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Synergistic Analgesic Effect of Salmon Calcitonin Loaded PLGA Nanoparticles – In Vivo Study

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Abstract

Salmon calcitonin (subcutaneous or intranasal) has been shown to have an analgesic effect in individuals suffering from a variety of painful skeletal diseases, including nontraumatic osteoporotic vertebral fractures, in multiple prospective clinical studies. Several assessments have determined that salmon calcitonin is a secure and efficacious option for osteoporosis therapy. The precise process through which calcitonin mitigates pain is not yet understood. It's theorized that there may be specific receptors in the brain for salmon calcitonin, or that alterations in serotonergic descending pathways affecting sensory transmission via C fibers account for calcitonin's pain-relief effects in osteoporotic patients. To study this substance, Salmon Calcitonin attached to PLGA nanoparticles (SC-PLGA NPs) has been developed using dual-emulsion (W/O/W) and solid-oil dipping techniques. The morphology of the particles was analysed by scanning electron microscopy, and their analgesic effectiveness was evaluated in vivo using the tail flick method. Male albino rats with body weights between 160 and 180 grams were used for the study. Nano-sized particles ranging from 400–550 nm, mostly spherical with a limited size variance, have been synthesized. Studies confirmed that PLGA nanoparticles carrying salmon calcitonin effectively diminished pain swiftly. Groups treated with SC-PLGA nanoparticles experienced significant pain relief, confirmed by the tail flick test. Statistical evaluation via the Mann-Whitney test demonstrated that 93.33% of mice that underwent subcutaneous-PLGA nanoparticle treatment exhibited improvements. The creation of these nanoparticles carrying Salmon calcitonin correlates strongly with positive results.

Keywords**:** *Microscopy and Metastasis, Opioids, Plga Nanoparticles, Salmon Calcitonin, Subcutaneous-Plga, Scanning Electron.*

Introduction

Pain is a painful sensory and emotional experience linked to existing or potential tissue damage or characterized in terms of such harm [1]. It is frequently triggered by unpleasant stimuli and relayed to the CNS via specific neural networks, where it is recognized. It is a method of protecting the body against damage [2]. Standard pain therapies including opioids, NSAIDs and acetaminophen are usually effective in managing pain especially nociceptive types with different intensities ranging from mild to moderate and even severe [3]. These agents however pose risks such as psychological adverse effects, hepatic gastrointestinal and renal or interactions with different medications like bisphosphonates and different antihypertensives [4]. Osteoporosis is an age-associated systemic disease that is characterized by a progressive loss of bone mass, decreased bone strength and increased fracture risk [5]. The onset of the disease often has no warning sign or symptoms, and therefore may remain undiagnosed until the pain induced by an osteoporotic fracture attracts attention.

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Postmenopausal women are particularly susceptible to this disorder of bone metabolism. During child-bearing years, estrogen aids the maintenance of bone mass in women by stimulating osteoblast activity. The reduced estrogen levels in postmenopausal women lead to reduced rates of bone formation by osteoblasts, resulting in a net increase in bone resorption [5]. This goal could be effectively addressed by the application of nano/microtechnology to create local drug depots directly at the target site thus reducing the frequency of administrations while improving patient compliance with the treatment.

Calcitonin is a hormone known for its capacity to alleviate pain and preserve bone density, thus reducing the risk of fractures due to disease. Issues like hypercalcemia and nerve compression are also minimized. Calcitonin is thought to inhibit bone resorption, raise circulating endogenous opioid levels, and serve as an endorphin receptor agonist [6].

Salmon calcitonin [SC] could serve as a viable option for individuals whose bone pain remains unrelieved by other therapies. Calcitonin is considered an effective analgesic in many patients unable to tolerate NSAIDs drugs and narcotics [3] Extensive research has been conducted on salmon calcitonin, revealing its heightened potency compared to the human variant, thus establishing it as the preferred option in clinical application [7]. Having been utilized in medicine for over three decades, salmon calcitonin demonstrates positive clinical outcomes in managing metabolic bone disorders like osteoporosis and Paget's disease, in addition to treating bone metastases. Calcitonin plays a significant role in maintaining calcium balance and in the process of bone renovation. Use of formulation additives in the medicinal product to transiently change the intestinal environment or targeting particular intestinal areas with favourable peptide delivery qualities are two possible techniques to improve SC absorption (e.g., low residual volume, high absorptive surface area or reduced enzymatic activity) [8] NPs assure (i) increased drug solubility; (ii) prolonged drug stability as a result of the protective shield that reduces drug metabolism after administration and avoids fast clearance by filtering organs; (iii) enhanced transport ability across cell membranes; (iv) reduction of drug resistance mediated by extrusion pumps; (v) specific delivery of therapeutics to targeted tissue; (vi) controlled release of therapeutic cargo; (vii) reduced systemic adverse effects on healthy tissues or organs; and (viii) multimodal therapies.[9]All these properties aid in successful drug delivery.

This study examined the uptake in the gastrointestinal (GI) tract of therapeutic salmon calcitonin (SCT), contained within poly(lactide-co-glycolide) (PLGA), aimed at treating bone metastases. The research explored two organic compounds, bile acids and transferrin, for their potential to enhance absorption [11]. This study aims to assess how well subcutaneously administered salmon calcitonin-loaded PLGA Nanoparticles work in managing metastatic bone pain.

Materials and Methods

Salmon calcitonin (SCT) was obtained from Sisco Research Laboratories in Chennai. The D, L-lactide-co-glycolide polymers (PLGA) with a 50:50 molar ratio (molecular weight between 40,000 and 75,000) as well as Polyvinylalcohol (PVA, molecular weight 86,000, in a 99-100% hydrolyzed form) were sourced from Sigma Aldrich in Chennai. All additional chemicals used in this research were of analytical grade. Further materials like Glacial acetic acid, obtained from Sigma-Aldrich in Germany, and aspirin sourced from the Ethiopian Pharmaceutical Manufacturing Factory in Ethiopia, were also utilized for the experiment.

Preparation of Salmon Calcitonin loaded PLGA Nanoparticles.

Salmon calcium-loaded PLGA nanoparticles were fabricated using the double emulsion-solvent evaporation technique. Briefly, 10 mg of SCT was dissolved in 1 mL of de-ionized water (W1 phase). Then, 300 mg of PLGA was dissolved in 20 mL of methylene chloride (O phase), to which 100 µL of polysorbate-20 was added. The W1 phase was then merged with the O phase and homogenized at 13,000 rpm for 1 minute to form a primary W1/O emulsion. This emulsion was injected into 200 mL of 1% PVA solution (W2 phase) under strong stirring and further homogenized for 30 minutes at 5,200 rpm. The solvent methylene chloride was extracted from the emulsion using an evaporator. The resulting suspension, derived from the final emulsion, was centrifuged at 13,000 rpm for 30 minutes to isolate the SC-loaded PLGA nanoparticles.

Physical Characterization of Nanoparticles

The surface morphology of particles was assessed using scanning electron microscopy (SEM; Model S4300-SE, Hitachi, Japan). This study was done in the Department of Pharmacology at Saveetha Institute of Medical and Technical Sciences between May 2013 and May 2016.

Animal and Maintenance Condition

Male albino rats with body weights between 160 and 180 grams were supplied by the Biomedical Research Unit and Laboratory Animal Centre, BRILAC/SDCH/SIMATS/IAEC/3-2015/049 Chennai. All procedures conformed to CPCSEA guidelines, overseen by the Animal Ethical Committee. The animals were kept in standard laboratory conditions (25±1°C, with a 12-hour light/dark cycle), and provided with unlimited access to food and water. They underwent a 7-day acclimatization period in the lab before any experimental procedures began.

Glacial Acetic Acid-Induced Toxicity in Male Albino Rats [11]

Glacial Acetic acid was diluted in water and given once a day at a level of 10ml/kg body weight. There was no induction of the control rat.

Albino rats of either sex weighing 160 - 80 g were randomly divided into 5 groups of 4 mice per group. Group I was assigned as negative control and received vehicles. Group II served as positive control and was treated with standard drugs; aspirin (10 mg/kg) for the tail-flick test Groups III–V were used as test groups and given the test nanoparticles of 100, 200 and 400 mg/kg respectively. All treatment administrations were performed orally, and the maximum volume administered “was 0.015 ml/kg.”

Tail Flick Test

The antinociceptive (analgesic) activity of the extract was evaluated by the tail-flick method. About 5 cm from the distal end of the tail of each rat was immersed in warm water maintained at 50°C. The reaction time (in seconds) was the time taken by the rat to flick its tail due to pain. The first reading was omitted and reaction time was taken as the average of the next two readings. The reaction time was recorded before (0 min) and at 15, 30, 45, and 60 min after the administration of the treatments. The maximum reaction time was fixed at 15 sec to prevent any tail tissue injury.

If the reading exceeds 15 sec, it would be considered as maximum analgesia. The maximum possible analgesia (MPA) of the three test groups was calculated by the below formula [12].

Statistical Analysis

The Mann-Whitney test was performed to evaluate different parameters between the three test groups at different periods using statistical analysis [13].

Results

Physical Characterization of Nanoparticles

Regardless of the nomenclature, nanoparticles have a spherical form. As a result, adding Salmon calcitonin to PLGA particles had no discernible effect on the surface shape. The particle size assessed by SEM, on the other hand, showed a substantial variation. The mean diameters of SC-PLGA were 420 nm, as illustrated in Figure 1.

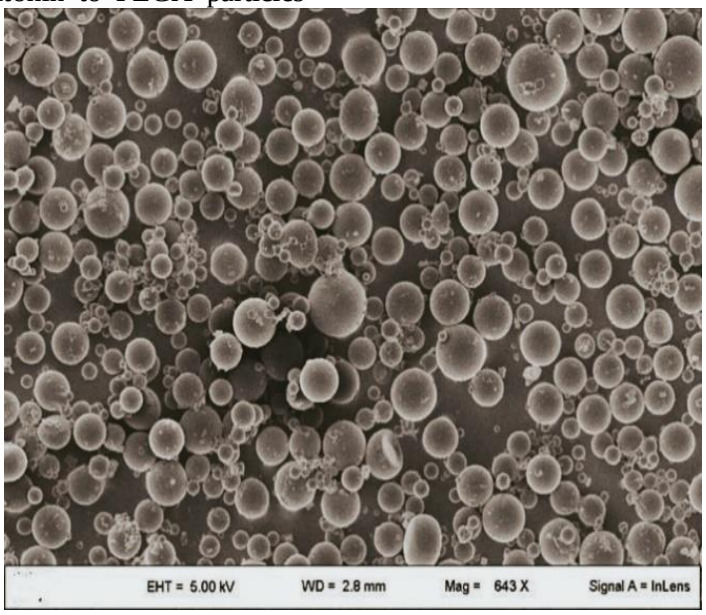

Figure 1. SEM Analysis

Tail Flick Test

The results of the analgesic activity of Salmon calcitonin PLGA Nanoparticles are shown in Table 1. A control group of mice (injected by vehicle) did not show any significant difference in the reaction time on tail flicking throughout the whole observation. Salmon Calcitonin PLGA NPS (100, 200 and 400 mg/kg) revealed a significant dose-dependent increase in the latency time when compared to the control. The maximum reaction time for Aspirin (standard) was 11.31 secs reached at 30 min, which returns to normal after four hrs (3.77 secs). On the other hand, the maximum activity of Salmon Calcitonin PLGA nanoparticles appeared after 3 hours (7.4, 8.2, and 10 secs for 100, 200 and 400 mg/kg doses, resp.) as shown in Table 1. These increases in latencies (SC-PLGA NPs) remain significant after 8 hours and even after 24 hours. The relative activity of this nanoparticle concerning aspirin in the tail-flick test shows significant changes [figure 2].

Subjective improvement was reported in 95% of mice in the salmon-calcitonin PLGA Nanoparticles group. In the Standard treatment group, however, 60% of mice reported no improvement [Table 2].

Table 1. Analgesic Effect of Different Doses of Subcutaneous PLGA NP's by Tail-Flick Method In Mice

No	Group	Body weight	Basal reaction time before drug administration					Basal reaction time after drug administration			
			1hr	2hr	3hr	4hr	5hr	1hr	2 hrs	3 hrs	4 hrs
I	Control 5ml/kg p.o	H-130	1	2	1	1	2	2	2	3	2
		B-150	2	1	2	1	1	1	1	1	1
		T-140	3	3	3	2	2	2	3	2	1

		C-150	1	1	3	2	3	1	2	1	1
II	**Standard (Aspirin) 10mg/kg i.p**	**H-140**	1	2	1	2	2	5	8	11	13
		B-150	1	2	3	2	1	6	9	13	12
		T-140	2	2	2	1	2	5	6	8	10
		C-130	1	2	1	2	2	5	7	9	11
III	**Sample A 100mg/kg i.p**	**H-150**	2	1	2	2	1	5	6	7	6
		B-140	1	2	1	1	2	4	6	6	5
		T-160	1	1	2	1	1	6	8	7	7
		C-150	1	1	1	2	2	5	6	5	6
IV	**Sample A 200mg/kg i.p**	**H-150**	1	2	2	1	2	5	7	7	6
		B-160	2	1	2	2	2	6	7	8	7
		T-120	2	1	1	2	2	6	6	7	8
		C-130	1	1	2	1	1	7	8	9	8
V	**Sample B 400mg/kg i.p**	**H-160**	2	1	2	1	1	6	7	7	6
		B-130	2	2	2	2	1	7	8	8	7
		T-140	1	1	2	2	1	5	6	5	5
		C-150	1	2	1	1	2	6	7	7	6

Figure 2. Tail Flick Method

Table 2. Subjective Assessment of Efficacy by the Investigator

	Salmon-Calcitonin PLGA NPs group	**Aspirin group**
Extremely useful	19	8
Not useful	1	12

Discussion

Nanoparticles, regardless of their name, have a spherical shape. Salmon Calcitonin PLGA has a mean diameter of 420 nm.

According to Jung et al., 2009, the subcutaneous loaded PLGA nanocapsules were made using a W/O/W emulsification method. A prescribed content additive was combined with Salmon Calcitonin dissolved in methanol. The amount of bile acids (0–7.5 mg to 6 mg) was shown to have a significant impact on both the emulsification and encapsulation processes. When 1.5 mg of bile acids were added, Salmon Calcitonin-loaded PLGA nanocapsules with a diameter of roughly 700 nm and an encapsulation effectiveness of more than 35% were generated [14].

When compared to the control, the analgesic activity of subcutaneous-PLGA Nanoparticles (100, 200, and 400 mg/kg) demonstrated a substantial dose-dependent increase in latency time. At 30 minutes, the maximal reaction time for Aspirin (standard) was 11.31 seconds, which returned to normal after four hours (3.77 seconds). In the tail-flick test, the relative activity of these Nanoparticles compared to aspirin shows considerable variations.

The mechanism of calcitonin's analgesic action is unknown. Particular salmon calcitonin binding sites probably exist in the brain. Another theory is that calcitonin's analgesic effects on pain in osteoporotic individuals are due to alterations in descending serotonergic modulation of sensory transmission mediated by C afferents. This pain-ameliorating effect is irrelevant to its osteoclastic inhibitory effect and mechanisms like altering the Na+ channel and serotonin receptor expression or hypotheses including the endorphin-mediated mechanism were used to explain this effect [4]. The analgesic action of calcitonin is advantageous throughout the medical treatment of osteoporotic patients from a clinical standpoint [15]. Oral consumption has been associated with some side effects like runny nose, nosebleed, sinus pain, nose symptoms such as crusts, dryness, redness, swelling, back pain, joint pain, upset stomach, flushing (feeling of warmth) etc [16]. Further research is needed to investigate its adverse outcomes.

In the salmon-calcitonin PLGA NPs group, 95 percent of mice showed subjective improvement. However, 60% of mice in the Standard therapy group showed no improvement. Salmon Calcitonin was integrated into nanoparticles with encapsulation efficiencies ranging from 69 to 83 percent, according to Glowka et al., 2010 In vitro release tests done in 5% acetic acid revealed significant variations in salmon Calcitonin release time patterns. Within a few hours, nanoparticles with a quick-release profile released 80–100% of the encapsulated medication. Salmon calcitonin release from pure PLGA nanoparticles, on the other hand, was delayed and incomplete, reaching just 20% after 4 weeks. After subcutaneous delivery of PLGA nanoparticles to Wistar rats, an in vivo investigation revealed that could be maintained for 3 days [17].

$$Percentage = Test\ mean - control\ mean / control\ mean\ x\ 100$$

Conclusion

The present study demonstrates that salmon calcitonin-loaded PLGA nanoparticles exhibit a significant analgesic effect in vivo. Calcitonin may be deemed a suitable option for managing acute pain associated with vertebral fractures and offers a viable substitute in the management of acute and chronic neuropathic pain where alternative therapies are ineffective. The encapsulation of salmon calcitonin within PLGA nanoparticles not only enhances the drug's stability and bioavailability but also allows for a more controlled and sustained release, thereby improving the overall therapeutic efficacy. Sustained release of drugs from PLGA-based nanoparticles could thus potentially improve the treatment efficacy [18]. Calcitonin was shown to be a useful additive to

local anaesthesia in the case of controlling postoperative pain or trigeminal neuralgia [19]. Nanotechnology has started a new era in pain management and many promising results have been achieved [20]. However, its application as an analgesic will require further pharmacological and toxicological research. Further studies are recommended to optimize the formulation and to explore the long-term safety and efficacy of this novel delivery system in clinical settings.

Conflict of Interest

There is no conflict of interest.

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