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Original Article

Formulation and Characterisation of Melatonin *in situ* Gel for Oral Application

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ABSTRACT

Background: Melatonin *in situ* gel was formulated and tested to use in the bony defects. Intrabony defects are clinical presentation in some periodontal disease. **Materials and Methods:** *In situ* gel containing 0.1% w/v Melatonin and chitosan as polymer was formulated by cold method using different concentration of chitosan and beta glycerol phosphate. Based on gelling time and gelling capacity *in situ* gel was optimized for further evaluation. Evaluation of the product for appearance, pH, gelling capacity, viscosity, *in vitro* release studies, drug content estimation, cytotoxicity test was performed. **Results:** Examination revealed drug release, gelation temperature considerably decreased with increasing concentration of polymer. The resulting solution contained 1.5% (w/v) chitosan, 12-18% (w/v) β-Glycerol phosphate and 1.2% (w/v) drug and was maintained at 4°C. **Conclusion:** Periodontal regeneration involves activity of bone and fibroblasts**.** Melatonin impacts this action of the cells. It promotes bone formation and suppresses resorption. Clarity, pH, drug content of the prepared product was satisfactory. The formulations showed sustained drug release. This is essential accomplishment in treatment of periodontitis for bone regeneration.

Keywords: Chitosan, Glycerol Phosphate, *in situ* Gel, Melatonin, Periodontal Disease.

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INTRODUCTION

Periodontal diseases are highly prevalent dental condition among dental diseases affecting oral cavity. It affects nearly 90% of the population worldwide. Periodontitis is preceded by gingivitis which affects only the soft tissues of the periodontium. It is thereby mildest form of disease whereas periodontitis affects the hard tissues like alveolar bone along with gingiva, periodontal ligament and cementum. Gingivitis presents with common inflammatory symptoms such as redness, swelling of gingiva, pain which is mild form and resolves on routine treatment like scaling. Gingivitis is caused due to accumulation of microorganisms and their products on tooth and supporting tissues of periodontium called dental plaque. If untreated gingivitis progresses to periodontitis resulting in loss of tooth and reduced quality of life (Pihlstrom *et al.*, 2005). Inflammation of the supporting tissues of the teeth is called periodontitis. Group of non-specific and specific microorganisms affects periodontal tissues. This results in attachment loss. The host immune response plays pivotal role in disease progression, systemic diseases, certain genetic factors and smoking acts as risk factors for disease modification. Treatment of periodontitis aims at elimination of etiological factors namely plaque and management of risk factors. Supportive periodontal therapy at regular intervals is mandatory for patients at risk.

Periodontal regeneration is one of the rationales of periodontal treatment wherein renewal of lost periodontal tissues is attempted. Regeneration opens wide arena for periodontal research as more than one type of tissue are involved. Biomaterials are commonly used in periodontal regeneration therapy as it improves the outcome. periodontal tissue regeneration encompasses tissue engineering concept; therefore, new biomimetic materials and scaffolding fabrication technologies have been proposed for the same (Deng *et al.*, 2022). Incorporating scaffolding biomaterials in the form of *in situ* gel formulation for periodontal regeneration is one such approach.

To obtain sustained or prolonged release of drugs for periodontal regeneration, Oral liquid *in situ* gelling systems can be used as targeted approach. The therapeutic level of the drug can be maintained over an extended period of time using this system.

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This ensures minimal dose, minimizing the adverse effect. Solution of the polymer containing drug in dispersed or dissolved form is used in this *in situ* gel system which forms a gel owing to sol-gel transition. This change from sol to gel may be either due to change in pH, temperature-induced, or ionic interaction in the area of interest (Nagarwal and Pandit, 2008). Oral *in situ* gelling system is administered in the form of a solution which transforms itself in to a gel. The nature of polymers also determines the transition of sol to gel. In the present study, this system is used for oral use and so pH induced, temperature induced change is anticipated. Diffusion-controlled release explains the linear drug release pattern of *in situ* gel (Kubo *et al*., 2003). The polymer used in the present study is Chitosan. Alkaline *N*-deacetylation of chitin produces a hydrophilic cationic polyelectrolyte called chitosan. Chitosan is biodegradable and biocompatible with low toxicity (Ahmadi and Bruijn, 2008). Chitosan is degraded by lysosomes. The allergenic property of chitosan is low with moderate immunostimulant effects. In acidic water up to pH 6.2 Chitosan stays clear and liquid. Above 6.2, it becomes jelly like solid; a hydrated gel-like precipitate is formed on further basification systemically.

The biomaterial incorporated in the *in situ* gel is Melatonin. It is a hormone secreted mainly by pineal gland and derived from an essential amino acid tryptophan (Claustrat *et al*., 2005). It has local and systemic effects in controlling inflammatory reactions. Melatonin is claimed as a broad-spectrum antioxidant. Melatonin acts by scavenging reactive oxygen species in the inflamed area and thereby minimizing the damage. Additionally, melatonin influences fibroblast activity and bone regeneration. Synthesis of type I collagen fibres is stimulated (Martin *et al*., 2000). These wide spread functions of melatonin are beneficial in treating periodontal diseases. Melatonin was considered for the formulation. *In situ* gel systems prolong the residence time of the agent (Garala *et al*., 2013). Owing to the advantage of *in situ* gel system and therapeutic benefits of melatonin, this paper aims the development and optimisation of melatonin *in situ* gel for periodontal application.

MATERIALS AND METHODS

Melatonin was obtained from sigma Aldrich. Other materials required are chitosan, beta glycerol phosphate, are accordingly obtained. The method of preparation of melatonin *in situ* gel was carried out by using 0.25%, 0.5%, 0.75%, 1%, 1.25% and 1.5% chitosan and 3%, 6%, 9%, 12%, 15% and 18% of β-Glycerol phosphate to evaluate the *in situ* gelling capacity (Pakzad and Ganji, 2016). Among these different concentrations evaluated 1.5% chitosan and 12%, 15% and 18% of β-Glycerol phosphate formed *in situ* gel (Kolawole *et al*., 2019). Hence these concentrations were selected for *in situ* gel preparation. These three formulations were mentioned as F1, F2, F3 respectively and considered for the analysis. The preparation methodology involves addition of chitosan, methyl paraben and propyl paraben in required amount of 1% acetic acid and left for 24 hr and filtered. Accurately weighed quantity of Melatonin and β-Glycerol phosphate were dispersed separately in the above solution continuously stirring to obtain clear homogeneous liquid. The entire process was carried out in an ice water bath maintained at 4ºC. The resulting solution contained 1.5% (w/v) chitosan, 12 - 18% (w/v) β-Glycerol phosphate and 1.2% (w/v) drug and was maintained at 4ºC for further study (Khodaverdi *et al*., 2012).

The clarity of prepared solutions was examined visually. It was observed in white and black backgrounds in light. The viscosity determination of the optimized *in situ* gel was determined using DV-E Brookfield Viscometer. The optimized formulation was taken in a beaker. It was kept at room temperature. Measurements were carried out using spindle number 64. Speed of 50 rpm was maintained in the sample, The corresponding dial reading was noted (Vandana and Shweta, 2019). Using a calibrated pH meter at 4ºC the pH of the gel was determined. Average of three readings were recorded.

To examine the gelation. 2 mL aliquot of gel was transferred to a test tube. Test tubes were immersed in a water bath. Gradually, temperature of water bath was increased. At new setting, equilibration was done for 5 min. Non movement of the meniscus even after tilting through 90ºC, confirms gel formation (Mustafa *et al*., 2022).

The *in situ* gel formulation equivalent to 1mg of drug was pipetted and transferred to a 10 mL volumetric flask containing 6.8 pH phosphate buffer. The solution was shaken vigorously for complete dissolution of the drug. Drug absorbance was recorded for diluted filtrate using UV-visible spectrophotometer - UV Shimadzu, Japan at 278 nm using phosphate buffer as blank. The prepared gel formulations were transferred into an identical plastic syringe to constant volume (1 mL) and the ability of the gel to flow under standard handling pressure was assessed (Nasra *et al*., 2017). The *in vitro* drug release study of Melatonin was studied using a modified diffusion testing apparatus. Prepared phosphate buffer (pH 6.8) was used as a diffusion medium. Gelatin sheet as a semi permeable membrane was previously soaked in the diffusion medium for overnight. It was tied to one end of a specially designed glass cylinder -open at both ends. The cylinder had inner diameter of 3.4 cm. 1 mL of formulation was precisely pipetted into the donor chamber. The cylinder was suspended in a acceptor chamber. The chamber contained 50 mL of diffusion medium. The membrane just touched the surface of the medium. Acceptor chamber was maintained at a temperature of 37±2ºC using magnetic stirrer. About 5 mL of sample was withdrawn at a time interval of 1 hr. It was replaced with fresh diffusion medium. They were analysed at 278 nm using UV spectrophotometer (Aslani *et al*., 2016).

The*in vitro* cytotoxicity of the melatonin *in situ* gel formed was evaluated on periodontal fibroblast cells. All materials were sterilized by gamma irradiation. Fibroblast cells were seeded in cultural vessel of 35 mM dish plates at $2x10^5$ Cells/mL as cell seeding capacity. It was incubated at 37°C under a 5% carbon dioxide humidified atmosphere in Dulbecco's Modified Eagle's Medium. It was kept for the duration of 48 hr pretesting (Halkai *et al.*, 2019). The gel was added into each plate which was placed for 48 hr in an incubator. An inverted microscope - Olympus, Tokyo, Japan was used to obtain the microscopic images as shown in Figures 1 and 2.

RESULTS

The results of pH measurements are shown in Table 1. pH of formulations was fairly constant. The pH of all the formulations was approximately 6.02 to 6.84, which is close to pH of the oral cavity. The viscosity of the Melatonin *in situ* gel is an important evaluation parameter and the results are revealed in Table 2. Viscosity of the formulation is dependent on the concentration of the β-Glycerol phosphate used. The formulated *in situ* gel was rated for the *in vitro* cumulative release pattern of Melatonin which was entrapped within the chitosan/Glycerol phosphate gel. The results showed F3 formulation release was slow and sustained up to 24 hr while F1 release was 10 hr and F2 release was 15 hr, which is dependent on the gel characteristics shown in Figure 3. The prepared formulations should pass the syringeability test for easy instillation to the periodontal pocket by applying small amount of pressure. Thus, the prepared formulations were synringeable. Results are depicted in Table 1. All the *in situ* gel formulations were assessed for Gelation time and temperature, the results are depicted in the Table 3. The results concluded that the as the concentration of *in situ* gelling agent increases the formulation F_3 gelled at 37°C within 90 sec. The Melatonin *in situ* gel was subjected to percentage drug content studies. All the formulations showed good percentage drug content from 91% to 96.6% as shown in Table 4. Figure 1 represents the periodontal fibroblast cells coated with melatonin gel in Dulbecco's Modified Eagle's Medium containing media incubated for 48 hr, the results revealed that 90% of cells are viable. Figure 2 represents the cell morphology of fibroblast cell with contact with melatonin gel after the incubation for 48 hr.

DISCUSSION

The Melatonin *in situ* formulations were subjected to pH determination for stability and maintenance with oral pH. The pH of all the *in situ* gel was in between 6.02 to 6.84, reflecting the pH of oral cavity (Dabhi *et al.*, 2010). Viscosity of the formulation is dependent on the concentration of the β-Glycerol phosphate used. As the concentration of the thermoresponsive *in situ* gelling agent increases, there will be increase in the viscosity due to intermolecular hydrogen bonding. Initially the viscosity of all the *in situ* gel formulations should be low for easy instillation to the periodontal pocket that it should be in the sol phase at 25°C. And when the *in situ* gel comes in contact with the body temperature at 37°C there will be increase in the viscosity due to the electrostatic cross-linking present in the Chitosan/ β-Glycerol phosphate hydrogel network. This increase in the viscosity enables the formulation to stay in application area for longer time. This enables the innate contact and leads to localized action of Melatonin in oral cavity (Ganguly and Dash, 2004).

Melatonin release was decreased by increasing the concentration of β-Glycerol phosphate, this may be due to modulation of

Table 1: pH and Syringeability of Melatonin *in situ* gel formulations.

Formulation code	pH±SD	Syringeability
F1	6.02±0.115	Easily
F2	6.45±0.057	Dropwise
F3	6.84±0.057	Dropwise

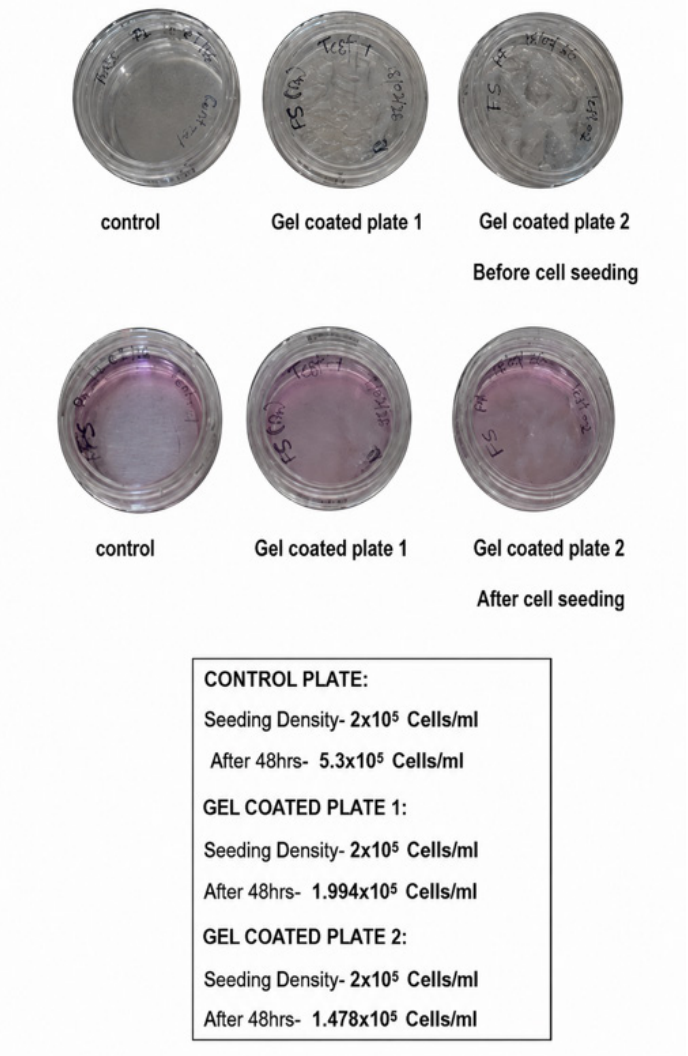

Figure 1: Periodontal fibroblast cells coated with melatonin gel in DMEM containing media (full page width).

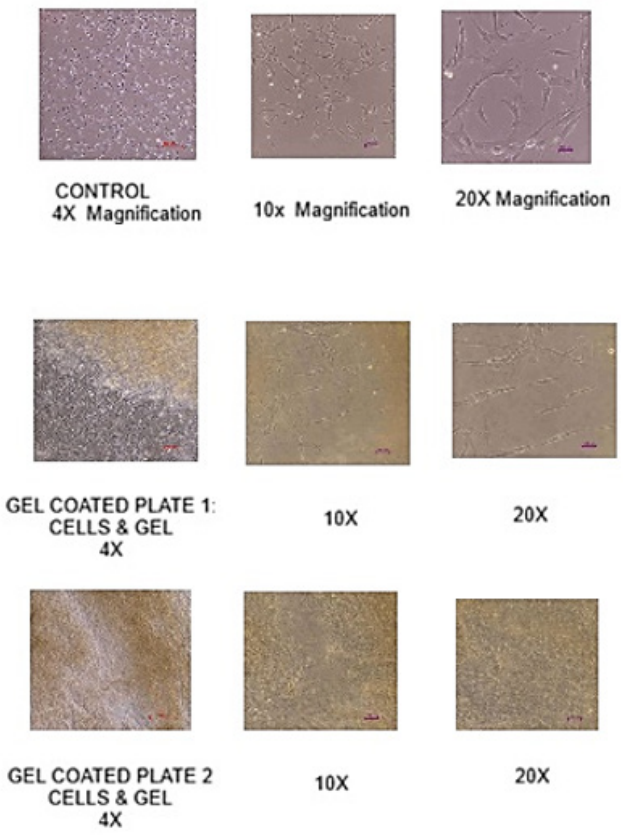

Figure 2: Cell morphology of fibroblast cell with contact with melatonin gel after the incubation for 48 hr. (full page width).

Table 2: Viscosity of Melatonin gel formulations.

Formulation code	Viscosity in cps (Mean ±SD) Before gelation	Viscosity in cps (Mean ±SD) After gelation
F1	3368±61.100	10753±20.810
F2	4173±52.493	11531±13.29
F3	5240±52.915	11946±51.310

Table 3: Gelation time and temperature of melatonin *in situ* gel formulations.

Formulation code	Gelation time	Gelation temperature
F1	250 sec	55.05°C
F2	188 sec	48.96°C
F3	100 sec	37.56°C

Table 4: Percentage Drug content of *in situ* gel formulations.

Code	% Drug content
F1	91%
F2	94.2%
F3	96.6%

electrostatic, hydrophobic interactions, mainly the hydrogen bonding between the chitosan chains which were involved in the gel formation as the β-Glycerol phosphate is added to the aqueous solution of chitosan. Thus, increase in β-Glycerol phosphate concentration will result in hydrogen bonding via OH-NH and O-HN of the chitosan. This bonding encompasses tightest network and hinders the release of melatonin from the formulation. The results concluded that the increase in concentration of β-Glycerol phosphate retarded the release of melatonin from the *in situ* gel, due to the formation of strong cross-linked network of intermolecular hydrogen bonding between OH and NH and O-HN groups of chitosan/ β-Glycerol phosphate gel network (Zhou *et al.*, 2015).

Syringeability of an *in situ* gel is an important factor to be considered in delivery of the thermoresponsive formulations at the specific site. Syringeability is the force required for the injection of the thermosensitive formulation through the predetermined gauge to the target site. This syringeability mainly depends upon the viscosity and the concentration of gelling agent. As the concentration of the *in situ* gelling agent increases, the viscosity of the formulations also increases and thus requires more pressure in expelling out the formulation through the syringe. The prepared formulations should pass the syringeability test for easy instillation to the periodontal pocket by applying small amount of pressure (Goud and Acharya, 2020) Thus, the prepared formulations were synringeable.

The temperature at which a sharp increase in the viscosity is observed is termed as gelation temperature. Time required for this is known as gelation time. This sharp increase in viscosity at the body temperature is dependent on the concentration of the thermoresponsive agent used (Parvathy *et al.*, 2015). Gelation temperature is required to be in the range of body temperature. The results showed that as the concentration of the β-Glycerol phosphate increases, the time and temperature required for the sol phase to convert into gel phase decreases. The underlying mechanism involves as the temperature increases; the physical junction zones of the chitosan chain segments start to progress. It occurs throughout the solution to form a hydrogel (Dumortier *et al.*, 2006). This happens due to the attractive hydrophobic and hydrogen bonding forces than the repulsive interchain forces. The shift to attractive forces is observed. This shift from the repulsive interchain forces is thermally induced. This mechanism can be attributed to following three main reasons:

Reduced chitosan chain polarity and increased hydrophobicity upon change or increase in temperature.

Reduced polarity and increased structuring of the free water by the glycerol moiety of β-Glycerol phosphate dehydrating chitosan chains and thus causing increased interaction interchain hydrophobic interaction.

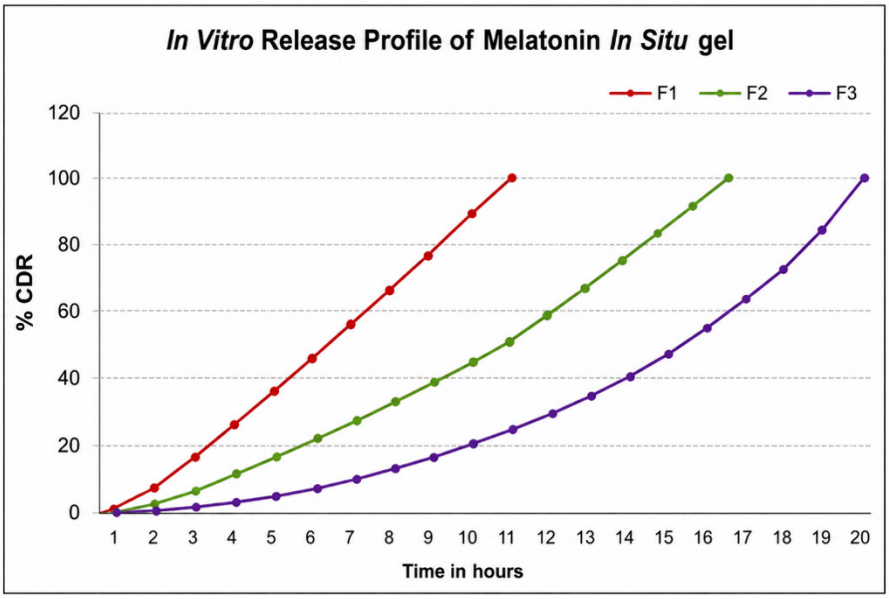

Figure 3: *In vitro* drug release profile (column width).

A thermally induced transfer of protons from chitosan amine groups to the phosphate moiety of β-Glycerol phosphate thus reducing the chain charge density and allowing attractive interchain hydrophobic and hydrogen bonding forces (Chenite *et al.*, 2001).

The gel coating showed non-uniform distribution across the culture surface after approximately time of the cell seeding the gel started float and indicating it weak adherence to the surface on the non-consistent area. Few cells settled on the gel did not attach and cells were not clear observed under microscope. Around 50% cells got attached to the culture surface below the gel and showed cell proliferation (Yoshino *et al.*, 2013). These results confirm that the resultant melatonin *in situ* gel is non-cytotoxic and potentially suitable for biomedical applications. Melatonin *in situ* gel is non-cytotoxic and potentially suitable for biomedical applications.

CONCLUSION

Melatonin for the treatment of periodontal disease is justified. It was successfully formulated in the form of *in situ* gel. This formulation was prepared by cold method. Beta glycerol phosphate and chitosan were used as gelling agent. Chitosan was used to sustain Melatonin release. The mucoadhesive property of chitosan prolongs residence time of Melatonin. Also, the contents of the present formulation like chitosan, glycerol phosphate and melatonin individually contribute to periodontal regeneration. this adds to the benefit of the formulation. Melatonin is a free radical scavenger and significant broad-spectrum antioxidant. Melatonin influences fibroblast activity and bone regeneration by promoting osteoblast differentiation and bone formation and suppression of bone resorption. The present Melatonin *in situ* gel can be used effectively for the clinical application in periodontal bony defects.

ABBREVIATIONS

DMEM: Dulbecco's Modified Eagle's Medium; **UV:** Ultraviolet; **w/v:** Weight per volume; **rpm:** Revolutions per minute; **SD:** Standard Deviation.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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