

## Development of novel mucoadhesive gels containing nanoparticle for buccal administration of dexamethasone

Seda Rençber<sup>1\*</sup>, Fadime Aydın Köse<sup>2</sup>, Sinem Yaprak Karavana<sup>3</sup>

<sup>1</sup>Department of Pharmaceutical Technology, Faculty of Pharmacy, Izmir Katip Celebi University, Izmir, Turkey, <sup>2</sup>Department of Biochemistry, Faculty of Pharmacy, Izmir Katip Celebi University, Izmir, Turkey, <sup>3</sup>Department of Pharmaceutical Technology, Faculty of Pharmacy, Ege University, Izmir, Turkey

This study aimed to develop promising and innovative mucoadhesive gel systems containing dexamethasone-loaded nanoparticle to increase the effectiveness of treatment for oral precancerous lesions and to reduce side effects. In this respect, a dexamethasone-loaded nanoparticle formulation was prepared by using emulsification/solvent evaporation method. The nanoparticle has high zeta potential (-10.3±0.5 mV), low particle size (218.42±2.1), low polydispersity index (0.070±0.014) and high encapsulation efficiency (95.018±2.982%). To improve the mucosal retention time, the dexamethasone-loaded nanoparticle was dispersed in mucoadhesive gel using gellan gum. The developed gels offered appropriate pH value, high drug content, suitable mechanical and mucoadhesive performance and appropriate viscosity for mucosal administration. All formulations exhibited plastic flow and typical gel-type mechanical spectra after the determined frequency value. The developed formulations exhibited extended drug release as intended for these systems. Cytotoxicity was tested by MTT assay in human epithelioid carcinoma cell (HeLa) *in vitro*. The MTT assay showed that the blank formulations were non-toxic to cells. It was observed that the bioactivity of the free dexamethasone was potentiated by mucoadhesive gels containing dexamethasone-loaded nanoparticle in HeLa cells. Results from this study indicate that mucoadhesive gels are effective for the local treatment of precancerous lesions. Our findings showed that the developed formulations were worthy of further studies.

**Keywords:** Dexamethasone. Gellan gum. Mucoadhesive gel. Oral precancerous lesions. Cell culture.

### INTRODUCTION

Oral cancer represents approximately 3% of all malignancies in men and 2% of all malignancies in women worldwide (Cancer facts and figures, 2002). In 2018, oral cancer types occurred globally in about 355,000 people and resulted in 177,000 deaths ([www.cancer.org](http://www.cancer.org)). The main reasons for oral cancer include excessive alcohol intake and tobacco use (Gandini *et al.*, 2008; Goldstein *et al.*, 2010). The human papillomavirus is another risk factor for oral cancer (Kreimer *et al.*, 2005). Immunosuppressed patients like human immunodeficiency virus and renal

transplant cases are under the highest risk for oral cancer (Petersen, 2009).

In potentially malignant disorders, clinical examination reveals morphologically changed tissue in which cancer is more likely to occur than in healthy tissue. Oral lesions, oral leukoplakia, oral erythroplakia, oral lichen planus and submucous fibrosis could be premalignant and could exhibit dysplasia on histopathological examination (Yardimci *et al.*, 2014). The risk ratio for malignant transformation in oral dysplastic lesions is approximately 20%. Potential malignant disorders of the oral cavity can be divided into two groups as precancerous lesions and precancerous conditions. A precancerous lesion can be defined as “a benign, morphologically altered tissue that has potential

\*Correspondence: S. Rençber. Department of Pharmaceutical Technology, Faculty of Pharmacy, Izmir Katip Celebi University, Izmir, Turkey. E-mail: [sedarencber@gmail.com](mailto:sedarencber@gmail.com). ORCID: <https://orcid.org/0000-0003-0172-2120>

for malignant transformation.” The aim of identifying malignant disorders of the oral cavity is to initiate timely and adequate intervention and, if possible, to prevent malignant transformation, or to enable early detection of oral cancer (Sankar *et al.*, 2011)

Dexamethasone (DEX) is a highly potent and long-acting synthetic adrenal corticosteroid with potent anti-inflammatory properties. Corticosteroids are considered as first-line therapy and are effective in the management of symptomatic oral precancerous lesions (Chole, Patil, 2016). DEX is an immune suppressive drug with its antagonistic activity on the soluble factors released by the sensitized lymphocytes that are activated by nonspecific antigens. It also muzzles the inflammatory reaction. Thus, fibrosis is prevented by a reduce in fibroblastic proliferation and collagen deposition (James *et al.*, 2015).

Oral glucocorticoid therapy has severe systemic side effects, both in chronic and high dose application, and significantly decreases life quality, life expectancy and increases healthcare costs (Sarnes *et al.*, 2011; Manson *et al.*, 2009). Side effects include increased sensitivity to stomach acid, adrenal gland depression, immunosuppression, hypertension, psychological disturbances, osteoporosis, muscle atrophy, weight gain, exogenous Cushing’s syndrome with thin, fragile skin and steroid diabetes (Hopkins, Leinung, 2005).

Local administration of DEX strictly to precancerous lesions could constitute a useful means to improve treatment of oral cancer. Direct DEX delivery to precancerous lesions can increase the drug levels in the buccal mucosa and decrease drug resistance and undesired systemic side effects. Oral lesions can be effectively treated by local therapy thanks to the ease of applicability. Local drug delivery could provide a targeted and efficient drug delivery alternative than systemic treatment for oral mucosal diseases (Sankar *et al.*, 2011). However, constant salivary finding of the oral cavity makes it very difficult for the dosage to stay for an extended period (Scholz *et al.*, 2008; Sudhakar, Kuotsu, Bandyopadhyay, 2006).

For this reason, mucoadhesive gel formulations containing nanoparticles (NPs) have been proved as more convenient dosage forms for buccal applications in recent years. To increase the effectiveness of treatment

and to reduce side effects, drug delivery systems using colloidal particulate carriers such as NPs represent an essential option for buccal drug delivery (Westedt *et al.*, 2007). NPs are proposed for improving bioavailability, extending drug release and maintaining the local effect in the buccal mucosa. Their physical properties enable them to make intimate contact with a larger mucosal surface area. Particulates have the advantage of being relatively small and are more likely to be accepted by patients (Chinna Reddy, Chaitanya, Madhusudan Rao, 2011; Rençber, Aydın Köse, Karavana, 2020). However, buccal nanoparticle formulations need to be administered frequently due to short residence time at the buccal mucosa due to the self-cleansing action of saliva. For curbing this issue, mucoadhesive semi-solid systems can be used to prolong the residence time of nanoparticle formulation. Mucoadhesive formulations intended for buccal mucosa provide a promising and efficient approach for the treatment of oral diseases. Unlike conventional oral medications, these types of formulations offer an interaction between the mucoadhesive polymer and buccal mucosal lesion and retain for a more extended period. There are many kinds of mucoadhesive polymers, both synthetic and those from natural sources (Rençber *et al.*, 2017). Gellan gum is a high molecular weight, water-soluble bacterial exocellular polysaccharide produced by *Sphingomonas elodea*. At low concentrations, it is extremely effective in forming soft, elastic gels (Dabhi *et al.*, 2010; Mahdi, Conway, Smith, 2015). It is a mucoadhesive, hydrophilic, non-toxic, biocompatible and biodegradable polymer (Shaligram Mahajan *et al.*, 2017; Mythri *et al.*, 2011; Pereira Fernandes *et al.*, 2018).

The main objective of the present study was to develop mucoadhesive gels containing DEX-loaded nanoparticles (NPs) for increasing the effectiveness of treatment for oral precancerous lesions and to reduce side effects.

## MATERIAL AND METHODS

### Material

DEX was donated by the Pharmacia&Upjohn Company LLC (A Subsidiary of Pfizer Inc, USA). Poly(D,

L-lactic-co-glycolic acid) (PLGA) (Resomer® RG 502H) and polyvinyl alcohol (PVA) (ave. mol. wt.=30,000–70,000) was purchased from Sigma-Aldrich (St. Louis, MO). Gellan gum was obtained from Sigma-Aldrich (St. Louis, MO). Methanol was obtained from Sigma-Aldrich in high-performance liquid chromatography (HPLC) grade. Human cervical cancer epithelial carcinoma cell line (HeLa) was purchased from ATCC. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reagent was obtained from Invitrogen. All cell culture media and supplements were purchased from Thermo Fisher Scientific. All other chemicals were of analytical grade.

## Methods

### *Solubility Study of DEX*

Solubility studies of DEX were carried out by adding an excess of the drug to 10 mL of phosphate buffer (pH 6.8) in beaker maintained under stirring (400 rpm) at 25°C (48 h). The samples were analyzed by a validated high-performance liquid chromatography (HPLC) method (n=5).

The drug amount was determined using a validated HPLC method (Hewlett-Packard Agilent 1100, Agilent Technologies, Santa Clara, CA, USA) with a UV-Visible detector. A 250 mm × 4.6 mm (5 µm particle size) reversed-phase C18 column was used for separation and quantitation. The mixture of methanol: water: triethylamine (70:30:0.6, v/v/v) was used as the mobile phase and pH of the mixture was adjusted to 3.0 ± 0.05 with orthophosphoric acid. The mobile phase was degassed by sonication. A flow rate of 0.9 mL/min was maintained at 25°C. The injection volume and wavelength of the system were set up as 25 µL and 240 nm, respectively (Hazzah *et al.*, 2009; Desai *et al.*, 2013).

### *Preparation and Characterization of NP*

The PLGA NP was prepared by the emulsification/solvent evaporation method (Kima, Martin, 2006; Sengel Türk *et al.*, 2009). Production parameters such as mixing time, homogenization time, total formulation volume

and organic/aqueous phase ratio were established in accordance with the study of Rençber *et al.* in 2019. In this study, only the optimum NP formulation was dispersed in the mucoadhesive gel formulation. First, 2% of PLGA and 0.1% of DEX (w/v) were dissolved in 5 mL of acetone and used as an organic solvent. 3% of PVA (w/v) in 10 mL of ultrapure water was prepared using a heated magnetic stirrer. To prepare an organic solution, the specified quantities of PLGA and DEX were dissolved in 5 mL of acetone. Then, the PVA solution was brought to room temperature. The organic solution was added to the aqueous solution. The mixture was homogenized using a high-speed homogenizer (Silverson L5M) at 10000 rpm for 2 min. 15 mL of ultrapure water was added to the mixture and O/W emulsion was formed. This system was stirred with a magnetic stirrer to evaporate organic solvent at room temperature for 24 hours. The resulting mixture was centrifuged at 4750 rpm for 90 minutes. After centrifugation, the supernatant was separated. For removal of excess PVA, 10 mL ultrapure water was added and centrifuged at 4750 rpm for 30 min. After centrifugation, the supernatant was separated and re-suspended by adding 20 mL of ultrapure water to the NP.

The particle size (PS) and polydispersity index (PI) were measured by dynamic light scattering (Malvern Zetasizer-Nano ZS, Malvern Instruments Limited, Worcestershire, UK) at room temperature. The PS and PI values were obtained by averaging ten measurements at an angle of 173° using disposable cells (n=5). Mean values and standard deviations were reported (Imam *et al.*, 2015; Kapoor *et al.*, 2019; Moolakkadath *et al.*, 2020).

The zeta potential (ZP) of the NP was measured using disposable plain-folded capillary zeta cells (Malvern Zetasizer Nano-ZS) at room temperature. The ZP was calculated from the electrophoretic mobility using the Helmholtz–Smoluchowski equation under an electrical field of 40 V/cm. The processing was done using the software included within the system (n=5).

The drug amount in the NP was determined using a validated HPLC method. For drug encapsulation efficiency (EE) study, 1 mL of the DEX-loaded NP dispersion was ultracentrifuged at 10,000 rpm (Beckman Coulter Inc., Brea, CA, USA) for 90 min. The EE was determined based on the non-encapsulated DEX

recovered in the supernatant at 240 nm by a validated HPLC method. The encapsulated amount of DEX was calculated by subtracting the free amount of DEX from the total amount in the dispersion (n=5). The EE capacity was calculated according to the following equations (Imam et al., 2015; Kapoor et al., 2019; Rençber *et al.*, 2016; Şenyiğit *et al.*, 2010).

$$EE (\%) = \frac{\text{Total amount of DEX} - \text{The amount of free DEX}}{\text{Total amount of DEX}} \times 100$$

#### Preparation and Characterization of Mucoadhesive Gel Formulations

Mucoadhesive gels containing gellan gum at concentrations 0.8-1.4% w/v were prepared by dissolving gelling agent in the NP dispersion with vigorous stirring using a magnetic stirrer. Dry gellan gum was dispersed in the NP dispersion and maintained at 50°C. The dispersion was stirred at 50°C for 20 min to facilitate the hydration of gellan gum. The solution was left to cool at 25°C. The prepared gel formulations were stored in the refrigerator until the entire polymer got completely dissolved. The formulations were prepared with different ratios of gellan gum and the compositions of gel formulations containing DEX-loaded NP were given in Table I.

**TABLE I** - The composition of the gel formulations

Formulation Code	Gellan Gum (%)	DEX (%)
G1	0.8	-
G2	1	-
G3	1.2	-
G4	1.4	-
G1*	0.8	0.1
G2*	1	0.1
G3*	1.2	0.1
G4*	1.4	0.1

To investigate the compatibility of the prepared gels containing NP for mucosal surfaces, their pH values were

measured by a pH meter (Hana Instruments HI 221) at room temperature.

To determine the drug content, 0.25 g of gel sample was taken from top, middle and bottom of the gel and extracted by an addition of 10 mL of phosphate buffer (pH 6.8) followed by mixing for 48 hours. The drug content of the sample was analyzed using a validated HPLC method (n=5).

For the evaluation of the mechanical properties of the formulations, a textural analysis was performed using Software-controlled penetrometer (TA-TX Plus, Stable Micro System, UK) equipped with 500 g load cell in texture profile analysis (TPA) mode. Formulations were transferred into universal bottle (25 mL) at room temperature. In this, an analytical probe was compressed twice into each gel formulation to a defined depth (15 mm) and at a defined rate (2 mm/s), with a recovery period (15 s) between the end of the first compression and the beginning of the second one. Mechanical parameters were derived from the resultant force-time curve. Mechanical properties such as hardness, compressibility, elasticity, adhesiveness and cohesiveness were determined (Chang *et al.*, 2002a; Moolakkadath *et al.*, 2020). Experiments were carried out at least five times for reproducibility.

The mucoadhesive strength of the formulations was evaluated by measuring the force required to detach the gel from mucin dispersion under a 500 g load cell TPA in tension mode (Jones, Woolfson, Brown, 1997; Jones *et al.*, 2000).

50 µL mucin dispersion was attached to the lower end of the probe (P 10 Perspex, h: 10 mm). The gels were packed into the beaker. The upper probe (P 0.5 Perspex, θ: 12.5 mm) holding the mucin dispersion was lowered to the surface of the gel at a speed of 0.1 mm.s<sup>-1</sup> and a contact force of 0.05 N. The surfaces were kept in contact for 120 s and then the probe was moved upward at a constant speed. Maximum detachment force (F) was obtained from the force-distance graph. The area under the curve (AUC) was calculated from the force-distance plot as the mucoadhesion (M). The tests were conducted at 37±0.5°C and each experiment was carried out at least five times for reproducibility.

All rheological measurements were performed using a rheometer (TA Discovery HR-1 Hybrid Rheometer).

Continuous shear analysis of each formulation was performed in inflow mode and in conjunction with parallel steel plate geometry with a gap of 1 mm. Upward and downward flow curves were measured at room temperature ranging from  $10 \text{ s}^{-1}$  to  $1000 \text{ s}^{-1}$  (Rençber *et al.*, 2017; Sandri *et al.*, 2004; Chang *et al.*, 2002b).

Stress sweep studies were used to determine the yield stress of gel formulations containing nanoparticle to predict the stress required to initiate flow. The stress was gradually conducted over the range of 0.1-1000 Pa and at a frequency of 1 Hz. The resulting viscoelastic parameters were monitored and their linear viscoelastic regions were determined, where the stress was directly proportional to the strain and the storage modulus remained constant. Also, yield stress value was detected.

A rheological analysis for each formulation was performed after determination of its linear viscoelastic region. Frequency sweep analysis was performed over the frequency range of 0.1-10.0 Hz following the application of constant stress. Elastic (storage) modulus ( $G'$ ), viscous (loss) modulus ( $G''$ ) and the loss tangent ( $\tan \delta$ ) were determined (Rençber *et al.*, 2017; Andrews, Gorman, Jones, 2005; Andrews, Jones, 2006).

*In vitro* DEX release studies were performed using Spectra/Por Regenerated Cellulose Dialysis Membrane Tubes (12.000–14.000 MWCO) in 100 mL of phosphate buffer (pH 6.8) under sink conditions at  $37^\circ\text{C} \pm 0.5^\circ\text{C}$ , stirred continuously with a magnetic stirrer at 300 rpm (Gupta *et al.*, 2015). At defined time intervals, the samples were withdrawn and the DEX content of each sample was analyzed using a validated HPLC method. All the experiments were repeated five times and the data were expressed. The mass of DEX released from the formulations was calculated using a calibration curve. There was no analytical interference from the polymers.

For the determination of release mechanism, the data of *in vitro* drug release studies were analyzed according to Korsmeyer–Peppas release kinetics. *In vitro* drug release data was plotted against mucoadhesive gel containing DEX-loaded NP erosion and the obtained  $r^2$  values were used to evaluate the relation between gel erosion and the release of DEX (Ritger, Peppas, 1987).

In the stability studies, the formulations were stored at  $4^\circ\text{C} \pm 1^\circ\text{C}$  in the refrigerator and at  $25^\circ\text{C} \pm 2^\circ\text{C}$  with a

relative humidity of 60% for 6 months in the stability cabinet (Nuve ID 300, Ankara, Turkey). The stability of formulations was evaluated according to appearance, pH, viscosity and drug content ( $n=5$ ).

For cell culture studies, effects of the mucoadhesive blank gels and mucoadhesive gels containing DEX-loaded NP on cell proliferation were tested in HeLa by MTT assay (Mosmann, 1983). HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2 mM glutamine and 10 % fetal bovine serum (FBS) at  $37^\circ\text{C}$  in a humidified 5%  $\text{CO}_2$  incubator. For the experiment, the cells were seeded in 12-well plates at a density of  $1.5 \times 10^5$  cells/well. One day after the seeding, the cells were treated with 1: 1000 diluted formulations at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  incubator for 48 hours. The cells treated with only empty medium and DEX (1  $\mu\text{g}/\text{mL}$ ) were used as negative and positive control, respectively. After the treatment, the medium was replenished with fresh DMEM containing MTT solution (5 mg/mL). The plates were further incubated at  $37^\circ\text{C}$  for 4 hours and the medium was removed. 0.2 mL dimethyl sulfoxide was added to dissolve the blue-formazan crystals and was then transferred to a 96-well plate. The absorbance of the formazan solution was measured in a plate reader (Thermo Scientific Varioskan Microplate Reader) at 540 nm. The ratio of the absorbance of treated samples to the absorbance of the negative control (taken as 100%) was expressed as % cell viability. Cell survival was expressed as the percentage of formazan absorbance. Results were expressed as Mean  $\pm$  Standard Deviation (Mean  $\pm$  SD) from at least three different experiments.

Statistical differences among the data were analyzed using one-way analysis of variance (ANOVA) or Student's t test with  $p < 0.05$  as the minimal level of significance by GraphPad Prism 5.0 (GraphPad Software).

## RESULTS AND DISCUSSION

The frequency of oral cancer is increasing worldwide. Precancerous lesions of the oral mucosa (oral leukoplakia, oral submucous fibrosis, oral erythroplakia, etc.) can transform into malignant lesions. Despite the advances in therapy, the prognosis

of oral cancer remains poor. Nowadays, early diagnosis of premalignant lesions and primary and secondary chemoprevention strategies of oral cancers are investigated. There have been fewer studies about oral cancer prevention using corticosteroids (Hambly *et al.*, 2017; Ayushee Hebbale Mhapuskar, Agarwal, 2017). Intending to increase DEX performance in the local treatment of oral precancerous lesions, we designed and evaluated mucoadhesive gels containing DEX-loaded NPs to be administered directly to the region of oral precancerous lesions.

NPs have attracted considerable interest for therapeutic application in the oral cavity owing to their high drug loading, stability, specificity and sustained and extended release properties. Although NPs have several advantages as carriers, liquid dispersions can be rapidly removed from the oral cavity through the activity of saliva, which can lead to rapid elimination of drugs by involuntary swallowing and constant salivary scavenging can result in drug loss. Additionally, since NPs promote extended drug release, their buccal administration with a view to a local effect only makes sense if the retention on the buccal mucosa is prolonged. Therefore, the development of a mucoadhesive buccal delivery system containing NPs has a potential. Mucoadhesive gels are a promising option, since they allow close drug contact with the buccal mucosa, providing adhesiveness and prolonging the residence time of the dosage form. Also, gel formulations have the advantage of being deliverable with a syringe, with a consequent easy placement in dental pockets and easy dispersion throughout the lesion. In our previous study, DEX-loaded poly(D,L-lactic-co-glycolic acid) (PLGA) nanoparticles (NPs) were successfully prepared using the emulsification/solvent evaporation method for the local treatment of oral precancerous lesions (Rençber, Aydın Köse, Karavana, 2020). NPs with different rates of PLGA showed adequate properties in relation to pH, PS, PI, ZP, encapsulation efficiency, morphology, *in vitro* drug release, stability, *ex vivo* drug diffusion and cell culture cytotoxicity. In the present study, mucoadhesive gels containing optimum DEX-loaded NP were successfully prepared using gellan gum as mucoadhesive polymer.

## Solubility study of DEX

A solubility study of the DEX was carried out for preformulation study. Solubility plays a prime role in the *in vitro* drug release of a drug substance from the formulation. The solubility of DEX in pH 6.8 phosphate buffer was found to be 0.067 mg/mL. Similar results were found in the literature for DEX (Yalkowsky, Yan, 2003; The Merck Index, 2001).

An HPLC method for quantitative analysis of DEX was developed and validated according to the ICH guidelines. The curve showed a linear relationship between concentration and absorbance. The value of the regression coefficient ( $r^2$ ) was found to be 1 and the regression equation generated was  $y=63.934x-14.539$ . The limit of detection (LOD) and the limit of quantification (LOQ) of DEX were found to be 0.182 and 0.487  $\mu\text{g/mL}$ , respectively.

## Preparation and characterization of NP

DEX-loaded PLGA NP was successfully prepared by the emulsification/solvent evaporation method (Kima, Martin, 2006; Sengel Türk *et al.*, 2009). NP formulation showed translucent and uniform appearance. PS and PI are the most critical characteristics for determining the biocompatibilities, bioactivities and stability of NPs (Singh, Lillard, 2009; Sun *et al.*, 2015). The PS and PI of DEX NP determined by dynamic light scattering were  $218.42\pm 2.1$  nm and  $0.070\pm 0.014$ , respectively. Lower PI indicated good agreement in size distribution by intensity. ZP is a significant factor that gives an indication of the charge of the NPs in a specific medium and permits to evaluate the degree of repulsion between close and similarly charged particles in the dispersion. The ZP of the NP formulation had a negative value of  $-10.3\pm 0.5$  mV. The negative surface charge is attributed to the presence of free carboxylic acid groups at the chain ends of the PLGA polymer exposed to the surface of NP

In this study, the prepared DEX-loaded NP had high drug EE%, as expected ( $95.018\pm 2.982\%$ ) (Rençber *et al.*, 2019). Previous studies have shown that DEX can be successfully loaded into NPs (Butoescu *et al.*, 2008; Friedrich *et al.*, 2008). The high value can be a

consequence of the high solubility of DEX in the solvent used. In the studies of Butoescu *et al.* (2008) and Friedrich *et al.* in 2008, the EE were similarly 90% and 89.56%, respectively.

### Preparation of mucoadhesive gel formulations

The mucoadhesive gels containing DEX-loaded NP were prepared using various concentrations of gellan gum. Clarity of the gel may be used to indicate that all the ingredients were able to dissolve. All the prepared gel formulations were found to be transparent with a smooth and homogenous appearance. Gel formulations were characterized by pH determination, drug content, mechanical and mucoadhesion properties, viscosity measurement, rheological analyses, *in vitro* drug release and cell culture study.

### Determination of pH

It was known that the apparent viscosity of gellan gum aqueous solution could be markedly influenced by the pH (Dabhi *et al.*, 2010). Therefore, the pH was adjusted and maintained between 5-6 with the help of a non-ionic alkalizing agent, if necessary. The pH of all prepared gel formulations was observed in the range of 5.1-5.37 (Table II). Therefore, there was no need for pH adjustment by any alkalizing agent. Besides, an acidic or alkaline formulation may irritate buccal mucosa and hence, this parameter assumes importance in the formulation of mucoadhesive dosage forms (Gousia Begum, Sekar, 2017). The obtained pH values of the formulations are considered acceptable to avoid the risk of irritation upon application to the buccal mucosa.

### Drug content

The drug content of the mucoadhesive gels containing DEX-loaded NP was found to be within the acceptable range of 82.034% - 96.410%, which indicates content uniformity (Table II). In 2012 Jaya Raja Kumar *et al.* prepared a guar gum-based fluconazole gel formulation for oral thrush. In their study, the drug content of the formulations was found to be between 88.15-92.55%.

**TABLE II** - pH and drug content of gel formulations

Code	pH $\pm$ S.D.	Drug content (%) $\pm$ S.D.
G1*	5.30 $\pm$ 0.05	82.034 $\pm$ 2.107
G2*	5.37 $\pm$ 0.04	96.410 $\pm$ 1.165
G3*	5.34 $\pm$ 0.04	86.258 $\pm$ 1.445
G4*	5.10 $\pm$ 0.05	96.045 $\pm$ 1.349

### Mechanical properties of formulations

TPA was used to evaluate the buccal application of the gel formulations. The mechanical properties of the gel are essential to take off the gel from the primary packaging or an applicator, to apply to the desired region and for the retention at the buccal mucosa. Buccal gel formulations should have appropriate mechanical properties for the maximum benefit of the patient. The essential parameters for buccal administration are hardness, compressibility, elasticity, adhesiveness and cohesiveness. Table III represents the obtained results of the TPA analysis.

Hardness and compressibility values of formulations should be lower to take the formulation easily from the container and to apply it to the buccal mucosa. These values increased significantly due to the increases in gellan gum concentration. Gels containing DEX-loaded NP may be ranked as G4\* > G3\* > G2\* > G1\* in terms of hardness and compressibility values. However, in comparison with the hardness values, no significant differences were observed among the formulations ( $p \geq 0.05$ ). Although significant differences were observed between G1\* and G2\*, G1\* and G3\*, G1\* and G4\* in terms of compressibility ( $p < 0.05$ ), no significant differences were observed between G2\* and G3\*, G3\* and G4\* ( $p \geq 0.05$ ). If the hardness of the gel is low, the work required to take off the gel from the packaging and to apply it to the buccal mucosa decreases. However, if the hardness is too low, the retention time of the gel at the buccal mucosa decreases. Thus, for the appropriate therapeutic effect and easily application, gels should have optimum hardness and compressibility values (Amasya *et al.*, 2012; Özyazıcı *et al.*, 2015; Karavana *et al.*, 2012a).

Elasticity is defined as the reform of a deformed gel after a compression period. There is an inverse proportion between the numerical value of elasticity and the elasticity of the gel. If the numerical elasticity value of the gel increases, the elasticity of the gel decreases. It has been reported that gels including high elasticity components have increased tissue adhesion. The basic physical mechanism of bioadhesion is related to the elasticity of the polymer chains. Elastic polymer chains form stronger adhesive bonds by inclusion between the polymer and mucosal surface (Cevher *et al.*, 2008; Peppas, Buri, 1985; Rençber *et al.*, 2017; Kaplan *et al.*, 2018). It has been observed that the elasticity of all gel formulations was acceptable according to the literature and no significant differences were observed among the formulations ( $p \geq 0.05$ ) (Rençber *et al.*, 2017; Karavana *et al.*, 2012b)

The buccal mucosa is an ideal area for the application of systemic and local drug delivery systems. However, the adhesion of many buccal systems is limited due to

the application to a humid region such as an oral cavity, secretion of saliva and weak cohesion forces. The cohesion introduces the measure of the reconstruction of the gel after application (Peppas, Buri, 1985; Jones *et al.*, 1996a). A full structural recovery can explain the high value of cohesiveness, which can be described by the effects of repeated shearing stresses on the structural properties of formulations (Rençber *et al.*, 2017; Karavana *et al.*, 2012b; Cevher *et al.*, 2008). A significant difference was determined between all formulations ( $p < 0.05$ ).

Adhesiveness, a property related to mucoadhesion, was defined as the work required to overcome the attractive forces between the surface of the mucoadhesive gel and the surface of the probe. The increase in gel adhesiveness might result in a more exceptional ability of the polymer to interact with the probe chemically (Kaplan *et al.*, 2018; Karavana *et al.*, 2012b; Cevher *et al.*, 2008). No significant differences were observed among the formulations ( $p \geq 0.05$ ).

**TABLE III** - Mechanical properties of the formulations

Code	Hardness (N) $\pm$ S.D.	Compressibility (N.mm) $\pm$ S.D.	Elasticity $\pm$ S.D.	Adhesiveness (N.mm) $\pm$ S.D.	Cohesiveness $\pm$ S.D.
G1*	0.12 $\pm$ 0.009	0.38 $\pm$ 0.011	1.07 $\pm$ 0.030	0.06 $\pm$ 0.005	0.30 $\pm$ 0.008
G2*	0.18 $\pm$ 0.012	0.60 $\pm$ 0.007	1.06 $\pm$ 0.001	0.14 $\pm$ 0.008	0.58 $\pm$ 0.021
G3*	0.19 $\pm$ 0.050	0.84 $\pm$ 0.013	0.99 $\pm$ 0.005	0.19 $\pm$ 0.004	0.67 $\pm$ 0.011
G4*	0.21 $\pm$ 0.015	0.89 $\pm$ 0.016	0.97 $\pm$ 0.013	0.20 $\pm$ 0.009	1.75 $\pm$ 0.018

### Evaluation of mucoadhesive properties

The mucoadhesion value is an essential property for the maximum benefit of the formulation and local application to the buccal mucosa. In buccal application, mucoadhesion is determined as adhesion of the polymeric material to the buccal mucosa. It was considered that an increase in the residence time of the formulation at buccal mucosa would increase the effectiveness of the gel formulation.

Direct quantification of gel mucoadhesion cannot be performed, as the strength of the cohesive bonds

associated with prepared gels is frequently lower than gel-mucin adhesive bonds. This problem was primarily overcome by using a compressed mucin disc and mucin dispersion in conjunction with tensile analysis (Jones *et al.*, 2000). In the literature, mucin disc and mucin dispersion are also used instead of the natural mucosal membranes to determine the mucoadhesive strength of the formulation (Sandri *et al.*, 2004; Chang *et al.*, 2002b). Therefore, the mucoadhesive properties of gels were determined using mucin dispersion with a TA-XT-plus texture analyzer equipped with the A/MUC measuring system.

All the prepared gel formulations showed appropriate mucoadhesive force for buccal mucosa, as shown in Table IV. The mucoadhesion value of all formulations increased with the increase in the gellan gum concentration. However, in the comparison of the mucoadhesive values, no significant differences were observed among the formulations ( $p \geq 0.05$ ). In a study by Matapady et al. (Nairy Matapady *et al.*, 2009), a similar methodology was used to evaluate the

effect of gellan gum concentration in mucoadhesiveness of gels. They observed a positive relation between the concentration of gellan gum and the values of mucoadhesion. The mechanism behind mucoadhesion due to use of gellan gum is that it swells and facilitates the formation of an adhesive interaction between the gellan gum and the musin gel and contributes to the establishment of a more extensive cohesive layer, resulting in superior mucosal retention.

**TABLE IV** - Mucoadhesive properties of the formulations

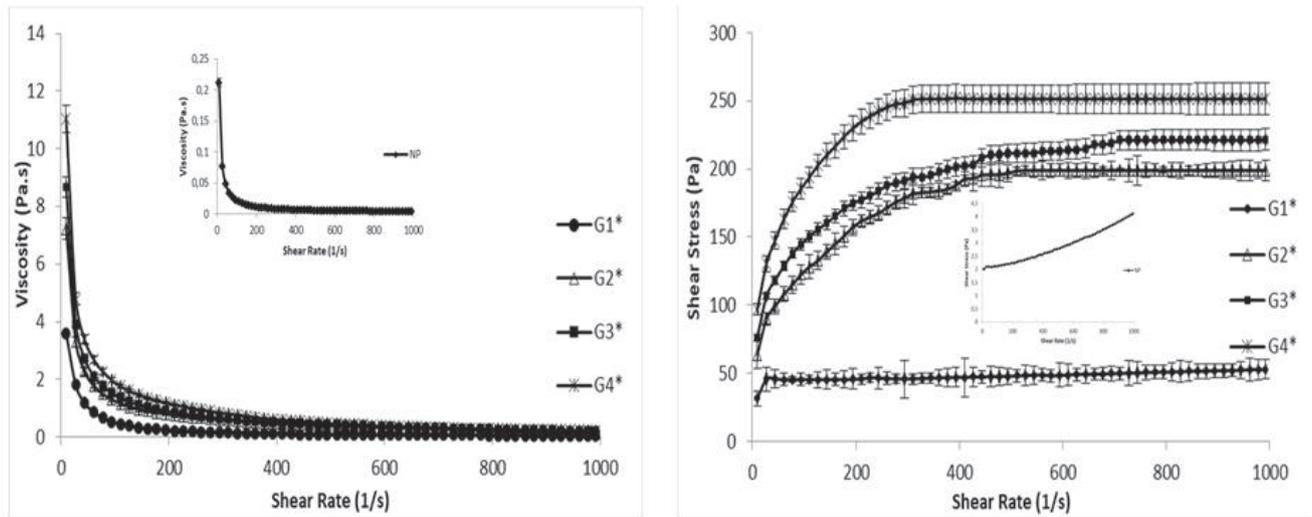
Code	Force (mN) $\pm$ S.D.	Mucoadhesion (mN.mm) $\pm$ S.D.
G1*	642.60 $\pm$ 1.896	274.184 $\pm$ 0.985
G2*	688.72 $\pm$ 2.155	324.802 $\pm$ 1.054
G3*	746.81 $\pm$ 2.465	368.016 $\pm$ 1.265
G4*	828.96 $\pm$ 3.512	480.444 $\pm$ 1.368

### Rheological studies

The best formulations were characterized to obtain a longer residence time in the buccal mucosa. It is thought that gellan gum gel containing NP, which has the highest viscosity, will show the longest stay time. NPs, which are aqueous systems, have concise residence times in the buccal mucosa. The dispersing of NPs in gellan gum gel, which has a high viscosity, will increase the duration of mucosa residence. Since the viscosity of the gellan gum gels containing NP is higher than the NP formulation, gel formulations remain longer in the buccal mucosa. Also, the increase in gellan gum concentration led to a somewhat increased viscosity and higher concentrations showed a slight increase in viscosity (Figure 1). In the

literature, Harish et al. (2009) have described a correlation between a formulation's viscosity and its gellan gum concentration.

The shear stress changes versus shear rates were used to determine whether the flow properties of the formulations were Newtonian or non-Newtonian. The NP formulation showed Newtonian flow. However, all the prepared gel formulations were determined to have a non-Newtonian plastic flow with a particular yield value (Figure 1). After these yield values, the formulations showed shear thickening behavior and their viscosity values decreased. This was expected due to its semisolid properties. Our results showed similarity with the literature (Karavana *et al.*, 2012a; Fernandez, Von Plessing, Cardenas, 2006).

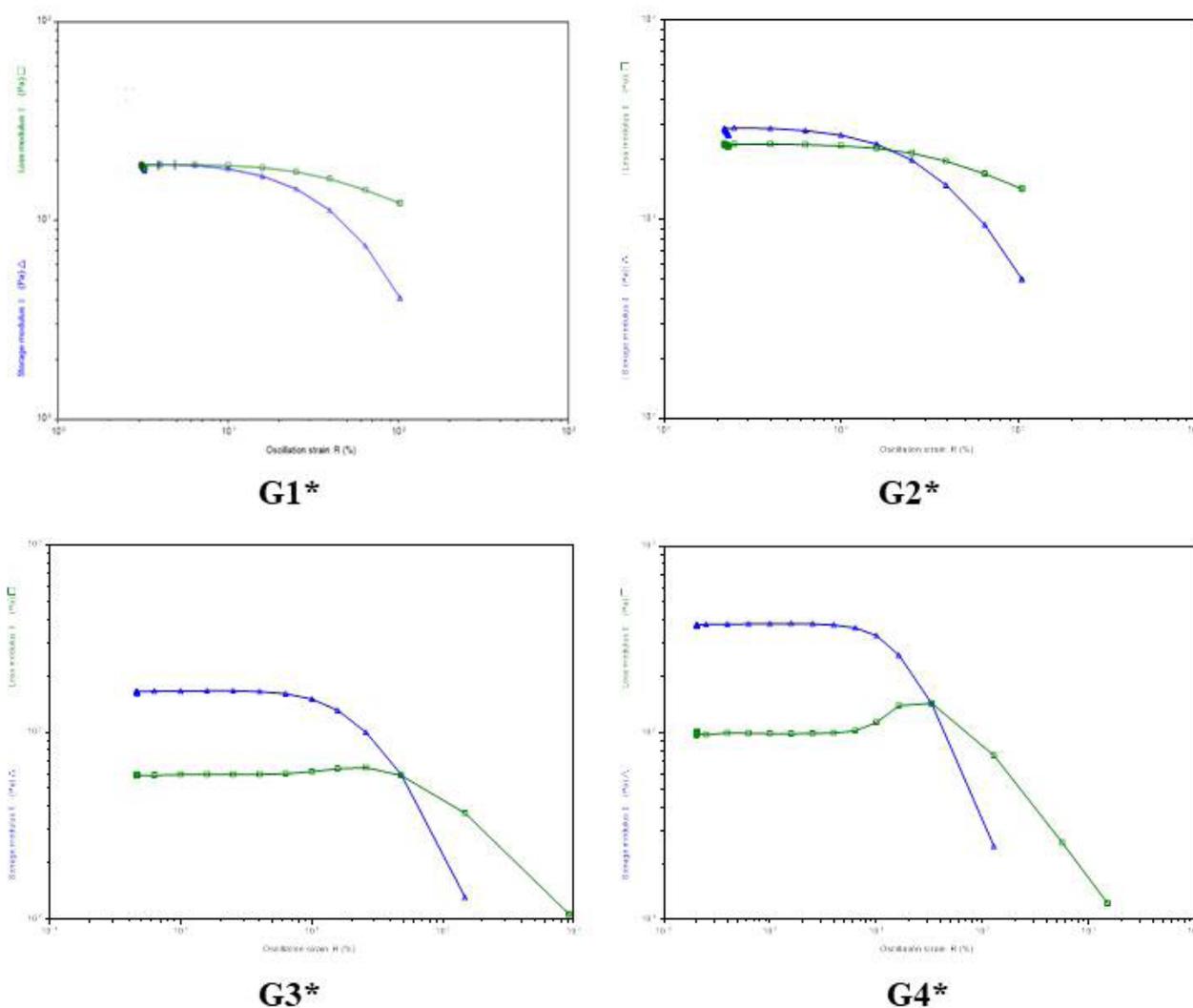


**FIGURE 1** - Viscosity and flow curves of the formulations at 25°C (n=5).

Rheological studies for gel formulations need to be controlled and understood since it is crucial for predicting their behavior in *in vivo* conditions. The flow characteristics of the formulation affect the residence time on the application site and anticipate the spreading and coverage of the formulation over the mucosal tissue.

Most rheological studies made on gel formulations use the viscosity of gel as a rheological parameter (Baloglu *et al.*, 2011). However, this does not give relevant data for the gel. A high speed applied during the viscosity measurements destroys the structure of the gel. Therefore, it is more appropriate to make oscillatory measurements through a low oscillatory angle to keep the gel structure intact during measurements.

The yield stress has a significant value in semisolid formulations. Depending on the yield stress, different filling and packing equipment are used and the final products are placed in bottles, tins, boxes, tubes or other individual containers. The critical stress also has a significant role while developing stable products (Rençber *et al.*, 2019). During the stress sweep test, a linear viscoelastic region and yield stress value can be determined. The prepared mucoadhesive gels containing DEX-loaded NPs exhibited linear viscoelastic behavior up to 100 Pa of oscillation stress. All the gel formulations had the same yield stress values (Figure 2).

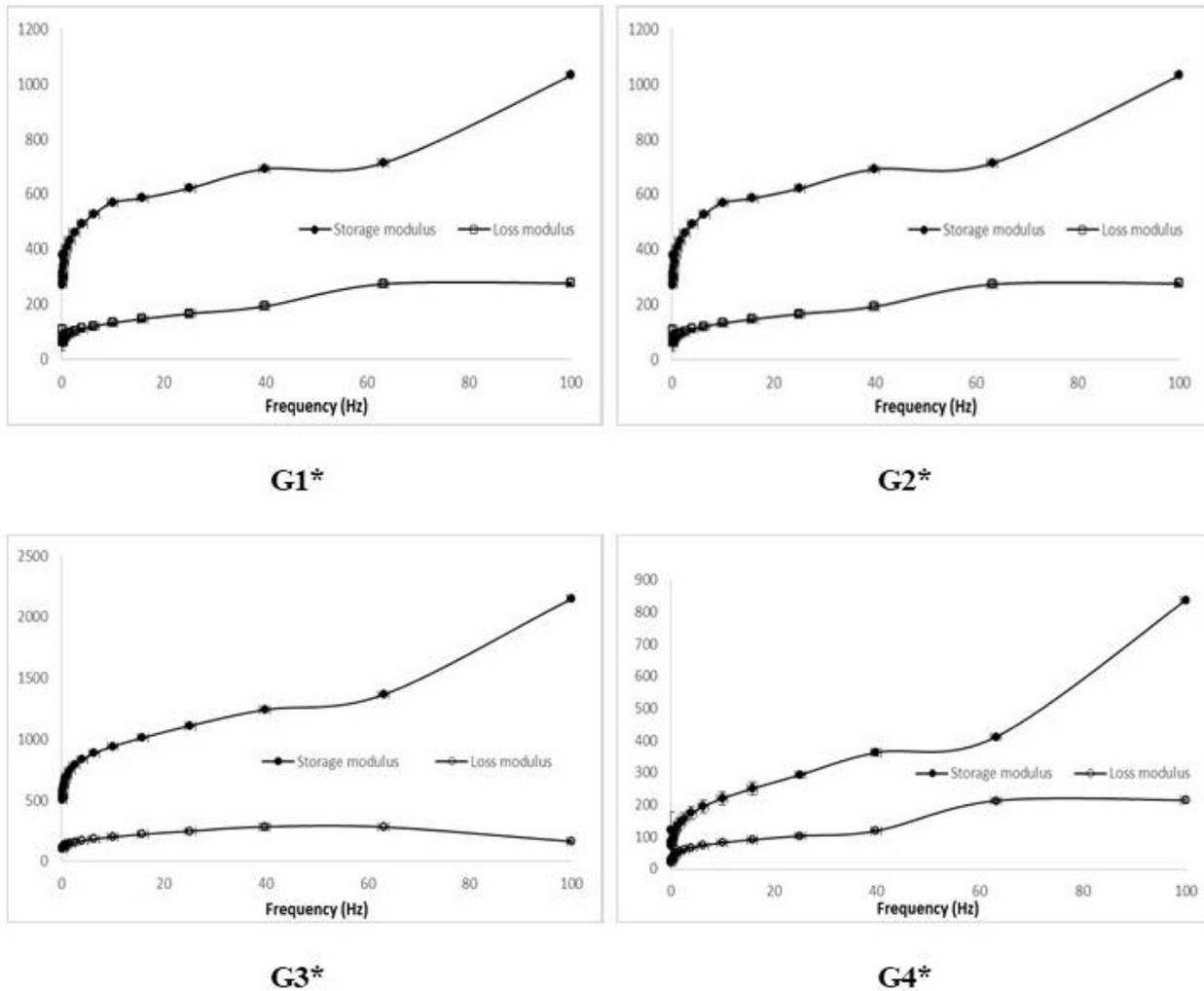


**FIGURE 2** - Stress sweep of the formulations.

The rheological properties of gel formulations affect their ease of application and retention within the buccal mucosa. In oscillatory rheometry, the effects of oscillatory stresses on the viscoelastic properties were measured, from which two dynamic moduli, namely, the storage modulus,  $G'$ , a measure of elasticity, and the loss modulus,  $G''$ , representing viscous components at a given frequency of oscillation, were obtained. If it is solid, interchain entanglements do not have sufficient time to come apart within the period of a single oscillation and  $G'$  becomes higher than  $G''$ . A gel should exhibit a solid-like mechanical spectrum, that is,  $G' > G''$  throughout the experimentally accessible

frequency range and there should be little frequency dependence of the moduli (Rençber *et al.*, 2017; Karavana *et al.*, 2012b; Rençber *et al.*, 2019). Figure 3 represents the frequency dependence of the  $G'$  and  $G''$  of advanced gel formulations at 25°C. According to the rheological studies, all the formulations were found to be frequency-independent and both exhibited typical solid-like mechanical spectra ( $G' > G''$ ).

The value of phase angle ( $\tan\delta$ ) is a measure of the relative contribution of viscous components to the mechanical properties of the formulations (Rençber *et al.*, 2017).  $\tan\delta$  was lower than 1 for all the formulations and a solid gel response was obtained (data not shown).



**FIGURE 3** - Frequency-dependent changes of viscoelastic properties of the formulations (n=5).

### ***In vitro* drug release studies**

The *in vitro* DEX release from mucoadhesive gels containing NPs were conducted using a dialysis bag in a diffusion medium with a pH of 6.8 for 48 h. The mechanism of drug release from gel formulations with gellan gum is due to water penetration, gelatinization and diffusion. The results were displayed in Figure 4. No burst effect has been observed, indicating that DEX was homogeneously dispersed in the formulation. As can be seen from the data obtained that after 8 hours, the release rates of G1\*, G2\*, G3\* and G4\* were 49.853, 47.116, 44.586 and 41.484%, respectively. In our previous DEX-loaded NP study, after 8 h, the drug release in formulations was found to be approximately 60% (data

not shown, Rençber, Aydın Köse, Karavana, 2020). As expected, mucoadhesive gel formulations containing NP showed a slower drug release profile than NPs. According to the results, the release of DEX decreases as the concentration of polymer increases. This was due to the increased viscosity of polymer concentration, as indicated by the results of the rheological study. Also, at the end of 48 hours, the release rates for G1\*, G2\*, G3\* and G4\* were 78.279, 82.321, 87.172 and 86.562%, respectively. Extended release was observed from mucoadhesive gels containing NP. Pandey *et al.* (2017) have optimized and developed mucoadhesive thermosensitive gels containing DEX 21-phosphate disodium salt, which possessed longer residence time and extended drug release, locally. A similar release pattern has been reported for clomiphene

citrate from mucoadhesive gel (Cevher *et al.*, 2008) and metronidazole from a periodontal bioadhesive gel

(Varshosaz, Tavakoli, Saidian, 2002), where the release decreased with an increase in polymer concentration.

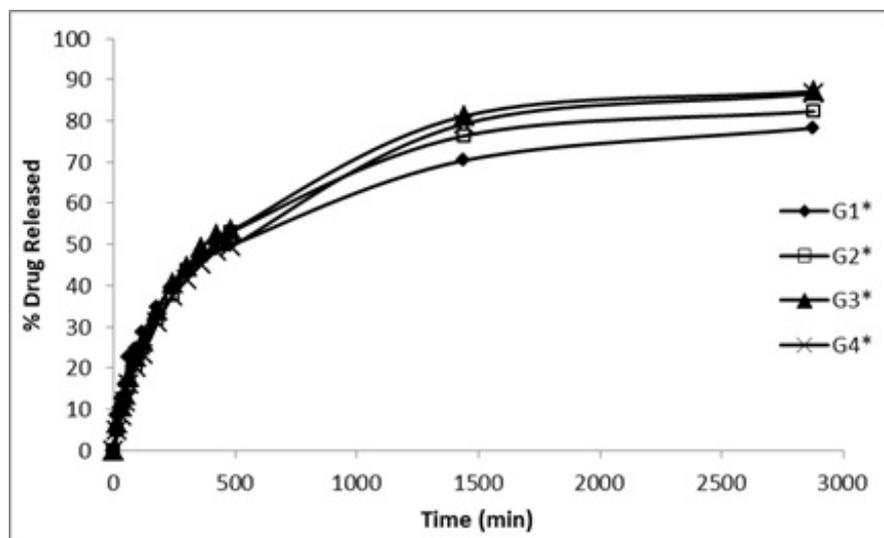


FIGURE 4 - *In vitro* drug release of formulations.

#### Determination of release mechanism

Data analysis for release kinetics showed that all the formulations generally fitted to Korsmeyer–Peppas kinetics, according to their high  $r^2$  values (Table V). High  $r^2$  values of Korsmeyer–Peppas kinetics can be used to describe the relationship of drug release and erosion for formulations. According to the obtained diffusional exponent values, all mucoadhesive gel formulations indicated non-Fickian ( $0.45 < n < 0.89$ ) diffusion mechanism of drug release, which was supported by their gel erosion profile. This behavior requires two parameters to describe the coupling of diffusion and relaxation phenomena. Similar kinetic

results have been investigated with gel formulations in the literature (Shaligram Mahajan *et al.*, 2017; Rençber *et al.* 2017; Rençber *et al.*, 2016; Yang *et al.*, 2016).

TABLE V - Release parameters of DEX from gel formulations

Code	n	Log k	$r^2$
G1*	0.509	0.376	0.981
G2*	0.662	-0.009	0.988
G3*	0.611	0.134	0.994
G4*	0.671	-0.048	0.987

## Stability studies

The stability studies of formulations were performed at  $4^{\circ}\text{C}\pm 1^{\circ}\text{C}$  and  $25^{\circ}\text{C}\pm 2^{\circ}\text{C}$  for 6 months. No significant changes were observed in terms of the appearance, pH, viscosity or drug content of the formulations (data not shown).

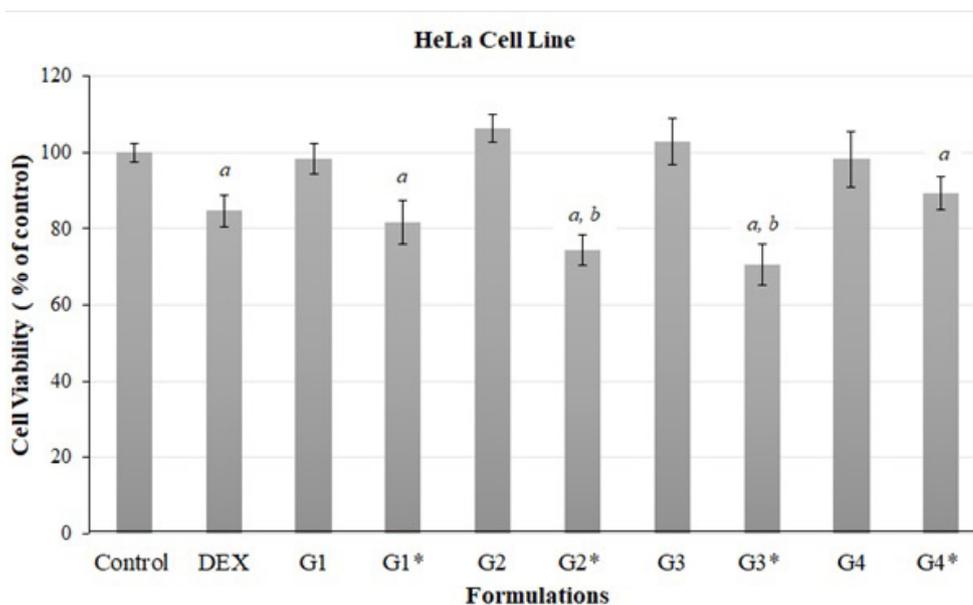
## Cell culture studies

Investigation of safety and efficiency of formulations is an essential step before starting *in vivo* studies. For this purpose, novel formulations were tested in HeLa cell culture, which is commonly used as an epithelial cell model to test new buccal formulations and/or anti-cancer drugs on cell proliferation (Sato *et al.*, 2014; Ujhelyi *et al.*, 2015; Guan *et al.*, 2016).

HeLa cells were treated with a free DEX solution, G1, G2, G3, G4, G1\*, G2\*, G3\* and G4\* formulations for 48 h. Then, the cell proliferation was determined using the MTT method (Figure 6). The results of the MTT assay showed that DEX treatment ( $1\ \mu\text{g}/\text{mL}$ ) caused a significant decrease ( $84.72 \pm 4.24\%$ ) in cell proliferation compared to untreated control cells

( $p < 0.05$ ). As shown in Figure 5, treatment with empty formulations (G1, G2, G3, and G4) did not affect the cell proliferation rate ( $98.31 \pm 3.87\%$ ,  $106.27 \pm 3.72\%$ ,  $102.84 \pm 6.22\%$ ,  $98.19 \pm 7.36\%$ , respectively) ( $p > 0.05$ ). However, it was determined that mucoadhesive gel formulations containing DEX-loaded NP (G1\*, G2\*, G3\* and G4\*) caused a significant decrease in cell proliferation compared to control cells ( $81.64 \pm 5.64\%$ ,  $74.42 \pm 4.11\%$ ,  $70.51 \pm 5.34\%$ ,  $89.34 \pm 4.21\%$ , respectively) ( $p < 0.05$ ). Furthermore, it was observed that G2\* and G3\* treatment caused a significant decrease in cell viability compared to free DEX-treated cells ( $p < 0.05$ ).

Gellan gum is a biodegradable material that is frequently used in cell culture for several applications such as drug and gene delivery systems, tissue engineering and 3D cell culture model (Afewerki *et al.*, 2019; Goyall *et al.*, 2011; Smith *et al.*, 2007). Similar to our results, it was reported that the gellan gum and PLGA constructs in the formulations were not cytotoxic in a dose-dependent manner (Ranch *et al.*, 2017). Also, gels containing DEX-loaded NP treatments have shown a similar decrease in cell viability to free DEX-treatment, which supports our foresight that these formulations may be a suitable and efficient buccal carrier for DEX.



**FIGURE 5** - Cell viability of the formulation. Cell viability was measured using the MTT assay. (a)  $P \leq 0.05$  vs untreated control cells, (b)  $P \leq 0.05$  vs. free DEX-treated cells.

## CONCLUSION

This study focused on the development of mucoadhesive gel formulations containing DEX NPs for the treatment of oral precancerous lesions. The prepared NP formulation was uniformly spherical with a PS of  $218.42 \pm 2.1$  nm, a PI of  $0.070 \pm 0.014$  and a ZP of  $-10.3 \pm 0.5$  mV. Gels containing NP obtained using mucoadhesive polymers, which have desirable rheological, texture and mucoadhesiveness properties, can benefit the therapeutic efficacy of DEX administered by buccal route, increasing the retention time and ease of local application in the buccal mucosa. Cell culture findings indicate that the novel formulations were non-toxic to HeLa cell. This drug delivery system can be a better alternative to the conventional drug delivery by virtue of its site-specific absorption as we have increased the residence time of drug at its target site. It can be concluded from the current study that G2\* and G3\* formulations in particular are a better and more effective approach to have an extended mucoadhesive drug delivery system of DEX for the treatment of oral precancerous lesions.

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## DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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