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Rasagiline mesylate mucoadhesive buccal microsphere-loaded gel formulations: A new candidate for non-oral Parkinson's treatment

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ABSTRACT

Parkinson's disease (PD) is one of the most common neurological diseases worldwide and affects over 10 million people around the world. Mucosal administration as an alternative to oral administration is very important for the patient's compliance with the treatment. The purpose of this study Rasagiline mesylate (RM) microspheres (MS) and RM MS-loaded gel formulations were developed and evaluated. Particle size, encapsulation efficiency, and loading capacity of MS were evaluated. For buccal administration, mucoadhesive RM MS was dispersed in chitosan (Chi) gels and characterized from the point of viscosity, mechanical, mucoadhesive, rheological, and release properties. The *in-vitro* cytotoxic effects of RM microspheres and RM MS-loaded gel formulation were tested against human embryonic kidney epithelial cells (HEK-293T) and mouse embryonic fibroblast cells (NIH/3T3) lines. The tested formulations did not have toxic effects on cells after 12 hours. RM MS-loaded gel formulation was successfully prepared and evaluated.

Keywords: Parkinson's disease, Rasagiline mesylate, mucoadhesive, buccal delivery, microspheres

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INTRODUCTION

Parkinson's disease (PD), also known as Paralysis Agitans, is characterized as an idiopathic neurodegenerative disorder affecting the central nervous system. It arises from the demise of dopamine-containing cells in the substantia nigra, a central region of the brain. Furthermore, the monoamine oxidase (MAO) enzyme diminishes dopamine levels by catalyzing its breakdown. MAO inhibitors are commonly employed to impede dopamine degradation. In the early stages of Parkinson's disease, monoamine oxidase type B (MAO-B) inhibitors may assist in maintaining synaptic dopamine levels, potentially delaying the initiation of levodopa intake by patients. Particularly in advanced PD cases with levodopa-induced response fluctuations, MAO-B inhibitor drugs could enable the use of lower levodopa doses. Rasagiline mesylate (RM) is a potent, selective, irreversible MAO-B inhibitor devoid of tyramine-enhancing effects and exhibiting neuroprotective activity[1]. It is utilized either as monotherapy in early PD without dose titration or as an adjunct to levodopa in later stages, administered at a daily dose of 1 mg. Literature suggests that rasagiline has an oral bioavailability of 35%, with a relatively short half-life ranging from 1.5 to 3.5 hours. Additionally, RM undergoes extensive liver metabolism mediated by cytochrome P450 type 1A2 (CYP1A2)[2]. Many researchers and clinicians acknowledge that oral therapies for symptom control in Parkinson's disease may become less effective as the condition progresses. Dysphagia is often implicated, but the impact of gastrointestinal issues is increasingly emphasized. Gastrointestinal dysfunctions, including delayed gastric emptying and reduced absorption of oral medications, can contribute to motor and non-motor fluctuations in patients. Given these challenges, physicians must explore alternative treatment approaches for PD patients[3,4]. To address this need, numerous studies have been conducted to develop non-oral drug delivery systems. Non-oral drug delivery through mucosal linings of the nasal, rectal, vaginal, ocular, and oral cavities provides distinct advantages over traditional oral administration for achieving systemic drug delivery. Key benefits include the potential avoidance of presystemic elimination within the gastrointestinal tract and the circumvention of the first-pass hepatic effect[5,6]. The buccal area is a highly acceptable route for patients with excellent accessibility in comparison with other mucous membranes. Buccal mucosa shows good permeability as it contains quite a lot of blood vessels. In addition, it is known to have a robust and rapid healing feature[7]. Therefore, it is an attractive region for drug administration[8]. Because the buccal mucosa has large smooth muscle and relatively immobile mucosa, it is a suitable site for the administration of controlled-release dosage forms. Disadvantages of drug delivery by this route are the low perme-

ability and a smaller surface area. Due to saliva secretion, the persistency of the drug in the region is shortened, and swallowing the saliva prevents the loss of the active substance and its absorption from the targeted area. In addition, involuntary ingestion of the dosage form may lead to suffocation. It is also possible that when using the dosage form, the patient may feel uncomfortable while eating[9]. The use of mucoadhesive drug delivery systems can prevent these problems, and efficiency can be increased by preparing vesicular or particulate systems such as liposomes, nanoparticles, or microspheres (MS) with mucoadhesive properties[10]. Among these systems, MS are larger in size and can provide higher loading capacity. In addition, MS prepared with mucoadhesive polymers can adhere strongly to the mucosa and remain in that area for a long time. However, mucoadhesive gels are seen as a suitable carrier for MS in order to prevent separation from the mucosa by salivary secretion and to provide long-term drug release when applied to the buccal mucous. The main aim of this study was to develop bioadhesive RM MS prepared with Carbopol 2020 NF and Eudragit E100 (EE100) for PD non-oral treatment. Afterward, MS were dispersed in chitosan (Chi) gel to prolong buccal residence time, provide sustained release, and enhance efficiency and bioavailability. Finally, the biocompatibility of novel formulations was tested against mouse embryonic fibroblast (NIH/3T3) and human embryonic epithelial (HEK-293T) cell lines by *in-vitro* cell culture studies.

METHODOLOGY

RM was gifted from Ali Raif Pharmaceutical Industry, Turkey. Chi (viscosity 0,2-0,8 Pa.s) was purchased from Sigma-Aldrich (St Louis, MO, USA). Eudragit E100 and Carbopol 2020 NF were gifted from Evonik Industries (Essen, Germany) and Lubrizol (Wickliffe, OH, USA), respectively. Magnesium stearate was purchased from Sigma-Aldrich (St Louis, MO, USA). All other chemicals were of analytical grade. NIH/3T3 and HEK-293T cell lines were purchased by American Type Culture Collection (ATCC), USA. Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), L-glutamine, trypsin-EDTA were purchased by Biowest, France. Trypan Blue solution was purchased by Biological Industries (Israel), Sterile cell culture plastics and ThinCert inserts were purchased by Greiner Bio-One, Germany. WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitro-phenyl)-2H-5-tetrazolio]-1,3-benzene sulfonate) cell proliferation reagent was purchased by Roche Diagnostics, Germany.

Fourier transform infrared spectrum analysis

RM, Eudragit E100, Carbopol 2020 NF, Chi and their mixtures (1:1) were mixed homogeneously with potassium bromide and compressed under pressure. The compressed powder mixture was scanned using IR spectrometry at a wavelength of 400-4000 cm^{-1} (Spectrum 100; PerkinElmer, Waltham, MA, USA).

Preparation of rasagiline mesylate mucoadhesive MS

MS were prepared with the solvent-evaporation method[1,2]. After dissolving 3 g of EE100 in 16 mL of acetone, 2 g of Carbopol 2020 NF was then added to this solution as a mucoadhesive polymer. Magnesium stearate (0.6 g) and RM (1 mg of RM in 2 g of gel) were suspended in 8 mL of acetone and added to the polymer dispersion. The resulting dispersion was cooled to 5°C and added slowly to 80 mL of liquid paraffin at the same temperature with stirring at 750 rpm. It was then mixed at 40°C for 50 minutes at 750 rpm. Then it was cooled to room temperature and mixed at 750 rpm and 30 mL of *n*-hexane was added. MSs formed in liquid paraffin were filtered and washed five times with 50 mL of *n*-hexane and dried at room temperature overnight. Obtained MS were sieved with 75, 125 and 250 μm sieves, and MS at 125 μm were used for further testing due to high production efficiency.

Characterization of microspheres

Particle size distribution

Mean diameters of RM MS were concluded with a Malvern Mastersizer 2000 (Malvern Instruments, Malvern, UK). The measurement was made while the MS suspended in *n*-hexane were mixed at 2000 rpm. All trials were run in triplicate.

Scanning electron microscopy

First, MSs were coated with gold palladium (Au/Pd) using a vacuum evaporator, and the surface morphology was examined by scanning electron microscopy at 5 kV pressure. (Thermo Scientific Apreo S, Germany).

Encapsulation efficiency and drug loading of microspheres

Drug-loaded MSs (0.5 g) were dissolved in 40 mL ammonium acetate buffer solution (pH 5.8) and mixed at 200 rpm for 24 hours at ambient temperature with horizontal shaker. The solution was filtered through a 0.45 μm syringe filter and the filtrate analyzed by high performance liquid chromatography (HPLC) (Thermo Scientific Accela, USA) to determine the amount of RM loaded in the MS. Encapsulation efficiency (%) and drug loading (%) were calculated according to the following equations.

Encapsulation efficiency (%)[1]: (Total Free RM-Total RM/ Total Free RM) 100

Drug loading (%)[2]: (Total RM-Free RM/Total amount of formulation components) 100

A fully validated HPLC method was used for RM assay using a Kromasil C18 (2504.6 mm, 5 µm) column at 25°C. Ammonium acetate buffer: Acetonitrile mixture (40:60) was used as mobile phase from HPLC studies. 50 µL of sample was injected into the system at a flow rate of 1 mL/min. The wavelength was 265 nm[3].

Preparation of Chi gels

To prepare mucoadhesive Chi gel, 3% (w/w) Chi was dissolved in 1% acetic acid solution. In order to obtain a clear gel, it was kept at ambient temperature for 24 hours and then, the calculated amount of MSs according to the encapsulation efficiency results were mixed and dispersed in the gel formulation until it became homogeneous (0.045 g MS/2 g gel)[2].

Characterization of MS loaded gel formulation

Mechanical and mucoadhesive properties

Mechanic tests and bioadhesion studies have been done with software-controlled penetrometer (TA-XT Plus texture analyzer; Stable Micro Systems, Godalming, UK). Test conditions are stated Table 1[2].

Table 1. Test conditions

Conditions of mechanic test	Conditions of mucoadhesion study
Prob: Perspex (10 mm diameter)	Prob: Perspex (10 mm diameter)
Cell load: 0.5 kg	Cell load: 0.5 kg
Speed of probe before test: 2 mm/s	Speed of probe before test: 1 mm/s
Speed of probe during test: 2 mm/s	Speed of probe during test: 1 mm/s
Speed of probe after test: 2.5 mm/s	Speed of probe after test: 2.5 mm/s
Target mode: Distance	Return distance of the probe: 20.000 mm
Penetration distance of the probe: 15.0 mm	Contact time between gel and buccal mucosa: 60 s
Force that the probe should feel to start the test: 0.05 N	Force applied during the contact of the gel with the buccal mucosa: 6000 mN

*Dx (10) refers the particle diameter of 10% of the sample volume, Dx (50) refers the particle diameter of 50% of the sample volume Dx (90) refers the particle diameter of 90% of the sample volume.

Rheological properties

The rheological properties of the formulations were characterized as described in the literature using a TA TX Discovery HR1 rheometer (TA, USA) at 25 and 37 ± 0.1°C[4].

To determine the storage modulus (G') and loss modulus (G"), oscillatory analysis of the formulations in the linear viscoelastic region was performed as indicated in the literature. Studies were carried out at 25 and 37 ± 0.1°C using a 40 mm diameter steel probe, 0.3 mm gap[5].

In-vitro release studies

Chi gels (2g) containing 1.56 mg of RM MS were placed into dialysis membranes (MW 12,000–14,000 Da). These membranes were then immersed in 35 mL of artificial saliva fluid (composed of disodiumhydrogenphosphate dihydrate 2.38 g, Potassium dihydrogenphosphate 0.19 g, sodium chloride 8 g, with distilled water q.s. to 1000 mL) to simulate the conditions of the buccal area and stirred at 300 rpm. The amount of artificial saliva fluid and the formulation introduced into the dialysis membranes was determined based on sink condition calculations.

At predetermined time intervals, samples were withdrawn. To maintain a constant volume of the medium, an equal volume of ambient liquid was added to the medium, thereby ensuring the maintenance of sink conditions[11]. Drug content was analyzed using the developed HPLC method (n=6).

Ex-vivo permeation studies

Permeability studies were performed using vertical jacketed Franz-type diffusion cells (Logan, Germany) with a diffusion area of 0.384 cm^2. As a result of the literature review, it is seen that the bovine cheek can be used to mimic the human buccal area in buccal permeation studies[6,7]. As the membrane model, mucosa samples taken from the inner cheeks (buccal area) of freshly slaughtered bovine for human consumption obtained from the local slaughterhouse were used. After collection, samples were immediately placed in PBS (pH 7.4), transferred to the laboratory in a refrigerated transport box within 1 hour, surgically processed to remove excess fat and connective tissue within 2 hours of animal sacrifice, and then stored at -20°C. Before starting the run, samples were equilibrated at room temperature and held for approximately 1 minute in pre-warmed 60°C PBS (pH 7.4). The connective tissue was then carefully peeled from the mucosa to obtain heat-separated epithelium along with the intact basal lamina. A digital micrometer was used to see if the mucosal thicknesses were homogeneous (250 μm). The samples were then incubated in PBS for approximately 3 hours at room temperature and the medium was refreshed

with fresh PBS every 15 minutes. Appropriately sized tissue sections were placed in vertical Franz-type diffusion cells, artificial saliva fluid was added to the recipient chambers, and allowed to equilibrate for 1 hour at 37 ± 0.1°C. Formulations weighing 0.3 g were placed on the mucous membranes and the study began. At certain time intervals, samples (1 mL) were taken from the receiving chamber and added to the medium containing 1 mL of artificial saliva fluid at 37 ± 0.1°C to maintain the sink conditions. The study was carried out at 37 ± 0.1°C for 24 hours. The amount of RM passing through the bovine buccal mucosa was analyzed by validated HPLC method. And membrane integrity was evaluated. A graph was drawn between the % cumulative amount of rasagiline mesylate passing through the buccal mucosa and the time (h). The steady state flux, Jss (μg/cm^2.h), was determined from the slope of straight line of the plot. All data were presented as mean ± standard deviation (n=6)[8].

In-vitro cytotoxic assay

Novel MS and MS-loaded Chi gel formulations' effects on cell viability against HEK-293T and NIH/3T3 cells by WST-1 reagent[9]. To cell culture treatments, formulations were extracted in DMEM. For this purpose, 0.025 g MSs and 5 mL DMEM were added into a sterile tube. In another tube, 2 g MSs-loaded Chi gel was added into a 4 mL DMEM medium. Tubes were incubated in an ultrasonic water bath for 8 hours at 37°C. Then, the tube contains filtered through a 0.22 μm sterile syringe filter.

Cells were grown in 2 mM L-glutamine and 10% FBS supplemented DMEM in conventional cell culture conditions. The day before the assay, cells were counted and seeded into the 12-well ThinCert™ plates at a density of 5x10^5 cells. Plates were incubated in a conventional CO_2 incubator overnight[10]. After the medium was replaced with 1 mL of fresh DMEM, ThinCert™ inserts (0.4 μm pore size) were placed into each well. Then 0.5 mL of filtered formulation extracts were added into inserts. Cells were treated with formulations for 12, 24 and 48 hours. Then inserts were discarded. The cell medium was replaced with a 0.5 mL WST-1 reagent prepared in fresh DMEM (10%) and incubated for four hours. The intensity of the red color formed in the wells, correlated with the number of viable cells, was measured in the spectrophotometer (CLARIOstar Plus, BMG LabTech) at 450 nm. Cell viability was expressed as the percentage of formazan absorbance. Results were given as mean of three different experiments plus minus standard deviation (Mean ± SD, n=3). Statistical analysis of the results was carried out by one-way ANOVA analysis in GraphPad Prism 5.0 statistical package program. The level of significance was accepted as $p<0.05$.

Stability studies

The stability of formulation was investigated at two temperatures and relative humidity (25°C ± 2°C/60% RH, 40°C ± 2°C/75% RH). Formulations were placed in the stability cabinet (Nüve TK 252, Türkiye) in a coated aluminum tube. Organoleptic properties, RM amount, flow properties and viscosity of the optimum formulation were evaluated at 0, 30 and 90 days.

RESULTS and DISCUSSION

It is widely recognized by researchers and healthcare professionals that with the progression of PD, conventional treatments for symptom control may become less effective. There may be different reasons for this result, but the importance of gastrointestinal system-related problems such as delayed gastric emptying and decreased absorption of oral treatments is better understood over time. If alternative treatment modalities for PD by-pass the gastrointestinal tract as with non-oral treatments, it may be valuable in patients who develop motor complications such as severe motor and/or non-motor fluctuations despite optimized oral therapy, particularly in patients with gastrointestinal absorption problems including gastroparesis. The buccal route can directly deliver a drug into the systemic circulation, avoiding gastrointestinal degradation and bypassing first hepatic metabolism. In addition, the buccal area can reach the area of 50 cm^2 for drug permeation. Nevertheless, efficient drug delivery through buccal mucosa has several disadvantages such as a low drug permeability and a low drug residence time. To overcome these limitations, the use of bioadhesive MS can be a useful approach to increase drug permeation and expand residence time[12].

FTIR analysis

According to the literature, the results obtained from the FTIR analysis were found to be appropriate and it was observed that there was no interaction between RM and the polymers (data not shown).

Preparation and characterization of RM MS

RM MS were efficiently prepared by solvent evaporation method[2]. At beginning, several bioadhesive polymers used for preparation of MS. The shapes of the MS were non-uniform and asymmetrical when prepared with Eudragit RS 100 and different type of HPMC (data not shown). When MS was prepared with Eudragit E100 and Carbopol 2020NF polymers, uniform and spherical particles with narrow size distribution and good surface properties were obtained. This is our final formulation was determined for future studies. The surface morphology of MS was visualized by scanning electron microscopy and

most MS were spherical shape with uneven surface morphology and the surface was free of drug crystals (Figure 1).

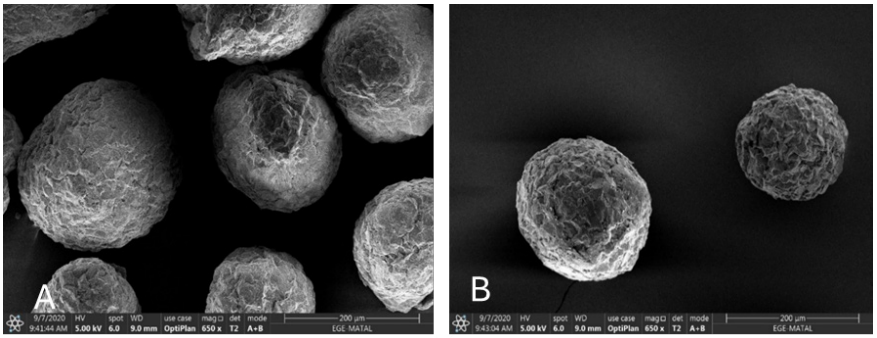

Figure 1. (A) Scanning electron microscopy of blank MS, (B) Scanning electron microscopy of RM loaded MS.

The particle size and distribution of MS were examined by laser-light scattering (Table 2).

Table 2. Particle-size distribution of MS

Formulation Code	Dx (10) µm	Dx (50) µm	Dx (90) µm	Span
Blank MS	150 µm ± 4.6	284 µm ± 5.2	503 µm ± 5.5	1.486
RM loaded MS	134 µm ± 3.2	196 µm ± 4.9	279 µm ± 5.0	4.839

When the results were examined, it was seen that 50% of the RM-loaded MSs had particle sizes of 196 µm, and 50% of the empty MSs were 284 µm in size. Span value was determined as 1,486 for blank MS and 4,839 for RM loaded MS. A span value close to 0 means that the particles are more uniform, and the dimensional consistency is better. However, the span value depends on the characteristics of the sample and there is no definite principle regarding this value. It is seen in the literature that some examples have significantly large span values[13].

MSs with RM-loaded high drug encapsulation efficiency and loading capacity could be produced. The encapsulation efficiency and loading capacity were found to be 85.20 ± 1.1% and 3.45 ± 0.04%, respectively. However, MSs were produced with high production efficiency (88.13% for total produced MSs and 59.75% for 125 µm size particles). In a study by Toksoy et al., RM solid lipid nanoparticles were prepared for nasal application. The encapsulation efficiency for RM solid lipid nanoparticles was found to be 37.8 ± 0.596%. It was deter-

mined that the remaining RM was in the aqueous phase of the formulation[14]. Upon examining the previous literature, it can be concluded that, based on the obtained values for encapsulation efficiency, loading capacity, and production efficiency, solvent evaporation is a simple and suitable technique for producing RM-loaded microspheres.

Preparation and characterization of MS loaded gel

It is requested that the applied gel adheres to the mucosa and remains there. Designing buccal formulations using bioadhesive biomaterials that strongly adhere to the buccal mucosa prevents washing of the carrier during eating and with tongue movements and increases the persistency of the drug in the target area. Therefore, MS were suspended in bioadhesive chitosan gel to prolong residence in the buccal mucosa. For this purpose, gel formulations of chitosan at different ratios and molecular weights (low, medium and high) were prepared. For this purpose, medium molecular-weight chitosan at 2% concentration was chosen as the gel base because of its strong mucoadhesiveness, proper mechanical and rheological characteristics (data not shown).

Flow properties, viscosity and oscillation properties of the formulation are important in terms of applicability. When it comes into contact with the buccal mucosa, it is desired that the formulation has a strong gel structure and high viscosity value. Thus, the formulation is desired to remain in the buccal mucosa for a long time and help the diffusion of the active substance.

Pseudoplastic flow is observed in systems whose viscosity decreases as shear rate increases. In this type of flow, a constant viscosity cannot be mentioned and generally observed in gel and emulsion systems. The selected formulation showed pseudoplastic flow (Figure 2), indicating that the viscosity of the gel formulations decreased as they were mixed, with shear thinning behavior demonstrated at both temperatures (Figure 3). When previous literatures examined, we observed that the viscosity of our MSs loaded gel formulation were suitable for buccal application[15].

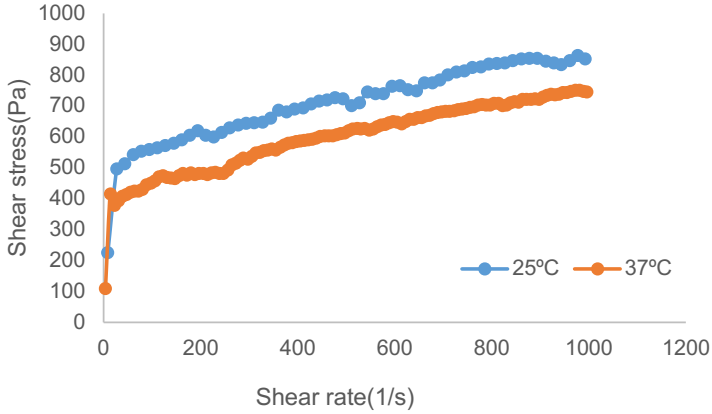

Figure 2. Shear rate–shear stress curve for the blank gel formulation at 25°C and 37°C

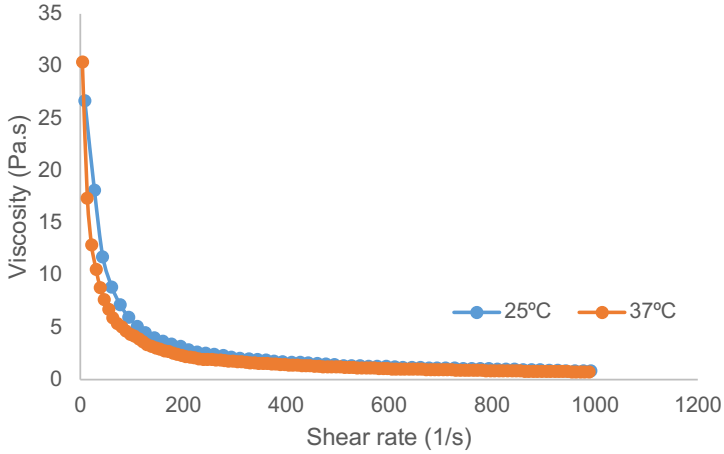

Figure 3. Shear rate–shear stress curve for the RM MSs loaded gel formulation at 25°C and 37°C

Additionally, within the scope of the rheological studies, oscillation tests that are a dynamic method were performed to determine about viscous and elastic properties of the formulations. During these measurements storage modulus G′ (elastic response) and the loss modulus G″ (viscous response) curves were obtained at two different temperatures. In Figure 4 and Figure 5 the plots of G′ and G″ were shown as a function of frequency at two different temperature values. G′ and G″ moduli of MSs loaded gel formulations were high at room temperature and decrease significantly at body temperature. At both temperature values, G′ dominated G″. It has been observed that the gap between the

two modules widens with increasing frequency. When these results were evaluated, it was seen that the prepared MS loaded gel formulation had a strong gel structure[2].

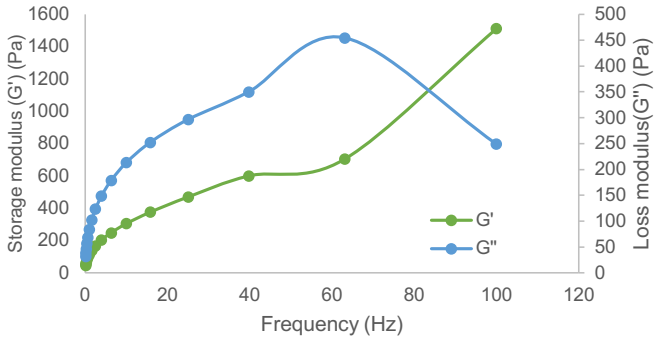

Figure 4. Frequency-dependent changes of the viscoelastic properties of RM MSs loaded gel formulation at 25°C

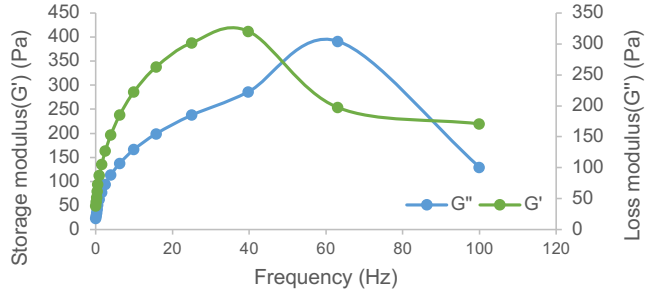

Figure 5. Frequency-dependent changes of the viscoelastic properties of RM MSs loaded gel formulation at 37°C

Gel formulations must have some suitable mechanical properties such as high adhesiveness, ease of application to the surface, low hardness and good adhesion in the application area to provide an effective treatment. TPA is a very useful device for evaluating these mechanical properties of gel formulations. Therefore, the mechanical properties of the blank and MS-loaded gel formulations were characterized in terms of hardness, compressibility, adhesiveness, elasticity and cohesiveness using TPA. The mechanical properties of the formulations are presented in Table 3.

Table 3. Mechanical properties of blank and RM MSs loaded gel formulations

Code	Hardness (g) ± SD	Compressibility (g.s) ± SD	Adhesiveness (g.s) ± SD	Cohesiveness ± SD	Elasticity ± SD
Blank gel	0.71 ± 0.01	0.99 ± 0.017	1.40 ± 0.07	0.84 ± 0.004	0.52 ± 0.059
MSs loaded gel	2.04 ± 0.01	4.31 ± 0.210	18.02 ± 1.520	0.97 ± 0.008	1.04 ± 0.06

It is important for the hardness value to be low, enabling easy application of the formulation and ensuring good spreadability. The compressibility value should be low so that the patient can easily take the formulation from the container during use. Higher adhesiveness behavior is significant to provide great adhesion and high drug retention in the buccal mucosa. Elasticity represents the return rate of the deformed sample to its beginning condition. Also, cohesiveness shows the effect of repeated shearing stresses on the formulations. As shown in Table 3, depending on adding MSs, hardness, compressibility and adhesiveness values of formulation increased significantly, and this was thought to be associated with the motion of the MSs. These results were in accordance with rheological evaluations. Based on the results, it appeared that the addition of MSs strongly improve the mechanical properties of gels. When the results of the mechanical properties were examined and their compatibility with the literature was evaluated, it was seen that prepared formulation had acceptable mechanical properties for mucosal application[16].

When the results of mucoadhesion studies presented in Table 4 were examined, adding the MSs in gel, caused to increase in the mucoadhesion. This result showed that the developed formulation can provide drug release at the buccal mucosa having the appropriate mucoadhesive properties.

Table 4. Mucoadhesive properties of blank and RM MSs loaded gel formulations

Code	Force (mN) ± SS	Mucoadhesion (mN.mm) ± SS
Blank gel	22.03 ± 2.01	12.45 ± 3.25
MSs loaded gel	39.57 ± 2.33	19.02 ± 1.21

The *in-vitro* release profile of RM MS-loaded gel formulations were examined in artificial saliva at 37 ± 0.1°C with dialysis bags, and results are displayed in Figure 6.

MS has the potential to be used for targeted and controlled release drug delivery in general; however, the addition of bioadhesive properties to MS also has significant additional advantages such as closer contact with the mucus layer,

efficient absorption of drugs and also improved bioavailability due to targeting of drugs to the absorption site[11]. Prolonged release of drugs and a reduction in frequency of drug administration can highly improve the Parkinson's patient compliance. Gel formulations are systems that reduce the release rate of the active substance compared to colloidal systems. For RM MS-loaded gel formulation, the gel system must first be eroded and then the active substance must be released from the MS. In one study, RM transfersome loaded in situ gel formulations were developed for nasal application. When RM *in-vitro* release was examined, RM release was found to be between 64.42-100.25% at the end of the 8th hour. In the same study, it was determined that the drug was completely released from the RM dispersion prepared after 30 minutes[17]. The formulation developed in our study were evaluated for their release rate. As seen in Figure 6, the formulation released ~80% after 24 hours and initial burst release was not apparent, which was related to MS structure. The slow release of the RM from the formulation suggests homogeneous entrapment of the drug throughout the systems and could have the potential to contribute to a lower dosing frequency.

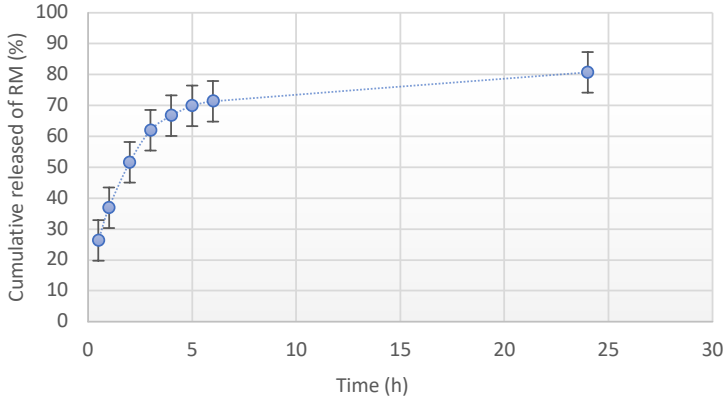

Figure 6. *In-vitro* release profile of RM MS-loaded gel formulation

Ex-vivo permeation study

Drug molecules diffuse through the buccal mucosa in two different ways: paracellular and transcellular. Paracellular transport takes place in the intercellular space between buccal epithelial cells. On the other hand, transcellular transport occurs by the transport of drug molecules from different cell layers[8]. It is known that RM is an active substance belonging to BCS class III. This shows that RM has high solubility and low permeability. In the results of permeability studies in the literature, it is seen that the pure RM solution has low flux val-

ues[18]. Within the scope of our study, the flux was found to be 86.44 μg/cm²/h ± 4.34 for the RM MS-loaded gel formulation. At the end of the 24th hour, the % cumulative permeated RM was determined as 20% ± 1.54. The permeation graph is shown in Figure 7.

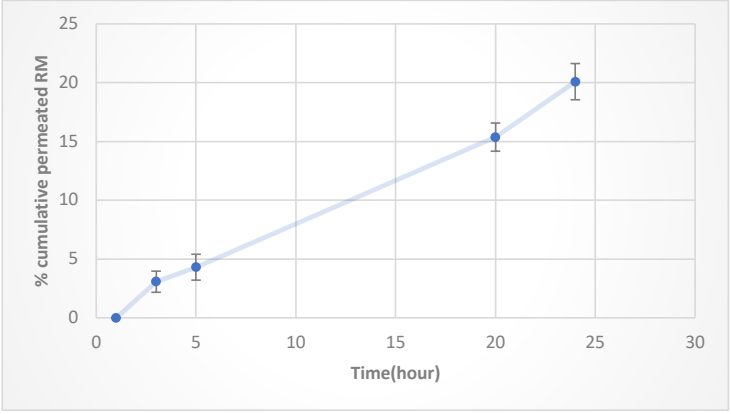

Figure 7. RM permeation profile through the buccal mucosa

During *ex-vivo* studies, it is thought that the small volume of dissolution environment in the donor chamber and the fact that the upper part of the mucosa is not moistened by saliva reduces swelling of the formulation, resulting in slower drug release in contrast to dissolution studies. Similar results have been obtained from different studies[19].

Cell culture and *in-vitro* cytotoxicity studies

To determine the biocompatibility of novel pharmaceutical carrier formulations, *in-vitro* cell culture cytotoxicity experiments provide valuable preliminary data[20]. Therefore, in this study, we investigated the cytotoxic potential of the novel MSs, RM loaded MSs, blank chitosan gel (Blank gel), blank MSs added chitosan gel (MSs-loaded gel), and RM MSs loaded gel formulations against HEK-293T cell and NIH/3T3 cells by WST-1 method. Obtained data are represented in Figure 8 and Figure 9. In experiments performed on HEK-293T cells, it was determined that the tested formulations did not show any cytotoxic effect on these cells at 12- and 24-hour incubation times ($p>0.05$). Similarly, at the end of the 48-hour treatment, no evidence of cytotoxicity was found in the blank gel and MSs-loaded gel formulations treated groups (97.68 ± 2.49% and 93.52 ± 3.19%, respectively) ($p>0.05$). In contrast, blank MSs, RM/MSs and RM/MSs-loaded gel treatment caused a significant decrease in HEK-293T cell viability compared to the control cells (93.47 ± 1.98%, 92.84

± 1.91%, and 93.52 ± 1.71%, respectively) ($p<0.05$). On the other hand, it was also determined that the cell viability in all tested groups was 90% and above.

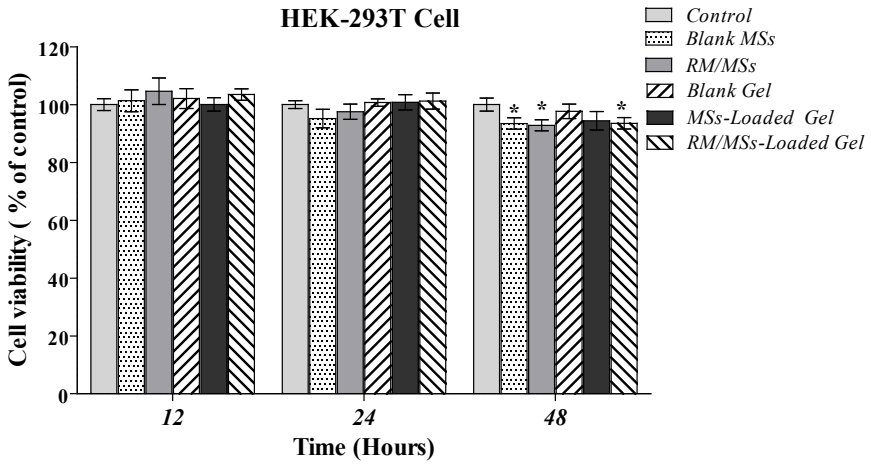

Figure 8. Cell viability evaluation following WST-1 assay on HEK-293T cells treated with tested formulations*

*The bars show the % cell viability compared to the control. Data expressed as mean ± S.D. (n=3). Cell viability significantly decreased compared to control cells, $p<0.05$.

As shown in Figure 9, in experiments performed on NIH/3T3 cells, similar to HEK-293T cell, after 12-treatment with formulations, % cell viability values were observed similar to control in all formulations tested ($p>0.05$). Among the cells treated for 24 hours, cell viability was determined to slightly decreased in the only blank gel treated group compared to the control (82.54 ± 2.58%, $p<0.05$). Controversially, it was observed that all the tested formulations were caused a significant decrease in NIH/3T3 cells at the 48 hours ($p<0.05$).

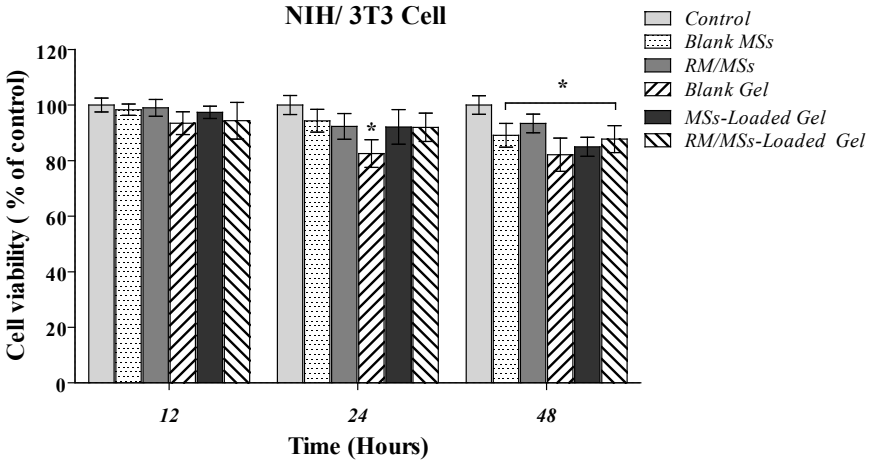

Figure 9. Cell viability evaluation following WST-1 assay on NIH/3T3 cells treated with tested formulations*

*The bars show the % cell viability compared to the control. Data expressed as mean ± S.D. (n=3). Cell viability significantly decreased compared to control cells, $p<0.05$.

Buccal formulations are drug delivery systems that stand out with their very short application times and rapid release of the active ingredient. The fact that no toxic effect of any formulation tested in the cytotoxicity studies we conducted was detected within 12 hours of the treatments reveals that these novel developed formulations are biocompatible for drug carrier systems.

Stability studies

Drugs must be effective and safe throughout their shelf life and until they reach patient use. Stability is one of the most important quality indicators. When the stability of a drug is mentioned, many factors are evaluated together. For example, the chemical properties of the active substance forming a dosage form, the interaction of the excipients in the formula with the drug molecule, the possibility of the product to encounter external environmental conditions such as high temperature, light and humidity determines the stability of the pharmaceutical product. Stability is a prerequisite for the pharmacological effect expected from an active substance[21]. In the light of the stability data obtained, it was observed that there was no significant change in the amount of active substance of the RM MS-loaded gel formulation. In addition, in the rheological examination, there was no significant difference in flow properties and viscosity (data not shown).

RM MS-loaded gel formulation could be a good alternative to conventional therapy of RM to overcome the limitations of the oral application and also reduce the dose-dependent side effects. A spherical and uniform RM MS with an

average diameter of 196 μm, a drug loading of 3.45 ± 0.04% and an encapsulation efficiency of 85.20 ± 1.1% could be successfully prepared by solvent evaporation. It was also possible to produce MS with 88.13% efficiency with this method. *In-vitro* dissolution experiments revealed that the dissolution of RM was slowly released from MS loaded gel formulation over 24 h duration. The results obtained revealed that MS-loaded gel formulations exhibited suitable properties for buccal administration of RM, with their strong gel structure, desirable mechanical, bioadhesive, and *in-vitro* properties. According to the results of *ex-vivo* permeation study, the flux was found to be 86.44 μg/cm^2/h ± 4.34. Additionally, the % cumulative permeated RM was determined as 20% ± 1.54 for the RM MS-loaded gel formulation. Permeation enhancers like Isopropyl myristate, Hyaluronidase, Ethanol, Oleic acid, Polyethylene glycol 400 and propylene glycol can be tried to increase the cumulative permeation of RM from the buccal mucosa, or it can be suggested to use a hydroalcoholic gel formulation as a gel formulation[21-22].

Cumulatively, the results suggested that mucoadhesive RM MS-loaded gel formulation could be used as a possible alternative to conventional treatment of PD disease. In addition to the results of *in-vitro* characterization studies and cell culture studies conducted in the current study, it is planned to demonstrate the effectiveness by performing *in-vivo* animal experiments in our future studies.

STATEMENT OF ETHICS

This article does not contain any studies with human participants or animals performed by any of the authors.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest, financial or otherwise.

AUTHOR CONTRIBUTIONS

MG, FAK, SYK designed the study. MG, GT, FAK worked on literature search. MG, GT, FAK, SYK conducted the experimental work and collected the data. MG, FAK, SYK analyzed and interpreted the data. MG, GT, FAK, SYK wrote the draft of manuscript. ÖÖ supervised the study. All authors involved in revising the final manuscript.

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