Comparison of hyaluronate/carboxymethylcellulose membrane and melatonin for prevention of adhesion formation in a rat model

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BACKGROUND: To investigate the effectiveness of melatonin in preventing post-operative adhesion formation and to compare it with the efficacy of hyaluronate/carboxymethylcellulose membrane in a rat model. MATERIALS and METHODS: Following pilot studies, 35 rats were operated on in the full study. In 15 animals (group one), 10 standard lesions were inflicted in each uterine horn (total 30 horns) and melatonin was applied before closure of the abdomen. In the second group, 20 animals were operated on and one of the uterine horns (total 20 horns) with standard lesions was treated with hyaluronate/carboxymethylcellulose membrane and the other uterine horn served as a control. Second-look operations were performed 1 week later and adhesion scores were compared. RESULTS: The adhesion scores of uterine horns treated with melatonin and of uterine horns treated with hyaluronate/carboxymethylcellulose membrane were significantly lower than the scores of the controls (P<0.001). There was no statistically significant difference between the adhesion scores of uterine horns treated with melatonin and of uterine horns treated with hyaluronate/carboxymethylcellulose membrane (P>0.05). CONCLUSIONS: Both melatonin and hyaluronate/carboxymethylcellulose membrane were effective in prevention of adhesion formation in a rat uterine horn model.

Key words: adhesion formation/hyaluronate–carboxymethylcellulose membrane/melatonin/surgical barriers/rat uterine horn model

Introduction
Postoperative peritoneal adhesion formation is still a major problem resulting in chronic pelvic pain, infertility, bowel obstruction and subsequent intraoperative complications (Ray et al., 1993; Strickler et al., 1994). Fifty to eighty percent of surgical procedures result in peritoneal adhesions (di Zerega, 1994; Monk et al., 1994). Although many methods have been used for the prevention of adhesion formation, no completely satisfactory solution is currently available.

Inflammatory response due to surgery starts with increasing peritoneal fluid including proteins and cells, which results in a fibrinous exudate and finally fibrin formation (Holmdahl et al., 1996) by transformation of prothrombin into thrombin. This is the first step of peritoneal healing and adhesion formation following surgery. During open surgery, the production of reactive oxygen species (ROS) is increased (Elkins et al., 1991). Although it is not certain, ROS can play a role in postoperative adhesion formation, since it was shown that ROS scavengers reduced adhesion formation (Tsimoyiannis et al., 1989, Tsimoyiannis, 1990; Ozcelik et al., 2000).

Melatonin (N-acetyl-5-methoxy-triptamine) is a very potent and efficient endogenous free radical scavenger (McCord, 1985; Kazez et al., 2000). In addition to the pineal gland, the liver, retina, salivary glands, thyroid and gastrointestinal system are also reported to be sites of melatonin synthesis (Raikhlin et al., 1975). However, it is accepted that it is mainly produced by the pineal gland. The mechanism of its stimulation is unclear. Melatonin can enter the subcellular compartments of cells easily because of its high diffusion ability. It can show its effects through its receptors and also without receptors by entering the cell, which makes it one of the most powerful antioxidants (Pentney and Bubenik, 1995; Reiter, 1997; Ustundag et al., 2000; Okatani et al., 2003).

A mixture of chemically derived sodium hyaluronate and carboxymethylcellulose (HA/CMC) membrane has been tested in multiple animal models and widely used in human surgery for the prevention of postoperative adhesion formation (Diamond, 1996; di Zerega, 1996; Leach et al., 1998). This translucent bioresorbable membrane reduces
the incidence, extent, and severity of abdominal and pelvic postoperative adhesions (Beck, 1997; Burns et al., 1997).

We carried out a study to compare the efficacy of HA/CMC membrane with that of melatonin in reducing postoperative peritoneal adhesion formation in a rat uterine horn adhesion model.

Materials and Methods
Fifty non-pregnant, female Wistar albino rats, weighing 180–210 g, were used in the study. The animal investigation committee of Gülhane Military Medical Academy approved this study. The animals were born and grown in a family system at the vivarium of the Research Centre of Gülhane Military Medical Academy.

Before the full study, a pilot study was carried out. The objective of this pilot study was to decide the methodology for inflicting standard lesions, which would result in standard adhesions. Different power settings were used to find out the appropriate power setting for standard lesions using 1 W, 5 W, 10 W, 20 W and 40 W as the power setting for bipolar coagulation in five rats (on 10 uterine horns). We decided to inflict 10 standard lesions during 1 s on uterine horns, since 10 lesions covered almost all the surface of the uterine horn and 1 s was easy to measure. There were no adhesions at all in the animal with lesions on both uterine horns inflicted using 1 W. There were no adhesions in one of the uterine horns of the animal with lesions inflicted on both uterine horns using 5 W. In the animal with lesions inflicted using 20 W there were very firm and vascular adhesions on both sides but the adhesions were not standard because of extensive carbonization. The animal in which we used 40 W died on the same postoperative day probably because of extensive carbonization. We inflicted lesions using 1 W in one animal and in this animal we found firm and vascular adhesions on both sides. The power setting we chose for coagulation was 10 W, which was well tolerated by the animals, resulting in standard adhesion formation. We collected specimens for histological examination and a simple histological examination was carried out, but a quantitative histological examination did not take place since the difference between the power settings was very clear.

In the second pilot study, which was carried out to assess the type, tenacity, extent and vascularity of adhesions caused by standard lesions, 10 rats were operated on and 10 standard lesions were inflicted in each uterine horn using 10 W for 1 s for each lesion. All the animals underwent second-look operations after 1 week.

A total of 35 rats were operated on in the full study. The rats were premedicated with xylazine hydrochloride (10 mg/kg im) and 5 min later anaesthesia was induced with ketamine hydrochloride (90 mg/kg im). The abdomen was shaved and prepared with povidone–iodine. A lower midline vertical incision, ~3–4 cm in length, was made. Ten standard lesions were inflicted in the uterine horns using bipolar coagulation. As an opposing lesion, an area of 1.5 × 1.5 cm between two main vascular branches of the lower sidewall at the level of the uterine horn was cauterized. All the surgical procedures were performed by the same researchers.

Following the completion of standard lesions, the animals were randomized before closure of the abdomen either to the melatonin or HA/CMC membrane group according to a randomization table. Melatonin was obtained as a dry powder (Sigma, St Louis, MO), dissolved in 99% ethanol and then diluted in saline just before injection. The final ethanol concentration was 5% and there was 2 mg of melatonin in 1 ml of solution. In the melatonin group (n = 15), 1 ml of solution was injected intraperitoneally into each rat. In the second group (n = 20), an HA/CMC membrane (Seprafilm; Genzyme Corp., Cambridge, MA) 2 × 2 cm in size was used as a barrier on one of the uterine horns. The surface of the HA/CMC membrane used in the study was adjusted so as to cover the whole uterine horn. The other uterine horn served as a control.

The abdominal incision was closed in two layers: the musculoperitoneum and fascia were closed together with simple interrupted sutures of 4.0 polyglycolic acid and the skin was closed with simple interrupted sutures of 3.0 polyglycolic acid. Following complete recovery, the rats were housed separately. Seven days later, the animals were euthanized using an overdose of pentobarbital sodium. Scoring of adhesions was performed through a transverse subcostal incision, evaluating the whole intraperitoneal cavity by an experienced researcher blinded to the groups of animals.

The criteria of scoring system for evaluating adhesions in this study were the same as those given by Leach et al. (1998). The type of adhesions was classified macroscopically as follows: 0 = no adhesions, 1 = filmy avascular, 2 = vascular or opaque, and 3 = cohesive attachment of the uterine horn to the abdominal sidewall. The tenacity of adhesions was evaluated according to the following: 0 = no adhesions, 1 = if the adhesion separated from tissue with gentle traction, 2 = if the adhesion separated from tissue with moderate traction, and 3 = requiring sharp dissection.

The extent of adhesions was scored as follows: 0 = no adhesion; 1 = 1–25% involvement, 2 = 26–50%, 3 = 51–75% and 4 = 76–100%. The sum of the three scores was used as the total adhesion score. The main outcome measures were, type, tenacity and extent of adhesions and total adhesion scores.

Statistical analysis was performed by Kruskal–Wallis test and Bonferroni adjusted Mann–Whitney U test. Data were expressed as mean values ± standard deviation.

Results
Our surgical procedures were tolerated well by all animals. There was no mortality in the study groups. All laparotomy sites were intact and none of the rats had an incisional hernia.

At the end of the second pilot study, the rat uterine horn model was shown to be a standard model for adhesions.

<table>
<thead>
<tr>
<th>Group</th>
<th>Type of adhesions (mean ± SD)</th>
<th>Tenacity of adhesions (mean ± SD)</th>
<th>Extent of adhesions (mean ± SD)</th>
<th>Total scores (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA/CMC (n = 20)</td>
<td>0.45 ± 0.94</td>
<td>0.35 ± 0.74</td>
<td>0.50 ± 1.05</td>
<td>1.30 ± 2.69</td>
</tr>
<tr>
<td>Melatonin (n = 30)</td>
<td>0.40 ± 0.72</td>
<td>0.36 ± 0.66</td>
<td>0.33 ± 0.60</td>
<td>1.10 ± 1.97</td>
</tr>
<tr>
<td>Control (n = 40)</td>
<td>2.67 ± 0.47</td>
<td>2.77 ± 0.42</td>
<td>3.50 ± 0.50</td>
<td>8.95 ± 0.90</td>
</tr>
</tbody>
</table>

Comparison of HA/CMC membrane and melatonin for reducing primary postoperative adhesions in the rat uterine horn model: P > 0.05 (not significant) for HA/CMC membrane vs melatonin and P < 0.001 for HA/CMC membrane vs control and melatonin vs control.
since firm and vascular adhesions were observed in all animals (n = 10, 100%) at the second-look operations.

After treatment with melatonin, there were no adhesions in 11 of 15 animals (22 of totally 30 uterine horns, 73.3%). The four animals with adhesions had them on both sides. In the HA/CMC membrane group 16 of 20 treated uterine horns were free of adhesions (80%), whereas all the untreated horns (n = 20), which served as internal controls with standard lesions, had firm adhesions (100%). Mean total adhesion scores in the 30 uterine horns of 15 rats treated with melatonin and in the 20 uterine horns of 20 rats treated with HA/CMC membrane were 1.10^±^0.90 and 1.30±2.69, respectively, which were significantly lower than the scores (8.95±0.90) in the 40 untreated uterine horns of 30 rats (20 uterine horns of 10 rats from the control group and 20 untreated uterine horns of 20 rats from the HA/CMC group) (P < 0.001 for all of them) (Table I). However, there was no significant difference between adhesion scores of the HA/CMC membrane and melatonin groups (P > 0.05).

**Discussion**

Postoperative peritoneal adhesions are the result of wound healing, and may develop following any injuries to the peritoneum (Yoldemir et al., 2002). Surgical trauma to the serosa or peritoneum is held to be responsible for >90% of peritoneal adhesions (Roy et al., 2004). There are also cofactors in adhesion formation such as desiccation, the effect of which was demonstrated in an endoscopic mouse model (Yesildaglar et al., 1999), and high intra-abdominal pressure during endoscopic surgery, which was studied in a rabbit model (Yesildaglar and Koninckx, 2000). Whatever the factors and cofactors are, the common denominator in all pathways for adhesion formation is the inflammatory response. Leukocyte-dependent inflammatory reactions may increase cellular and tissue injury through the actions of oxygen-derived free radicals and metabolites (Fantone and Ward, 1982; Dvorak et al., 1995). It has been demonstrated in vitro that free radicals contribute to the formation of cross-linked proteins that may be the first step in the development of adhesions (Dijkstra et al., 2003). These adhesions are generally lysed within 72 h of formation by the endogenous fibrinolytic activity and much of the healing is completed within 5 days. If there is an imbalance between fibrin deposition and fibrin dissolution, deposited fibrin may persist and fibrinous adhesions may develop (Gutmann et al., 1995; Hellebrekers et al., 2000).

Melatonin is a very potent scavenger of free oxygen radicals, which have been shown to be toxic to eukaryotic cells, including endothelial cells, erythrocytes, platelets and fibroblasts (Gutmann et al., 1995; Hellebrekers et al., 2000). Free oxygen radicals are produced during the first 5 min of ischaemia (Bertuglia et al., 1993). The protective effect may be due to the melatonin indole structure, which has been shown to scavenge free radicals, in particular the hydroxyl radical (Reiter et al., 1999), thereby giving rise to new structures that are resonance-stabilized and no longer toxic to macromolecules. Melatonin is normally found in the human circulation but the antioxidant effects of melatonin in humans probably occur only at pharmacological concentrations. No serious side effects have been reported with use of melatonin (Brzezinski, 1997). Advantages of melatonin are being suitable for use in endoscopic surgery and administration systematically in the prevention of postoperative intraperitoneal adhesions.

The antioxidant effects of melatonin were examined using different organ ischemia–reperfusion models such as rat bladder (Sener et al., 2003), rat heart (Lagneux et al., 1998) and rabbit heart (Dave et al., 1998). In these studies, the melatonin dose was reported as 10 mg/kg. To the best of our knowledge, the only study published on the prevention of intraperitoneal adhesion formation was carried out in 2003 (Ozcelik et al.). The researchers reported the dose of melatonin as 10 mg/kg and we decided to use the same dose in our study. However, the optimal dose of melatonin to prevent intraperitoneal adhesion formation is not known precisely and further studies are required in this field, particularly in large animal models.

The effect of different routes (intraperitoneal or subcutaneous) and treatment schedules (10 mg/kg; single dose or 5 days) of melatonin on postoperative adhesion formation was investigated by Ozcelik et al. (2003) in a rat uterine horn model. They administered melatonin immediately after, or 30 min prior to, injury. In the same study it was shown that a single dose of melatonin could provide a significant reduction in intraperitoneal adhesion formation. When the treatment groups were compared for prevention of adhesion formation, a statistically significant difference was not found. These findings may be important for future studies testing melatonin in open and endoscopic surgery in larger animal models and eventually in humans.

Physical barriers have been used in an attempt to prevent adhesion formation by limiting tissue opposition during the critical period of peritoneal healing, thereby minimizing the development of a fibrin matrix between tissue surfaces (di Zerega, 1996). The ideal barrier should be non-reactive, absorbable and easy to use, and it should remain in the lesion site during critical stages of healing. HA/CMC membrane has been extensively studied and approved for clinical use (Becker et al., 1996; Beck, 1997). In the field of gynaecology, it is also effective in reducing the areas of postoperative uterine adhesions after myomectomy (Diamond, 1996).

Our study was carried out to compare the efficacy of melatonin and HA/CMC membrane in the prevention of adhesion formation. Melatonin had a significant anti-adhesiogenic effect in the rat uterine horn adhesion model. Moreover, the results showed that the anti-adhesiogenic effects of melatonin and HA/CMC membrane were not significantly different in rats bred in a family system at the research centre where this study was carried out, which possibly minimized the inter-animal variability.

In conclusion, melatonin and hyaluronate/carboxymethylcellulose membrane were both effective in the prevention of adhesion formation in a rat uterine horn model. Further studies are warranted on the use of melatonin in humans and in endoscopic surgery.
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References

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