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CHITOSAN-STABILIZED LIPID VESICLES FOR SUSTAINED RELEASE AND PROLONGED ANALGESIC EFFECTS OF INDOMETHACIN IN A SOMATIC PAIN MODEL IN MICE

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CHITOSAN-STABILIZED LIPID VESICLES FOR SUSTAINED RELEASE AND PROLONGED ANALGESIC EFFECTS OF INDOMETHACIN IN A SOMATIC PAIN MODEL IN MICE (Abstract): The evolution of pharmaceutical nanotechnology enhances the flexibility of drug delivery by using carrier systems to target specific tissues, enabling controlled release at optimal concentrations. The inclusion of nonsteroidal anti-inflammatory drugs (NSAIDs) in nanocarriers presents a promising approach for achieving sustained release and improving the pharmacodynamic effects. We **aimed** to evaluate the in vivo kinetic release profile and the impact of indomethacin (IND) encapsulated in lipid vesicles on somatic nociceptive reactivity in mice. **Materials and methods:** Lipid vesicles were prepared using a molecular droplet self-assembly method, incorporating phosphatidylcholine and chitosan (CHIT) to encapsulate IND. The *in vivo* evaluation involved administering IND-loaded lipid vesicles to male Swiss mice and assessing their analgesic effects using the hot plate test, a standard method for measuring somatic pain. Blood samples were collected at different intervals to determine the release kinetics of IND, with concentrations measured by high-performance liquid chromatography. **Results** indicated that IND encapsulated in lipid vesicles exhibited a sustained release profile compared to the direct administration of the drug. The treatment with IND-loaded microvesicles resulted in significantly prolonged analgesic effects, as shown by extended latency times in the hot plate test. The release profile of IND from lipid vesicles, composed of phosphatidylcholine and stabilized with CHIT, demonstrated a gradual and controlled drug release, correlating with extended pain relief. **Conclusions:** These findings confirm the potential of lipid-based vesicles, as effective carriers for IND, offering sustained drug release and prolonged analgesic effects in a somatic pain model. **Keywords**: LIPID VESICLES, INDOMETHACIN, IN VIVO RELEASE, HOT PLATE, MICE.

INTRODUCTION

Nanotechnology applications in medicine, known as nanomedicine, involve the use of nanomaterials for diagnosing, monitoring, controlling, preventing, and treating diseases (1). Nanomaterials possess unique physicochemical properties distinct from their conventional counterparts due to their small size, making them valuable in drug development (2). These properties, such as altered pharmacokinetics, the ability to cross biological barriers, potential toxicity, and persistence in the environment and human body, must be carefully examined under use conditions (3, 4).

Lipid nanoparticles have emerged in the pharmaceutical industry as promising carriers for therapeutic agents. Liposomes, an early form of lipid nanoparticles, are versatile nanocarriers capable of transporting both hydrophobic and hydrophilic molecules, including small molecules, proteins, and nucleic acids. Many liposome-based drug formulations have been approved and successfully used in medical practice (5). Traditional drugs often face limitations in pharmacokinetics, low bioavailability, and high toxicity, which can restrict their use (6). Nanotechnology and nanomedicine have advanced in overcoming these issues, enhancing pharmacodynamic effects and improving disease detection, diagnosis, and treatment (5). With their small size, nanosystems reduce toxicity and improve pharmacokinetic parameters, such as distribution, prolonged circulation time, targeted release, higher intracellular concentration, and enhanced drug solubility and stability (6, 7).

A key area of research is enhancing the therapeutic profile of non-steroidal anti-inflammatory drugs (NSAIDs) with nanoparticles. NSAIDs are widely used to treat inflammatory diseases, but their long-term use can lead to side effects, especially gastrointestinal toxicity, due to repeated administration. Nanoparticle-based approaches offer an intriguing solution to improve NSAID safety and efficacy (8-10).

The 1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indole-3-acetic acid derivative indomethacin (IND) is a nonsteroidal anti-inflammatory drug (NSAID). Its structure is characterized by an indole acetic acid core with additional functional groups that contribute to its activity. The structure includes a chlorobenzoyl group attached to the indole ring, along with a methoxy group and a methyl group, which enhance the molecule's stability and potency as an anti-inflammatory agent. It is practically insoluble in water, which limits its absorption in aqueous environments (11). IND is rapidly absorbed from the gastrointestinal tract following oral administration, reaching peak plasma concentrations within 2 to 4 hours, though this can vary based on the formulation and presence of food. It has a bioavailability of approximately 90-95%, though high variability can occur among individuals. It is approximately 90% bound to plasma proteins, predominantly albumin, which limits its free fraction in circulation but also contributes to its longer half-life and tissue distribution. The drug undergoes extensive hepatic metabolism, primarily by demethylation and deacetylation, and is eventually excreted in urine and feces both as metabolites and in small amounts as the parent compound. Its elimination half-life typically ranges between 4 to 5 hours but can vary depending on patient-specific factors such as liver and kidney function (12).

IND exerts its effects primarily by inhibiting cyclooxygenase (COX) enzymes, specifically COX-1 and COX-2, thus reducing the inflammatory response, pain, and fever, making it effective in the treatment of conditions such as arthritis, gout,

and dysmenorrhea (13, 14). However, its inhibition of COX-1 also reduces the protective effects of prostaglandins in the gastrointestinal tract and kidneys, contributing to common adverse effects like gastrointestinal irritation, ulceration, and renal impairment. Its strong inhibition of COX enzymes also explains its potential cardiovascular risks, as prostaglandins involved in vasodilation and platelet inhibition are reduced, potentially leading to increased blood pressure and risk of thrombotic events (15). Incorporating IND into nanosystems may offer significant pharmacological benefits, including reduced gastrointestinal irritation and enhanced anti-inflammatory and analgesic effects.

The **purpose** of this study was to assess the impact of IND encapsulated in nanosystems on somatic nociceptive reactivity in rats.

MATERIALS AND METHODS

Substances. The materials used for nanoparticle preparation were procured from Sigma-Aldrich Chemical Co., Steinheim, Germany (www.sigma-aldrich.com). These included CHIT (catalog code: C3646; derived from crab shells, molecular weight: 310,000 g/mol 80% degree of N-deacetylation, polydispersity index of 3.26), IND (catalog code: I7378; 98.5% purity; molecular weight: 357.79 g/mol), phosphatidylcholine (catalog code: P5638; type II-S, soy-derived, containing 14–29% choline), cholesterol (catalog code: C8667; ≥99% purity; molecular weight: 386.65 g/mol), chloroform (catalog code: C2432; stabilized with 100–200 ppm amylene; ≥99.5% purity; molecular weight: 119.38 g/mol), glacial acetic acid (catalog code: 695092; ≥99.7% purity; molecular weight: 60.05 g/mol), and ethyl alcohol (catalog code: E7148; 95% purity; molecular weight: 46.07 g/mol).

Preparation of lipid vesicles entrapping IND. Lipid vesicles containing IND were prepared using a molecular droplet self-assembly method. Ethanol was used as the solvent for the lipids, cholesterol, and IND, while CHIT was dissolved in 0.5% glacial acetic acid.

In the preparation, 0.09 g of soy lipid was mixed with 0.66 mL of ethanol, and 0.015 g of cholesterol and 0.01 g of IND were each dissolved in 1 mL of ethanol. The combined lipid and IND solution (1.66 mL) were subjected to sonication at 25% amplitude for 10 minutes at 29°C using a Bandelin SONOPULS 2450 ultrasonic homogenizer, providing 20,000 kJ of energy (16). This sonication step transformed multilamellar structures into unilamellar vesicles, with their size controlled by ultrasound amplitude. The resulting mixture was then injected into 8.33 mL of double-distilled water (22-23°C), forming a slightly translucent suspension of IND-loaded lipid vesicles (IND-vl). Separately, 1.66 mL of the ethanol mixture was added to 8.33 mL of 0.25% CHIT solution under magnetic stirring (800 rpm for 20 minutes at 22°C), resulting in the formation of IND-loaded vesicles (IND-ves). Both dispersions were further stirred for an additional 5 minutes (16). The inclusion of CHIT altered the vesicle size and morphology, improving the colloidal stability. The pH of both dispersions was measured using a Sartorius Professional PP-50 pH meter. To remove excess lipids and unencapsulated drug, the dispersions were dialyzed for 2 hours using 12,000 Da MWCO tubular membranes (catalog code: D6191-25EA, Sigma-Aldrich). This process adjusted the pH to physiological levels, with the IND suspension reaching a pH of 7.0 and the CHIT-coated vesicles (IND-ves) achieving a pH of 6.7 (16).

Animals. The experiments were carried out on male white Swiss mice (20-25 g), acquired from the National Medical-Military Institute for Research and Development, Baneasa, Bucharest, Romania, the Biobase at "Grigore T. Popa" University of Medicine and Pharmacy of Iasi and CEMEX (Advanced Research and Development Center for Experimental Medicine) in Iasi, Romania. The mice were kept in individual plexiglass cages and given a 7-day acclimatization period in a controlled laboratory setting, where the temperature was maintained at 21±2°C, humidity ranged from 50% to 70%, and a 12-hour light/dark cycle was followed. Standard granulated food and tap water were available *ad libitum*, with daily food intake recorded and the animals' behavior closely monitored.

The protocol of the experiments. For the experiment, the animals were randomly assigned to groups of five and treated orally using an esophageal-gastric device with a single daily dose as follows: Group 1 (Control) received 0.1 mL of distilled water per 10 g of body weight; Group 2 (IND) was administered IND solution at 5 mg/kg body weight; and Group 3 (IND-ves) received CHIT-based vesicles containing IND at 5 mg/kg body weight.

The release profile of IND from the lipid vesicles was determined by conducting repeated measurements of the drug concentration in the blood. The mice were anesthetized with 1% isoflurane, and 0.3 mL of blood was collected prior to the administration of IND-ves. Subsequent blood samples were taken at intervals of 15 minutes, 30 minutes, 60 minutes, 90 minutes, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, and 8 hours post-administration. The concentration of IND released into the bloodstream was analyzed using high-performance liquid chromatography (HPLC) with an Agilent 1100 HPLC system (Santa Clara, CA, United States), employing ultraviolet (UV) absorbance detection at a wavelength of 254 nm.

The somatic pain model utilized in this research involved the hot plate test (Deuis, 2017). The baseline latency, measured before drug injection, was found to be 4.2 ± 0.2 seconds (mean ± standard deviation). A cut-off time of 10 seconds was implemented to prevent any potential tissue damage. The differences observed between experimental and baseline latencies were used as indicators of analgesia; specifically, longer latency times suggested analgesic effects, while shorter latencies indicated hyperalgesia (17,18). Latency times in response to the thermal stimulus applied to the paws were assessed at several points: before the experiment and at 15, 30, 60, 90, and 120 minutes, as well as at 4, 6, and 8 hours after administration of the substances. To quantify the intensity of the antinociceptive effect, the latencies were converted into a percentage of the maximum possible effect (%MPE), calculated using the formula: % MPE = (measured latency – baseline latency) x 100 / (cut-off time – baseline latency) (19). Changes in pain threshold following administration of the lipid vesicles with IND were considered significant when they exceeded 60% MPE; values below this threshold, while statistically notable, raised concerns regarding their biological significance. In this somatic pain model, an extension of reaction time compared to baseline measurements indicated an antinociceptive effect of the substances under study, whereas a decrease in latency suggested a hyperalgesic response to the compounds tested (18).

The protocol followed the guidelines established by the Ethics Commission of "Grigore T. Popa" University of Medicine and Pharmacy of Iasi (Ethical Approval Certificate No. 362/28.11.2023; Project

Authorization No. 69/15.01.2023), in accordance with current European ethical standards (20).

Statistical processing of data. Data were reported as mean values with standard error of the mean (± SEM). Statistical significance was evaluated using *SPSS Statistics for Windows version 13.0,* employing the ANOVA method. A P-value of less than 0.05 was interpreted as statistically significant when compared to the control group.

RESULTS

In our previous studies, we developed novel polymeric vesicles that encapsulate IND, with an average size of 317.6 nm and a polydispersity index of 0.364, demonstrating good suspension stability (Zeta potential of 24 mV). In vitro studies revealed a sustained release of IND from the lipid vesicles, unlike the non-encapsulated drug. Furthermore, the IND-loaded vesicles exhibited excellent in vitro hemocompatibility (16).

We continued the researches investigating the in vivo release profile of IND from carrier systems and evaluating the effects of the IND-loaded lipid vesicles administration on the nociceptive reactivity in mice.

The in vivo kinetic assessing of IND incorporated into IND-ves shows no detectable release of the drug during the initial 15 minutes. At 30 minutes, a small percentage begins to appear, marking the onset of release. By 2 hours, approximately 75% of the total release is achieved. The release reaches its peak at 3 hours, corresponding to 100% of the maximum release (fig. 1). Following this peak, the drug concentration decreases gradually due to metabolism and clearance from the bloodstream. By 6 hours, about 50% of the maximum release remains, dropping further to 16% at 8 hours. After 10 hours, the concentration declines close to baseline, indicating nearly complete clearance of the drug (fig. 1). This release profile highlights a controlled and sustained release of IND over time, demonstrating the efficiency of the nanoparticles in prolonging drug availability compared to free IND.

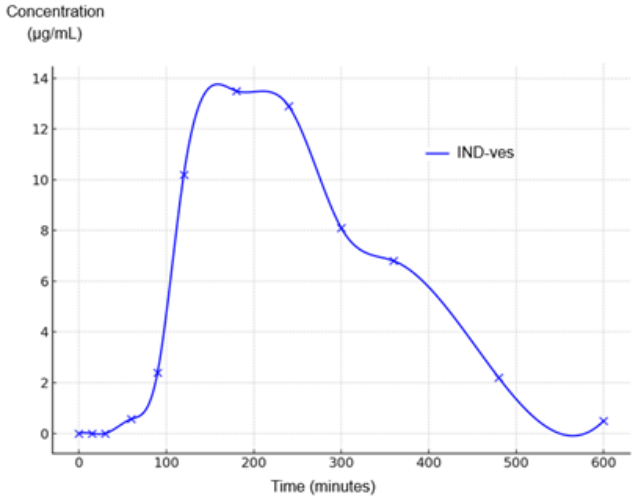

Fig. 1. The *in vivo* kinetic release profile of IND from IND-ves

The statistical analysis of the hot plate test demonstrates that free IND significantly increased the latency time of the animals' response following thermal noxious stimulation. A statistically significant increase was observed between 15 minutes and 90 minutes (**p > 0.01), as well as at 2 hours (*p > 0.05) post-administration (fig. 2). However, the effect did not persist beyond this period, as the response latency rapidly decreased and the antinociceptive effect was entirely lost by 180 minutes (fig. 2). These findings are consistent with previous studies showing the antinociceptive properties of IND in various pain models.

When IND was encapsulated in soft vesicles, it resulted in a notable prolongation of the animals' response latency to experimentally induced pain. The significant effects (**p > 0.01) were observed between 90 minutes and 6 hours, with the response latencies lasting longer compared to free IND. The antinociceptive effect of IND-ves peaked during this period, but the effects diminished after 8 hours.

Free IND significantly increased %MPE during the first 2 hours, with the peak effect observed at 60 minutes post-administration. During the initial hour of the experiment, the effect was notable, exceeding 50% MPE, indicating a submaximal antinociceptive response. However, the effect waned after 120 minutes, with %MPE dropping below 50% (fig. 3).

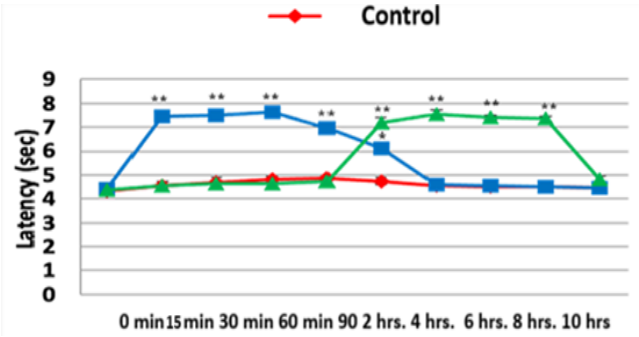

Fig. 2. The latency time response to thermal noxious paw stimulation in hot plate test in mice

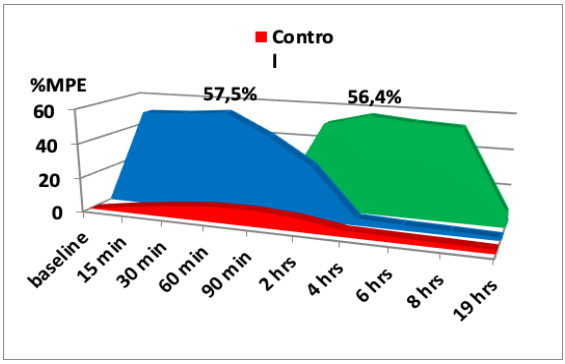

Fig. 3. Quantification of the analgesic activity of IND and IND-ves in hot plate test in mice

In contrast, IND encapsulated in soft vesicles demonstrated a delayed onset of %MPE, becoming evident around 2 hours post-administration. The %MPE exceeded 50% between 120 minutes and 8 hours during the experiment, before abruptly declining thereafter. These results indicate that vesicle-encapsulated IND offers a more prolonged antinociceptive effect compared to the free drug form.

DISCUSSION

The advancement of pharmaceutical nanotechnology expands drug application possibilities, as carrier systems enable the targeted delivery of active compounds to specific tissues, releasing them within an effective concentration range to increase the accumulation of anti-inflammatory agents. In recent years, nanosystems incorporating NSAIDs have shown the ability to enhance anti-inflammatory effects while reducing side effects (21). A diverse array of hybrid nanoparticles with anti-inflammatory agents has been synthesized, characterized, and studied. These nanoparticles act as carriers, loaded with anti-inflammatory drugs to create a controlled delivery system for active substances. Each nanoparticle structure has unique properties that optimize delivery to target tissues (22, 23).

The use of biocompatible and biodegradable polymers is a major advantage, as they allow the carrier to degrade naturally, minimizing toxicity (24, 25). Natural polymers like starch, collagen, alginate, cellulose, chitosan, and chitin are particularly appealing for developing systems to incorporate pharmacologically active agents. Among these, chitosan is a biocompatible, biodegradable, and non-toxic polymer with exceptional properties. It exhibits antibacterial effects, film-forming ability, high mechanical strength, and good thermal stability. Numerous chitosan-based bio nanocomposites with enhanced physical and chemical properties have been developed to create novel drug-loaded nanoformulations (26).

Research has presented numerous innovative approaches for creating IND-loaded systems, including phospholipid conjugates designed to improve IND's transport and controlled release. These methods focus on enhancing the solubility, stability, and release profile of IND within nanoformulations (27). Lipid-based nanocarriers, such as lipid emulsions, leverage phospholipids and surfactants have been developed to improve the solubility and bioavailability of IND (28, 29). Liposomal formulations, incorporating sterically stabilized vesicles with stearyl amines and cholesterol, enhance drug stability and provide sustained release (30).

Other biocompatible carrier systems for modified release of IND were developed by embedding IND within copolymeric networks of poly(2-hydroxyethyl methacrylate-co-3,9-divinyl-2,4,8,10-tetraoxaspiro [5.5]-undecane) and poly (aspartic acid) as a stabilizing colloid, or hyaluronic acid and four variants of poly(itaconic anhydride-co3,9-divinyl-2,4, 8,10-tetraoxaspiro[5.5] undecane) via poly-merization methods (31-33).

Some researchers developed original systems based on indomethacin-loaded microparticles composed of poly(lactic-co-glycolic acid), prepared using solvent evaporation and freeze-drying for enhanced stability (34). Keßler *et al*. prepared amorphous solid dispersions loading indomethacin by embedding the drug into polymer matrices such as poly(vinyl pyrrolidone) or poly(lactic-co-glycolic acid), employing

techniques like hot melt extrusion or solvent evaporation (35). Moreover cyclodextrin-based systems for loading IND were obtained using various techniques, such as solvent evaporation, co-precipitation, or spray-drying, for controlled and targeted drug delivery uses (36-38).

In our researches, the in vivo assessment revealed that IND, when encapsulated in lipid vesicles stabilized with CHIT, exhibited a significantly prolonged and controlled release compared to the direct administration of the drug. The IND-loaded micro-vesicles not only enhanced the duration of the analgesic effect but also extended the overall therapeutic response in the hot plate test, a common method for evaluating pain sensitivity in animals. In particular, the release profile of IND from lipid vesicles, composed of phosphatidylcholine and stabilized with CHIT, showed a sustained and gradual release of the drug over time, which correlated with a longer-lasting antinociceptive effect. This extended analgesic activity was in stark contrast to the rapid onset and quick decline of pain relief observed with the non-encapsulated drug. The onset of the antinociceptive effect of IND-vesicles after a two-hour latency period can be attributed to the release mechanism of IND from the lipid vesicles, which aligns with the in vivo drug release kinetics.

The results demonstrated a clear relationship between the in vivo release kinetics of IND from the lipid-based vesicles and the prolonged pain relief observed in the somatic pain model.

CONCLUSIONS

The administration of IND-loaded lipid vesicles demonstrated a sustained release profile in comparison to direct drug administration. Treatment with the micro-vesicles containing IND resulted in a longer-lasting analgesic effect in the hot plate test, unlike the non-encapsulated drug. A clear correlation was found between the in vivo release of IND from lipid vesicles made of phosphatidylcholine and CHIT, and the extended antinociceptive activity in mice. Overall, our results support the effectiveness of lipid vesicles as carriers for IND, facilitating controlled drug release and prolonged analgesic effects in this somatic pain model.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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