THE EFFECTS OF TWO POLYMERIC MATRICES FOR INDOMETHACIN IN CUTANEOUS NOCICEPTIVE REACTIVITY IN MICE

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THE EFFECTS OF TWO POLYMERIC MATRICES FOR INDOMETHACIN IN CUTANEOUS NOCICEPTIVE REACTIVITY IN MICE (Abstract): **Aim.** This paper is focused on the investigation the effects of two polymeric matrices for indomethacin in cutaneous pain models in mice. There were used two different co-polymers polyhydroxyethyl methacrylate and undecan, polymerization reactions being conducted under nitrogen, at 80°C. **Material and methods:** The experiments were carried out on white Swiss mice (20-25 g), divided into 6 groups of 7 animals each, treated orally. Biocompatibility properties of indomethacin-loaded co-polymeric matrices ware evaluated by assessing the effects on the blood parameters, the serum biochemical tests of animals treated. The nociceptive somatic testing was performed using hot plate assay and tail immersion test. The latency (second) response to paw, respectively tail thermal noxious stimulation, was measured before the experiment and 15, 30, 60, 90, 120 minutes, 4, 6, 8, 10, 12 hours after the substances administration. **Results:** Laboratory analysis did not show significant differences of blood parameters, serum biochemical tests between control mice group (IND) and groups treated with 1 M, 1 IND, 3 M, 3 IND. In our experimental conditions IND determined a significant increasing of the latency period response, in hot plate and also in tail immersion tests. Using two different co-polymers for indomethacin incorporation we obtained an increasing of the latency time pain reaction in hot plate assay, respectively in tail immersion test, statistically significant (*p< 0.05) compared with the simple co-polymers administration. **Conclusions:** We demonstrated that indomethacin co-polymeric matrices 1 IND and 3 IND determined similar immune responses with indomethacin and simple co-polymers after oral administration in mice, indicative of good *in vivo* biocompatibility. Oral administration of both 1 IND and 3 IND resulted in prolonged antinociceptive effects in hot plate assay and also in tail immersion test in mice. **Keywords:** INDOMETHACIN, POLYMERIC MATRICES, HOT PLATE, TAIL IMMERSION.

According to its colossal potential in medicine and medical technologies, the actual directions of nanomedicine are represented by the development of the basic sci-
ences experiments according to the multidisciplinary vision of the nanoscience, and training programs particularly for young researchers in this field. The importance of nanomedicine is represented by the opportunity of understanding the molecular and genetic level of pathophysiological mechanisms of the diseases, the improvement of pharmacotherapeutic management due to targeted action of drug loaded nanoparticulate systems, and reducing the side effects of substances. Nowadays the use of nanoparticulate formulations is under research in experimental studies and in clinical trials (1, 2). Nanoparticle drug carriers consists of solid biodegradable particles in size ranging from 10 to 1000 nm in which the substances is dissolved, entrapped or encapsulated, and/or to which the active principle is absorbed or attached (3). The development of analgesic drugs loaded nanoparticulate systems may represent a future challenge to realize promising agents for local drug delivery in inflammatory and painful conditions (4, 5).

The supposition that the utilization of various materials at the nanometer scale, will determine the improvement of different pharmaco-dynamic actions of active substances entrapped in them is based on different experimental reports indicating the possibility of reaching otherwise less accessible sites in the body and also the sustained release of various drugs (3, 4).

To directly prove this hypothesis we investigated the effects of two polymeric matrices for indomethacin in hot plate and tail immersion tests. The performed studies are significant because they offer a fundamental perspective on the self-assemble processes from the bio-molecular systems and also represent a basis for controlled release applications which can be valuable for the biomedical and pharmaceutical fields.

We aimed to investigate the effect of two polymeric matrices for indomethacin in two different experimental cutaneous pain models in mice.

**MATERIAL AND METHODS**

**Materials:** 2 - Hydroxyethyl Methacrylate (HEMA) (from Fluka, purity>96%) was purified by passing it through an inhibitor removal column. The inhibitor - remover replacement packing (for removing hydroquinone (HQ) and hydroquinone monomethyl ether (MEHQ) was purchased from Aldrich. 3,9-divinyl-2,4,8,10-tetraoxaspiro[5.5]undecane (U) (Aldrich, 98%), sodium lauryl sulfate (C12H25O4SNa) – (SLS) from Sigma (c > 95 wt %), PAV (polyvinyl alcohol) and the radical initiator 4,4’-azobis(cyanopentanoic acid) (ACPA) (Fluka, 98%), were used without further purification. In all experiments it was used twice distilled water which contained no foreign ions. The water used in all experiments was purified using an Ultra Clear TWF UV System.

Male white Swiss mice (20-25g) were used. Lighting was on a 12-h light/dark cycle (lights on at 6:00 a.m.), with standard laboratory food and tap water freely available, except during the time of the experiments. Before the experiment, mice were placed on a raised wire mesh, under a clear plastic box and allowed 2 hours to acclimate to the testing room.

**Methods:** The drug powder (about 10 wt% of the copolymers weight) was dissolved in ethanol/phosphate buffer solution (pH=7.2) 50:50. The obtained drug concentration in the initial loading solution was c=1.9005×10^{-5} mol/ml. The co-polymers (approximately of the same weight) were immersed in this solution and swollen to
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equilibrium. Polymerization reactions were conducted under nitrogen atmosphere (nitrogen purged to remove oxygen), at 80°C in a constant temperature bath, with a mechanical stirring rate of 180 rpm (6). The swollen drug-loaded samples were then dried at ambient temperature for several days to constant mass and used for the release experiments. After synthesis the polymeric particles were precipitated three times with methanol from water solution and finally freeze-dried by lyophilization during 24 hours (7).

The experiments were carried out on white Swiss mice (20-25 g), divided into 6 groups of 7 animals each, treated orally (using an eso-gastric device) as follows groups: I (Control): 0.1 ml/10 g weight; II (IND): indomethacin 5 mg/kbw (IND); III (1 M): polyhydroxyethyl methacrylate (HEMA) 10% + polyvinyl alcohol (APV); IV (1 IND): HEMA 10% + APV + IND 5 mg/kbw; V (3 M): HEMA 10% + undecan 3.5% + APV; VI (3 IND): HEMA 10% + undecan 3.5% + APV + IND 5 mg/kbw. The experiment was performed according to the “Grigore T. Popa” University of Medicine and Pharmacy guidelines, for handling of experimental animals, also to the recommendations and policies of the International Association for the Study of Pain. Each animal was used once only and sacrificed immediately after the experiment (8).

RESULTS AND DISCUSSION

Biocompatibility evaluation. We evaluate the acute toxicity of indomethacin-loaded co-polymeric matrices, by assessing the effects on the blood count, the serum biochemical tests (glutamic oxaloacetic transaminase [GOT], glutamic pyruvic transaminase [GPT] and lactic dehydrogenase [LDH] activity) and on some immune system parameters (serum opsonic capacity [OC], phagocytic capacity [PC] and bactericidal capacity [BC] of peritoneal macrophages). Data were expressed as mean ± S.E.M. and statistically analyzed using t-student of Windows EXCEL program test.

Hot plate test. The nociceptive somatic testing was performed using hot plate assay to determine the latency of nocifensive reactions evoked by thermal heat stimuli applied on the paws. In this test, animals were individually placed on a hot plate maintained at a constant temperature (55±0.3°C). The latency to first sign of hind paw licking or jump response to avoid heat nociception was taken as an index of nociceptive threshold (9). Pre-dosing latencies (seconds) were measured before administration of any drug or vehicle. The baseline latency (before drug injection) in hot plate test was 4.2±0.2 seconds (mean ± standard error of mean - SEM) (10). The recommended cut-off time of 12 seconds was used to prevent tissue damage. Latency time response is measured at 15, 30, 60, 90, 120 minutes, 4, 6, 8 hours after the substances administration. Differences between the experimental and baseline latencies are interpreted as an index of analgesia. Increases in the latency time response for the mouse to avoid thermal stimulus are indicative of analgesia, while decreases in the latency reaction are indicative of hyperalgesia (11). Response latency data from paw reaction latency were converted to percent of maximum possible effect (%MPE) according to the formula: %MPE = [(observed latency-baseline latency)/(cut off time-baseline latency)] × 100 (12).

Tail immersion test. The effects of these two polymeric matrices for indomethacin were assessed in tail immersion test. The apparatus consists of a water bath heated to
a nociceptive temperature of approximately 54°C. The animal is placed into individual restraining devices leaving the tail hanging out freely. The lower 5 cm segment of the tail is immersed in the bath hanging out freely. The lower 5 cm segment of the tail is marked. This part of the tail is immersed in the water bath. Within a few seconds the animal reacts by flicking its tail and the latency to withdrawal the tail as a response to noxious thermal stimulation is measured by stop watch (9, 12). The baseline latency (before drug injection) in the tail immersion test was 5.5 ± 0.3 seconds (mean ± standard error of mean - SEM). The recommended cut-off time of 15 seconds was used to prevent tissue damage. Differences between the experimental and baseline latencies are considered as an index of analgesia. Prolongation of the reaction time to the typical tail-withdrawal reflex is an indicative of analgesia, while reduction in tail-flick latency corresponds to a hyperalgesic effect. The tail-withdrawal reactivity (seconds) was recorded before administration of any drug or vehicle. Latency time response was determined 15, 30, 60, 90, 120 minutes, 4, 6, 8, 10, 12 hours after substances administration. Response latency data from tail withdrawal measurements were converted to per cent of maximum possible effect (%MPE) according to the formula: % MPE = [(observed latency - baseline latency) / (cut off time – baseline latency)] × 100 (11).

In both experiments, results of time response from each group were calculated as mean ± standard error of mean (SEM) and significance was tested by SPSS 13.0 Statistics and ANOVA method, followed by Neumann Keuls test as post hoc. p-values less than 0.05 are considered statistically significant comparing with those of control group. Laboratory analysis did not show significant differences of leucocyte formula elements, GOT, GPT and LDH levels, nor immune parameters (OC, PC, BC), between control mice group (IND) and groups treated with 1 M, 1 IND, 3 M, 3 IND relevant elements for a good in vivo biocompatibility. The behavioral reflex evoked by noxious heat stimuli is a relatively good predictor of pain sensitivity and for the study of the antinociceptive actions of different substances.

In our experimental condition in models of hot plate and tail-immersion in mice, which are measures of thermally-induced pain, the two polymeric matrices for indomethacin increased nociceptive pain threshold, actions that are prolonged comparing with the effects of simple drug administration. Statistical analysis of the results obtained in hot plate test shows that indomethacin (IND) determined a rapidly and statistically significant (p<0.05) increasing of the latency time period of the response, effect prolonged 4 hours after oral administration of the substance. Then the effect of indomethacin was gradually decreased (p<0.05) to 8 hours after its administration. The results of our experimental study are congruent with the literature communicated data, regarding the effects of the nonsteroidal antiinflammatory drug indomethacin, in this experimental cutaneous pain model in mice (13). The administration of co-polymers 1 M and 3 M did not influence the latency time response to thermal noxious stimulus in this cutaneous pain model. Using two different co-polymers for indomethacin incorporation we obtained an increasing of the latency time pain reaction in hot plate test, statistically significant compared with simple co-polymers (*p< 0.05) (fig. 1).
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![Graph](attachment:image.png)

**Fig 1.** The latency time of IND, 1 M, 1 IND, 3 M, 3 IND response to thermal noxious paw stimulation, in hot plate test. Each point is the mean ± SEM of latency time (seconds) for seven mice. *p<0.05, **p<0.01 vs. control. Both 1 IND (6.7±0.1 after 6 hours, 6.6±0.7 after 8 hours, 5.5±0.3 after 10 hours) and 3 IND (5.9±0.7 after 6 hours, 6.2±0.9 after 8 hours, 5.3±0.5 after 10 hours) increases the latency time period, statistically (*p<0.05) significant vs. control.

Both 1 IND and 3 IND proved the retard release of the non-steroidal anti-inflammatory indomethacin from the synthesized polymer networks, 1 IND eliciting a higher effect than 3 IND in the somatic pain model used.

![Graph](attachment:image.png)

**Fig. 2.** Time course of the maximum possible effect (%MPE) of IND, 1 M, 1 IND, 3 M, 3 IND on the paw latency response in hot plate test. Each point is the mean ± SEM of percentage of maximum possible effect (%MPE) for seven mice.

Moreover, we used the percentage of maximum possible effect (%MPE), to quantified the intensity of antinociception which is important to corroborate and confirm these previous results. Indomethacin administration resulted in an increase in %MPE in hot plate assay, statistically significant in a first 2 hours after substance administration. The higher percent of inhibition was obtained in the interval between 60 minutes (39.3±1.7) and 90 minutes (36.4±2.3) in the experiment. Oral administration of 1 IND determined an increase of response latency in the hot plate
test, after 2 hours, effects prolonged around 8 hours, with a higher values in the interval between 6 hours (23.1%±1.3) and 8 hours (22.2%±2.5), after substance administration. 3 IND produced a significant increase of response latencies between 4 hours (12.2%±2.1) and 8 hours (17%±0.9) after substance administration, but less than submaximal level in the hot plate test (< 60% of MPE) (fig. 2).

![Fig 3](image)

**Fig 3.** The latency time of IND, 1 M, 1 IND, 3 M, 3 IND response to thermal noxious tail stimulation, in tail immersion test. Each point is the mean ± SEM of latency time (seconds) for seven mice. *p<0.05, **p<0.01 vs control. Both 1 IND (7.2±0.7 after 4 hours, 8.8±0.3 after 6 hours, 8.9±0.5 after 8 hours, 8.6±0.9 after 10 hours) and 3 IND (6.8±0.3 after 4 hours, 8.6±0.5 after 6 hours, 8.5±0.1 after 8 hours, 8.0±0.7 after 10 hours) increases the latency time period, statistically (*p<0.05) significant vs control.

Indomethacin oral administration produced a rapidly and statistically significant (p<0.05) increasing of the latency time period of the response, effect prolonged 2 hours in the experiment. After this time its effect was gradually decreased (p<0.05) to 6 hours after its administration. These results are in concordance with data existing in literature, regarding the effects of indomethacin, in this experimental somatic pain model in mice (14). The treatment with 1 M and 3 M co-polymers did not influence the latency time response to thermal noxious stimulus in tail immersion test. The administration of co-polymers including indomethacin resulted in an increasing of the latency time response, statistically significant (*p<0.05) compared with simple co-polymers (fig. 3).

Both 1 IND and 3 IND used demonstrated the prolonged release of the indomethacin from the polymer matrices. The effect of 1 IND was most intense than the effect of 3 IND in tail immersion model.

In these two experimental pain models used the retard release of the drug from the polymer network may be explicated as it follows: 2 - Hydroxyethyl Methacrylate is coupling indomethacin by hydrogen bonds through hydroxyl groups and release the active substance after bursting the physical links. The association of 3,9-divinyl-2,4,8,10-tetraoxaspiro[5.5]undecane facilitated the formation of both intra and intermolecular physical bonds and at the same time induced steric hindrances generated by the spiroacetal groups. Much more it functioned as cages which coupled and retained of the drug into the network meshes, slowing down of the bioactive substance release from them (15).

The treatment with indomethacin de-
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determined an increase in %MPE statistically significant in a first 2 hours after substance administration, in tail immersion test. The higher percents of inhibition were obtained in the interval between 30 minutes (36.5%±0.9) and 60 minutes (41.7%±1.3) after substance administration. 1 IND administration resulted in a significant increase in inhibition percent between 6 hours (36.1%±1.2) and 10 hours (34%±0.7), but with a peak value 8 hours (37.1%±1.3) in the experiment. 3 IND produced an increase in inhibition percent after 2 hours, effects prolonged around 10 hours, with a higher values in the interval between 6 hours (32.6%±0.7) and 8 hours (31.6%±1.1), after substance administration (fig. 4).

**CONCLUSIONS**

We evaluated the in vivo biocompatibility and investigated the effects of different copolymeric networks based on 2- Hydroxyethyl Methacrylate in association with 3,9-divinyl-2,4,8,10-tetraoxaspiro[5.5]undecane, using polyvinyl alcohol as protective colloid for indomethacin incorporation in somatic nociceptive reactivity in mice.

Indomethacin co-polymeric matrices 1 IND and 3 IND determined similar immune responses with indomethacin and copolymers after oral administration in mice, indicative of good in vivo biocompatibility.

The administration of both indomethacin co-polymeric matrices 1 IND and 3 IND proved antinociceptive effects in hot plate test in mice. Oral administration of these two co-polymeric networks including indomethacin 1 IND and 3 IND resulted in a significant and prolonged analgesic effect in tail immersion test.

The good in vivo biocompatibility and the antinociceptive evident effects of different copolymeric systems including indomethacin attests the possibility for using these compounds as innovative drug-carriers with applicability in biological and medical fields.

**REFERENCES**

NEW APPROACHES AGAINST ANTIFUNGAL DRUG RESISTANCE

Fluconazole (FLC) is widely used in the treatment of candidiasis because of its efficacy and low toxicity. The emergence of FLC-resistant Candida albicans isolates and biofilm-associated infections pose a great challenge to antifungal treatment. According to Liu et al., in order to increase the susceptibility of C. albicans to FLC different combinations of FLC with non-antifungal agents have been proposed, such as: calcineurin inhibitors, antibacterials, calcium homeostasis regulators, heat shock protein 90 inhibitors and traditional Chinese medicine drugs. The modes of action of these compounds and the mechanisms of their synergistic effects against resistant strains of C. albicans are: increased membrane permeability, reduced efflux of antifungal drugs, interference with intracellular ion homeostasis, inhibition of enzymes required for fungal survival, and inhibition of biofilm formation. The study concludes that the reversal of fungal resistance can be achieved through various mechanisms (Liu S1, Hou Y2, Chen X3.et al. Combination of fluconazole with non-antifungal agents: A promising approach to cope with resistant Candida albicans infections and insight into new antifungal agent discovery. Int J Antimicrob Agents. 2014 pii: S0924-8579(14)00002-8. doi: 10.1016/j.ijantimicag.)

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