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THERAPEUTIC APPROACHES FOR OBSERVING THE ANTI-INFLAMMATORY AND ANTI-NOCICEPTIVE EFFECTS OF GLYCYRRHIZIN AND KETOROLAC IN CFA-INDUCED INFLAMMATORY PAIN IN MICE

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ABSTRACT

Inflammatory mediators and cytokines are responsible for the inflammatory pain responses and their inhibition represents as the therapeutic approach in controlling chronic pain. Glycyrrhizin and ketorolac act as anti-inflammatory and anti-nociceptive. In this present study, the combination of both the drugs are used as the treatment for inflammatory pain. Male Swiss mice were treated with this combination after the 24hours of CFA (Complete freunds adjuvant) induction. CFA is used as inducer in mice for inflammatory pain model. Both these drugs inhibit the mechanical and thermal hyperalgesia. Glycyrrhizin is used to block the High Mobility box protein-1 (HMGB1) and Ketorolac acts on the Transient receptor potential vanilloid-1 (TRPV1) receptors thus they further block the inflammatory pathway. Indomethacin is used as a standard and it is the most used Non-steroidal anti-inflammatory drugs (NSAIDS). Indomethacin when used for prolonged time, might cause gastric injury. So, the new treatment must come into picture for treating the inflammatory pain.

KEYWORDS: Inflammatory pain, Complete freunds adjuvant, HMGB1, TRPV1.

INTRODUCTION

Pain is a clinical issue which is highly associated with various diseases and it affects the individuals of all the ages.[1] The International Association for the Study of Pain defines pain as "an unpleasant sensory emotional experience with actual or potential tissue damage, and is

described in terms of damage".[2] Tissue injury causes a signaling threat, which gives rise to a pain.[3] Pain acts as an alarm that helps the body to defend itself from potential or actual tissue damage thus avoiding noxious stimuli.[4] The sensitivity of the nerve free endings of sensory neurons(nociceptors) increases, when the tissue activity is distorted by heat, infections, toxins, and other related stress which then aids in the Inflammatory pain. The increased in sensitivity of nociceptors are termed as peripheral sensitization. Peripheral sensitization helps to cause pain hypersensitivity. Pain hypersensitivity is also caused due to central sensitization. In central sensitization, tissue releases neurotransmitters from central nociceptors which results in the production of proinflammatory mediators in the spinal cord.[5]

A complex biological immune response occurs in the vascular tissues due to harmful stimuli such as pathogens, cellular damage and irritants. Activation of immune cells (ex. macrophages) occurs when the inflammatory responses are initiated. This plays a crucial role in regulation of immune responses and helps in secretion of the mediators.[6] Pain and Inflammation are very related to each other and it also process the release of common mediators.[7] For Inflammatory pain, new treatments are the need of the hour which decreases the acute pain and helps in overall prevention of chronic pain.[8]

High mobility box protein-1 (HMGB1), a nuclear protein that acts as the main mediator in acute and chronic pain. It is earmarked as the main biomarker for the inflammatory pain. HMGB1 act as the alarm clock for the DNA and participates in intracellular and extracellular activities. It also alerts the nearby cells during harmful stimuli and triggers the inflammation. Active and Passive are the two pathways through which HMGB1 is released from the cells. Passively, HMGB1 is secreted from all dead cells and actively it is secreted during the stress and inflammatory conditions.[9] Recent studies indicate that Glycyrrhizin, a major active component of roots and rhizomes of licorice *(Glycyrrhiza glabra*) acts mainly by inhibiting high mobility box protein.[10]

The transient receptor vanilloid potential 1 (TRPV1), is a pathological receptor which on sensitization plays an important role in inflammatory pain.[11] Thermal and mechanical stimuli are sensitized, when the inflammatory mediators bind to the TRPV1 receptor. As a result TRPV1 acts as a central player for thermal sensitization.[12] One of the best treatments to inactivate the TRP channels are by using NSAIDS. Most commonly used NSAID's like Ketorolac, attenuates the nociceptive and hyperalgesia effects which arises due to activation of TRPV1 receptor.

1. MATERIALS AND METHODS

1.1 DRUGS AND CHEMICALS

Glycyrrhizin (Gly), Indomethacin was purchased from the TCI chemicals, India. Complete freunds adjuvant (CFA) was purchased from sigma Aldrich chemicals company, India. Ketorolac (Ket) was provided as a gift sample from symed labs, India. Ketorolac and Glycyrrhizin were dissolved in distilled water and Indomethacin was dissolved in sodium bicarbonate.[13] All other chemicals were of analytical grade and obtained from the standard suppliers. CFA (10µl) was induced intra- plantar, Indomethacin (2.5mg/kg)[14], Glycyrrhizin (10mg/kg)[10] and ketorolac (3.5mg/kg, 5mg/kg) were induced intra-peritoneally (i.p).

1.2 ANIMALS

The experiments were carried using male Swiss albino mice weighing 20-30g from National Institute of Bioscience. They were housed in Perspex cages (4 mice per cage) and maintained under standard laboratory conditions [14h:10h dark/light cycle, a temperature of (22±2 ^{O}C), and 50-70% humidity]. The animals were fed on standard food pellets and drinking water ad libitum. Before starting an experiment, animals were acclimatized for the period of one week. The entire experimental protocol was reviewed and approved by an Institutional Animal Ethics Committee (Approval Number: CPCSEA/IAEC/P-31 /2018) registered under "Committee for the purpose of Control and Supervision of Experiments on Laboratory Animals"(CPCSEA), Ministry of Environment and Forests, Government of India.

1.3 INFLAMMATORY PAIN MODEL

For induction of inflammatory pain, mice received an intraplantar injection of CFA (10µl) in their right hind paw. They were randomly divided into eight groups (n=7). Group 1 served as a normal control receiving the same volume of saline on right hind paw. Group 2 served as a negative group receiving only CFA. Group 3 served as the standard group receiving indomethacin (2.5mg/kg) intraperitoneally for consecutive seven days after 24hrs of CFA induction.[15] Group 4 served as the monotherapy group receiving Ketorolac (3.5mg/kg). Group 5 served as the ketorolac (5mg/kg) receiving group. Group 6 served as the monotherapy group receiving Glycyrrhizin (10mg/kg). Group 7 served as a combination group receiving the Ketorolac (3.5mg/kg) along with Glycyrrhizn (10mg/kg). Group 8 receiving ketorolac (5mg/kg) + Glycyrrhizin (10mg/kg). All these groups were administered with treatments intraperitoneally in mice for consecutive 7 days after the 24 hours of induction. After the completion of the administration of the drugs, the animals were sacrificed and their

spinal cord and serum was isolated for biochemical parameters and paw tissue was removed for histopathology.

1.4 EVALUATION OF LOCOMOTER ACTIVITY

2.4 i Actophotometer

Actophotometer test was performed to examine the effects of drugs and its combination on spontaneous locomotor activity.

Procedure

Animals were kept in a darkened room for at least 1 hour before the experiment. Mice were treated with inducer and also with treatment prior to test and transferred them to respective home cages for the required injection-test interval, and then individually shifted to the actophotometer for 5 minutes test.[16, 17] Same animals were used for the test. It consists of six built in photo-sensors and 4-digit digital counter. Digital counter measures the movements of mice. When the beam of light fall on, the photocell is cut off by the animal, the count is recorded in the digital counter. The locomoter activity was measured by placing animal in actophometer and studied for 5 minutes and the number of counts was recorded. The locomoter activity was stated in terms of total photo beam interruption counts/animals. Test was performed 24 hours after CFA injection and after the treatment on 1st day and on 7th day.

2.5 EVALUATION OF NOCICEPTIVE ACTIVITY-

2.5 i Hot-plate

This parameter was performed to examine the effects of drugs and its combination on the thermal nociceptive threshold. It is mainly done to see the effects of pain response in animals.

Procedure

It is used for behavioral model of nociception where the behaviors such as jumping and hind paw licking are considered. A transparent glass cylinder is used to keep the animal on the heated surface of the plate. Mice were placed on a metallic surface apparatus, maintained at 55^{O}C.[18,19] The hindpaw reflex was registered as paw withdrawal latency (PWL). The reaction time was recorded when the animals licked their hindpaw or jumped. Maximum latency (cut-off) was set at 10 s to avoid tissue damage. Time was noted and was compared with the normal group. Test was performed 24 hours after CFA injection and after the treatment on 1st day and on 7th day.

2.5 ii Randall Selitto

The nociceptive withdrawal threshold was assessed using randall selitto. Before the test, each animal received 5min of handling to get acclimatized.

Procedure

The mechanical hyperalgesia was evaluated by using randall selitto. Mice were placed in the soft cotton cloth and carefully with the hand the tested paw was hold. The tip of the instrument was applied perpendicular to the central part of the paw with the maximum pressure. The removal of paw or jerk movement by animal was seen and the intensity of pressure was recorded. The maximum pressure of the treatment, standard and negative group was compared with the normal control group. The baseline evaluation was recorded for each group before induction.[20] Test was performed 24 hours after CFA injection and after the treatment on 1st day and on 7th day.

2.6 PAW EDEMA MEASUREMENT

Paw edema was measured as the difference between paw thickness in mm (millimeter) from baseline evaluation (before induction) using digital caliper.[21] The difference between groups indicated the degree of pain due to inflammation. Test was performed 24 hours after CFA injection and after the treatment on 1st day and on 7th day.

2.7 EVALUATION OF BIOCHEMICAL ASSAYS

2.7 i Tumor necrosis factor-alpha (TNF-alpha) level determination

After completion of study the animals were sacrificed and spinal cord and serum were collected. They were rinsed in cold saline to remove excess blood and then stored at -80^{O}C before homogenization. Both the tissues were minced and homogenized in glass homogenizer with PBS. Thawed at 2-8^{O}C or freeze at -20^{O}C. Homogenates were then transferred to 1.5ml eppendrof's tubes, centrifuged at 3000g for 10min. Further procedure for determining the level of TNF-alpha was followed by using ELISA as per the manufacturer's protocol. The absorbance was measured using a microplate reader at 450nm.

2.7 ii High mobility group box 1(HMGB1) level determination

After completion of study the animals were sacrificed and spinal cord and serum were collected. They were rinsed with cold saline to remove excess blood and stored at -80^{O}C. The tissues were minced and homogenized in glass homogenizer with PBS and 1% of triton X-100. Thawed at 2- 8^{O}C. Homogenates were then transferred to 1.5ml eppendrof's tubes,

centrifuged at 3000g for 15min. Further procedure for determining the level of HMGB1 was followed by using ELISA as per the manufacturer's protocol. The absorbance was measured using a microplate reader at 450nm.

2.8 HISTO-PATHOLOGY STUDY

At the end of the studies two mice from each group were sacrificed and their paw were in fixed in 10% formalin and given for histopathological evaluation by staining with Haematoxylin and Eosin (H & E).

2.9 STATISTICAL ANALYSIS

The statistical analysis was performed with the assistance of GraphPad prism 5 for 32bit Windows version. All the experiment groups were compared to assess the statistical significance using One way and Two way ANOVA (Analysis Of Variance) with dunnet test and Bonferroni test respectively.

2. RESULTS

Complete freunds adjuvant induction showed some behavioral changes. Treatment with drugs alters these changes. Different concentration of ketorolac, glycyrrhizin, and indomethacin showed significant changes in motor functions.

2.1 DRUGS EFFECT IN MOTOR ACTIVITY

3.1 i Actophotometer

The locomotor activity of mice was altered due to CFA induction. On the day 0, 1 and 7 they showed the different activities. CFA induced inflammatory pain in negative group was associated with decreased in locomotor counts as compared to normal group ($^{###}p<0.001$). Standard group showed significant increase in locomotor counts than the negative group (***$p<0.001$). In monotherapy groups mice also showed some changes in counts as compared to negative group but not as significant as combination groups. However, Combination groups showed significant improvement in locomotor counts as compared to the negative group.

Table no 1: Depicts the effects of treatments on locomotor counts (day0).

SR.NO	GROUPS	LOCOMOTOR COUNTS
1.	Normal	559 ± 27.575
2.	Negative	540.166 ± 59.973
3.	Standard	526.666 ± 27.796
4.	CFA+ Ketorolac(3.5 mg/kg)	545 ± 26.437
5.	CFA+ Ketorolac(5mg/kg)	529.166± 28.193
6.	CFA+ Glycyrrhizin(10mg/kg)	518.166± 67.017
7.	CFA+Gly(10mg/kg)+Ket(3.5mg/kg)	580 ±6.350
8.	CFA+ Gly(10mg/kg) + Ket (5mg/kg)	554.666 ±28.032

Data was analyzed by using two way ANOVA followed by Bonferroni test. Values are expressed as Mean ± SEM (n=7). The value indicates the effect of drugs in locomotor counts on day 0.

Table no 2: depicts the effects of different treatments on locomotor counts (day1).

SR.NO	GROUPS	LOCOMOTOR COUNTS
1.	Normal	608.6667 ± 2.973961$^{###}$
2.	Negative	312 ± 37.57215$^{###}$
3.	Standard	302 ± 16.0333$^{###}$
4.	CFA+ Ketorolac(3.5 mg/kg)	295.8333 ± 27.01779$^{##}$
5.	CFA+ Ketorolac(5mg/kg)	298.8333 ± 23.2141$^{###}$
6.	CFA+ Glycyrrhizin(10mg/kg)	279.3333 ± 49.9097$^{###}$
7.	CFA+Gly(10mg/kg)+Ket(3.5mg/kg)	273.6667 ± 3.323318$^{###}$
8.	CFA+Gly (10mg/kg) + Ket (5mg/kg)	298.5 ± 19.14288$^{###}$

Data was analyzed by using two way ANOVA followed by Bonferroni test. Values are expressed as Mean ± SEM (n=7). $^{#}p<0.05$, $^{##}p<0.01$, $^{###}p<0.001$ when compared with control group. The value indicates the effects of drugs in locomotor counts on day 1.

Table no 3: depicts the effects of different treatments on locomotor counts (day7).

SR.NO	GROUPS	LOCOMOTOR COUNTS
1.	Normal	533.6667 ± 42.71274****
2.	Negative	244.3333 ± 43.47617
3.	Standard	447.6667 ± 29.30719***
4.	CFA+ Ketorolac(3.5 mg/kg)	203 ± 18.980
5.	CFA+ Ketorolac(5mg/kg)	313.8333 ± 20.01402
6.	CFA+ Glycyrrhizin(10mg/kg)	329 ± 48.93737
7.	CFA+Gly(10mg/kg)+Ket(3.5mg/kg)	362.5 ± 7.6278
8.	CFA+Gly (10mg/kg) + Ket (5mg/kg)	396 ± 15.418*

*Data was analyzed by using two way ANOVA followed by Bonferroni test. Values are expressed as Mean ± SEM (n=7). *p<0.05, **p<0.01, ***p<0.001 when compared with Negative group. The value indicates the effects of drugs in locomotor counts on day 7.*

3.2 DRUGS EFFECT IN NOCICEPTIVE ACTIVITY-

3.2 i Hotplate

The behavioral activity of mice was altered due to CFA induction. On the day 0, 1 and 7 they showed the different activities. CFA induced inflammatory pain in negative group was associated with decreased in time spend on hot plate as compared to normal group ($^{****}p<0.001$). Standard group showed significant increase in jump/lick time (secs) than the negative group ($^{###}p<0.001$). In monotherapy groups they also showed some improvement changes in licking time as compared to negative group but not as significant as combination groups. However, Combination groups showed improvement in jumping/licking time as compared to the negative group.

Table no 4: depicts the effects of different treatments on licking time in seconds (day0).

SR.NO	GROUPS	LICKING/JUMPINGTIME
1.	Normal	6.408333 ± 0.237465
2.	Negative	6.353333 ± 0.429159
3.	Standard	6.426667 ± 0.366103
4.	CFA+ Ketorolac(3.5 mg/kg)	6.345 ± 0.310631
5.	CFA+ Ketorolac(5mg/kg)	6.418333 ± 0.478159
6.	CFA+ Glycyrrhizin(10mg/kg)	6.361667 ± 0.417448
7.	CFA+Gly(10mg/kg)+Ket(3.5mg/kg)	6.31 ± 0.341086
8.	CFA+Gly (10mg/kg) + Ket (5mg/kg)	6.261667 ± 0.175431

Data was analyzed by using two way ANOVA followed by Bonferroni test. Values are expressed as Mean ± SEM (n=7).The value indicates the time taken by mice for licking/ jumping in seconds on day 0.

Table no 5: depicts the effects of different treatments in time taken by mice for licking/jumping in seconds (day1).

SR.NO	GROUPS	LICKING/JUMPING TIME
1.	Normal	6.398333 ± 0.410645
2.	Negative	2.45± 0.132332****
3.	Standard	2.138333 ± 0.172905****
4.	CFA+ Ketorolac(3.5 mg/kg)	2.013333 ± 0.205112****
5.	CFA+ Ketorolac(5mg/kg)	2.081667 ± 0.109344****
6.	CFA+ Glycyrrhizin(10mg/kg)	2.161667 ± 0.26909****
7.	CFA+Gly(10mg/kg)+Ket(3.5mg/kg)	4.401667 ± 0.500582****
8.	CFA+Gly (10mg/kg) + Ket (5mg/kg)	1.988333 ± 0.179061****

Data was analyzed by using two way ANOVA followed by Bonferroni test. Values are expressed as Mean ± SEM (n=7). $^{****}p<0.0001$ *when compared with control group. The value indicates the time taken by mice for licking/ jumping in seconds on day 1.*

Table no: 6 depicts the effects of different treatments in time taken by mice for licking/jumping in seconds (day7).

SR.NO	GROUPS	LICKING/JUMPING TIME
1.	Normal	6.45±0.161576$^{###}$
2.	Negative	5.321667±0.417001
3.	Standard	1.9±0.080333$^{###}$
4.	CFA+ Ketorolac(3.5 mg/kg)	3.023333±0.157917
5.	CFA+ Ketorolac(5mg/kg)	3.27±0.0999$^{#}$
6.	CFA+ Glycyrrhizin(10mg/kg)	3.055±0.623521
7.	CFA+Gly(10mg/kg)+Ket(3.5mg/kg)	4.711667±0.2525$^{##}$
8.	CFA+Gly (10mg/kg) + Ket (5mg/kg)	5.16±0.247911$^{##}$

Data was analyzed by using two way ANOVA followed by Bonferroni test. Values are expressed as Mean ± SEM (n=7). $^{#}p<0.05$, $^{##}p<0.01$, $^{###}p<0.001$ when compared with negative group. The value indicates the time taken by mice for licking/ jumping in seconds on day 7.

3.2 ii Randall Selitto

The behavioral activity of mice was altered due to CFA induction. On the day 0, 1 and 7 they showed the different activities. CFA induced inflammatory pain in negative group was associated with decreased in maximum force in gram as compared to normal group ($^{****}p<0.0001$). Standard group showed significant increase in maximum force than the negative group ($^{###}p<0.001$). In monotherapy groups mice also showed some improvement in maximum force as compared to negative group but not as significant as combination groups. However, Combination groups showed significant improvement in maximum force as compared to the negative group.

Table no: 7 depicts the effects of different treatments in mice by randall sellito (day0).

SR.NO	GROUPS	MAXIMUM FORCE(g)
1.	Normal	85.43333 ± 1.473695
2.	Negative	81.23333 ± 2.649109
3.	Standard	93.13333 ± 1.325812
4.	CFA+ Ketorolac(3.5 mg/kg)	83.53333 ± 3.14406
5.	CFA+ Ketorolac(5mg/kg)	84.91667 ± 4.146759
6.	CFA+ Glycyrrhizin(10mg/kg)	95.21667 ± 1.708297
7.	CFA+Gly(10mg/kg)+Ket(3.5mg/kg)	89.2 ± 2.381736
8.	CFA+Gly (10mg/kg) + Ket (5mg/kg)	88.98333 ± 1.92413

Data was analyzed by using two way ANOVA followed by Bonferroni test. Values are expressed as Mean ± SEM (n=7). The value indicates the maximum force in gram on day 0.

Table no 8: depicts the effects of different treatments in mice by randall sellito (day1).

SR.NO	GROUPS	MAXIMUM FORCE(g)
1.	Normal	84.58333 ± 1.234346
2.	Negative	12.25 ± 0.311716****
3.	Standard	12.83333 ± 0.196073****
4.	CFA+ Ketorolac(3.5 mg/kg)	13 ± 0.375943****
5.	CFA+ Ketorolac(5mg/kg)	13.56667 ± 0.623253****
6.	CFA+ Glycyrrhizin(10mg/kg)	9.733333 ± 0.573101***
7.	CFA+Gly(10mg/kg)+Ket(3.5mg/kg)	12.05 ± 0.384491***
8.	CFA+Gly (10mg/kg) + Ket (5mg/kg)	10.8 ± 0.554377***

Data was analyzed by using two way ANOVA followed by Bonferroni test. Values are expressed as Mean ± SEM (n=7). $^{****}p<0.0001$**,** $^{***}p<0.001$ *when compared with control group. The value indicates the maximum force in gram on day 1.*

Table no 9: depicts the effects of different treatments in mice by randall sellito (day7).

SR.NO	GROUPS	MAXIMUM FORCE(g)
1.	Normal	$84.8 \pm 1.327655^{###}$
2.	Negative	10.5 ± 0.334664
3.	Standard	$77.31667 \pm 1.011078^{###}$
4.	CFA+ Ketorolac(3.5 mg/kg)	$30.85 \pm 0.530252^{#}$
5.	CFA+ Ketorolac(5mg/kg)	$38.91667 \pm 0.735942^{#}$
6.	CFA+ Glycyrrhizin(10mg/kg)	$45.63333 \pm 0.943634^{##}$
7.	CFA+Gly(10mg/kg)+Ket(3.5mg/kg)	$50.83333 \pm 2.369763^{##}$
8.	CFA+Gly (10mg/kg) + Ket (5mg/kg)	$63.6\pm 0.811172^{###}$

Data was analyzed by using two way ANOVA followed by Bonferroni test. Values are expressed as Mean ± SEM (n=7). $^{#}p<0.05$, $^{##}p<0.01$, $^{###}p<0.001$ *when compared with negative group. The value indicates the effects of drugs on maximum force applied to mice paw on day 7.*

3.3 EFFECTS ON PAW EDEMA-

The paw edema of mice was altered due to CFA induction. On the day 0, 1 and 7 there were difference in paw edema. CFA induced inflammatory pain in negative group was associated with increased in paw volume as compared to normal group ($^{****}p<0.0001$). Standard group showed significant decrease in paw edema than the negative group ($^{###}p<0.001$). In monotherapy groups mice also showed some improvement in paw edema as compared to negative group but not as significant as combination groups. However, Combination groups showed significant improvement in paw volume as compared to the negative group.

Table no 10: depicts the effects of different treatments on paw edema (day0).

SR.NO	GROUPS	PAW EDEMA(mm)
1.	Normal	1.856667±0.009888
2.	Negative	1.816667±0.006146
3.	Standard	1.801667±0.009098
4.	CFA+ Ketorolac(3.5 mg/kg)	1.871667±0.004773
5.	CFA+ Ketorolac(5mg/kg)	1.808333±0.011949
6.	CFA+ Glycyrrhizin(10mg/kg)	1.813333±0.006146
7.	CFA+Gly(10mg/kg)+Ket(3.5mg/kg)	1.806667±0.011738
8.	CFA+Gly (10mg/kg) + Ket (5mg/kg)	1.84±0.014376

Data was analyzed by using two way ANOVA followed by Bonferroni test. Values are expressed as Mean ± SEM (n=7). The value indicates the paw volume in mm on day 0.

Table no 11: depicts the effects of different treatments on paw edema (day1).

SR.NO	GROUPS	PAW EDEMA(mm)
1.	Normal	1.856667±0.009888
2.	Negative	2.65±0.011547****
3.	Standard	2.621667±0.011949****
4.	CFA+ Ketorolac(3.5 mg/kg)	2.691667±0.006009***
5.	CFA+ Ketorolac(5mg/kg)	2.68±0.013904****
6.	CFA+ Glycyrrhizin(10mg/kg)	2.698333±0.006009****
7.	CFA+Gly(10mg/kg)+Ket(3.5mg/kg)	2.661667±0.015147****
8.	CFA+Gly (10mg/kg) + Ket (5mg/kg)	2.685±0.006191****

Data was analyzed by using two way ANOVA followed by Bonferroni test. Values are expressed as Mean ± SEM (n=7). $^{****}p<0.0001$**,** $^{***}p<0.001$ *when compared with control group. The value indicates the paw volume in mm on day 1.*

Table no: 12 depicts the effects of different treatments on paw edema (day7)

SR.NO	GROUPS	PAW EDEMA(mm)
1.	Normal	1.856667±0.009888$^{###}$
2.	Negative	2.775±0.015438
3.	Standard	2.003333±0.01453$^{###}$
4.	CFA+ Ketorolac(3.5 mg/kg)	2.605±0.005$^{#}$
5.	CFA+ Ketorolac(5mg/kg)	2.52±0.009661$^{#}$
6.	CFA+ Glycyrrhizin(10mg/kg)	2.383333±0.008028$^{#}$
7.	CFA+Gly(10mg/kg)+Ket(3.5mg/kg)	2.176667±0.013824$^{##}$
8.	CFA+Gly (10mg/kg) + Ket (5mg/kg)	2.075±0.014083$^{##}$

Data was analyzed by using two way ANOVA followed by Bonferroni test. Values are expressed as Mean ± SEM (n=7). $^{#}p<0.05$, $^{##}p<0.01$, $^{###}p<0.001$ *when compared with negative group. The value indicates the effects of drugs on paw volume in mm on day 7.*

3.4 EFFECTS ON BIOLOGICAL PARAMETERS

3.4 i Estimation of HMGB1 levels

Fig 1 & 2: Indicates the level of HMGB1 in mice spinal cord tissue homogenate and in serum (ng/ml). HMGB1 levels were elevated in the group receiving only complete freund's adjuvant. Standard group showed the decrease in the HMGB1 level compared to the treatment groups. Combination groups showed the significant decrease in the HMGB1 level compared to monotherapy groups. Normal group showed minimum HMGB1 levels as compared to remaining groups.

HMGB1-SPINALCORD

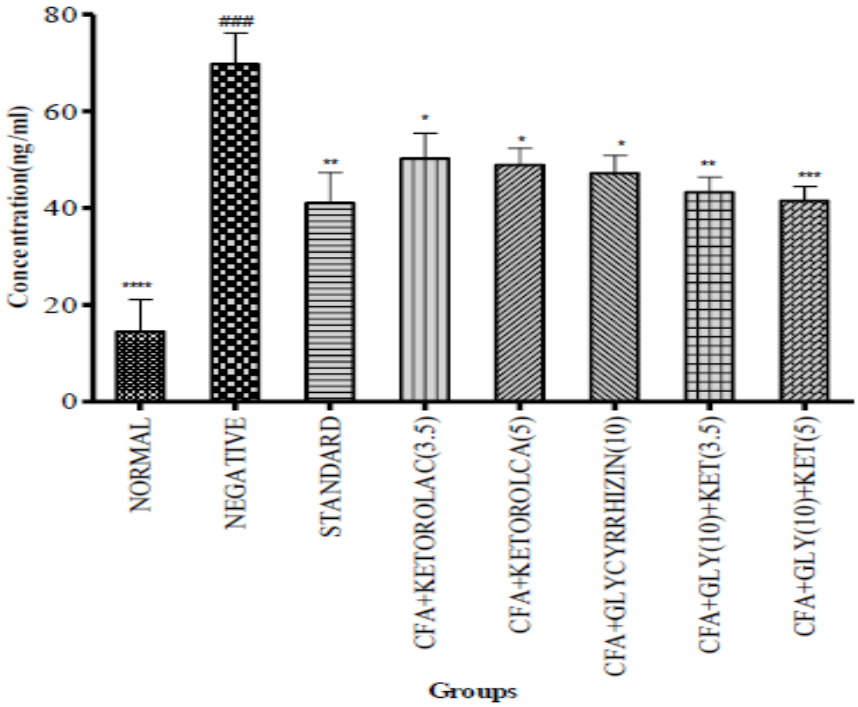


Fig 1: HMGB1 level in spinal cord of animals..

Data was analyzed by one way analysis of variance (ANOVA) followed by Dunnett test. Values are expressed as Mean ± SEM (n=4). $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ when compared with Normal group. *p<0.05, **p<0.01, ***p<0.001 when compared with Negative group.

HMGB1-SERUM

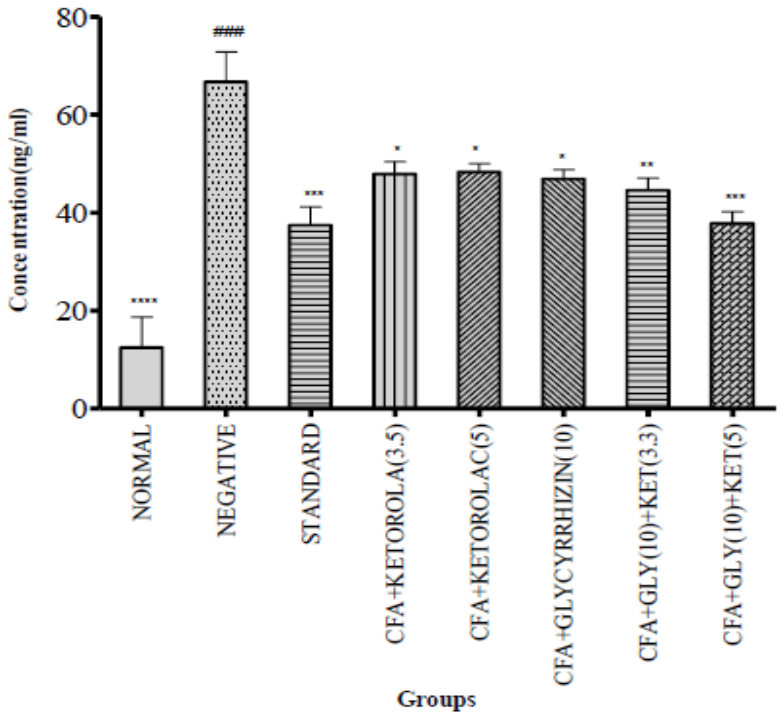


Fig 2: HMGB1 level in serum of animals.

Data was analyzed by one way analysis of variance (ANOVA) followed by Dunnett test. Values are expressed as Mean ± SEM (n=4). $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ when compared with Normal group. *p<0.05, **p<0.01, ***p<0.001 when compared with Negative group.

3.4 ii Estimation of TNF-α levels

Fig 3 & 4: Indicates the level of TNF-α in mice spinal cord tissue homogenate and in serum (ng/ml). TNF-α levels were elevated in the group receiving only complete freund's adjuvant. Standard group showed the decrease in the TNF-α level compared to the treatment groups. Combination groups showed the significant decrease in the TNF-α level compared to monotherapy groups. Normal group showed minimum TNF-α levels as compared to remaining groups.

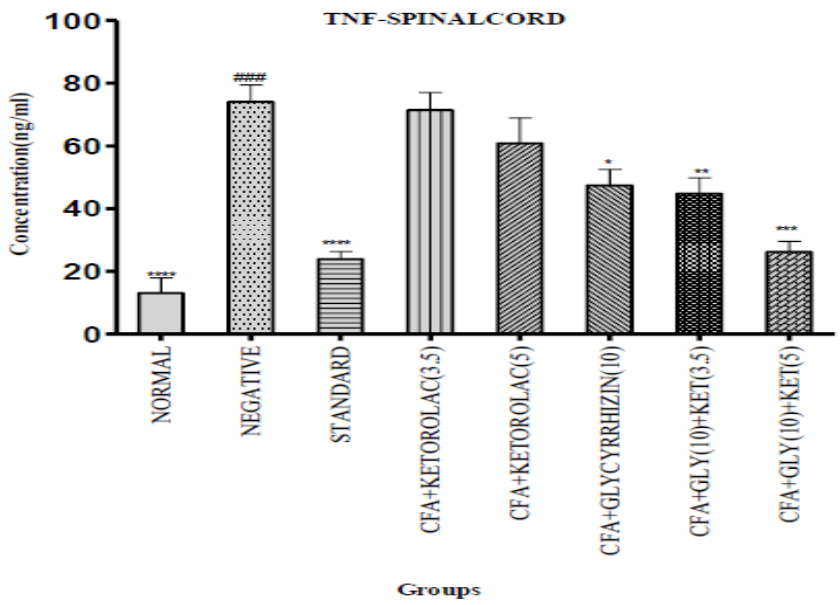


Fig 3: TNF-α level in spinal cord of animals.

The value are expressed as Mean±SEM (n=7). Data was analysed by one way analysis of variance (ANOVA) followed by Dunnett test. #p<0.05, ##p<0.01, ###p<0.001 when compared with Normal group. *p<0.05, **p<0.01, ***p<0.001 when compared with Negative group.

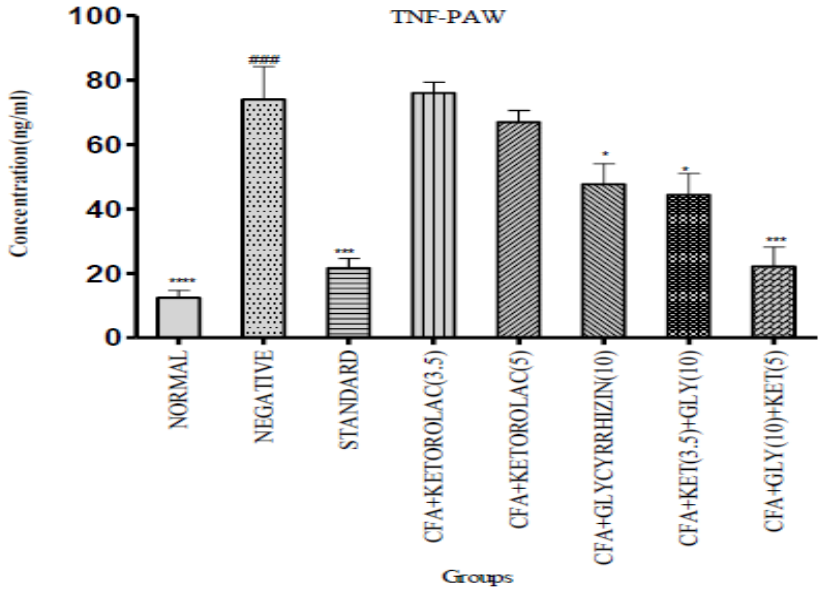
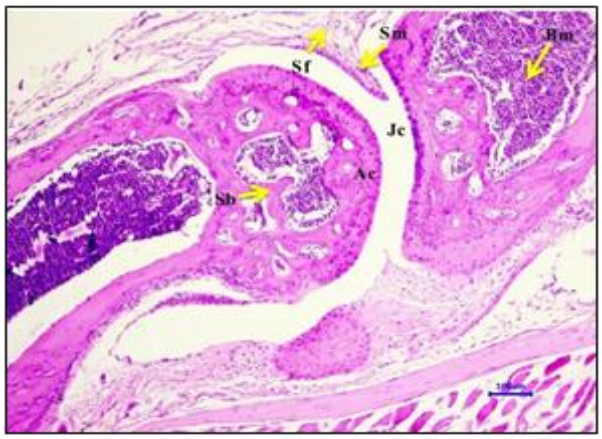


Fig 4: TNF-α level in paw of animals.

Data was analysed by one way analysis of variance (ANOVA) followed by Dunnett test. #p<0.05, ##p<0.01, ###p<0.001 when compared with Normal group. *p<0.05, **p<0.01, ***p<0.001 when compared with Negative group.

3.5 HISTOPATHOLOGICAL EXAMINATION OF PAW EDEMA

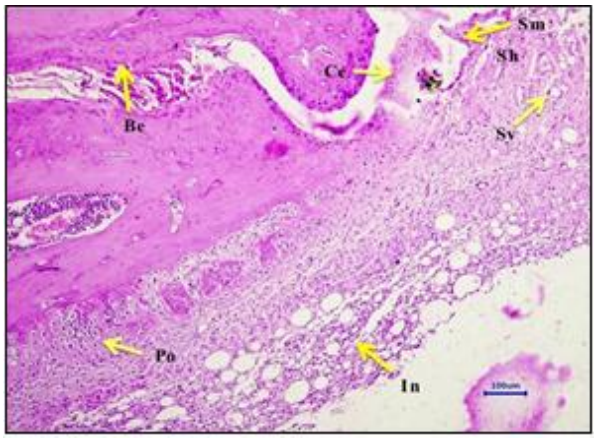
- Mice of normal group did not reveal any lesion of pathological significance.
- Mice of negative group showed multifocal mild to moderate enlargement of synovial lining cell layer, multifocal marked to severe synovial hyperplasia, multifocal moderate increased synovial vascularity, diffuse severe infiltration of inflammatory cells in synovial area.
- Mice treated with standard drug showed multifocal minimal enlargement of synovial lining cell layer, multifocal minimal to mild synovial hyperplasia and multifocal mild to moderate infiltration of inflammatory cells in synovial area.
- Mice of treated with Test drug Glycyrrhizin at 10 mg/kg showed multifocal minimal enlargement of synovial lining cell layer, multifocal mild to moderate synovial hyperplasia, multifocal minimal increased synovial vascularity, multifocal mild to diffuse moderate infiltration of inflammatory cells in synovial area.
- Mice of treated with Test drug Ketorolac at 5 mg/kg showed focal minimal enlargement of synovial lining cell layer, multifocal mild synovial hyperplasia, multifocal minimal to mild increased in synovial vascularity, multifocal minimal to moderate infiltration of inflammatory cells in synovial area.
- Mice of treated with combination of Test drug Glycyrrhizin & Ketorolac at 10 + 5 mg/kg showed multifocal minimal enlargement of synovial lining cell layer, multifocal minimal to mild synovial hyperplasia, multifocal minimal increased synovial vascularity, multifocal mild infiltration of inflammatory cells in synovial area.
- Mice of treated with Test drug Ketorolac at 3.5 mg/kg showed multifocal moderate enlargement of synovial lining cell layer, multifocal mild to moderate synovial hyperplasia, multifocal mild increased synovial vascularity, multifocal moderate to diffuse moderate infiltration of inflammatory cells in synovial area.
- Mice of treated with combination of Test drug Glycyrrhizin & Ketorolac at 10 + 3.5 mg/kg showed multifocal mild enlargement of synovial lining cell layer, multifocal moderate to marked synovial hyperplasia, multifocal minimal to mild increased synovial vascularity, diffuse moderate infiltration of inflammatory cells in synovial area.



Normal Group: Normal histology

Fig 5: Paw tissue of normal group.

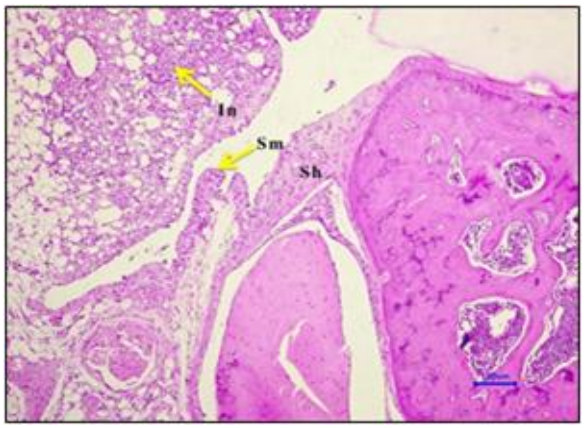
Note the synovial membrane (Sm), synovial fold (Sf) showing normal histology.



Negative Group: Enlarged synovial membrane Infiltration of cells

Fig 6: Paw tissue of Negative group.

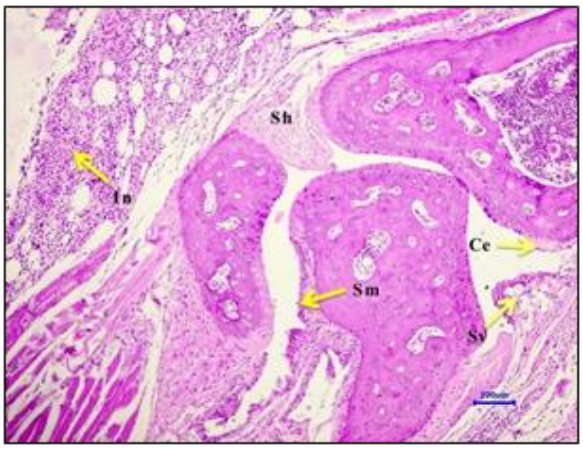
Note the enlarged synovial membrane(Sm), hyperplastic synovium (Sh) and inflammation (In).



Standard Group Enlarged synovial membrane Moderate infiltration of cells

Fig 7: Paw tissue of Standard group.

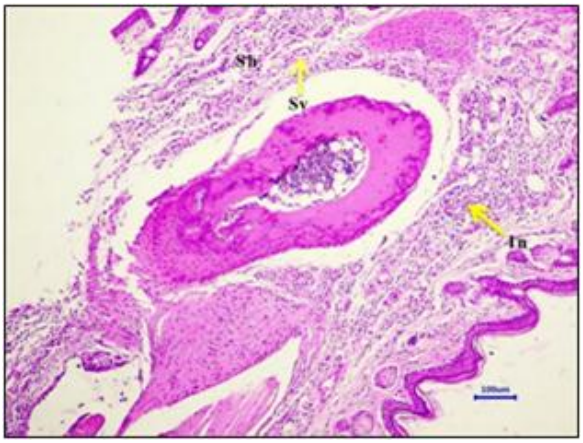
Note mild enlarged synovial membrane(Sm), moderate infiltration of cells and moderate synovial hyperplasia.



Ketorolac-3.5mg/kg Enlarged synovial membrane Moderate infiltration of cells

Fig 8: Paw tissue of ketorolac (3.5mg/kg).

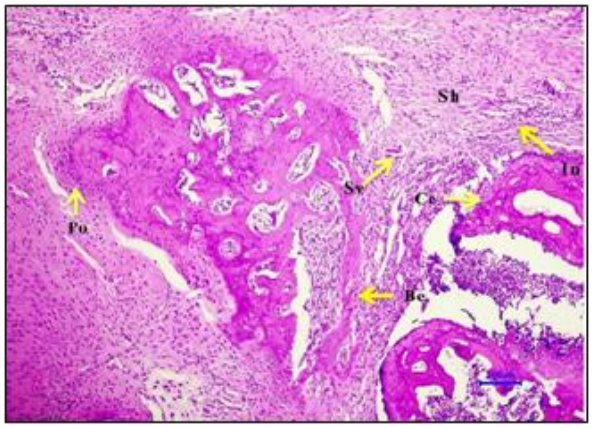
Note enlarged synovial membrane(Sm), moderate infiltration of cells and moderate synovial hyperplasia.



Ketorolac – 5mg/kg Enlarged synovial membrane Moderate infiltration of cells

Fig 9: Paw tissue of a Ketorolac (5mg/kg).

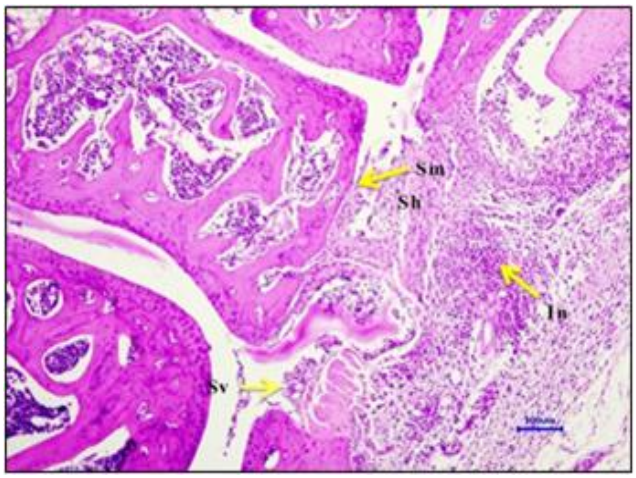
Note enlarged synovial membrane(Sm), moderate infiltration of cells and moderate synovial hyperplasia.



Glycyrrhizin-10mg/kg Minimal enlargement of synovial membrane Minimal infiltration of cells

Fig 10: Paw tissue of a Glycyrrhizin (10mg/kg).

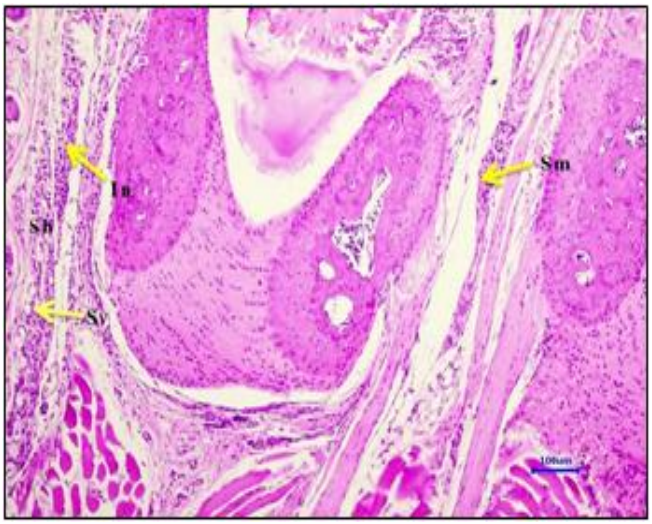
Note minimal enlargement of synovial membrane(Sm), minimal of infiltration of cells and minimal of synovial hyperplasia.



Ketorolac- 3.5mg/kg+Glycyrrhizin-10mg/kg Minimal enlargement of synovial membrane Moderate infiltration of cells

Fig 11: Paw tissue of ketorolac (3.5mg/kg) + Glycyrrhizin (10mg/kg).

Note minimal enlargement of synovial membrane(Sm), minimal of infiltration of cells and minimal of synovial hyperplasia.



Ketorolac-5mg/kg+ Glycyrrhizin-10mg/kg Mild enlarged of synovial membrane Mild infiltration of cells

Fig 12: Paw tissue of ketorolac (5mg/kg) + Glycyrrhizin (10mg/kg).

Note mild enlargement of synovial membrane(Sm), mild of infiltration of cells and mild of synovial hyperplasia.

3. DISCUSSION

Presently, the treatments available for the management of the inflammatory pain are not adequate. The most used therapy for inflammatory pain, nonsteroidal anti-inflammatory drugs (NSAIDS), and they results in toxicities when used in high dose and for maximum period of time. Therefore the search of new treatments are in need.[22] In this study it is shown, that the combination of Glycyrrhizin with Ketorolac shows the anti-inflammatory and anti-nociceptive effects.

The anti-inflammatory and anti-nociceptive effects of glycyrrhizin and ketorolac were evaluated using CFA induced inflammatory pain test. CFA induced inflammatory pain test is simple model for screening anti-inflammatory drugs. The protocol was set as per pain studies for 7 days with slight modifications. Treatment drugs were administered after the 24hours of induction by complete freund's adjuvant.[14] CFA induced model showed that Glycyrrhizin and ketorolac had significant anti-inflammatory and anti-nociceptive effects. Complete friends adjuvant group showed the paw edema. Changes in morphology of paw tissue illustrated enlarged synovial membrane, enlarged synovial fold, hyperplasic synovium, and higher level of diffusion of infiltration of cells.

HMGB1 is the intracellular pathway involved in the inflammatory pain response. It has been reported that expression of HMGB1 is elevated in injured mouse spinal cord, and therefore elicits neurotoxic inflammation.[23] In this study, Glycyrrhizin attenuate the production of pro- inflammatory cytokines (TNF-alpha, IL) by blocking the HMGB1-TLR pathway. Most important glycyrrhizin inhibit the inflammatory response in the spinal cord. Therefore, glycyrrhizin act as a potent drug for inflammatory responses. Activation of TLR pathway is widely recognized as a critical signaling for inflammatory progression during the development of various disease including inflammatory pain.[24, 25] We further elaborated the molecular mechanism involved in glycyrrhizin-mediated inhibition in HMGB1 signaling. Evidence confirmed that the progression of TLR pathway is mainly due to HMGB1.[25, 26]

Hyperalgesia, peripheral sensitization and central sensitization are triggered by adverse stimulus. During this time, nociception are delivered from peripheral tissues to central tissues, which then results in persistant pain.[27, 28] As a result, inflammation further develop to pain. As from earlier work, we already know that TLR and TRPV1 are related to one other, as TLR4 potentiated TRPV1 activation in sensory neurons.[29] Also it is stated that the NSAIDS work as the blockers for the TRP channel.[30] It inhibit the production of the pro-inflammatory cytokines by acting on TRPV1 receptor thus further inhibit the thermal hyperalgesia and mechanical hyperalgesia.[31]

Concerning the TRPV1 channel we have found that commonly used NSAIDs, such as ketorolac attenuate hyperalgesia induced by TRPV1.

In our present study, we suggest that the presented data indicate the new treatment involving the anti-inflammatory and anti-nociceptive effects of glycyrrhizin and ketorolac, which may

directly or indirectly involve in the attenuation of the HMGB1 and TRPV1 channels and could be use as the treatment.

4. CONCLUSION

In conclusion, by considering the dose of the standard indomethacin, it was seen to be effective in this study when compared to other treatment drugs. It might be because, indomethacin is more potent than ketorolac as NSAIDS. Glycyrrhizin has the action as anti-inflammatory and can reduce HMGB1 and also the ketorolac has the activity that can reduce hyperalgesia thus reducing pain. Also, more research has to be done on the dose accuracy of the treatment drugs.

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