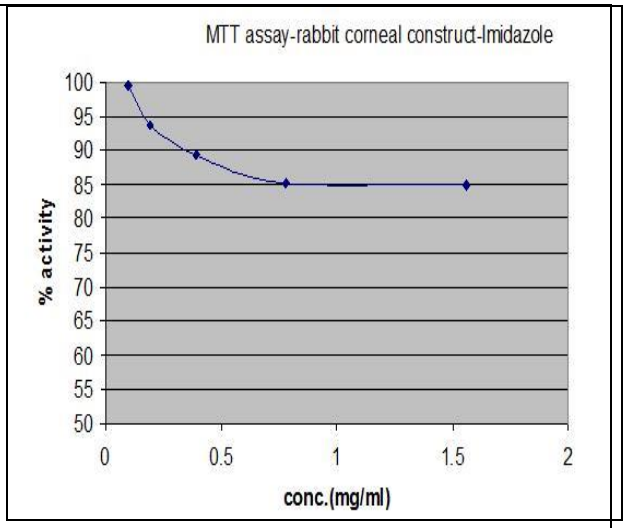


Figure 16.Percentage activity of Rabbit corneal construct after treatment with Imidazole

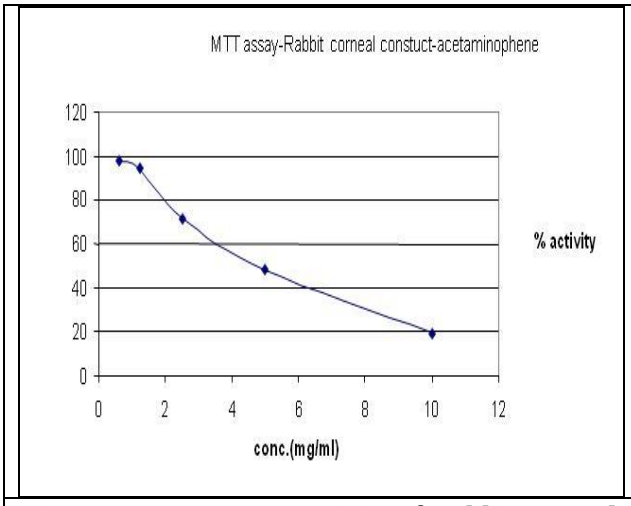


Figure 17. Percentage activity of Rabbit corneal construct after treatment with Acetaminophen

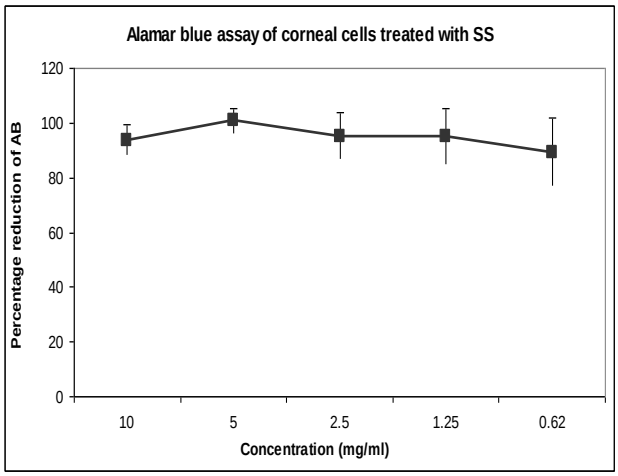


Figure 18. Alamar blue assay of corneal construct treated with Sodium Salylcilate

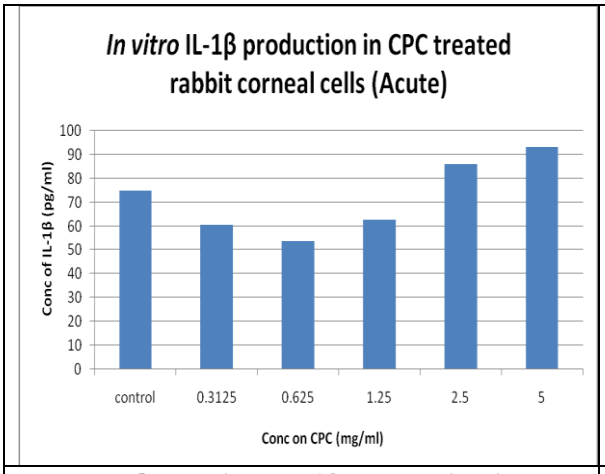


Figure 21b: *In vitro* IL-1β production in CPC treated rabbit corneal cells (acute)

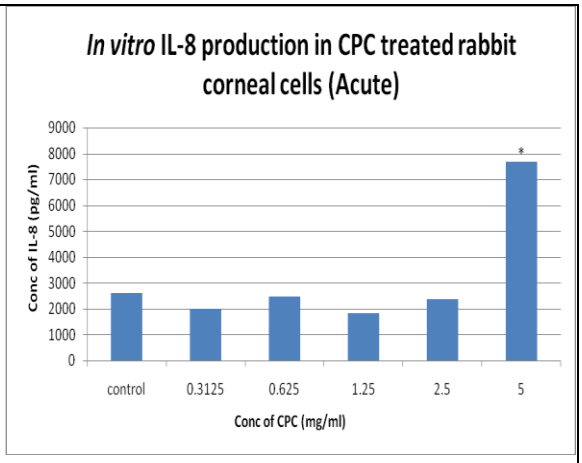


Figure 21c: *In vitro* IL-8 production in CPC treated rabbit corneal cells (acute). *p≤0.05

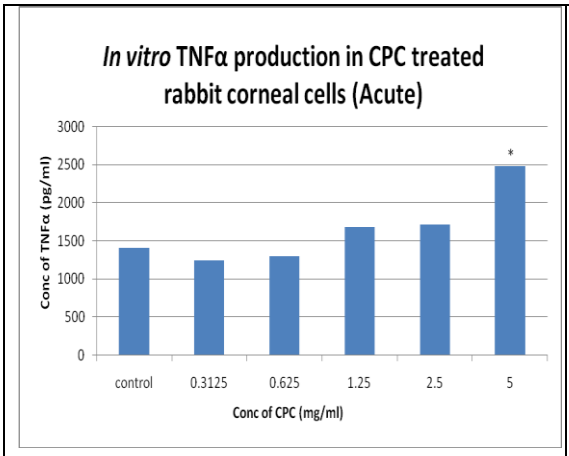


Figure 21d: *In vitro* TNFα production in CPC treated rabbit corneal cells (acute). *p≤0.05

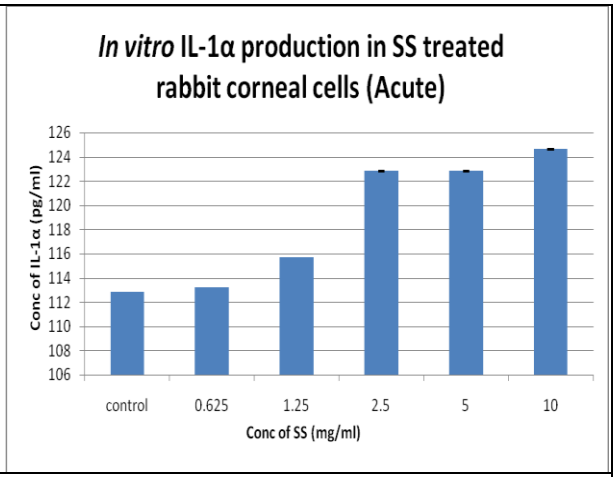


Figure 22a: *In vitro* IL-1α production in SS treated rabbit corneal cells (acute)

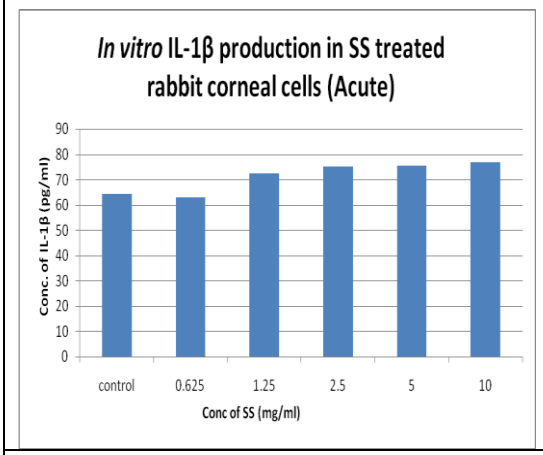


Figure 22b: *In vitro* IL-1β production in SS treated rabbit corneal cells (acute)

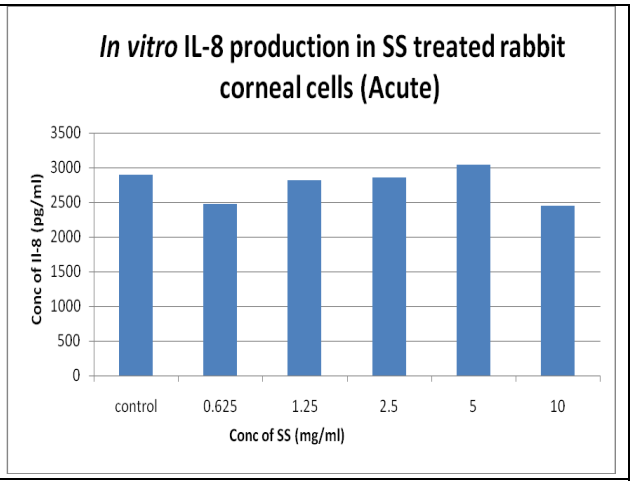


Figure 22c: *In vitro* IL-8 production in SS treated rabbit corneal cells (acute).

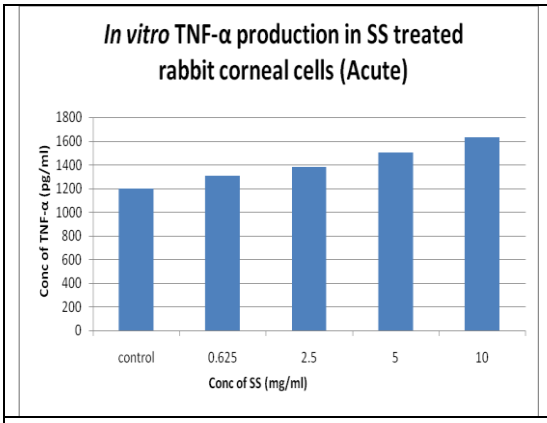


Figure 22d: *In vitro* TNF-α production in SS treated rabbit corneal cells (acute)

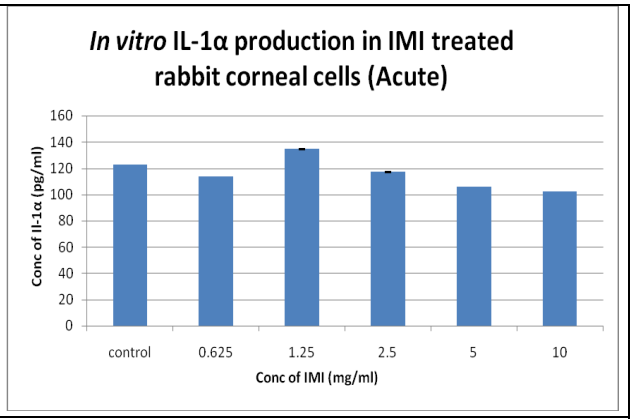


Figure 23a: *In vitro* IL-1α production in IMI treated rabbit corneal cells (acute)

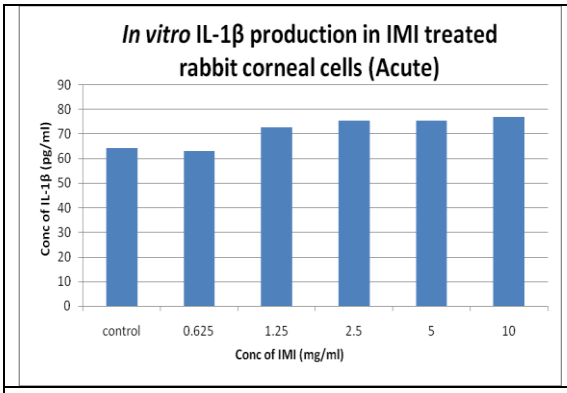


Figure 23b: *In vitro* IL-1β production in IMI treated rabbit corneal cells (acute)

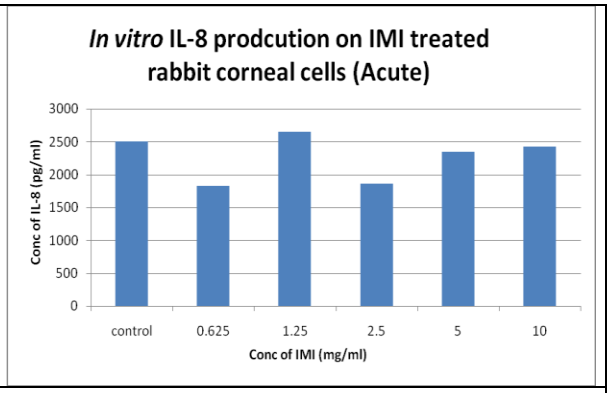


Figure 23c: *In vitro* IL-8 prodcution on IMI treated rabbit corneal cells (acute)

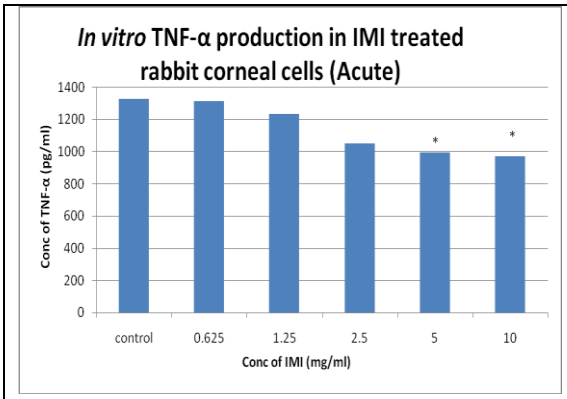


Figure 23d: *In vitro* TNF-α production in IMI treated rabbit corneal cells (acute). *p≤0.05

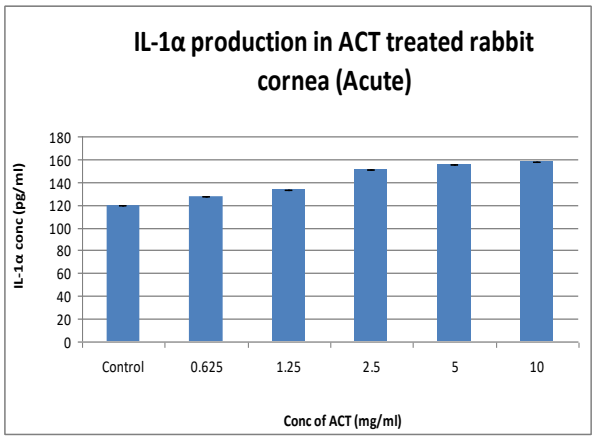


Figure 24a: *In vitro* IL-1α production in ACT treated rabbit corneal cells (acute)

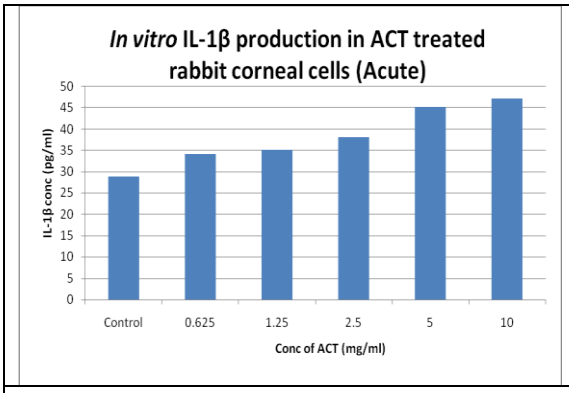


Figure 24b: *In vitro* IL-1β production in ACT treated rabbit corneal cells (acute)

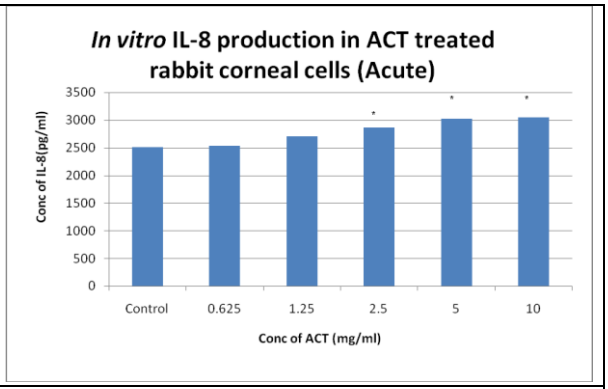


Figure 24c: *In vitro* IL-8 production in ACT treated rabbit corneal cells (acute). *p≤0.05

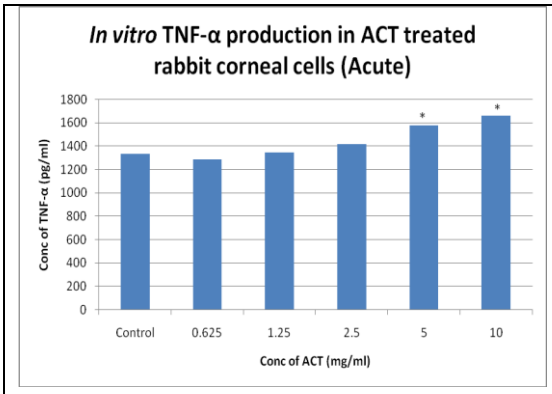


Figure 24d: *In vitro* TNF-α production in ACT treated rabbit corneal cells (acute). *p≤0.05

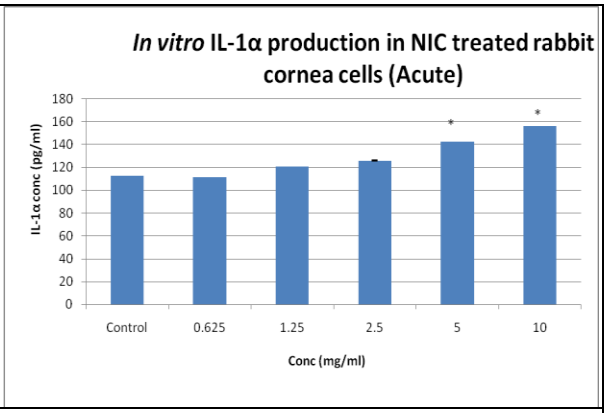


Figure 25a: *In vitro* IL-1α production in NIC treated rabbit cornea cells (acute). *p≤0.05

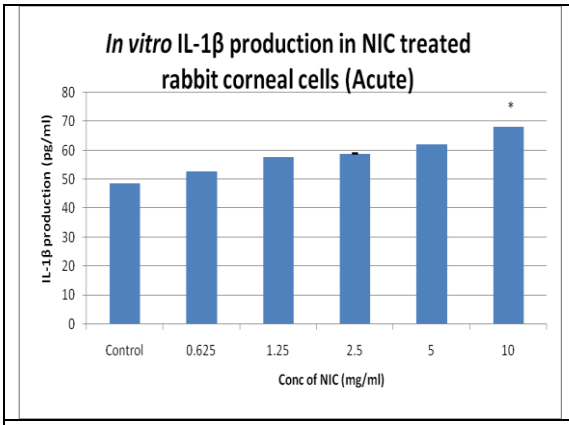


Figure 25b: *In vitro* IL-1β production in NIC treated rabbit corneal cells (acute). *p≤0.05

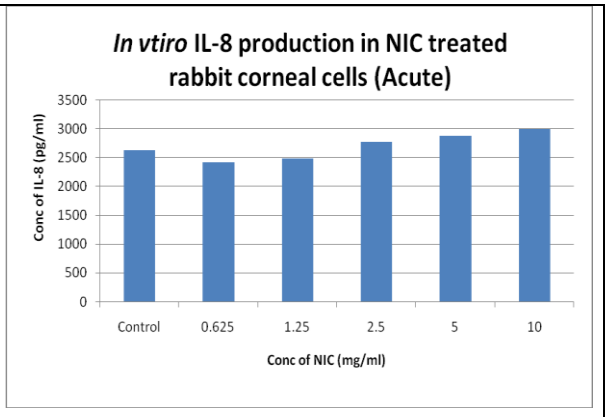


Figure 25c: *In vitro* IL-8 production in NIC treated rabbit corneal cells (acute)

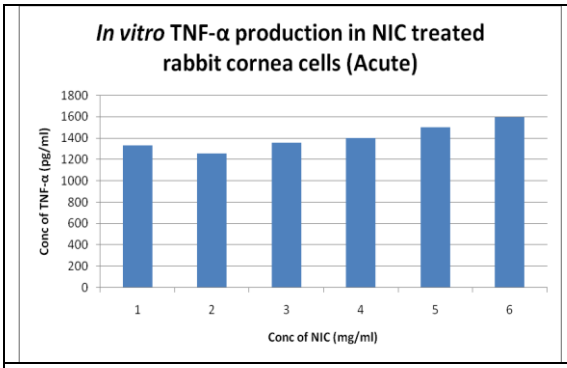


Figure 25d: *In vitro* TNF-α production in NIC treated rabbit cornea cells (acute)

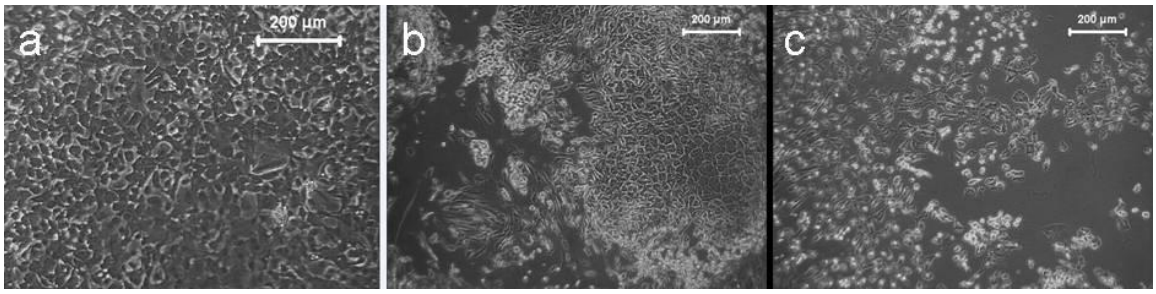


Figure 26.(a)Rabbit corneal tissue construct after 15min. treatment with 0.2mg/ml CPC, (b) 2 mg/ml CPC and 20mg/ml CPC

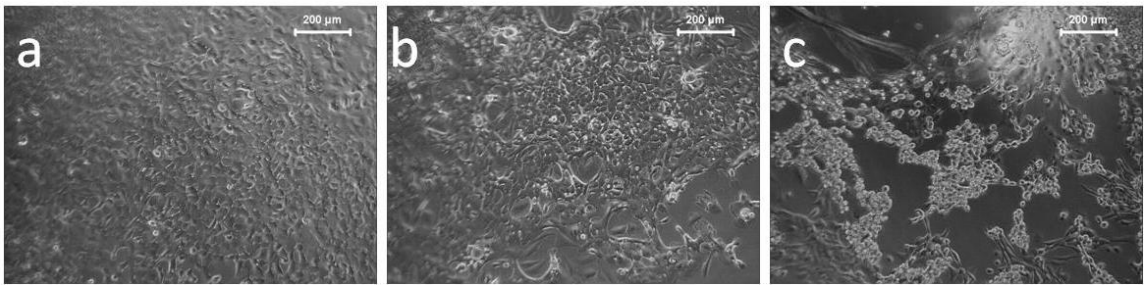


Figure 27. (a) Rabbit corneal tissue construct after 15min. treatment with 0.5mg/ml Imidazole, (b) 5mg/ml Imidazole and (c) 50mg/ml Imidazole

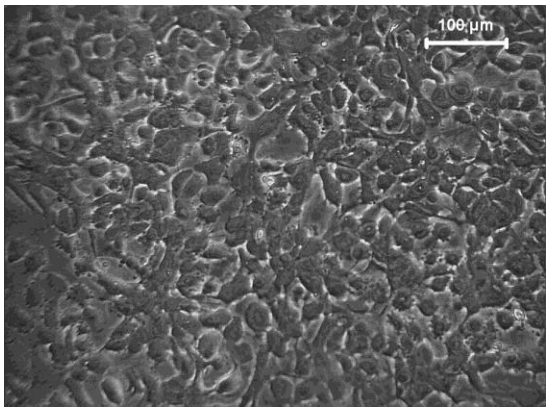


Figure 28. Phase contrast image of Rabbit corneal tissue construct on 10th day

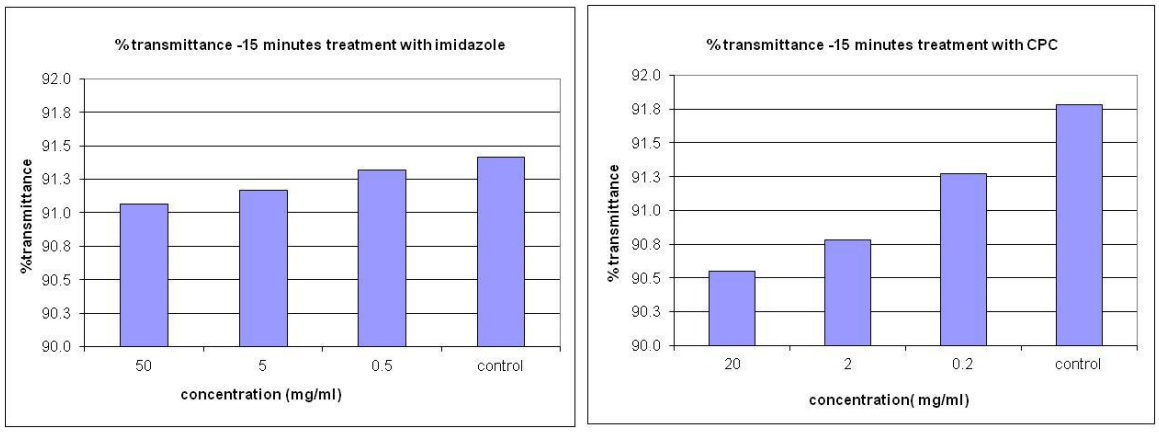


Figure 29: In vitro Corneal opacity measurement after treatment with Imidazole and CPC

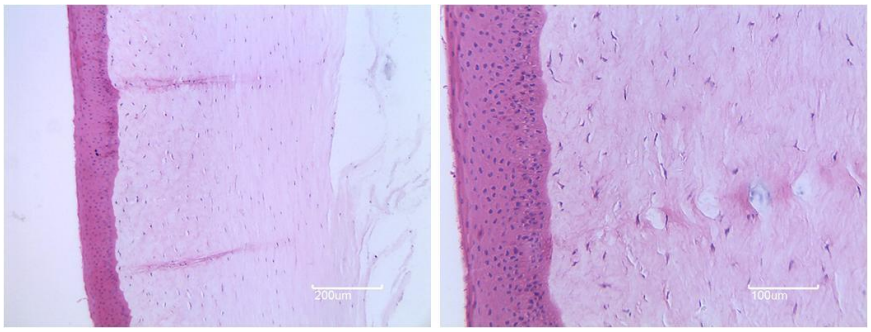


Figure 30. Paraffin section of goat corneal tissue stained with hematoxyline-eosin showing corneal epithelium.

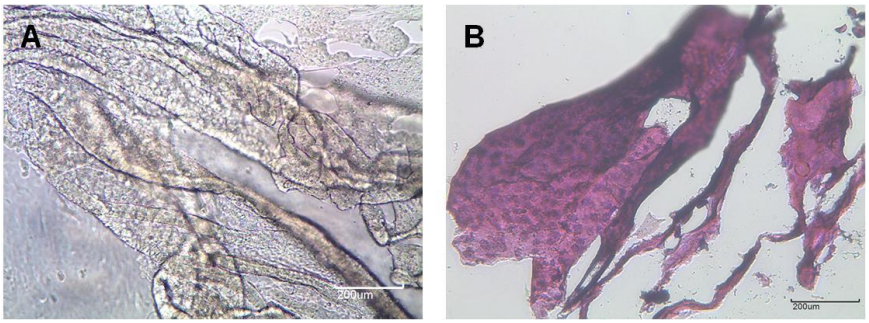


Figure 31. (A) Phase contrast image of Rabbit corneal tissue construct and (B) hematoxyline-eosin stained construct

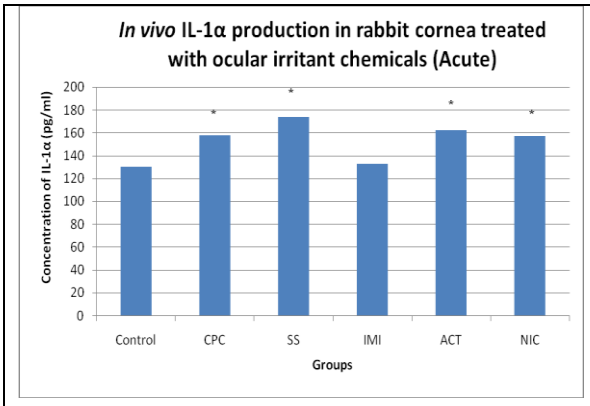


Figure 32: *In vivo* IL-1α production in rabbit cornea treated with ocular irritant chemicals (acute). *p≤0.05

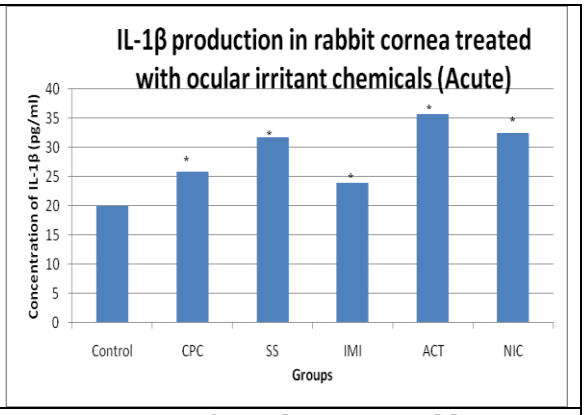


Figure 33: IL-1β production in rabbit cornea treated with ocular irritant chemicals (Acute). *p≤0.05

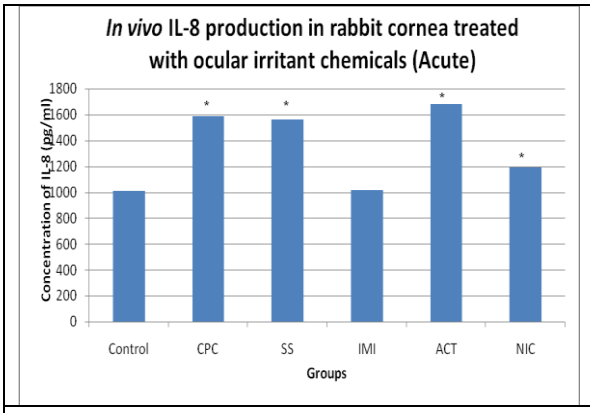


Figure 34: *In vivo* IL-8 production in rabbit cornea treated with ocular irritant chemicals (acute). *p≤0.05

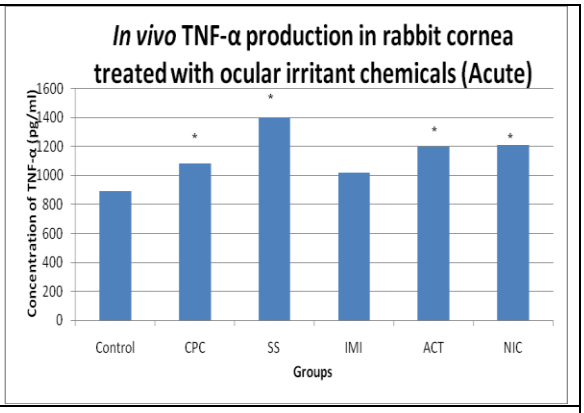


Figure 35: *In vivo* IL-8 production in rabbit cornea treated with ocular irritant chemicals (acute). *p≤0.05

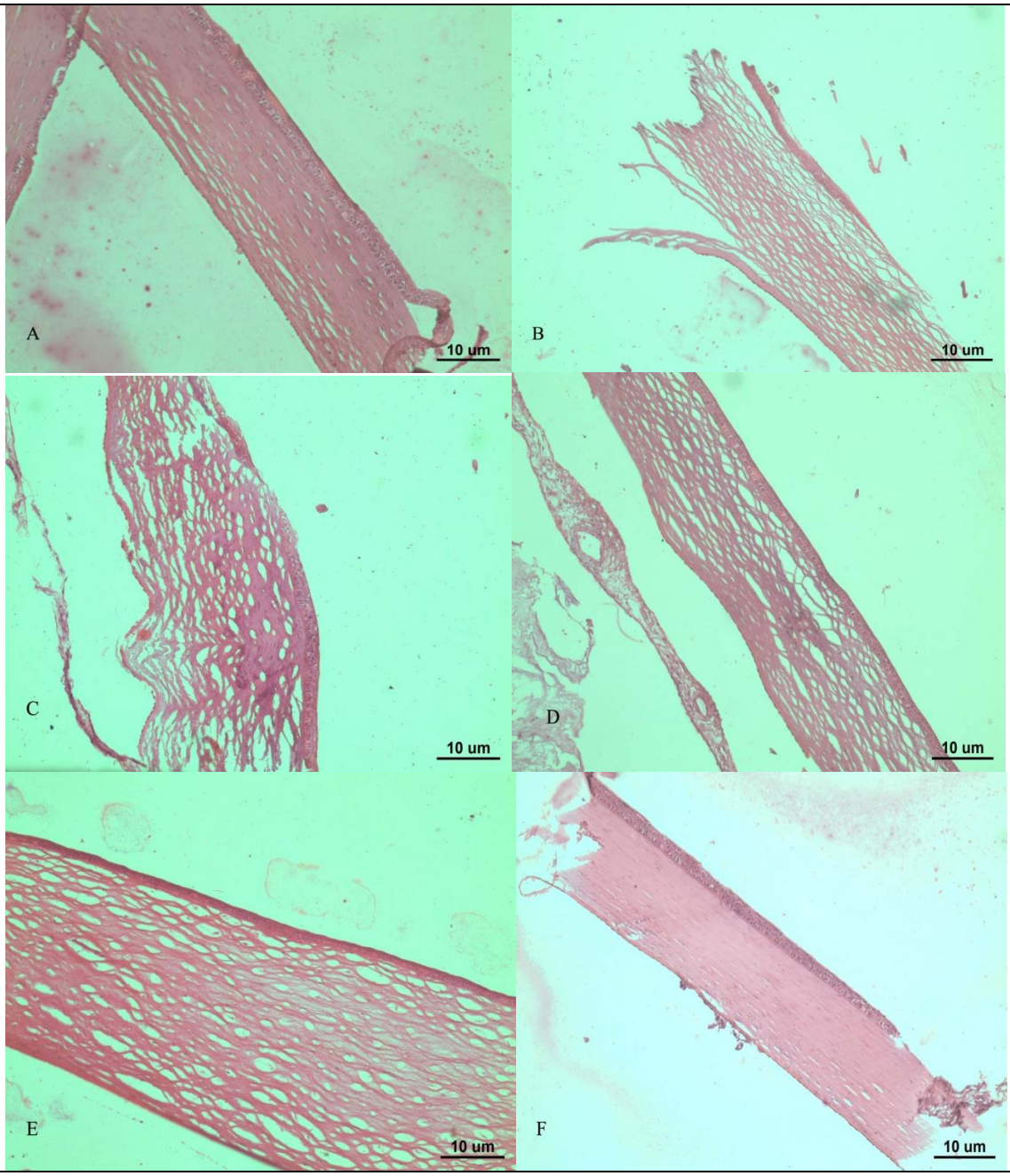


Figure 36: Histopathology of cornea using H&E staining. A)control; B) CPC treated; C) SS treated; D) IMI treated; E) ACT treated; and F) NIC treated

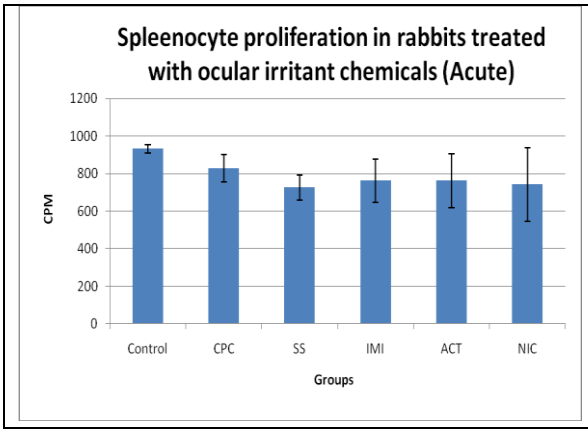


Figure 37: Spleenocyte proliferation in rabbits treated with ocular irritant (acute)

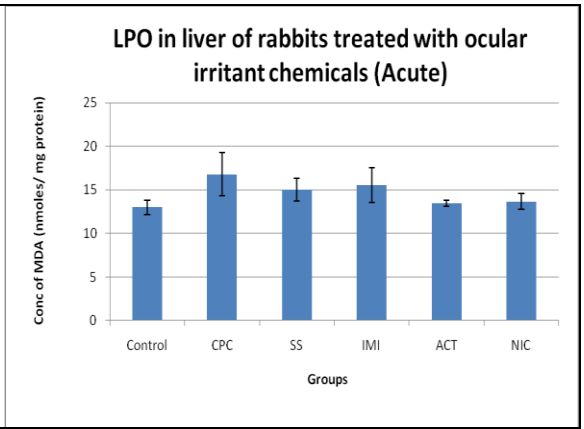


Figure 38: LPO in liver of rabbits treated with ocular irritant chemicals (acute)

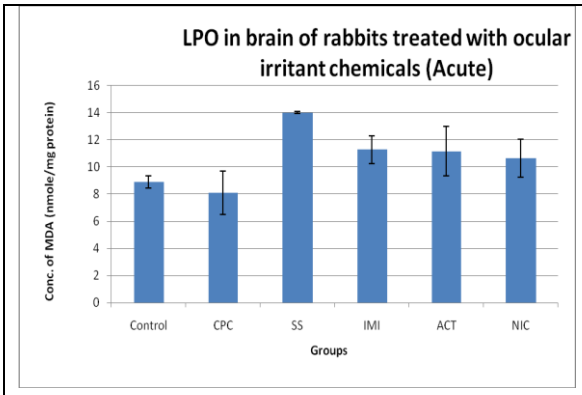


Figure 39: LPO in brain of rabbits treated with ocular irritant chemicals (Acute)

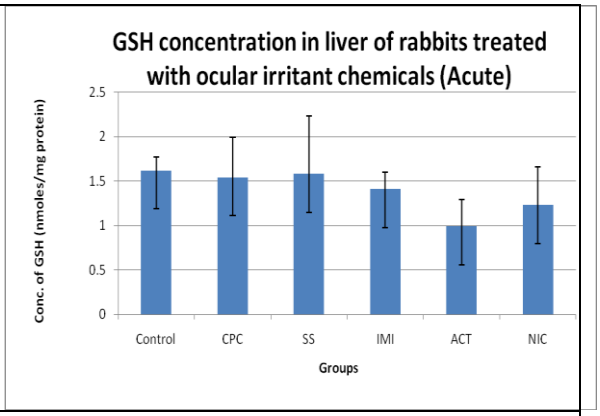


Figure 40: GSH concentration in liver of rabbits treated with ocular irritant chemicals (acute)

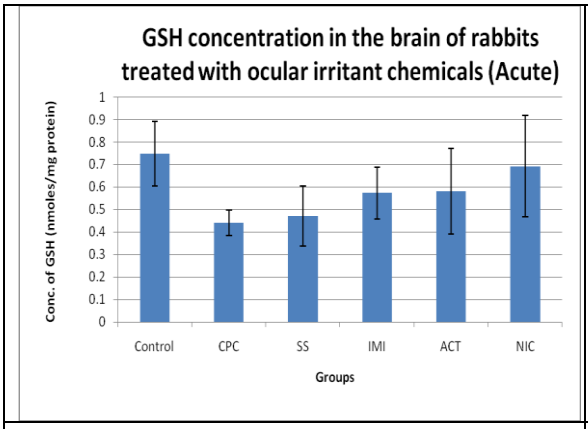


Figure 41: GSH concentration in the brain of rabbits treated with ocular irritant chemicals (Acute)

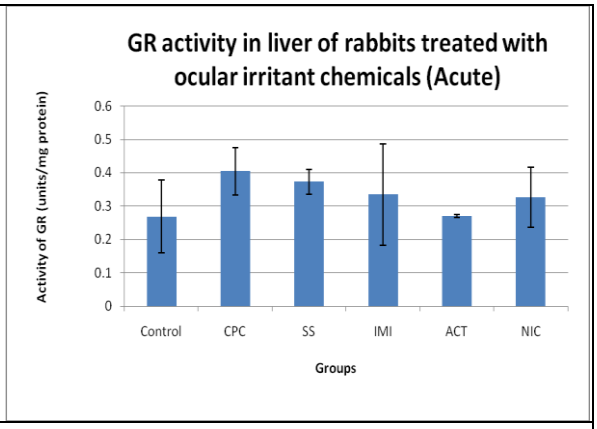


Figure 42: GR activity in liver of rabbits treated with ocular irritant chemicals (acute)

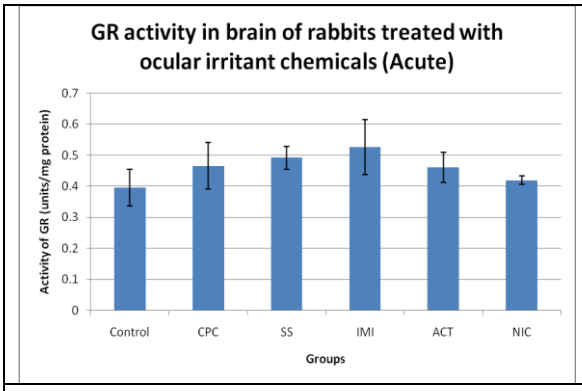


Figure 43: GR activity in brain of rabbits treated with ocular irritant chemicals (Acute)

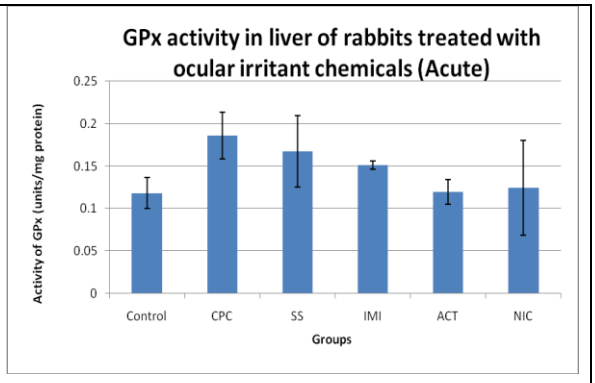


Figure 44: GPx activity in liver of rabbits treated with ocular irritant chemicals (Acute)

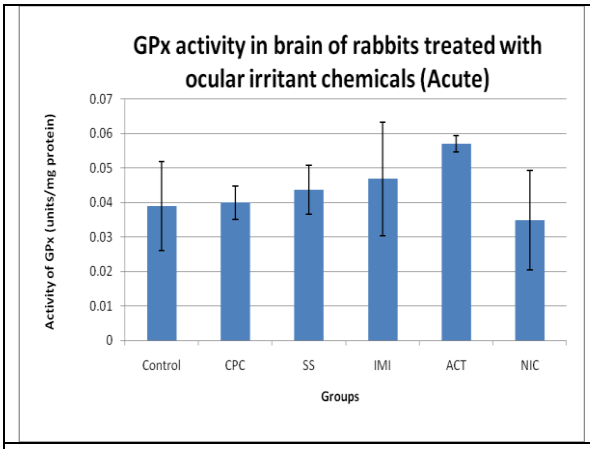


Figure 45: GPx activity in brain of rabbits treated with ocular irritant chemicals (Acute)

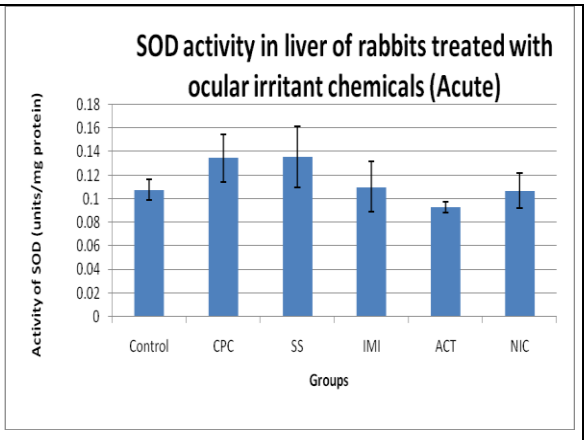


Figure 46: SOD activity in liver of rabbits treated with ocular irritant chemicals (acute)

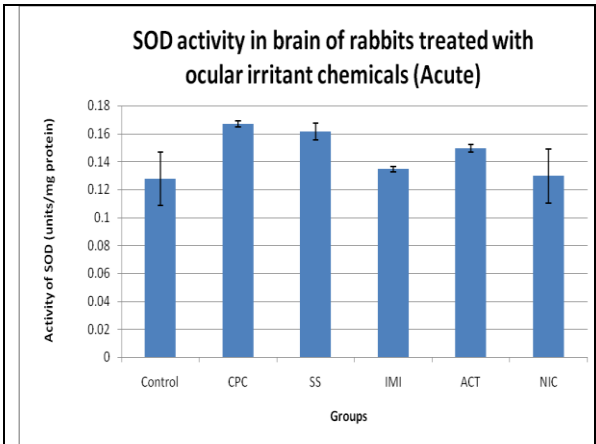


Figure 47: SOD activity in brain of rabbits treated with ocular irritant chemicals (Acute)

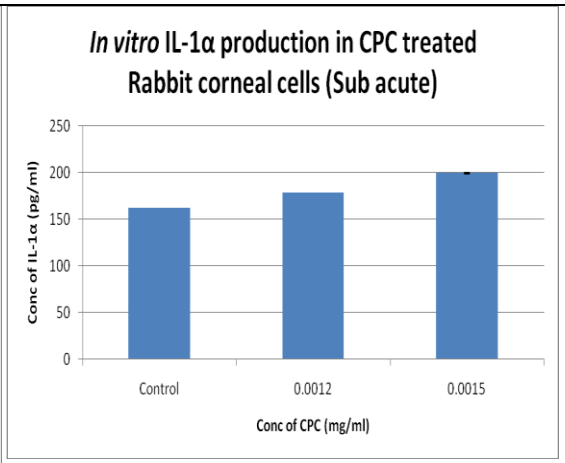


Figure 49a: *In vitro* IL-1α production in CPC treated Rabbit corneal cells (Sub acute)

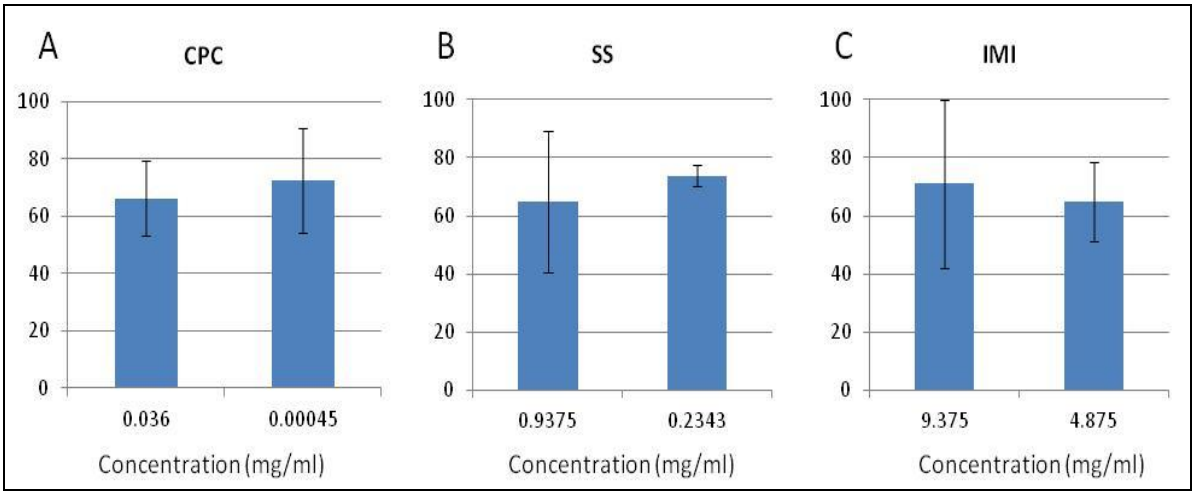


Figure 48. In vitro sub-acute test on corneal construct exposed to chemicals.

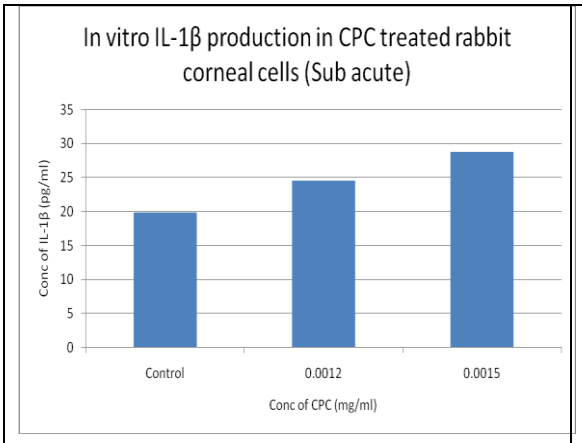


Figure 49b: *In vitro* IL-1β production in CPC treated rabbit corneal cells (Sub acute)

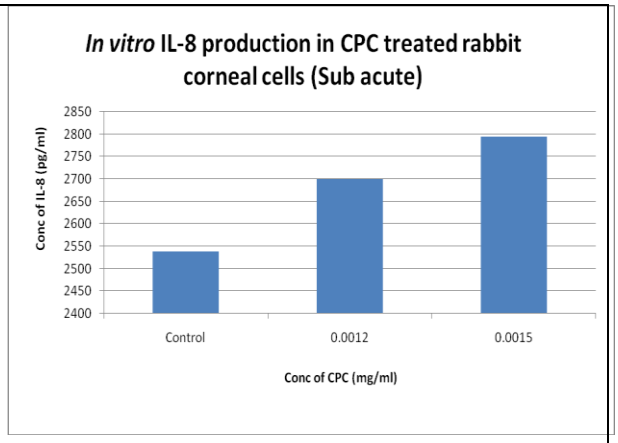


Figure 49c: *In vitro* IL-8 production in CPC treated rabbit corneal cells (Sub acute)

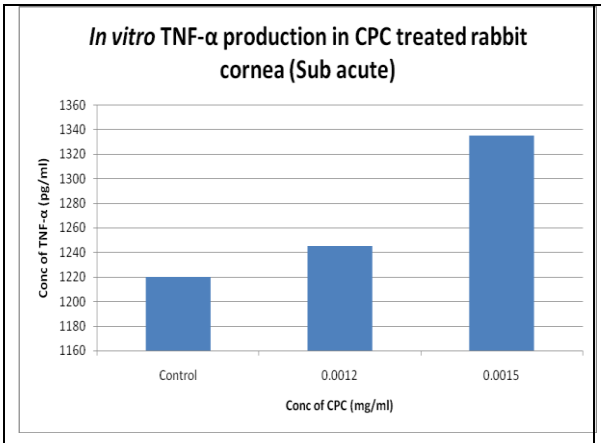


Figure 49d: *In vitro* TNF-α production in CPC treated rabbit cornea (Sub acute)

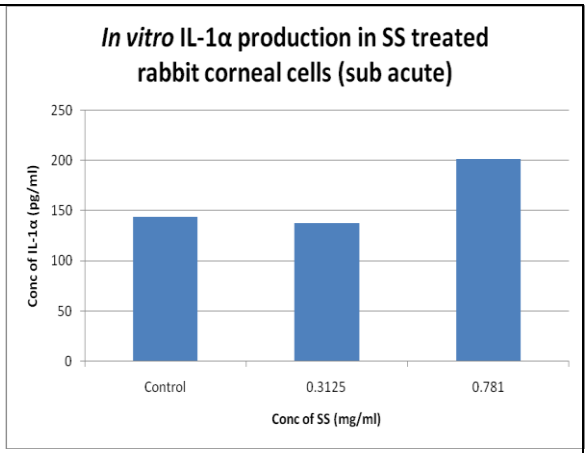


Figure 50a: *In vitro* IL-1α production in SS treated rabbit corneal cells (sub acute)

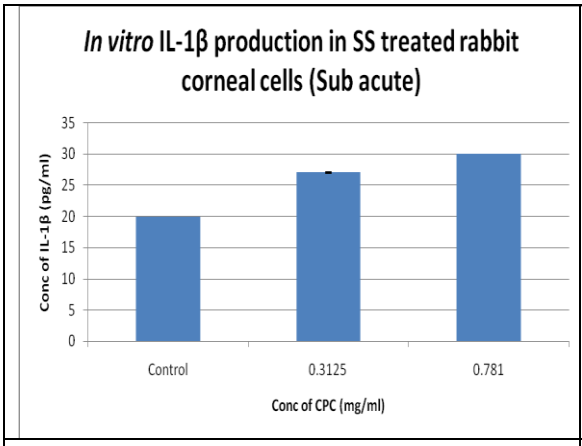


Figure 50b: *In vitro* IL-1β production in SS treated rabbit corneal cells (Sub acute)

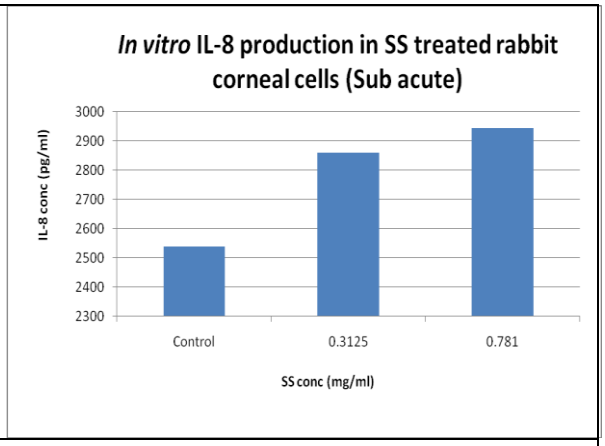


Figure 50c: *In vitro* IL-8 production in SS treated rabbit corneal cells (Sub acute)

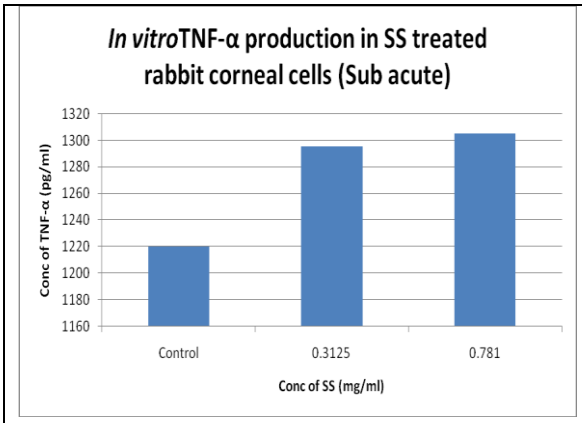


Figure 50d: *In vitro* TNF-α production in SS treated rabbit corneal cells (sub acute)

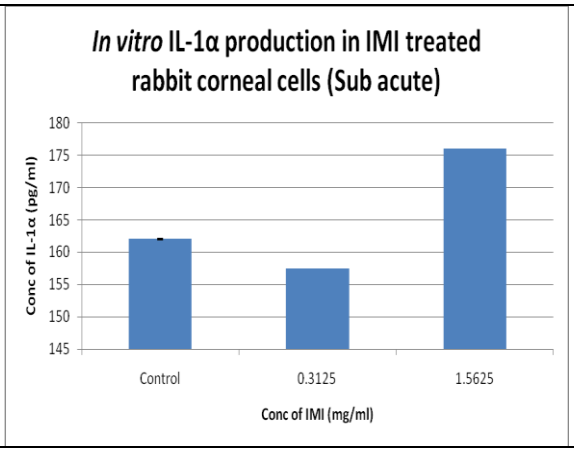


Figure 51a: *In vitro* IL-1α production in IMI treated rabbit corneal cells (Sub acute)

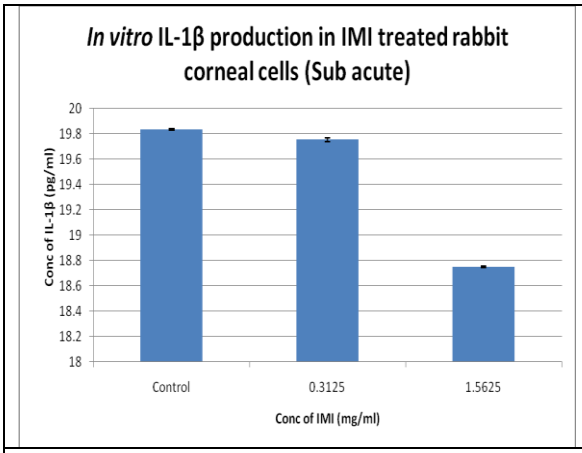


Figure 51b: *In vitro* IL-1β production in IMI treated rabbit corneal cells (sub acute)

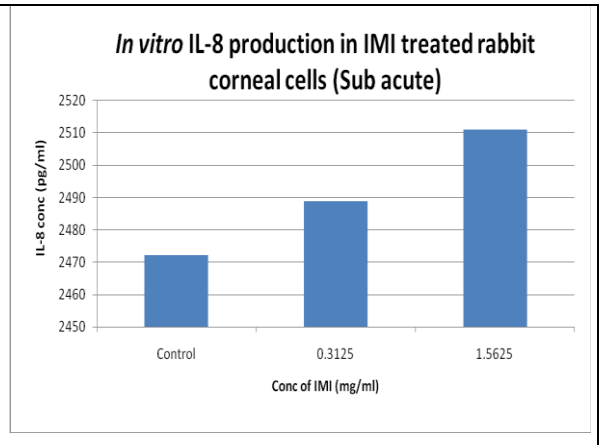


Figure 51c: *In vitro* IL-8 production in IMI treated rabbit corneal cells (Sub acute)

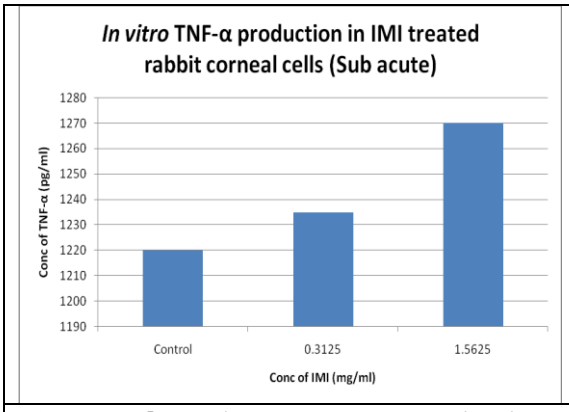


Figure 51d: *In vitro* TNF-α production in IMI treated rabbit corneal cells (sub acute)

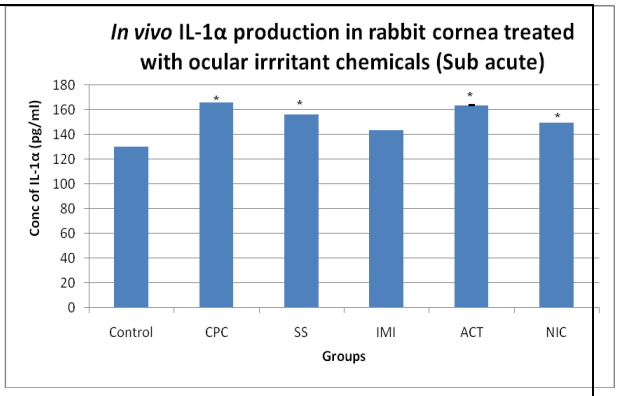


Figure 52: *In vivo* IL-1α production in rabbit cornea treated with ocular irrritant chemicals (Sub acute). * p≤0.05.

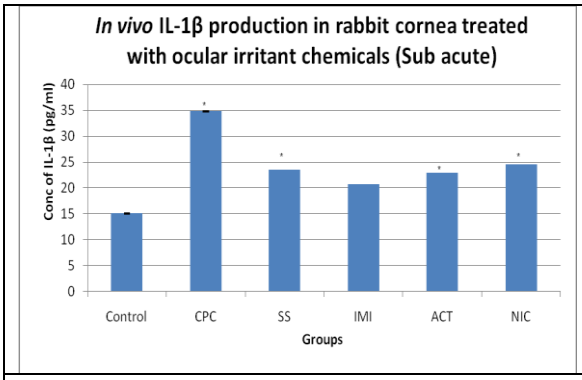
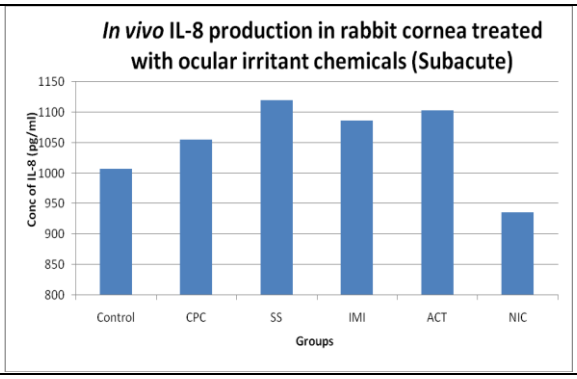


Figure 53: In vivo IL-1β production in rabbit cornea treated with ocular irritant chemicals (Sub acute). *p≤0.05



54: In vivo IL-8 production in rabbit cornea treated with ocular irritant chemicals (Subacute).

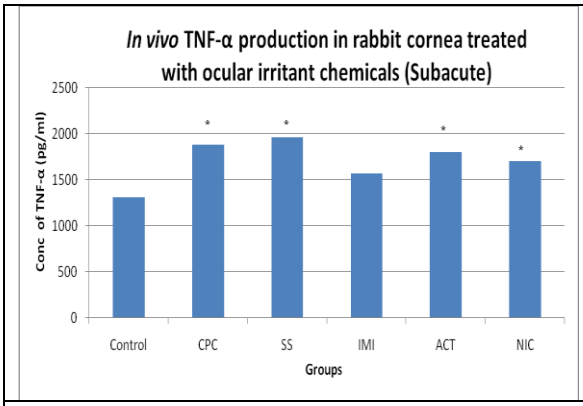


Figure 55: *In vivo* TNF-α production in rabbit cornea treated with ocular irritant chemicals (sub acute). *p≤0.05

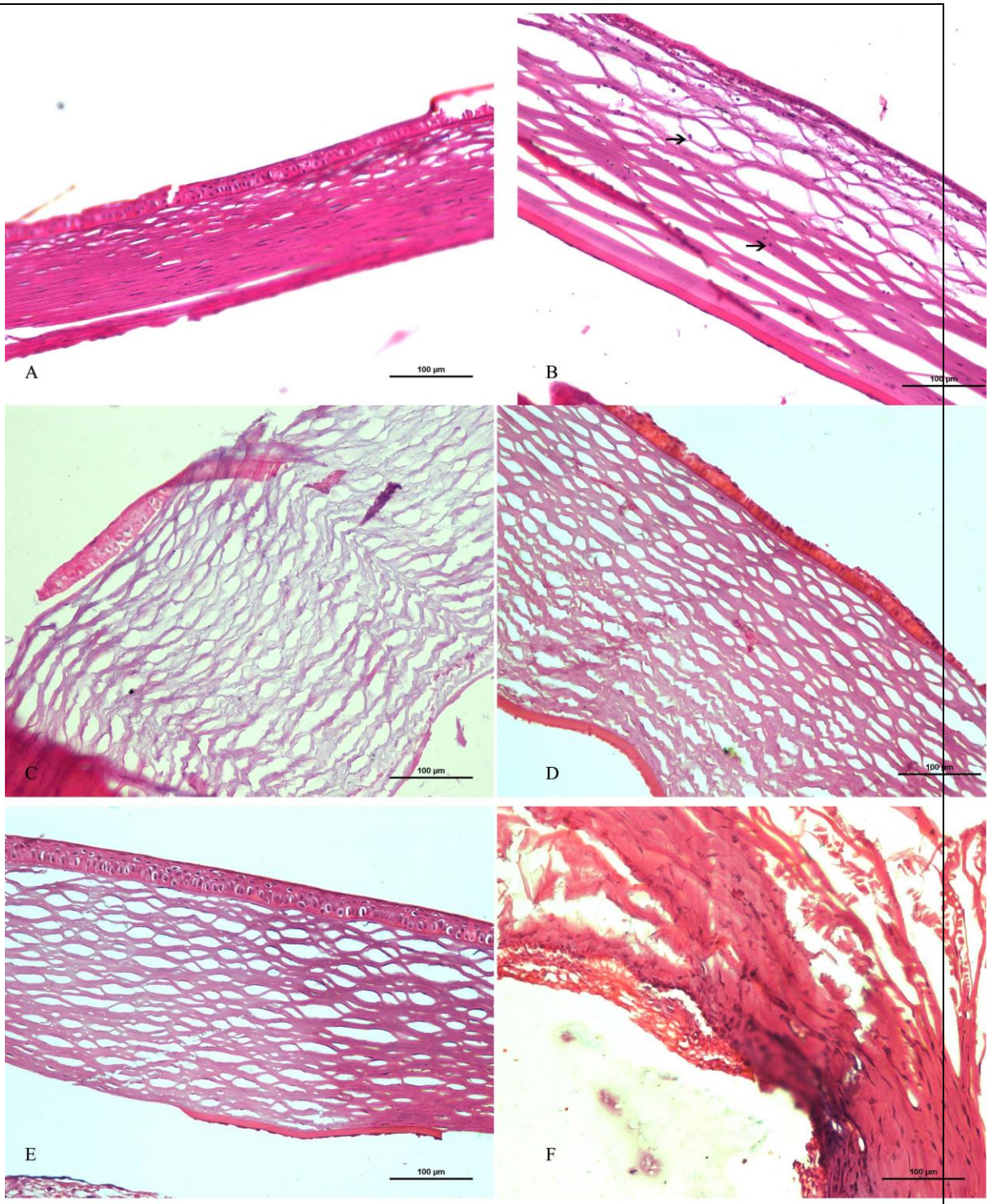


Figure : Histopathology of cornea using H&E staining. A) Control; B) CPC treated , arrows indicate macrophage infiltration; C) SS treated; D) IMI treated; E)ACT treated; and F) NIC treated.

Figure 56

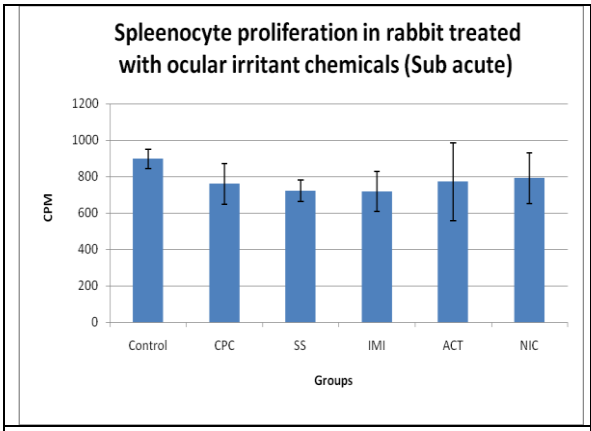


Figure 57: Spleenocyte proliferation in rabbit treated with ocular irritant chemicals (Sub acute)

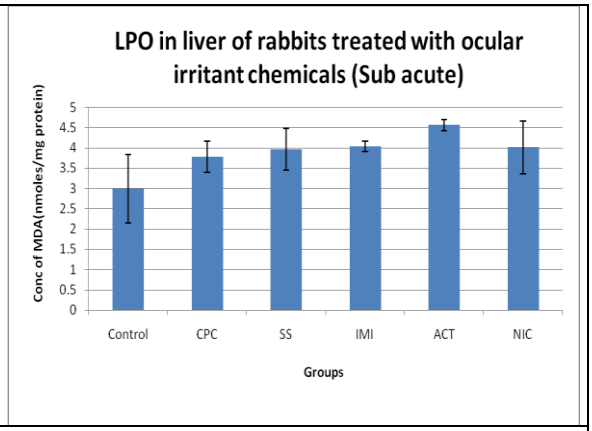


Figure 58: LPO in liver of rabbits treated with ocular irritant chemicals (Sub acute)

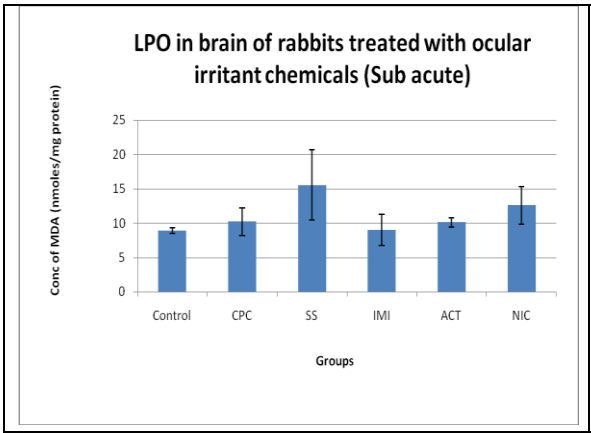


Figure 59: LPO in brain of rabbits treated with ocular irritant chemicals (Sub acute)

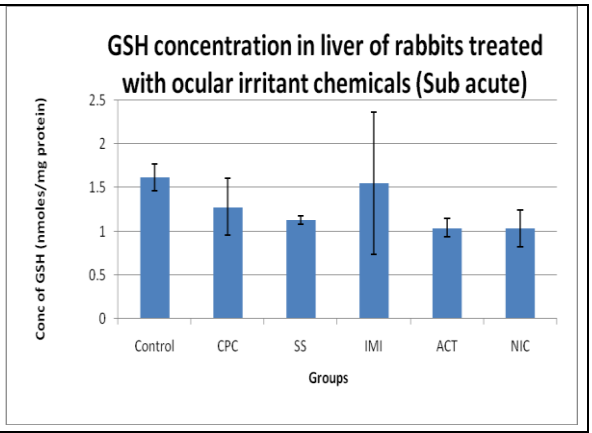


Figure 60: GSH concentration in liver of rabbits treated with ocular irritant chemicals (Sub acute)

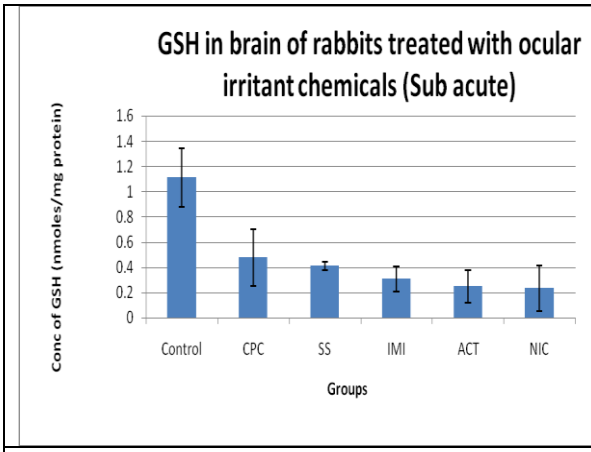


Figure 61: GSH in brain of rabbits treated with ocular irritant chemicals (Sub acute)

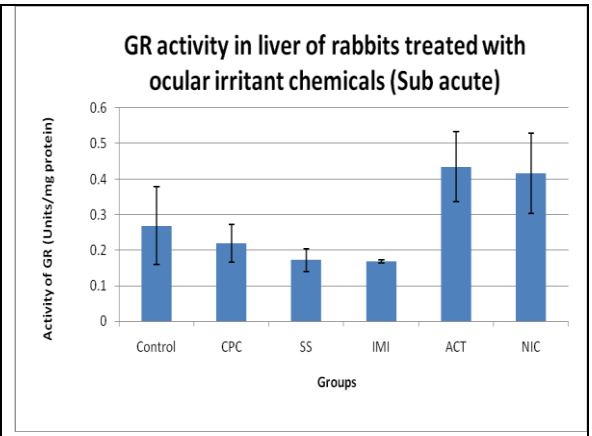


Figure 62: GR activity in liver of rabbits treated with ocular irritant chemicals (Sub acute)

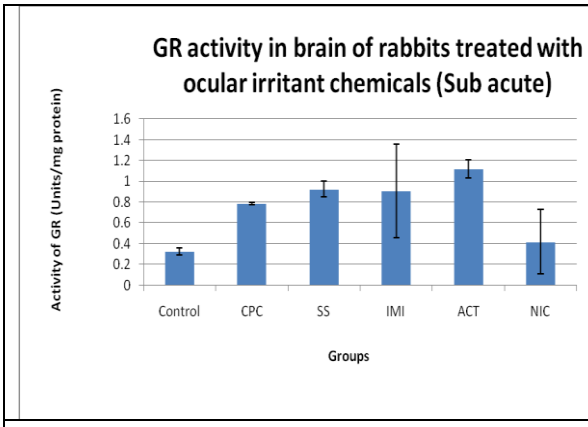


Figure 63: GR activity in brain of rabbits treated with ocular irritant chemicals (Sub acute)

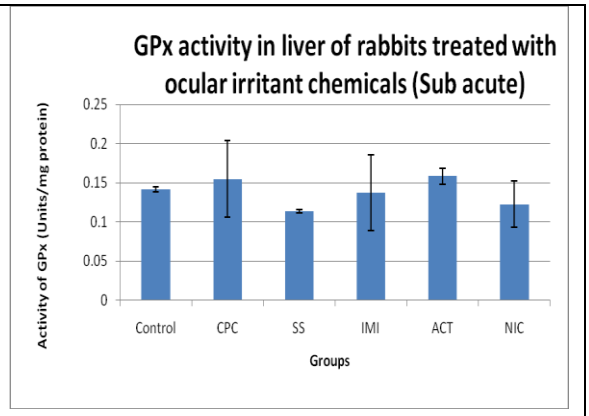


Figure 64: GPx activity in liver of rabbits treated with ocular irritant chemicals (Sub acute)

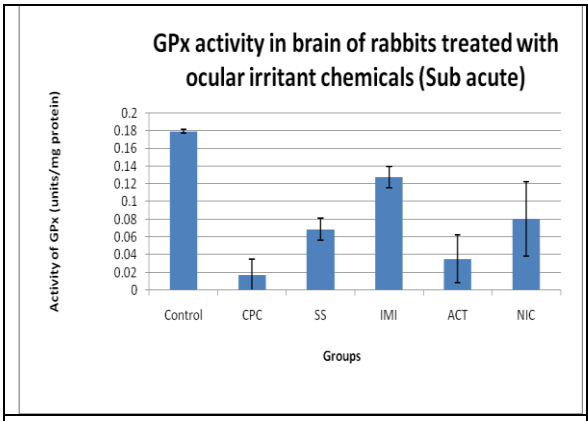


Figure 65: GPx activity in brain of rabbits treated with ocular irritant chemicals (Sub acute)

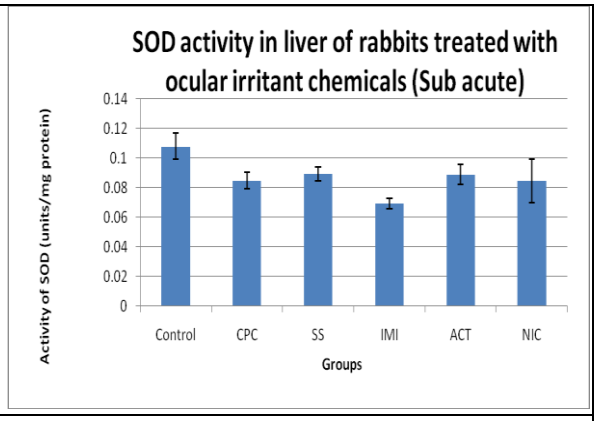


Figure 66: SOD activity in liver of rabbits treated with ocular irritant chemicals (Sub acute)

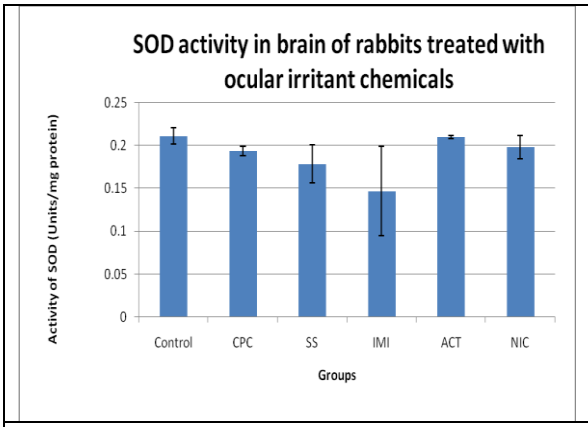


Figure 67: SOD activity in brain of rabbits treated with ocular irritant chemicals

		Control	CPC	SS	IMI	ACT	NIC
Cornea	Degree of opacity	0	0	1	0	0	0
	Area of cornea	0	3	2	2	2	2
Iris		0	2	2	1	2	2
Conjuctiva	Redness	0	3	2	2	2	2
	Chemosis	0	2	2	2	2	2
	Discharge	0	2	2	2	1	2

Table 1: *In vivo* scoring of rabbit eyes, after exposure to ocular irritant chemicals (acute).
CPC: Cetyl pyridinium chloride, SS: Sodium Salicylate, IMI: Imidazole ACT: Acetaminophen, NIC: Nicotinamide. All values are Mean±SD, n=5.
0: normal, 1: slight, 2: moderate, 3: severe

Parameters	Control	CPC	SS	IMI	ACT	NIC
HB (g/dL)	15.05± 2.62	12.07± 1.26	13.77± 1.10	13.50± 0.40	15.85± 3.18	13.70± 0.28
WBC X 10^3 /mm^3	5.70± 1.41	6.80± 3.54	4.63± 2.66	7.30± 2.35	5.55± 1.91	3.40± 1.13
RBCX 10^6/mm^3	7.08± 1.12	5.50± 0.76	6.42± 0.46	5.34± 1.63	7.18± 1.74	6.38± 0.17
PLT	229.0± 26.87	665.3± 55.43	642.3± 220.29	402.0± 49.51	533.0± 200.82	330.0± 90.51
MCV (µm^3)	69.50± 0.71	69.37± 2.78	67.13± 2.92	68.2± 1.90	69.45± 1.34	67.25± 0.92
MCH (pg)	21.70± 0.28	22.03± 0.74	21.43± 1.10	21.63± 0.85	22.25± 0.92	21.40± 0.14
MCHC (g/dL)	31.10± 0.14	31.70± 0.72	31.93± 0.25	31.73± 0.38	31.95± 0.63	31.85± 0.21

Table 2: Hematological parameters of rabbits treated with ocular irritant chemicals (acute)
CPC: Cetyl pyridinium chloride, SS: Sodium Salicylate, IMI: Imidazole ACT: Acetaminophen, NIC: Nicotinamide. All values are Mean±SD, n=5

Parameters	Control	CPC	SS	IMI	ACT	NIC
SGPT (IU/L)	44.00± 3.53	118.65 ±5.16	79.16± 6.81	85.06± 23.42	84.90± 2.88	84.90± 18.80
SGOT (IU/L)	21.30± 1.41	19.35± 6.15	22.86± 7.47	25.06± 4.77	20.33± 4.12	168.15± 19.70
ALP (IU/L)	61.00± 2.82	40.00± 5.65	35.66± 26.38	84.33± 11.06	54.33± 19.50	62.5± 19.09
GGT (IU/L)	10.85± 0.77	7.50± 0.84	5.33± 4.16	3.20± 2.36	12.26± 5.02	1.50± 0.56
Uric acid (mg/dL)	2.52± 3.06	0.23± 0.01	1.03± 0.49	2.41± 1.91	0.29± 0.07	2.21± 0.65

Calcium (mg/dL)	13.40± 0.14	13.75± 0.07	13.80± 0.79	14.83± 0.41	13.93± 1.10	14.20± 0.28
Phosphorous (mg/dL)	3.43± 0.17	4.32± 0.03	4.64± 0.80	5.33± 0.63	4.65± 0.16	5.26± 0.12
Chlorides (mmol/L)	104.3± 9.33	115.4± 1.34	110.7± 3.08	116.5± 1.47	109.2± 2.51	110.5± 1.62
Creatinine (mg/dL)	1.22± 0.24	1.05± 0.01	1.28± 0.42	1.09± 0.05	1.61± 0.82	0.92± 0.04

Table 3: Biochemical parameters of rabbits treated with ocular irritant chemicals (acute)
CPC: Cetyl pyridinium chloride, SS: Sodium Salicylate, IMI: Imidazole ACT: Acetaminophen, NIC: Nicotinamide. All values are Mean±SD, n=5

		Control	**CPC**	**SS**	**IMI**	**ACT**	**NIC**
Cornea	Degree of opacity	0	0	1	0	0	0
	Area of cornea	0	3	3	2	2	2
Iris		0	2	2	1	2	2
Conjuctiva	Redness	0	3	3	2	2	2
	Chemosis	0	2	2	2	2	2
	Discharge	0	3	2	2	1	2

Table 4: *In vivo* scoring of rabbit eyes, after exposure to ocular irritant chemicals (sub acute).
CPC: Cetyl pyridinium chloride, SS: Sodium Salicylate, IMI: Imidazole ACT: Acetaminophen, NIC: Nicotinamide. All values are Mean±SD, n=5.
0: normal, 1: slight, 2: moderate, 3: severe

Parameters	**Control**	**CPC**	**SS**	**IMI**	**ACT**	**NIC**
HB (g/dL)	15.05± 2.62	12.00± 0.87	13.13± 0.59	12.90± 0.57	13.47± 1.19	13.27± 1.27
WBC X $10^3/mm^3$	5.70± 1.41	6.27± 1.57	5.73± 2.52	8.05± 2.19	4.17± 1.93	6.07± 2.99
RBCX $10^6/mm^3$	7.08± 1.12	5.78± 0.25	5.97± 0.48	5.97± 0.36	6.21± 0.68	6.40± 0.79
PLT	229.00± 26.87	552.00± 378.71	339.46± 289.35	495.5± 238.29	316.33± 65.58	335.59± 291.77
MCV (μm^3)	69.50± 0.71	65.87± 1.83	69.60± 3.12	68.20± 0.84	68.00± 1.35	66.67± 2.87
MCH (pg)	21.70± 0.28	20.73± 0.55	22.07± 1.17	21.55± 0.35	21.70± 0.61	20.77± 0.76
MCHC (g/dL)	31.10± 0.14	31.50± 0.10	31.70± 0.44	31.60± 0	31.87± 0.50	31.20± 0.17

Table 5: Hematological parameter of rabbits treated with ocular irritant chemicals (sub acute)
CPC: Cetyl pyridinium chloride, SS: Sodium Salicylate, IMI: Imidazole ACT: Acetaminophen, NIC: Nicotinamide. All values are Mean±SD, n=5

Parameters	Control	CPC	SS	IMI	ACT	NIC
SGPT (IU/L)	44.00± 3.53	74.43± 21.60	99.16± 16.46	106.03± 18.33	103.93± 20.11	111.96± 13.47
SGOT (IU/L)	21.30± 1.41	18.20± 3.47	18.06± 4.82	17.70± 0.95	23.53± 5.11	16.66± 3.84
ALP (IU/L)	61.00± 2.82	108.66± 37.52	72.33± 50.33	102.00± 56.78	64.33± 9.71	89.66± 39.31
GGT (IU/L)	10.85± 0.77	6.13± 5.82	11.83± 2.97	11.26± 1.71	5.36± 1.12	8.20± 3.11
Uric acid (mg/dL)	2.53± 3.06	0.50± 0.54	0.11± 0.16	0.05± 0.09	0.68± 0.72	0.72± 0.01
Calcium (mg/dL)	13.40± 0.14	12.76± 0.50	13.86± 2.04	13.00± 0.78	13.20± 0.61	13.13± 0.11
Phosphorous(mg/d	3.43± 0.17	4.72± 0.78	4.96± 0.38	4.87± 0.69	4.92± 0.07	4.76± 0.27
Chlorides(mmol/L)	104.3± 9.33	112.3± 3.60	108.7± 3.43	115.4± 3.72	112.36± 2.35	110.7± 1.58
Creatinine(mg/dL)	1.22± 0.24	0.97± 0.12	1.35± 0.70	1.146± 0.08	1.12± 0.13	1.04± 0.07

Table 6: Biochemical parameter of rabbits treated with ocular irritant chemicals (sub acute)
CPC: Cetyl pyridinium chloride, SS: Sodium Salicylate, IMI: Imidazole ACT: Acetaminophen, NIC: Nicotinamide. All values are Mean±SD, n=5