Preparation and Evaluation of Bioadhesive Inserts Containing Verapamil Hydrochloride for Nasal Delivery

Özlem YENIL 1, Emine KAHRAMAN 1, Evren ALGIN YAPAR 2,3, Erdal CEVHER 1, Sumru ÖZKIRIMLI 4 & Yıldız ÖZSOY 1*

1 Istanbul University, Faculty of Pharmacy, Department of Pharmaceutical Technology, Istanbul, Turkey
2 Ankara University, Faculty of Pharmacy, Department of Pharmaceutical Technology, Ankara, Turkey
3 The Ministry of Health of Turkey, Turkish Medicines and Medical Devices Agency, Ankara, Turkey.
4 Istanbul University, Faculty of Pharmacy, Department of Pharmaceutical Chemistry, Istanbul, Turkey

SUMMARY. Verapamil HCl is an antihypertensive agent which have a low oral bioavailability (20-35%) due to its high first pass metabolism. The objective of the present study is to develop a verapamil HCl nasal insert of which would enable to improve the bioavailability and prolonged release of drug. As a result of textural analyses, sodium alginate gel was chosen to fabricate nasal inserts. In vitro drug release studies performed on Franz-diffusion cell showed that nasal insert gave prolonged drug release which was fitted to Higuchi kinetic model. PEG 400 was used as a penetration enhancer in formulation to increase the release of drug from insert. Ex vivo permeation studies with excised bovine nasal mucosa were carried out on inserts (with or without PEG 400). Ex vivo studies showed that PEG 400 increased the release of verapamil significantly. As a result, nasal inserts may be an alternative of oral route.

INTRODUCTION
The nasal mucosa has been considered as a potential administration route to achieve faster and higher level of drug absorption. This is due to the large surface area, porous structure of endothelial membrane, high total blood flow, and the avoidance of first-pass metabolism. The nasal administration of drugs including numerous compounds such as low molecular weight drugs, peptides and proteins has been widely investigated for systemic medication in recent years 1-2. Mucociliary clearance is the most crucial factor affecting the nasal absorption of drugs administrated due to their insufficient contact time to the nasal mucosa. Therefore, addition of mucoadhesive polymers into the formulations extends the drug residence time on the nasal mucosa 3-4 and enhance the bioavailability of drugs 5. Several theories have been put forward to explain the mechanism of polymer-mucus interactions that lead to mucoadhesion. To start with, the sequential events that occur during mucoadhesion include an intimate contact between the mucoadhesive polymer and the biological tissue due to proper wetting and swelling of the polymer. Inserts remaining in the nasal mucosa for a long period have been recommended to increase the nasal bioavailability 6,7. Nasal inserts, which are the novel dosage forms including hydrophilic polymer matrix in their structure, are prepared by lyophilisation technique. Chitosan 8, chitosan/pectin 9, chitosan/hyaluronate 10, hydroxypropyl methylcellulose 6,11-13, xanthan/guar gum 14, polyvinylpyrrolidone 11, sodium alginate 11, carrageenan 11, carbomer 11, sodium carboxymethyl cellulose 11 and xanthan gum 11 were used in inserts as mucoadhesive polymers. When the inserts contact with the nasal mucosa, the polymer in its spongy matrix structure turns into gel form quickly by absorbing the water in mucus and releases the active substance for long time period 12. The inserts are shown to supply more accurate dosages compared with drops and gels. Also, it is reported that they can be easily manu-
factured in industrial scale. McInnes et al. reported that the lyophilized inserts based on hydroxypropyl methylcellulose extended the nasal residence time (4-5 h).

Verapamil hydrochloride is an antihypertensive agent which is used via oral and parenteral routes. Verapamil HCl is class 1 drug according to Biopharmaceutical Classification System (BCS). More than 90% of its orally administered dose is absorbed and it reaches its maximum plasma concentration between 1 and 2 h after oral administration. Unfortunately, it has extremely low oral bioavailability (20-35%) due to rapid biotransformation during its first pass through the portal circulation. Furthermore, because of a linear inverse correlation between the absorption of drugs and molecular weight less than 1000 Da, highly soluble verapamil HCl with a low molecular weight of ~491 Da is also a good candidate for its systemic delivery by nasal route.

For the mentioned reasons, the study was developed to design a prolonged release mucoadhesive nasal inserts of verapamil HCl for nasal delivery to avoid the bypass effect of liver.

Nasal inserts comprising verapamil HCl as an alternative to current verapamil dosage forms in the market were fabricated by lyophilisation technique, which is a simple and reproducible method, and different hydrophilic bioadhesive polymers were investigated to improve the mechanical properties of inserts and to extend their retention time in the nasal cavity as well as to improve intimacy of contact of the inserts with absorptive membranes of nasal mucosa and to open the tight junctions between the cells to increase the permeability of water soluble verapamil HCl through the paracellular route. In order to evaluate the effect of polyethylene glycol 400 addition on nasal absorption of verapamil HCl. In vitro release and ex vivo permeation studies were carried out.

**MATERIALS AND METHODS**

**Materials**

The following chemicals were obtained from commercial suppliers: Verapamil hydrochloride (Recordati, Turkey), Polyethylene glycol 400 (Merck, Germany), Sodium alginate (A-2033, Sigma, USA), Acrylic acid homopolymer (Carbopol®940, B.F.Goodrich, USA), Xanthan gum (Jungbunzlauer, Austria AG), Pectin (P-9135, Sigma, USA), κ-Carrageenan (C-1013, Sigma, USA), and all other chemicals were of analytical grade.

<table>
<thead>
<tr>
<th>Polymers</th>
<th>Concentration (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbopol® 940</td>
<td>1.5</td>
</tr>
<tr>
<td>Xanthan gum</td>
<td>2.5</td>
</tr>
<tr>
<td>Sodium alginate</td>
<td>4.5</td>
</tr>
<tr>
<td>κ-Carrageenan</td>
<td>3.0</td>
</tr>
</tbody>
</table>

**Table 1. Selected polymers and their concentrations.**

**Preformulation studies**

Gel formulations containing different concentrations of Carbopol®940, xanthan gum, sodium alginate and κ-carrageenan were prepared in order to select the polymer to be used in the preparation of the nasal inserts and its concentration. Carbopol®940, xanthan gum, sodium alginate and κ-carrageenan were weighed at concentrations given in Table 1 and dispersed in distilled water and then, kept at room temperature for 24 h until homogeneous gels were obtained.

In order to determine the most appropriate gel formulations to be used in the preparation of the inserts, their mechanical properties were determined using a software-controlled penetrometer (TA.XTPlus, Stable Micro Systems, UK) with a 5 kg load cell. Each gel formulation was transferred into universal bottle (25 mL) to a fixed height of 8 cm and kept in an ultrasonic water bath to remove air bubbles for 20 min, and the temperature was adjusted to 37 ± 2 °C. The Perspex probe of 10 mm diameter was twice compressed into each formulation at a defined rate of 2 mm/sec to a depth of 15 mm. A delay period of 15 s was allowed between the two compressions. A trigger force of 0.01 N was applied. At least three replicate analyses of each sample were performed. Data collection and calculation were performed using the Texture Exponent 6.0.4.0 software package of the instrument. From the resultant force-time plot, mechanical parameters such as hardness, compressibility, cohesiveness and elasticity of the gel formulations were defined.

**Preparations of nasal inserts**

Nasal insert formulations containing Verapamil HCl at a concentration of 10.0% (w/w) were fabricated using sodium alginate as a polymer which was selected as a result of the preliminary formulation studies. Polymers were dispersed in distilled water and let to hydrate for 24 h. 10% PEG 400 as a penetration enhancer was added to INS-1 formulation. 0.150 g gel with PEG 400 (INS-1) and without PEG 400
(INS-2) was poured into blister moulds and frozen for 24 h at -35 °C (Electrolux MRF 120/35, Sweden) then lyophilized (Lyovac GT 2, Leybold-Heraeus, Germany) for 24 h and stored in desiccator until use.

Physical properties of nasal inserts

Inserts were examined in terms of physical properties such as appearance, homogeneity and measured its thickness by using a micrometer calliper (Mitutoyo, Japan), and its weight (CPA2P, Sartorious, Germany). Each experiment was carried out ten times.

Water uptake studies

Water absorptivity of inserts was performed according to the methods in the literature. A sponge (3 cm × 5 cm × 3 cm) was fully soaked in the hydration medium (phosphate buffer with pH of 7.4) and placed in a petri plate filled with the same buffer to a height of 1 cm in order to keep the sponge soaked during the experiment. Circular filter paper (55 mm, Whatman No.41, USA) was also soaked in the medium and positioned on the top of the sponge. This experimental setup was equilibrated for 15 min. Accurately weighed inserts were then placed on the filter paper, and the water uptake was determined as the increase in the weight of insert according to Eq. (1):

\[
\text{Water uptake} (\%) = \frac{(W_{\text{hp}} - W_{\text{dp}} - W_{\text{di}}) \times 100}{W_{\text{di}}} \tag{1}
\]

where \(W_{\text{hp}}\) is the weight of hydrated insert and wet filter paper, \(W_{\text{dp}}\) is the weight of wet filter paper and \(W_{\text{di}}\) is the initial weight of the dry insert. All measurements were performed under ambient conditions. Each experiment was carried out three times.

Mucoadhesion studies

The mucoadhesion properties of the inserts were determined by measuring the force required to detach the inserts from cellulose acetate membrane filter (0.22 µm, Millipore) which was wetted with 10% of gastric mucin by using Texture Analyser (TA.XTPlus, Stable Microsystems, UK) at 37 ± 0.5 °C. Insert was attached to the upper probe of the instrument, and cellulose acetate membrane filter was placed down of the instrument. The upper probe was lowered at a speed of 3 mm/sec to touch the surface of the membrane. A force of 3N was applied for 3 min, to ensure intimate contact between the insert and membrane, and then the upper probe was removed at a speed of 3 mm/sec. The surface area of insert exposed membrane was 1 cm². Work of mucoadhesion (mJ/cm², Eq. (2)) and peak detachment force (N/cm²) were calculated from force-distance plot using Texture Exponent 6.0.4.0 software package of the instrument. Each experiment was carried out three times.

\[
\text{Work of mucoadhesion (mJ/cm²)} = \frac{\text{AUC}}{\pi r^2} \tag{2}
\]

where \(\pi r^2\) is the surface area of insert and AUC area under the force-distance curve.

In vitro release studies

In vitro drug release from nasal inserts (INS-1, INS-2) was investigated on modified Franz diffusion cell with permeation area of 1.77 cm² by using pH 7.4 phosphate buffer as receptor phase with a volume of 12 mL and cellophane membrane (D-9777, Sigma, USA) as diffusion membrane which was hydrated with distilled water for one night before using. One insert (150 mg) were put onto the cellophane membrane and receptor fluid was stirred continuously with a Teflon-coated magnetic stirrer at 600 rpm. The temperature was kept at 37 ± 2 °C using a water bath, circulator, and jacket surrounding the cells. At predetermined time points (0.5 to 24 h), 0.25 mL samples were withdrawn from the receptor compartment, replacing the sampled volume of pre-warmed (37 ± 2 °C) with phosphate buffer pH 7.4, after each sampling. The in vitro release studies were carried out during 24 h. The samples withdrawn were filtered and used for analysis. The amount of diffused drug was determined with high performance liquid chromatography (HPLC) according the literature. The chromatographic system was composed of a Waters, Germany, detector set at 254 nm. Separation was obtained with ACE 5 C18, (150 x 4.6 mm I.D., 5 µm) column. The mobile phase was composed of a mixture of 0.05 M potassium dihydrogen phosphate-phosphate-ace-tonitrile (60:40, v/v). Fluoxetine was used as an internal standard. The flow rate was 1.3 mL/min and injection volume was 40 μL. A calibration curve was set up in the 1-9 µg/mL range; good linearity was found (r² = 0.9996). Repeatability assays were carried out on verapamil HCl standard solutions, at concentrations corresponding to the lower and upper limit and the middle point of the calibration curve. Method precision was satisfactory: RSD% values of 0.44, 0.06, and 1.5 were calculated for verapamil HCl of 3, 5,
and 7 µg/mL, respectively. In vitro release experiments were carried out three times. The data of drug release from the nasal inserts were subjected to theoretical analysis to determine the order of kinetic release according to zero order, first order and Higuchi models.

**Ex vivo permeation studies**

Bovine nasal mucosa was obtained from local slaughterhouse. The turbinate was fully exposed by a longitudinal incision through the nose. The mucosa was carefully removed from the underlying bone by cutting with homeostatic forceps and pulling the mucosa off. In order to maintain the freshness of the specimen as far as possible, permeation studies were started immediately after the mucosa samples were excised. The permeation study was performed in a modified Franz diffusion cell with the procedure described before. The samples withdrawn at a predetermined time intervals were filtered through 0.45 µm membrane filter and analysed by HPLC as detailed above. The ex vivo permeation studies were also carried on for 24 h. The cumulative amount of permeated drug was plotted against time.

**Data analysis**

The cumulative amount of drug permeated through the bovine nasal mucosa was plotted against time. Steady-state drug flux (Jss) is defined by Eq. [3]:

\[ Jss = \frac{dQ}{A \cdot dt} \]  

where \(dQ\) is the change in the quantity of drug passing through the bovine nasal mucosa expressed in µg, \(A\) is effective diffusion area (cm²), and \(dt\) is time (h). \(Jss\) (µg/cm²·h) was estimated from the slope of the straight line portion (1-24 h) of the cumulative amount of drug permeated versus time.

**Statistical analysis**

All the experiment was done in triplicate. Results are expressed as mean±SD. Statistical analysis of studies was one-way analysis of variance (ANOVA). The criteria for statistical significance was \(P < 0.05\).

**RESULTS AND DISCUSSION**

Polymers and their concentrations are important factors affecting the mechanical properties and drug release characteristics of inserts. Gel formulations which will be used to fabricate films should possess appropriate mechanical properties such as good flowability, pourability and spreadability. In preliminary studies, the gels prepared with different polymers were examined to evaluate their effect on the structural properties of nasal inserts. Type and concentration of each polymer was chosen by measuring hardness, compressibility, cohesiveness and elasticity of the gel formulations using Texture Analyser. Firstly, gels prepared with different polymers at different concentrations (from 1 to 5% for all the polymers, data not given) and then ideal concentration for each polymer was chosen by measuring the mechanical properties of the gels and finally each polymer with an optimal concentration was compared to each other. The optimal formulations that were determined as a result of the preliminary formulation studies were given in Table 2.

Hardness is the force acquired for deformation of gel and significantly influences the pourability and spreadability of the gel into the Petri dishes. Cevher et al. investigated the hardness of vaginal gel formulations and found that an increasing in polymer concentration raised the hardness value and reduced the pourability of gel formulations. They indicated that ideal gel formulations prepared by polyacrylic acids had between 0.033-0.046 N hardness values. Their findings verify our hardness results. In our study, the hardness values of gels were found to be between 0.043 and 0.219 N (Table 3). Results showed that spreadability increased in the rank of sodium alginate > xanthan gum > Carbopol®940 > κ-carrageenan, and sodium alginate gel formulation had appropriate hardness value enabling the gel to flow properly, to be poured into the mould and to spread homogeneously.

<table>
<thead>
<tr>
<th>Content</th>
<th>INS-1 (% w/w)</th>
<th>INS-2 (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verapamil hydrochloride</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Sodium alginate</td>
<td>4.5</td>
<td>4.5</td>
</tr>
<tr>
<td>PEG 400</td>
<td>-</td>
<td>10.0</td>
</tr>
<tr>
<td>Distilled water</td>
<td>85.5</td>
<td>75.5</td>
</tr>
</tbody>
</table>

Table 2. Optimal nasal insert formulations.
Compressibility defines the work required to deform the gel during the first compression of the probe. This parameter expresses the simplicity of the spreadibility of gel formulations in the mold affecting homogeneity of formulations. A low compressibility value is required to enable the ease of the spreadibility of the gel in the film mold. In this study, while the lowest compressibility value was obtained in gel prepared with sodium alginate (0.351 ± 0.044 mJ), the compressibility value raised in the rank of sodium alginate < xanthan gum (0.518 ± 0.005 mJ) < Carbopol® 940 (0.618 ± 0.004 mJ) < κ-carrageenan (1.699 ± 0.161 mJ) (Table 3). These results were in accordance with literature.

Cohesiveness defines the ratio of the area under the force-time curve produced on the second compression cycle to that produced on the first compression cycle, where successive compressions are separated by a defined recovery period. Cohesiveness is a critical parameter that affects the strength and the elasticity of the inserts. It is predicted that the strength of the insert could be increased by using gels with high cohesiveness. As seen in Table 3, the cohesion of the gel prepared with sodium alginate (0.875) was found to be significantly higher than those of other polymers (between 0.764 and 0.806).

The elasticity of gels is a parameter that would significantly affect the elasticity of the inserts to be prepared by using these gels. When gel formulations are poured into the moulds to form inserts, deformation will be shown depending on the force that is applied for enabling the flowing of gels. If this deformation is reversible, the gel shows proper spreading in the mould in which it is poured and a homogeneous insert is obtained. Therefore, it is important that the elasticity of the gels should be high enough to obtain a homogeneous inserts. Elasticity is defined as the direction of re-construction of the gel after its deformation by compression in a defined period of time. The increase in the numerical value of elasticity obtained during texture profile analysis shows the decrease in the elasticity of the gel. Our study showed that the gel containing sodium alginate had a higher elasticity (0.888 ± 0.011) when compared to gels containing Carbopol®940 (0.899 ± 0.025), xanthan gum (0.916 ± 0.013) and κ-carrageenan (0.928 ± 0.023).

Results of textural analyses of gel formulations demonstrated that sodium alginate at a concentration of 4.5% was more suitable for fabrication of nasal inserts due to its better mechanical properties such as lower hardness (high pourability) and compressibility (high spreadibility) values and higher elasticity and cohesiveness (better film formability with a high tensile strength) values than those of other polymers.

Due to these reasons, nasal inserts (INS-1 and INS-2) were prepared by using sodium alginate (4.5% w/w) as a matrix forming polymer and PEG 400 (10% w/w) as a plasticizer (Table 3) and used for further studies.

INS-1 and INS-2 inserts containing 10% w/w verapamil HCl were homogeny, sponge like and white in colour. The thicknesses of both insert formulations were around 0.025 mm, and weights were 0.024 and 0.041 g for INS-1 and INS-2, respectively. The standard weight deviation of the sections taken from inserts was found to be less than 2% confirming to be compliant with the pharmacopoeia limits.

Water uptake studies showed that while INS-1 reached maximum water uptake capacity in 3.5 h, INS-2 formulation containing PEG 400 reached in 2.5 as seen in Fig. 1. Furthermore, INS-1 formulation absorbed around three-times higher water then formulation containing PEG 400 (INS-2).

The results of the bioadhesion study of nasal insert formulations were given in Figs. 2a and 2b. The formulation without PEG 400 (INS-1: 0.81 ± 0.06 mJ/cm²) showed higher bioadhesion then the formulation containing PEG 400 (INS-2: 0.61 ± 0.13 mJ/cm²). Addition of penetration en-

<table>
<thead>
<tr>
<th>Gel Formulation</th>
<th>Hardness (N)</th>
<th>Compressibility (mJ)</th>
<th>Adhesiveness (mJ)</th>
<th>Cohesiveness</th>
<th>Elasticity (mJ/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbopol® 940 (1.5%, w/w)</td>
<td>0.063 ± 0.001</td>
<td>0.618 ± 0.004</td>
<td>0.352 ± 0.016</td>
<td>0.805 ± 0.021</td>
<td>0.899 ± 0.025</td>
</tr>
<tr>
<td>Xanthan gum (2.5%, w/w)</td>
<td>0.045 ± 0.001</td>
<td>0.518 ± 0.005</td>
<td>0.023 ± 0.003</td>
<td>0.764 ± 0.011</td>
<td>0.916 ± 0.013</td>
</tr>
<tr>
<td>Sodium alginate (4.5%, w/w)</td>
<td>0.035 ± 0.001</td>
<td>0.351 ± 0.044</td>
<td>0.045 ± 0.001</td>
<td>0.875 ± 0.001</td>
<td>0.888 ± 0.011</td>
</tr>
<tr>
<td>κ-Carrageenan (3.0%, w/w)</td>
<td>0.219 ± 0.012</td>
<td>1.699 ± 0.161</td>
<td>0.354 ± 0.028</td>
<td>0.806 ± 0.113</td>
<td>0.928 ± 0.023</td>
</tr>
</tbody>
</table>

Table 3. Mechanical properties of gel formulations. Mean ± standard deviation.
hancer slightly reduced the bioadhesiveness of inserts.

In vitro drug release profiles of INS-1 and INS-2 insert formulations are presented in Fig. 3 and verapamil HCl release from INS-2 formulation including PEG 400 was higher than that of INS-1 formulation without permeation enhancer (p < 0.05). Higher drug release profiles from inserts containing PEG 400 could be attributed to the hydrophilic character of PEG 400 which increases penetration of water into inserts with accordingly increased drug release. Gaikwad showed that PEG 400 could enhance drug absorption from nasal membrane and could facilitate to reach the effective plasma concentration of drug. Additionally, in vitro release studies (Fig. 3) showed that drug release from inserts (INS-1 and INS-2) had an initial lag time which could be explained by a hydration time of lyophilised inserts to form a gel matrix. Higher drug release from inserts compared to gels could be explained by the structure of inserts. Inserts had high porosity that allows water penetration effectively to the inner parts of the inserts thus accelerates the release of drug.

The data of in vitro drug release from inserts (INS-1 and INS-2) were subjected to theoretical analysis to determine the order of kinetic
release according to zero order, first order and Higuchi models (Table 4). Highest determination values for Higuchi kinetic model were obtained for insert formulations of verapamil HCl which corroborated by the study of Arnold et al.30.

Ex vivo permeation studies were carried out on INS-1 and INS-2 nasal insert formulations. Drug permeation profiles from excised bovine nasal tissue and data analyses of drug permeation were presented in Fig. 4 and Table 5, respectively. The flux of INS-1 and INS-2 inserts was calculated as 0.208 ± 0.02 and 0.217 ± 0.013 µg/cm².h, respectively (Table 5). The cumulative amount of verapamil HCl (Q24) released after a period of 24 h from INS-1 and INS-2 inserts were found 4.939 ± 0.478 and 5.214 ± 0.338 µg/cm², respectively. In a light of in vitro drug release results, it was predicted that PEG 400 would enhance release and absorption of drug through bovine nasal mucosa. But as seen in Fig. 4, flux and Q24 were not significantly different (p > 0.05) between INS-1 (without PEG 400) and INS-2 (with PEG 400, Table 5). In a study of Gaikwad 29, addition of PEG 400 as a penetration enhancer into gels containing metoprolol tartrate prepared with the mixture of Pluronic®127 and Carbopol®934 increased in vitro release of the drug from formulation. In another study, the effect of PEG 400 on the nasal absorption of verapamil and nicardipine was evaluated in rats 19. Nasal bioavailability of the drugs increased with an increase on concentration of PEG 400 (1-5%). In our study, while a significant increase observed on drug release in in vitro studies, unfortunately similar release characteris-

tics could not be obtained in ex vivo studies due to the structural difference between synthetic membranes and mucosal membranes (Table 5).

Nasal inserts, new dosage forms, are effective to overcome the disadvantages of gel preparations (like dosage inaccuracy, difficulty of administration etc.). These also offer the advantages of solid dosage forms. In this study, the nasal inserts were prepared by lyophilisation of natural polymeric gels including verapamil HCl. Lyophilisation led to the formation of a highly porous dosage form. The spongy matrix structure of the nasal inserts ensures in situ gel formation in the nasal cavity. In situ gelling inserts can be prepared with various natural and synthetic hydrophilic polymers such as chitosan, sodium alginate, pectin, hyaluronic acid, xanthan gum and guar gum. Nasal insert swells immediately as soon as it contacts with nasal mucosa and release of the drug continues for longer periods. Thus, the drug passes directly through the systemic circulation without undergoing first-pass effect. Due to this advantage, it may be an alternative dosage form for verapamil HCl with a low oral bioavailability.

**CONCLUSION**

In situ gelling mucoadhesive nasal inserts were successfully designed for prolonged delivery of verapamil HCl. The best nasal insert formulation was obtained with sodium alginate at a concentration of 4.5% (w/w). Nasal insert showed higher mucoadhesion in the nasal mucosa. Insert formulations with or without PEG 400 showed prolonged release of verapamil HCl in ex vivo studies. Results will be supported by performing in vivo studies of inserts in animal models.

**Acknowledgement**: This study was supported by Research Fund of the Istanbul University. Project Number: 3802

**REFERENCES**