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(as of August 25, 2009)
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Jun Miura, Atsuhiro Kikuchi, Akira Fujii, Tomonori Tateishi, Sunao Kaneko
Evaluation of the effects of freeze-dried soybean curd intake on cholesterol levels using a novel biomarker

Setsuo Hasegawa*, Michiko Kainuma, Kazunori Saito, Naoko Imanishi

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ABSTRACT: To evaluate the effects of freeze-dried soybean curd intake on serum cholesterol levels, we examined the subclasses of cholesterol for healthy adult volunteers who continued to eat a piece of freeze-dried soybean curd each day along with their ordinary diet for four weeks. Of 12 subjects, the soybean curd diet proved effective in 2 cases; small dense LDL (sd-LDL) cholesterol levels were significantly reduced in association with the decrease in LDL cholesterol levels. These results suggested that daily intake of freeze-dried soybean curd may lead to an improvement in cholesterol metabolism in subjects with originally higher cholesterol levels and that sd-LDL cholesterol can be a novel biomarker for evaluation of the cholesterol lowering-action of functional food.

Keywords: Freeze-dried soybean curd, small dense LDL cholesterol

1. Introduction

The treatment strategy for metabolic syndrome has received increased attention because of the need to reduce cardiovascular events. Smoking cessation, lowering the levels of LDL-C, and blood pressure management are primary targets for risk reduction. Lifestyle interventions are the initial therapies recommended for treatment of metabolic syndrome. If lifestyle changes are not sufficient, then drug therapies for abnormalities in terms of individual risk factors may be indicated (1). Soybean protein is known to have components to help reduce total cholesterol (2) and the risk of myocardial infarction (3). Freeze-dried soybean curd (tofu) in particular has an even greater effect on reducing cholesterol and appears to be a beneficial food in terms of preventing lifestyle-related chronic diseases (4).

Recently, small dense low-density lipoprotein (sd-LDL) has been highlighted as a new risk factor for coronary heart disease (5,6). Sd-LDL is closely associated with insulin resistance and hypertriglyceridemia, suggesting a high prevalence of these atherogenic particles in metabolic syndrome. Therefore, sd-LDL cholesterol can be a useful marker for metabolic syndrome in patients with coronary artery disease. The effect of soybean protein intake on sd-LDL cholesterol has not been previously investigated. A preliminary study was conducted to examine the subclasses of cholesterol, including sd-LDL cholesterol, for healthy adult volunteers who continued to eat a piece of freeze-dried soybean curd each day over the course of 4 weeks.

2. Materials and Methods

2.1. Study subjects

A total of 12 volunteer subjects ages 24 to 58 was recruited after providing written informed consent, and this study was approved by the Ethics Committee of Sekino Clinical Pharmacology Clinic. All of the subjects had a wide range of serum lipid levels and were essentially healthy with no evidence of diseases related to atherosclerosis. None had been treated with lipid-lowering drugs.

2.2. Study diet

Seasoned, bite-size freeze-dried soybean curd was provided by Asahimatsu Foods Co., LTD (Nagano, Japan) for repeated daily intake over 28 consecutive days.

2.3. Study design

The subjects continued to eat a piece of freeze-dried soybean curd (18.5 g) each day for four weeks. Lifestyle and dietary habits were unchanged except for the equivalent caloric restriction corresponding to intake of the study diet. The subjects were also requested to
maintain a stable lifestyle throughout the duration of the trial with no intensive physical activity. Blood samples were collected after fasting (before breakfast) once every week for up to 4 weeks, including the start date. Serum was separated to measure serum levels of total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides by standard laboratory procedures. Serum was also provided to assess liver functional tests such as ALT, AST, γ-GT, and Total bilirubin in order to evaluate the safety of food intake. Sd-LDL cholesterol assay was performed using a kit purchased from Denka-Seiken (Tokyo, Japan) (7).

3. Results and Discussion

The present study was conducted to make a preliminary assessment of the effect of freeze-dried soybean curd intake on the levels of subclasses of cholesterol. Although statistical analysis of inter-individual variations was difficult due to the small number of subjects, two subjects showed significant changes over time (Figure 1). For both, the levels of total cholesterol decreased to an extent. Of note is the fact that sd-LDL cholesterol levels decreased significantly in association with the decrease in LDL cholesterol levels. That said, HDL cholesterol levels were fairly constant over time. Results for the subject depicted in Figure 1A also revealed a pronounced decrease in triglyceride levels. Laboratory data with regard to liver function showed no particular changes for all of the subjects, indicating the safety and tolerability of the study diet. A meta-analysis of a randomized controlled study revealed that soy isoflavones significantly reduced serum total cholesterol and LDL cholesterol but did not change HDL cholesterol. Reductions in LDL cholesterol were greater in hypercholesterolemic subjects than in normocholesterolemic subjects (8). The present study, using sd-LDL cholesterol as a new marker, suggested that daily freeze-dried soybean curd intake may be beneficial for lipid metabolism in hypercholesterolemic subjects.

Sd-LDL cholesterol was identical to cholesterol in the denser LDL fraction. An increased concentration of small LDL particles is reported to be predictive of an increased risk of coronary heart disease (5,9). The therapeutical modulation of sd-LDL cholesterol has been shown to significantly reduce cardiovascular risk (10). Therefore, measurement of sd-LDL-C is useful at evaluating overall atherogenic risks associated with metabolic syndrome and may be applicable to routine clinical examination (6). To date, there is insufficient evidence for primary use of drugs that target the underlying causes of metabolic syndrome (1). If functional food such as soy products can be effective in reducing the sd-LDL-cholesterol level, it may prove beneficial as a form of first-line therapy. Although more detailed studies are required to verify the effectiveness of freeze-dried soybean curd intake on cholesterol homeostasis, sd-LDL cholesterol can be a novel biomarker for evaluation of the effect of functional food on improving lipid metabolism.

![Figure 1. Changes in cholesterol levels during intake of the study diet.](www.ddtjournal.com)
References


(Received April 23, 2009; Accepted April 25, 2009)
Effects of a hyaluronic acid and low molecular weight heparin injection on osteoarthritis in rabbits

Peixue Ling¹,* , Lina Zhang², Yan Jin¹, Yanli He¹, Tianmin Zhang¹

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ABSTRACT: An osteoarthritis (OA) model was created in the knees of rabbits by injecting them with 0.3 mL of sterile papain solution in order to evaluate the effects of a hyaluronic acid (HA) and low molecular weight heparin (LMWH) injection on osteoarthritis. HA-LMWH, LMWH, and HA were injected into animals once weekly. After 5 weeks of treatment, the animals were sacrificed and the effects of the injections on osteoarthritis were evaluated by histological assessment. HA levels in cartilage and the levels of IL-1β and TNF-α expression in synovial fluids were determined. As shown by histological observation, recovery of the synovium and cartilage of animals injected with HA-LMWH was better than that in animals injected with HA or LMWH. HA levels in cartilage of animals injected with HA-LMWH were much higher than those of the control group. The levels of IL-1β expression in synovial fluids of animals injected with HA-LMWH were lower than those in other animals. The levels of TNF-α expression in synovial fluids of animals injected with HA-LMWH were lower than those in other animals. In conclusion, HA-LMWH injection had a favorable anti-inflammatory and therapeutic effect on experimental OA.

Keywords: Hyaluronic acid, osteoarthritis, low molecular weight heparin, IL-1β, TNF-α

1. Introduction

Osteoarthritis (OA) is among the most frequent and symptomatic medical problems for the middle-aged and elderly. The exact etiology, pathogenesis, and progression of this disease have yet to be determined (1). Studies have indicated that inflammation of the synovium may play an important role in its pathogenesis (2,3).

Hyaluronic acid (HA) is a glycosaminoglycan (GAG) composed of D-glucuronic acid and D-N-acetylglucosamine with versatile biological activity, such as matrix construction, water retention and lubricating action, and wound healing. HA and its derivatives have been widely utilized in the medical field. HA is a major component of synovial fluid and plays a central role in the formation of the synovial joint (4). High molecular weight HA has been used in the treatment of human (5-7) and animal OA (8-11). Intra-articular HA treatment of the knee of patients with OA has been shown to reduce painful symptoms and improve joint mobility. The purpose of intra-articular HA therapy is to make up for the loss of viscoelasticity of synovial fluid due to inflammation and to protect the degradation of cartilage (12).

Heparin is a sulfated glycosaminoglycan that is the most widely studied natural anticoagulant (13). A number of physiological effects have been ascribed to heparin since its discovery, many of which are independent from its first-described and best-characterized activity as an anticoagulant. Heparin is believed to possess many forms of biological activity that include the ability to modulate embryonic development, neurite outgrowth, tissue homeostasis, wound healing, metastasis, cell differentiation, cell proliferation, and inflammation (14). The potential anti-inflammatory effects of heparin are supported by several modestly-sized clinical trials that have included patients with rheumatoid arthritis, bronchial asthma, and inflammatory bowel disease, and these effects have been corroborated by its prevention of macroscopic inflammatory lesions in animal models. Recent studies have indicated that heparin and related glycosaminoglycans can modulate the activity of a number of inflammatory cells, including T-cells and neutrophils (15).

Low molecular weight heparins (LMWH) have been developed by several manufacturers and have advantages in terms of pharmacokinetics and convenience of administration. LMWH has been shown to be at least as effective and safe as unfractioned heparin and has
replaced the latter in many applications (16). LMWH also has a marked inhibitory effect on inflammation in animal models and clinical practice (17,18).

Combinations of HA and NSAIDs have been used to treat OA patients. Although NSAIDs are effective at mitigating inflammation, many reports have noted that they may accelerate disease progression. In order to avoid the adverse effects of NSAIDs and obtain a drug with favorable anti-inflammatory activity to treat OA, the current study prepared an injection of HA and LMWH and evaluated its effect on OA in rabbits.

2. Materials and Methods

2.1. Preparation of injection

HA with $M_r$ of $1.56 \times 10^6$ was obtained from a bacterial strain of *Streptococcus zooepidemicus* and was provided by Shandong Freda Biochem Co., Ltd. (Ji'nan, China). LMWH with $M_r$ of $4.25 \times 10^5$ was purchased from Hangzhou Jiuyuan Gene Engineering Co., Ltd. (Hangzhou, China).

An injection of HA and LMWH (HA-LMWH injection) was prepared according to the following steps: $2.0 \times 10^5$ anti-FXa IU of LMWH was dissolved in 100 mL of distilled water containing 0.73 g of NaCl. One gram of HA was added to the solution and left to swell for several hours. Then, the compound solution was sterilized with flowing steam.

An injection of LMWH was prepared by dissolving $2.0 \times 10^5$ anti-FXa IU of LMWH in 100 mL of distilled water containing 0.73 g of NaCl followed by flowing steam sterilization. One g of HA was added to 100 mL of PBS (pH 7.4) followed by flowing steam sterilization to prepare an injection of HA.

2.2. Induction and treatment of osteoarthritis in rabbits (animal experiment)

Papain was from Sigma-Aldrich (St Louis, MO, USA). Adult skeletally mature New Zealand White rabbits (body weight 2.5–3.0 kg) provided by the Center for Drug Safety Evaluation of Shandong Province were housed individually in cages. Osteoarthritis was induced through injection with 0.3 mL of sterile papain solution into the knees (1 mL of the solution containing 4.0 mg of papain and 50 mg of cysteine hydrochloride) under general anesthesia. After osteoarthritis induction, 34 rabbits were randomized into four groups: a control group, a 'HA-LMWH' group, a 'LMWH' group, and a 'HA' group. After 7 days, rabbits in the control group ($n = 8$) were injected with 0.3 mL of NS in the knees. Rabbits in the 'HA-LMWH' group ($n = 9$), 'LMWH' group ($n = 8$) and 'HA' group ($n = 9$) were respectively injected with HA-LMWH, LMWH, or HA in the knees. All animals were injected once weekly. The animals were injected continuously for 5 weeks.

2.3. Histological evaluation

Seven days after the last treatment, the animals were sacrificed and both knees were collected. Synovial fluids were also collected. Routine histological methods, involving fixation in 10% formaldehyde, were followed by decalcification in 10% nitric acid. Standard haematoxylin-eosin staining was performed, and the specimens were assessed by an independent pathologist who was experienced in the examination of osteoarthritis specimens.

2.4. Biochemical evaluation

HA levels in cartilage of animals in different groups were collected after sacrifice and evaluated by radioimmunoassay. The levels of IL-1β and TNF-α in synovial fluids were determined with an enzyme-linked immunosorbent assay kit (RapidBio Lab., Shanghai, China).

2.5. Statistical analysis

A T-test was used to analyze data. $p < 0.05$ was considered significant.

3. Results

As shown in Figure 1, the structure of the normal synovial membrane was intact. Lamination of the synovial membrane of animals in the control group disappeared. Some of the epithelial cells swelled and displayed hyaloid degeneration or shedding. There was slight vascular proliferation in the synovial membrane and focal ischemic necrosis in the synovial cavity. The fibrous tissue swelled and large amounts of capillaries expanded. The synovial membrane of animals in the 'HA-LMWH' group was in the early stages of recovery. The synovial membrane recovered in patches and in layers, similar to normal tissue. The proliferation of capillaries was obvious. The 'LMWH' group had large amounts of fibroblasts. The infiltration of inflammatory cells was not obvious. In the 'HA' group, the epithelial cells proliferated in patches. There was slight vascular proliferation. Recovery of the synovial membrane of animals in the 'HA-LMWH' group was better than that of animals in other groups.

As shown in Figure 2, the normal chondrocytes were vacuolar and regularly aligned. In the control group, there was slight focal hyperplasia of the fibrous tissue on the surface of the cartilaginous tissue. The chondrocytes obviously shrank. There was focal chondronecrosis and the strip-funicular fibrous tissue was more abundant than normal tissue. In the 'HA-LMWH' group, large amounts of capillaries proliferated around the margin of the cartilage. There were some chondrocytes in the early stage of proliferation and some mature chondrocytes.
There was a slight increase in the amount of neutral GAG on the surface of the cartilage. In the 'LMWH' group, there was no obvious proliferation of chondrocytes and little acid GAG. The chondrocytes were irregularly aligned. There was little granulation tissue. In the 'HA' group, the chondrocytes proliferated in patches. On the surface of the cartilaginous tissue there was punctiform proliferation of fibrous tissue.

HA levels in cartilage and levels of IL-1β and TNF-α expression in synovial fluids of animals are shown in Table 1. HA levels were much higher in the cartilage of animals in the 'HA-LMWH,' 'LMWH,' and 'HA' groups than those of the control group ($p < 0.05$). Meanwhile, the levels of IL-1β expression in synovial fluids of animals in the 'HA-LMWH' and 'HA' groups were much lower than those of the control group ($p < 0.05$). The level of IL-1β expression in synovial fluids of animals in the 'HA-LMWH' group was lower than that of the 'LMWH' and 'HA' groups ($p < 0.05$). The levels of TNF-α expression in synovial fluids of animals in the 'HA-LMWH,' 'LMWH,' and 'HA' groups were also much lower than those of the control group ($p < 0.05$), and there was no difference in the levels of TNF-α expression among the groups receiving injections.

4. Discussion

OA is not a simple wear-and-tear phenomenon but is an active process that is part of the reparative response to injury (19). The disease affects not only cartilage but has also been shown to cause damage to the entire joint structure, including the subchondral bone, synovium, and joint capsule. The exact cause of OA is not yet known. Studies have indicated that inflammation of the synovium may play an important role in the pathogenesis of OA (2,3). Pro-inflammatory cytokines, particularly IL-1β and TNF-α, are synthesized by synoviocytes, chondrocytes, and infiltrating leukocytes during the disease process (20).

Evidence suggests that intra-articular administration
The current study prepared HA-LMWH and injected it into rabbits to treat osteoarthritis. The levels of IL-1β expression in synovial fluids of animals injected with HA-LMWH were lower than those in other groups, to some extent indicating that the inflammation of the synovium was attenuated by injection of HA-LMWH. The levels of TNF-α expression in synovial fluids of animals injected with HA-LMWH, HA, or LMWH were lower than those of the control group, indicating the anti-inflammatory effects of injecting HA-LMWH, HA, and LMWH. However, there was no difference in the TNF-α levels of the three groups receiving injections. The effect of HA-LMWH injection on pro-inflammatory cytokines involved in the process of osteoarthritis still needs to be explored.

There are reports indicating that proteoglycan levels in the cartilage of OA patients or animals decreased in comparison to normal levels (25,26). In the current study, the HA levels of the groups receiving injections of HA improves symptoms of OA in selected patients and has few adverse effects (27). Anti-inflammatory effects suggest a potential mechanism of HA in osteoarthritis. LMWH has been widely used as an anticoagulant to treat diseases that feature thrombosis as well as for prophylaxis in situations that lead to a high risk of thrombosis (22). LMWH also possesses anti-inflammatory activity supported by experimental data and clinical application (23,24).

<table>
<thead>
<tr>
<th>Groups</th>
<th>HA (pg/mg)</th>
<th>IL-1β (pg/mL)</th>
<th>TNF-α (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.89 ± 0.84</td>
<td>153.08 ± 52.33</td>
<td>71.26 ± 9.17</td>
</tr>
<tr>
<td>'HA-LMWH'</td>
<td>13.63 ± 1.75*</td>
<td>49.90 ± 16.64*</td>
<td>50.22 ± 6.79*</td>
</tr>
<tr>
<td>'LMWH'</td>
<td>17.81 ± 2.90*</td>
<td>109.29 ± 14.39</td>
<td>43.54 ± 7.99*</td>
</tr>
<tr>
<td>'HA'</td>
<td>16.93 ± 1.01*</td>
<td>79.82 ± 11.35*</td>
<td>50.71 ± 7.05*</td>
</tr>
</tbody>
</table>

* Compared to the control group, p < 0.05.

Figure 2. Pathological sections of cartilage stained with HE. (A) Section of normal cartilage (×10); (B) Section of cartilage from animals in the control group (×20); (C) Section of cartilage from animals in the 'HA-LMWH' group (×20); (D) Section of cartilage from animals in the 'LMWH' group (×20); (E) Section of cartilage from animals in the 'HA' group (×20).
were much higher than those of the control group, indicating that injection of HA, LMWH, or HA-LMWH may promote the production of HA in cartilage, thus improving the symptoms of OA.

5. Conclusion

HA-LMWH injection had a favorable effect on experimental OA. However, the effect and mechanism of HA-LMWH injection on OA must still be further explored.

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References


5. Conclusion


(Received May 19, 2009; Accepted July 3, 2009)
**Original Article**

**Pterocarpus marsupium** extract reveals strong *in vitro* antioxidant activity

Mahnaaz Mohammadi¹, Swati Khole¹, Thomas Paul Asir Devasagayam², Saroj S. Ghaskadbi¹,*

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**ABSTRACT:** Diabetes mellitus is a complex chronic disease characterized by hyperglycemia, which via several mechanisms leads to an increase in production of reactive oxygen species (ROS) leading to various secondary complications. Thus, a drug having both antidiabetic and antioxidant properties would have great therapeutic value for overcoming the oxidative load in diabetes. The present study was aimed at extensively evaluating the antioxidant properties of an anti-diabetic plant extract of stem bark of *Pterocarpus marsupium* using various *in vitro* radical scavenging assays as well as by using liver slice cultures as a model system. Our results demonstrate that the whole aqueous extract showed high antioxidant activity in all different assays used and also protected mitochondria against oxidative damage. Ethanol was used as an inducer of oxidative stress in liver slice culture and cytotoxicity was estimated by quantitating release of cytotoxicity marker enzymes such as lactate dehydrogenase (LDH). Additionally, levels of antioxidant enzymes (AOEs) namely superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase were also estimated. The whole aqueous extract significantly reduced LDH release along with reduction of lipid peroxidation compared to ethanol treated slices. These results indicate that the *P. marsupium* extract may serve as a potential source of natural antioxidant for treatment of diabetes.

**Keywords:** Antioxidant activity, *P. marsupium*, liver slice culture, *in vitro* antioxidant assays

1. Introduction

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia and insufficiency of secretion or action of endogenous insulin. It is a common disease affecting over 124 million individuals worldwide.

Persistent hyperglycemia during diabetic conditions leads to production of free radicals or impaired antioxidant defenses via several mechanisms (1). Also there is strong evidence that diabetes induces changes in the activity of antioxidant enzymes in various tissues (2). Many studies reveal that antioxidants capable of neutralizing free radicals are effective in preventing experimentally induced diabetes in animal models (3,4) as well as reducing the severity of diabetic complications (5). Additionally, supplementation with antioxidants in diabetic patients provided greater protection against free radical induced damage (6). Recent reviews suggest that certain herbal plants possess both antidiabetic and antioxidative activities and that their proper use in the diet may help decrease the oxidative load in diabetes mellitus. Therefore, various plant or herb extracts having both antidiabetic and antioxidant activity would have a better therapeutic value than other treatments for overcoming the oxidative load in diabetes.

*Pterocarpus marsupium* Roxb (from the family Leguminosae) known in the vernacular as "vijaysar" of "Bijasar" is a large tree that commonly grows in the central, western, and southern parts of India and in Sri Lanka. Various portions of the bark are used as astringent, anti-diarrheal, antacid, for treatment of toothache and for the management of diabetes (7). The heartwood of *P. marsupium* is known to be useful in arthritis, gout, bronchitis, skin infections asthma, diabetes, etc. The flavonoids marsupiun, pterosupin, and liriquiritigenin are reported to posses antihyperglycemic (8) and antihyperlipidemic (9) activities. The aqueous extract of stem bark was found to reduce the blood glucose level in alloxan-induced diabetic rats (10). In one laboratory study, pancreatic beta-cell regeneration was observed in alloxan-induced diabetic rats that received the flavonoid fraction from the bark of *P. marsupium* (11).

In view of this, the present study evaluated the antioxidant activity of the stem bark of *Pterocarpus marsupium* against ethanol induced oxidative stress
in liver slice cultures and by using different in vitro biochemical assays.

2. Materials and Methods

2.1. Chemicals

Chemicals were from one of the following companies: SRL (Mumbai, India), BDH (Mumbai, India), Hi-media (Mumbai, India), Sigma-Aldrich (St Louis, MO, USA), Merck (Mumbai, India), Accurex (Mumbai, India), and EMD (Madison, WI, USA) assay kits. Ethanol was obtained from Fluka, Buchs, Switzerland. Stem bark of *P. marsupium* was obtained and authenticated from the pharmacy.

2.2. Biologicals

Adult Swiss albino mice (6-8 weeks old) of either sex, bred in the animal house of Department of Zoology, University of Pune, were used for preparations of liver slices. Prior approval for the protocols involving animals during this work was obtained from Pune University Institutional Animal Ethical Committee. Animals were maintained at 12:12 h light/dark cycle and were given food *ad libitum*.

2.3. Preparation of sample

Stem bark of *P. marsupium* was used for preparing 10% aqueous extract. Briefly, 10 g of powder was dissolved in 100 mL of distilled water and stirred for 1 h. The suspension obtained was centrifuged and the supernatant was collected and used as the whole aqueous extract. Sequential extraction of *P. marsupium* bark powder was done using petroleum ether, chloroform, and methanol using Soxhlet apparatus. The powder was extracted for 16 h with each solvent to remove the soluble matter. The extract was evaporated to dryness in vacuum desiccators. Finally, the powder was extracted with distilled water, centrifuged, dried, and used as the soxhlet aqueous extract.

2.4. In vitro antioxidant capacity assays

Whole aqueous extract (Aq), soxhlet methanolic extract (Met) and soxhlet aqueous extract (Aqs) of *P. marsupium* were used for these assays. All the assays were replicated at least three times and values expressed are an average of these replicates.

DPPH (1,1′-diphenyl-1-picrylhydrazyl) radical scavenging ability of the extracts was checked according to Aquino, et al. (12). The ability of the extracts to reduce ferric complex was assayed according to the protocol developed by Alzorkey, et al. (13), whereas the potential of the extracts to inhibit 2,2′-azobis-3-ethylbenzthiazoline-6-sulfonic acid radical cation (ABTS⁺) formation was evaluated by measuring the lag time in formation of the radical by the ABTS⁺/ferrylmyoglobin assay (14). In all these assays, antioxidant activity is expressed as equivalent of μg/mL of standard antioxidant L-ascorbic acid.

2.5. ABTS⁺ and CO₃⁻ radical anions scavenging assay by pulse radiolysis

The pulse radiolysis experiments were carried out at National Centre for Free Radical Research, University of Pune. The ability of the extracts to scavenge ABTS⁺ and CO₃⁻ radicals was determined by pulse radiolysis. ABTS⁺ radical was produced by the reaction of radiolytically generated azide radicals with ABTS⁺. CO₃⁻ radicals were generated using a reaction mixture containing 0.05 M NaHCO₃ and 0.05 M Na₂CO₃ saturated with N₂O. In the presence of the extract, the decay of ABTS⁺ and CO₃⁻ were correlated with the concentration of ascorbic acid equivalents (15).

2.6. Isolation of rat liver mitochondria and exposure to oxidative stress

Three month old female Wistar rats were used for the isolation of mitochondria from liver (16). Oxidative stress to mitochondria was generated by 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH), which generates peroxyl radical and ascorbate-Fe²⁺ which generates 'OH like radicals (16).

2.7. TBARS assay

The ability of the extracts to protect lipids from oxidative damage was assessed by measuring inhibition of lipid peroxide and hydroperoxide formation. Briefly, mitochondria were treated with AAPH and or ascorbate-Fe²⁺ in the absence or presence of extract and were incubated at 37°C in a shaker water-bath. These were then reacted with TBA reagent, boiled for 30 min and the pink color developed due to thiobarbituric acid reactive substance (TBARS) formed was estimated at 532 nm spectrophotometrically and expressed as malonaldehyde (MDA) equivalents after accounting for appropriate blanks. Malonaldehyde was prepared by acid hydrolysis of tetramethoxypropane (16).

2.8. Lipid hydroperoxide assay

Oxidative damage to mitochondria by AAPH and ascorbic acid-Fe²⁺ was measured in terms of lipid hydroperoxides formed using FOXII reagent (17). The working reagent was routinely calibrated against solutions of cumene hydroperoxide of known concentration. Samples were incubated at 37°C for 30 min, and then reacted with FOXII reagent, spun and
absorbance was read at 560 nm.

2.9. Estimation of protein carbonyl

Protein carbonyls were measured using 2,4-dinitrophenyl hydrazine (DNPH) (18) and are expressed as nmoles of protein formed per mg protein.

2.10. Measurement of protein sulphydryl

Protein sulphydryls were quantitated using Ellman's reagent (5,5-dithiobis-2-nitrobenzoic acid) and are expressed as nmoles protein sulphydryls per mg protein (19).

2.11. Liver slice culture

Liver slice cultures were as described earlier (20,21). Liver slices were incubated with or without whole aqueous extract and damage was induced by ethanol. At the end of incubation, the culture medium was collected and used for estimation of lactate dehydrogenase (LDH), glutamate pyruvate transaminase (GPT), and glutamate oxaloacetate transaminase (GOT), which were used as cytoxicity markers. The slices were homogenized and the homogenates were centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was assayed for LDH, GPT, GOT and antioxidant enzymes catalase, superoxide dismutase (SOD), and glutathione peroxidase (GPx), and antioxidant molecules glutathione and uric acid. Oxidative damage induced by ethanol was estimated by measuring lipid peroxidation. Cholesterol, triglyceride, and bilirubin were assayed as lipid metabolic markers.

2.12. Measurement of cytotoxicity markers

Samples were assayed for cytotoxic marker enzymes namely LDH, GPT, and GOT. LDH activity was assayed according to the protocol of Whalefeld (22). GPT and GOT were estimated using Accurex kits (Accurex Biomedical Pvt. Ltd., Mumbai, India) (23). Enzyme units in both the supernatant and tissue homogenate were measured. Release of enzymes from liver slices was calculated as the ratio of enzyme activity found in the supernatant to the total enzyme (supernatant + homogenate) activity.

2.13. Measurement of antioxidant enzymes

Superoxide dismutase (SOD) activity was measured spectrophotometrically using the method of Beauchamp and Fridovich (24). The activity of catalase was measured spectrophotometrically at 240 nm as described by Aebi (25). Glutathione peroxidase activity was determined according to the protocol described by Lawrence and Burk (26). Glutathione reductase (GR) was measured by the method of Goldberg (27). Assays were repeated three times and the values given are an average of these replicates. Values are expressed as μmoles/100 mg tissue.

2.14. Measurement of antioxidant molecules

Levels of uric acid were determined using an Accurex kit (Accurex Biomedical Pvt. Ltd.) (28). Uricase converts uric acid to allantoin and hydrogen peroxide. The generated hydrogen peroxidase degrades a phenolic chromagen in the presence of peroxidase to form a red colored compound, which is measured at 510 nm. The values are expressed as mg/100 mg tissue and the values given are replicates of three experiments.

Total glutathione levels were estimated in liver slice homogenates following the protocol of Teixeria and Meineghini (29). For the determination of GSSG, the cell extract was treated with 4-vinyl pyridine to a final concentration of 0.1% (v/v) and then incubated for 1 h. The glutathione content was determined as above. The amount of GSH could be determined by subtracting the GSSG content from the value of total glutathione. Values are expressed as micromoles/100 mg of tissue and the values are an average of three different experiments.

2.15. Measurement of lipid peroxidation

Lipid peroxidation in liver slices was estimated in terms of thiobarbituric reactive substances formed (30). Tissue homogenate was prepared in 5% TCA. To 1 mL of homogenate, 4 mL of 0.5% TBA in 20% TCA was added and this was incubated at 95°C for 30 min. This was immediately cooled on ice, during which the color changed from orange to pink. After centrifugation at 4,000 rpm for 10 min, the difference in the absorbance of the supernatant at 532 nm (specific) and 600 nm (non-specific) was measured to estimate pmol of malonaldehyde (MDA).

2.16. Measurement of lipid metabolic markers

Cholesterol was estimated using an Accurex kit (Accurex Biomedical Pvt. Ltd.) using a three-step reaction (31). Cholesterol esterase hydrolyses cholesterol esters into free cholesterol and fatty acids. The free cholesterol is acted upon by cholesterol oxidase to form cholest-4-en-3one and hydrogen peroxide. In the presence of peroxidase, hydrogen peroxide oxidatively couples with 4-amino-antipyrine and phenol to produce red quinoneimine dye which has an absorbance maximum at 510 nm. Triglyceride levels were estimated by an Accurex kit (32). Hydrolysis of triglycerides by lipoprotein lipase releases glycerol, which is converted to glycerol-3-phosphate by glycerol kinase. This glycerol-3-phosphate is oxidized to dihydroxyacetone phosphate and hydrogen peroxide. In the presence of peroxidase, hydrogen peroxide oxidizes...
the phenolic chromogen to a red colored compound, which is measured at 510 nm. Bilirubin levels were measured by an Accurex kit (33). In the presence of dimethyl sulfoxide, bilirubin reacts with diazotized sulphanilic acid to produce a violet colored compound azobilirubin, which is measured at 546 nm. The values given are an average of three replicates and are expressed as mg/100 mg tissue.

3. Results

3.1. P. marsupium extracts exhibit high in vitro antioxidant activity

Antioxidant activities of whole aqueous, soxhlet aqueous and soxhlet methanolic extracts was evaluated by their radical scavenging ability, ferric reducing ability, and radical formation inhibition activity.

In DPPH and ferrylmyoglobin/ABTS$^\cdot$ assays, the whole aqueous extract exhibited the highest radical scavenging as well as inhibition of ABTS$^\cdot$ radical formation, with a value of 1.22 ± 0.01 and 3.74 ± 0.05 μg/mL, respectively, of ascorbic acid equivalent antioxidant capacity (AEAC) (Figures 1 and 2). In Ferric reducing antioxidant power (FRAP) assay, the maximum ferric reducing ability of 0.803 ± 0.03 μg/mL ascorbic acid equivalent was shown with the 0.05% soxhlet methanolic extract (Figure 3).

Additionally, pulse radiolysis was used to quantitate radical scavenging activity of these extracts. Decay of ABTS$^\cdot$ (Figure 4a) and CO$_3$$^\cdot$ (Figure 5a) radicals was monitored in the presence of ascorbic acid and the linear plot of pseudo-first order rate constant ($K_{abs}$) versus ascorbic acid concentration was used to calibrate the standard curve for estimation of ascorbate equivalents in the extracts. The pseudo-first order rate constants for the decay of ABTS$^\cdot$ and CO$_3$$^\cdot$ were determined for the known concentrations of extracts and from the calibration curve, the ascorbate equivalents, present in different extracts were determined. Figures 4b-d and Figures 5b-d show ABTS$^\cdot$ and CO$_3$$^\cdot$ radical scavenging activities by whole aqueous, aqueous soxhlet, and methanolic soxhlet extracts, respectively. Maximum ABTS$^\cdot$ radical scavenging activity was exhibited by the 1% soxhlet methanolic extract with a value of 2.3 μg/mL of AEAC and maximum CO$_3$$^\cdot$ radical scavenging activity was exhibited by 0.1% methanolic soxhlet extract with a value of 3.9 μg/mL of AEAC.

3.2. P. marsupium extracts protect biomolecules from oxidative damage

These extracts were checked for their ability to protect rat liver mitochondria against oxidative damage induced by AAPH and ascorbate-Fe$^{2+}$. Lipid peroxides and hydroperoxides were used as a measure of damage to lipids, while protein sulphydryl and protein carbonyl were quantitated as a measure of damage to protein.

The whole aqueous extract showed the highest ability to inhibit formation of lipid peroxides followed by soxhlet aqueous against ascorbate-Fe$^{2+}$ and AAPH induced oxidative damage respectively (Table 1). Similar results were obtained for lipid hydroperoxide formed in response to damage by ascorbate-Fe$^{2+}$ and AAPH. The nmoles of lipid hydroperoxide formed was significantly ($p < 0.05$) reduced against ascorbate-Fe$^{2+}$ and AAPH as compared to oxidatively damaged mitochondria (Table 2).

Protein carbonyl and protein sulphydryl were quantitated as a measure of oxidative damage to proteins induced by AAPH. Protein carbonyl formation was reduced substantially ($p < 0.05$) by 1% whole extract. The phenolic chromogen to a red colored compound, which is measured at 510 nm. Bilirubin levels were measured by an Accurex kit (33). In the presence of dimethyl sulfoxide, bilirubin reacts with diazotized sulphanilic acid to produce a violet colored compound azobilirubin, which is measured at 546 nm. The values given are an average of three replicates and are expressed as mg/100 mg tissue.

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aqueous extract as compared to damaged mitochondria. Similarly, the 1% whole aqueous extract was able to protect mitochondria significantly ($p < 0.05$) by inhibiting depletion of the protein sulphhydrals against damaged mitochondria (Table 3).

3.3. Protection of liver slices from ethanol induced oxidative stress

The ability of whole aqueous extract of *P. marsupium* to protect liver cells against ethanol induced oxidative
Table 1. Inhibition of lipid peroxidation by P. marsupium measured in terms of nmoles of TBARs formed/mg protein in rat liver mitochondria damaged by ascorbate-Fe$^2+$ and AAPH. Lipid peroxidation in controls and oxidatively damaged mitochondria by ascorbate-Fe$^2+$ and AAPH was $0.5 \pm 0.1$ and $1.93 \pm 0.37$ nmoles of TBARs/mg protein, respectively. Lipid peroxidation in controls and oxidatively damaged mitochondria by AAPH were $0.44 \pm 0.04$ and $1.01 \pm 0.08$ nmoles of TBARs/mg protein, respectively. Values are represented as mean ± SD of three different experiments.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Nmoles of TBARs formed/mg protein by Ascorbate-Fe$^2+$</th>
<th>Nmoles of TBARs formed/mg protein by AAPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous extract 1%</td>
<td>0.32 ± 0.07$^a$</td>
<td>0.27 ± 0.04$^b$</td>
</tr>
<tr>
<td>Aqueous extract 0.1%</td>
<td>0.46 ± 0.05$^b$</td>
<td>0.34 ± 0.02$^a$</td>
</tr>
<tr>
<td>Aqueous soxhlet extract 1%</td>
<td>0.41 ± 0.07$^d$</td>
<td>0.29 ± 0.04$^e$</td>
</tr>
<tr>
<td>Aqueous soxhlet extract 0.1%</td>
<td>0.55 ± 0.09$^e$</td>
<td>0.32 ± 0.05$^d$</td>
</tr>
<tr>
<td>Methanol soxhlet extract 1%</td>
<td>0.42 ± 0.06$^f$</td>
<td>0.31 ± 0.03$^c$</td>
</tr>
<tr>
<td>Methanol soxhlet extract 0.1%</td>
<td>0.49 ± 0.1$^f$</td>
<td>0.34 ± 0.07$^b$</td>
</tr>
</tbody>
</table>

$^a$ These values differ significantly ($p < 0.05$) from the AAPH and ascorbate-Fe$^2+$ induced damage to rat liver mitochondria (Student's t-test).

Table 2. Inhibition of lipid peroxidation measured in terms of nmoles of lipid hydroperoxides formed/mg protein in rat liver mitochondria damaged by ascorbate-Fe$^2+$ and AAPH by different extracts of P. marsupium. Lipid hydroperoxides formed in controls and oxidatively damaged mitochondria by ascorbate-Fe$^2+$ were $152.32 \pm 3.8$ and $455.05 \pm 5.71$ nmoles of lipid hydroperoxides/mg protein. Lipid hydroperoxides formed in controls and oxidatively damaged mitochondria by AAPH were $132.64 \pm 3.99$ and $405.55 \pm 11.86$ nmoles of lipid hydroperoxides/mg protein. Values are represented as mean ± SD of three different experiments.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Nmoles of lipid hydroperoxides formed/mg protein by Ascorbate-Fe$^2+$</th>
<th>Nmoles of lipid hydroperoxides formed/mg protein by AAPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous extract 1%</td>
<td>292.89 ± 17.58$^a$</td>
<td>254.18 ± 10.72$^b$</td>
</tr>
<tr>
<td>Aqueous extract 0.1%</td>
<td>326.85 ± 15.11$^b$</td>
<td>300.51 ± 11.9$^c$</td>
</tr>
<tr>
<td>Aqueous soxhlet extract 1%</td>
<td>296.07 ± 17.37$^d$</td>
<td>258.94 ± 10.81$^e$</td>
</tr>
<tr>
<td>Aqueous soxhlet extract 0.1%</td>
<td>300.51 ± 19.65$^e$</td>
<td>270.04 ± 15.3$^f$</td>
</tr>
<tr>
<td>Methanol soxhlet extract 1%</td>
<td>303.05 ± 16.82$^f$</td>
<td>261.79 ± 11.58$^g$</td>
</tr>
<tr>
<td>Methanol soxhlet extract 0.1%</td>
<td>309.71 ± 18.56$^g$</td>
<td>270.68 ± 14.25$^h$</td>
</tr>
</tbody>
</table>

$^a$ These values differ significantly ($p < 0.05$) from the AAPH and ascorbate-Fe$^2+$ induced damage to rat liver mitochondria (Tukey’s t-test).

Table 3. Protein sulphydryl formed in rat liver mitochondria damaged by AAPH by different extracts of P. marsupium. Protein sulphydryl in controls and oxidatively damaged mitochondria were $36.65 \pm 1.99$ and $23.13 \pm 1.09$ nmoles protein sulphydryl/mg protein, respectively. Protein oxidation measured in terms of nmoles of protein carbonyl formed/mg protein in oxidatively damaged rat liver mitochondria by AAPH by different extracts of P. marsupium. Protein carbonyl in controls and oxidatively damaged mitochondria were $7.59 \pm 0.54$ and $23.13 \pm 1.09$ nmoles protein carbonyl/mg protein, respectively. Values are represented as mean ± SD of three different experiments.

<table>
<thead>
<tr>
<th>Extract</th>
<th>nmoles Protein sulphydryl/mg protein</th>
<th>nmoles Protein carbonyl/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous extract 1%</td>
<td>31.6 ± 0.5$^a$</td>
<td>12.84 ± 0.28$^b$</td>
</tr>
<tr>
<td>Aqueous extract 0.1%</td>
<td>27.97 ± 0.59$^a$</td>
<td>17.25 ± 1.33$^b$</td>
</tr>
<tr>
<td>Aqueous soxhlet extract 1%</td>
<td>30.97 ± 0.68$^a$</td>
<td>14.56 ± 0.25$^a$</td>
</tr>
<tr>
<td>Aqueous soxhlet extract 0.1%</td>
<td>30.17 ± 0.85$^b$</td>
<td>14.78 ± 0.29$^b$</td>
</tr>
<tr>
<td>Methanol soxhlet extract 1%</td>
<td>30.72 ± 0.62$^c$</td>
<td>14.43 ± 0.63$^c$</td>
</tr>
<tr>
<td>Methanol soxhlet extract 0.1%</td>
<td>27.54 ± 0.58$^c$</td>
<td>16.30 ± 0.31$^c$</td>
</tr>
</tbody>
</table>

$^a$ These values differ significantly ($p < 0.01$) from the AAPH and ascorbate-Fe$^2+$ induced damage to rat liver mitochondria (Student’s t-test).

stress was checked using liver slice culture and assaying the release of intracellular enzymes namely LDH, GOT, and GPT. As shown in Figure 6a, the % release for LDH, GOT, and GPT was significantly decreased to $12.6 \pm 1.06$, $15.3 \pm 1.1$, and $16.4 \pm 0.79$, respectively, in slices treated with ethanol and 1% whole aqueous extract as compared to ethanol treated slices which had a percent release of $53 \pm 1.83$, $41 \pm 1.4$, and $41 \pm 0.7$ for LDH, GOT, and GPT, respectively. The time course...
of release of these enzymes showed that these enzymes were released over the period of 2 h and this release was inhibited within 30 min in the presence of 1% P. marsupium extract (Figures 6b-d).

3.4. *P. marsupium* extract protects liver slices by lowering lipid peroxide formation due to ethanol

It was observed that in ethanol-treated liver cells the amount of MDA formed was substantially higher (1.29 pmol/100 mg tissue) compared to the control (0.27 pmol/100 mg tissue) (Figure 7a). Following the time course for lipid peroxidation, a gradual increase in lipid peroxidation over a period of 2 h was observed, which was inhibited within 30 min in the presence of 1% *P. marsupium* extract (Figure 7b).

3.5. *P. marsupium* extract modulates antioxidant status in liver cells in response to ethanol induced oxidative stress

3.5.1. Modulation of antioxidant enzymes

Intracellular antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were quantitated. In the presence of ethanol, the activities of SOD and catalase were found to be significantly increased as compared to controls whereas a small decrease was seen in GPx as compared to control (Table 4). Activities of all these enzymes were normalized in the presence of *P. marsupium* extract in a concentration-dependent manner. Following the time course for all three enzymes, SOD and catalase showed a gradual increase, while GPx activity gradually dropped in ethanol treated slices compared to control. The activity of all the enzymes was restored in the presence of extract (Figure 8).

GR is involved in synthesis of reduced glutathione, which is a major antioxidant in the cell. Liver slices treated with ethanol showed a decrease in the activity of this enzyme, which was restored by whole aqueous extract in a concentration dependent manner (Figure 9a). Moreover time course revealed that within 30 min, activity of GR was restored (Figure 9b).

3.5.2. Modulation of antioxidant molecules

Glutathione and uric acid are small antioxidant molecules, which directly scavenge free radicals. Activity of both these molecules was decreased in liver slices treated with ethanol as compared to untreated slices (Figures 10a and 11a). The whole aqueous extract of *P. marsupium* normalized the levels of both these molecules within 30 min in a concentration dependent manner (Figures 10b and 11b).

3.5.3. *P. marsupium* regulates lipid metabolic markers

Cholesterol, triglyceride, and bilirubin levels were checked as major lipid metabolic markers. In liver slices treated with ethanol both cholesterol (0.9 mg/100 mg tissue) and triglycerides (3.09 mg/100 mg tissue) were found to be markedly decreased as compared to untreated slices (12.18 and 13.72 mg/100 mg tissue, respectively). 1% *P. marsupium* extract was able to restore these levels. Bilirubin levels in liver slices treated with ethanol showed a small increase (0.39 ± 0.01 mg/100 mg tissue), as compared to control (0.2 ± 0.02 mg/100 mg tissue). Slices treated with 1% *P. marsupium* extract did not show any change compared to control untreated slices (Table 5). Cholesterol and triglyceride levels decreased gradually over a period of 2 h during ethanol treatment and were normalized.

---

Table 4. Specific activity of catalase, SOD and GPx (U/mg protein) under different conditions

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Extract 1%</th>
<th>EX 1% + OH</th>
<th>EX 0.5% + OH</th>
<th>EX 0.1% + OH</th>
<th>OH</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT</td>
<td>1,014.1 ± 64.13</td>
<td>908 ± 57.52</td>
<td>1,198.2 ± 126.59⁶</td>
<td>1,366.4 ± 90.72⁶</td>
<td>1,724.5 ± 48.83⁶</td>
<td>2,040.7 ± 96.23⁶</td>
</tr>
<tr>
<td>SOD</td>
<td>338.12 ± 17.82</td>
<td>303.38 ± 19.80</td>
<td>551.36 ± 17.93⁶</td>
<td>636.88 ± 17.28⁶</td>
<td>707.61 ± 5.28⁶</td>
<td>762.6 ± 18.90⁶</td>
</tr>
<tr>
<td>GPx</td>
<td>1.37 ± 0.0344</td>
<td>1.38 ± 0.0459</td>
<td>1.33 ± 0.0571⁶</td>
<td>1.32 ± 0.058⁶</td>
<td>1.32 ± 0.204⁶</td>
<td>1.31 ± 0.197⁶</td>
</tr>
</tbody>
</table>

⁶ These values differ significantly (p ≤ 0.001) from the control group (student’s t-test); ⁷ These values differ significantly (p ≤ 0.001) from ethanol treated group (student’s t-test); ⁸ These values differ significantly (p ≤ 0.1) from ethanol treated group (student’s t-test).
Figure 8. Time course for various antioxidant enzymes in the presence of ethanol and ethanol with whole aqueous extract. (a) Catalase, (b) superoxide dismutase (SOD), and (c) glutathione peroxidase (GPx). Values are mean ± SD of three different experiments.

Figure 9. Effect of *P. marsupium* extract on glutathione reductase in liver slice culture. (a) Level of glutathione reductase in the presence of ethanol and ethanol along with different concentrations of whole aqueous extract. (b) Time course for glutathione reductase in the presence of ethanol and ethanol with 1% whole aqueous extract. Values are mean ± SD of three different experiments.

Figure 10. Effect of *P. marsupium* extract on glutathione amount in liver slice culture. (a) Amount of glutathione under different conditions. (b) Time course for glutathione measured at an interval of 30 min under various conditions. Values are mean ± SD of three different experiments.

Figure 11. Effect of *P. marsupium* extract on uric acid amount in liver slice culture. (a) Amount of uric acid under various conditions. (b) Time course for uric acid measured at an interval of 30 min under various conditions. Values are mean ± SD of three different experiments.

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within 30 min in the presence of 1% *P. marsupium* extract along with ethanol. Bilirubin levels increased gradually over a period of 2 h during ethanol treatment and was restored within 30 min in the presence of 1% *P. marsupium* extract (Figure 12).

4. Discussion

Reactive oxygen species have been implicated in the etiology of many human diseases such as cardiovascular ailments, cancer, diabetes, Alzheimer’s disease, arthritis, and neurodegenerative, and in the process of aging. Antioxidants confer protection either by inhibiting formation of radicals or by scavenging free radicals (34). Recently, a large number of plant extracts have been explored for their ability as strong antioxidants. *Pterocarpus marsupium* has been used in ayurvedic medicine for the treatment of diabetes over a long period of time. Two major phenolic constituents of *P. marsupium* namely pterostilbene and marsupium were able to significantly decrease plasma glucose levels by 42 and 33%, respectively, in streptozotocin induced diabetic rats. Though the exact mechanism of the antidiabetic effect of this extract is yet to be understood, some diabetics use it regularly as an antidiabetic drug. Demonstration of the strong antioxidant activity of this extract would certainly increase its potential as an anti-diabetic drug. Therefore, the aim of the present study was to evaluate antioxidant activity of *P. marsupium* using *in vitro* assays and liver slice cultures as a model system.

The total antioxidant activity of *P. marsupium* extracts was evaluated using different *in vitro* antioxidant activity assays. These assays correspond to an action of antioxidants at different levels. The DPPH’ radical scavenging assay corresponds to the primary radical scavenging activity of an antioxidant, whereas ferrylmyoglobin/ABTS’’ corresponds to the ability of an antioxidant to inhibit radical formation. The FRAP assay, on the other hand, evaluates the reducing ability of the antioxidant. The whole aqueous extract of *P. marsupium* exhibited high antioxidant potential in all of the above mentioned assays except the FRAP assay, wherein the soxhlet methanolic extract showed high reducing power (Figures 1-3). Pulse radiolysis reveals the ability of antioxidant to scavenge secondary radicals such as ABTS’’ and CO$_3^{\cdot-}$. The soxhlet methanolic extract showed high ABTS’’ as well as CO$_3^{\cdot-}$ radical scavenging activity. The whole aqueous extract on the other hand was best in inhibiting formation of lipid peroxides and lipid hydroperoxide induced by AAPH and ascorbate-Fe$^{2+}$ in rat liver mitochondria (Tables 1 and 2). Similarly, the whole aqueous extract inhibited formation of protein carbonyls as well as inhibited depletion of protein sulphydryls induced by AAPH in rat liver mitochondria (Table 3). This data clearly shows that the whole aqueous extract and soxhlet methanolic extract behave differentially in different assays. This differential activity of the extracts could be attributed to different components present in the extract.

The antioxidant activity of the extract was
additionally evaluated using liver slice cultures as a model system. Oxidative stress was induced by addition of ethanol to the liver slice culture. These cultures offer a very good test system because they provide a model of the considerable complexity of structurally and functionally intact cells. These cultures retain intact tissue architecture and more closely mimic the in vivo situation as compared to isolated hepatocytes.

Ethanol used to induce oxidative stress was cytotoxic to the cells as measured by the increase in LDH, GOT, and GPT release in treated cells. This cytotoxic effect was substantially lowered in a concentration dependent manner on addition of whole aqueous extract (Figure 6) probably through reduction of oxidative stress.

Increased lipid peroxidation has already been associated with liver injury in animals fed ethanol (35) or in hepatocytes treated with ethanol in vitro (36). In our studies when cultures were treated with *P. marsupium* extract along with ethanol, lipid peroxidation was substantially reduced as compared with control cells (Figure 7). The fact that lipid peroxidation was significantly reduced in *P. marsupium*-treated cultures indicates that this extract has strong antioxidant activity. Activity of four antioxidant enzymes namely catalase, SOD, GR, and GPx was measured. In the presence of ethanol the activities of SOD and catalase substantially increased, probably in response to oxidative stress induced by ethanol. Addition of whole aqueous extracts along with ethanol lead to a decrease in these enzyme levels, which was comparable to those seen in untreated cultures (Table 4). The levels of GPx (Table 4) and GR (Figure 9), on the other hand, decreased in the presence of ethanol and were normalized on addition of whole aqueous extracts in a concentration dependent manner. This result correlated to the levels of glutathione, which also decreased in the presence of ethanol and was restored in the presence of the extract (Figure 10). The levels of uric acid, another antioxidant molecule, were also decreased with ethanol treatment and were normalized within thirty minutes in the presence of *P. marsupium* extract (Figure 11).

Cholesterol, bilirubin, and triglyceride levels were estimated as markers of liver specific lipid metabolism. Both cholesterol and triglycerides decreased substantially in the presence of ethanol and in the presence of extract this decrease was prevented (Figures 12a and b). We also checked for bilirubin, the product of cholesterol degradation and it was found to be increased with ethanol treatment and was restored in the presence of the extract (Table 5). However, this increase was not as prominent as the decrease in cholesterol. This was a bit surprising because the majority of reports have shown association of fat accumulation with alcohol intake. However, all those observations were made on chronic treatment of tissues whereas in our system cells are given acute exposure to ethanol (37). Our data demonstrates that during acute exposure to alcohol *P. marsupium* extract helps to maintain cholesterol as well as triglyceride levels.

Both the reduction in lipid peroxidation and restoration of the activity of AOE s clearly demonstrate that in the presence of *P. marsupium* extracts free radical generation is significantly reduced. The antioxidant activity exhibited by *P. marsupium* extract in this system seems to be due to its interaction with free radicals leading to their inactivation rather than altering the activity of antioxidant enzymes, which scavenge them.

Antioxidant activity of the plant extracts is often attributed to the presence of flavonoids. *P. marsupium* is known to contain pterostilbene, an analogue of resveratrol, which is shown to have peroxyl radical scavenging activity (38). Other purified flavonoids, marsupin, pterostilbene, and epicatechin have also been shown to possess antidiabetic activity (39). Our data clearly show that *P. marsupium* extract has strong antioxidant activity in all in vitro assays and protects liver cells from ethanol induced oxidative stress. Thus the strong antioxidant activity shown in *P. marsupium* extracts substantially increases its therapeutic value.

**Acknowledgement**

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Antiaggressive activity of hyperforin: A preclinical study

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ABSTRACT: The aim of present study was to investigate the in vivo antiaggressive activity of hyperforin using defensive and offensive behavioral models in rodents. Adult male rats and mice were used for the present study. Animals were divided into three groups, with 6 animals in each. Lorazepam was used as standard antiaggressive agent. Animals were treated once daily, for seven consecutive days. Hyperforin (10 mg/kg, i.p.) was injected in a volume of 10 mL/kg for seven consecutive days. Standard group was treated with lorazepam (2.5 mg/kg, i.p.). The control group was treated with equal volume of vehicle (0.3% carboxymethyl cellulose suspension, i.p.). Animals were screened for aggressive behavior before dividing them into groups. At the end of 7 days, experiments were performed. Antiaggressive activity was evaluated using following validated models of aggression viz. foot shock-induced aggression, isolation-induced aggression, resident-intruder aggression and water competition test. Hyperforin treatment significantly (p < 0.001) reduced various aggressive parameters viz. latency to first attack and number of fights in isolation induced aggression, resident intruder aggression and foot shock induced aggression tests. In water competition test, hyperforin treatment significantly (p < 0.001) reduced the duration of water consumption and frequency of water spout possession. We conclude that hyperforin, the major lipophilic compound contained in extracts of Hypericum perforatum, is thus responsible for the antiaggressive activity, suggesting the therapeutic potential of hyperforin as an antiaggressive agent.

Keywords: Aggression, hyperforin, foot-shock, isolation-induced, resident-intruder

1. Introduction

Aggression is a significant public health problem that has received limited attention. Many psychiatric disorders (e.g., personality disorders, schizophrenia and bipolar disorder) are characterized by impulsive aggressive behavior, which often brings patients with psychiatric disorders to the attention of medical and forensic systems (1). Human aggression is defined as behavior directed towards another individual carried out with the proximate intent to cause harm. Pharmacological treatment of aggression poses several challenges. Although the agents that have been used successfully in the clinic or in trials encompass nearly the full range of psychotropic medications, from antidepressants and neuroleptics to mood stabilizers and even β-blockers, there is a huge need for psychotropic drugs, specifically influencing aggression, without interfering with other important modalities. The drugs used clinically as antiaggressive are not at all specific for aggression but induces sedation, motor disturbances or other unwanted effects (2).

Recent studies have expanded the list of neurotransmitters, hormones, cytokines, enzymes, growth factors, and signaling molecules that influence aggression (3). Nevertheless, the prevalence of aggressive and violent behavior today is sufficient to make it a social problem worthy of attention around the world. The behavioral biology of mouse aggression offers insights to understanding the neurobiological and molecular mechanisms mediating behavior in social conflict (4).

There is a growing increase in the popularity of herbal medicines, and one of the most popular herbal remedies is the perennial herb, St. John's wort (SJW), consisting of the leaves and the flowering top of Hypericum perforatum L. (Clusiaceae). SJW contains numerous biologically active constituents viz. naphthodianthrone derivatives, phloroglucinol derivatives (e.g. hyperforin), flavonoids, tannins, procyanidines, essential oils, phenylpropanes, amino acids, xanthones and other hydrosoluble compounds (5). Hyperforin was isolated in 1975 by Bystrov and co-workers (6). SJW has been used to fight against infections and for the treatment of respiratory...
and inflammatory diseases, peptic ulcers and skin wounds (7), it inhibits proliferation of peripheral blood mononuclear cells and tumor cells, and induces apoptosis of tumor cells (8-10). It has been used for the treatment of neuralgia, anxiety, neurosis and depression (11). We have earlier reported the efficacy of hyperforin and standardized extract of SJW in various neurobiological disorders (12-22). Recently, we have investigated antiaggressive activity of SJW extract standardized to contain 3% hyperforin (23). This later investigation has prompted us to further elucidate the role of hyperforin in the observed antiaggressive activity.

2. Materials and Methods

2.1. Animals

Adult male Charles Foster rats and male Wistar mice, were obtained from the Central Animal House, Institute of Medical Sciences, Banaras Hindu University, and were randomly distributed into different experimental groups. The animals were housed in groups of six in polypropylene cages at an ambient temperature of 25 ± 1°C and 45-55% relative humidity, with a 12:12 h light/dark cycle. They were provided with commercial food pellets and water ad libitum. Experiments were conducted between 09:00 and 14:00 h. Animals were acclimatized to laboratory conditions for at least one week before using them for experiments and were subjected only once to the experimental conditions. Principles of laboratory animal care (NIH publication number 85-23, revised 1985) guidelines were followed.

2.2. Drugs and chemicals

Hyperforin (99.9% pure) was procured from Dr. Willmar Schwabe, GmbH & Co. Karlsruhe, Germany. Lorazepam (Intas, Ahmadabad, India) was used as standard antiaggressive agent in all the experiments.

2.3. Drug treatments

Animals were treated once daily, for seven consecutive days. Animals were divided into three groups. Group I was treated intraperitoneally with equal volume of 0.3% carboxy methyl cellulose (CMC) suspension. Group II was treated with lorazepam (2.5 mg/kg, i.p.). Group III was treated with hyperforin (10 mg/kg, i.p.). Animals were screened for aggressive behavior before dividing them into groups. A fresh solution of hyperforin was prepared everyday immediately before treatment. Hyperforin was suspended in 1% dimethylsulfoxide (DMSO) that contained 0.3% CMC. Hyperforin was injected i.p. in a volume of 10 mL/kg. At the end of 7 days, experiments were performed.

2.4. Models of aggression

A battery of four rodent models often used to detect potential effects of therapeutically used anti-depressants and anxiolytics on aggression and violence was chosen to screen the effects of hyperforin. Potential effects of agents on defensive (foot shock-induced aggression and water consumption tests) as well as offensive (isolation-induced and resident-intruder aggression tests) aggressive behavior have been detected and quantified by the battery of behavioral models chosen.

2.4.1. Foot shock-induced aggression

Male mice of Wistar strain weighing 20-30 g were used. Mice were treated with vehicle, hyperforin or lorazepam for seven consecutive days. On day seven, 1 h after the last treatment, all pairs of mice were subjected to foot shock by placing them in a box with a grid floor consisting of steel rods with a distance of 6 mm. A constant current of 0.6 mA was supplied to the grid floor by a shocker with an associated scrambler. During 3 min observation period, every 5 sec a 60-Hz current was delivered for 5 sec. Each pair of mice was dosed and tested without previous exposure. The total number of fights was recorded for each pair. The fighting behavior consisted of leaping, running, rearing and facing each other with some attempt to attack by hitting, biting or boxing (24,25). Behavioral parameters quantified in this test were leaping, running, rearing, facing each other and total number of fighting bouts.

2.4.2. Isolation-induced aggression

Male mice of Wistar strain weighing 20-30 g were used. Mice were kept isolated in small cages for a period of 6 weeks. Prior to the administration of the test drug, the aggressive behavior of the isolated mouse was assessed against a male mouse (similar in weight to that of isolated mouse, and accustomed to live in a group) into the cage of an isolated mouse for 5 min. Immediately, the isolated mouse started to attack the "intruder". The aggressive behavior of the isolated mouse was characterized by hitting the tail on the bottom of the cage, screaming and biting. Isolated mice not exhibiting aggressive behavior were excluded from the test. One day after the initial trial, isolated animals were distributed into three groups (6 in each) and were treated with vehicle, hyperforin or lorazepam for seven consecutive days. One hour after the last dose, aggressive behavior of isolated mouse against a male mouse was evaluated again for 5 min (25-27). Aggressive behavior related parameters assessed during this test were latency to first attack, screaming, pursuit frequency, tail rattle, aggressive posture and total number of fighting bouts.
2.4.3. Resident-intruder aggression

Resident male rats (400 ± 20 g) were tested in their home cages for aggression against a smaller (200 ± 20 g) male intruder. Before the start of the experiments, each resident male rat was kept in pair with one female rat in a polypropylene cage for 15 days, and they were randomly divided into three groups (6 pair in each). Drug treatment was started 16th day onward, and only male rats of each pair were administered with vehicle, hyperforin or lorazepam for seven consecutive days. Resident female was removed from the cage 30 min prior to the start of the test. One hour after the last treatment, a male intruder (~200 g) was placed in the territorial cage of the resident male, and behavior of the resident male was observed for the next 15 min. During this period, the time until the first attack (in seconds), number of attacks, and duration of each attack (in seconds) were recorded by a blind observer (25).

2.4.4. Water competition test

Two male rats of equal body weight (200 ± 20 g) were paired and housed in one cage for 6 days. After 6 days, the animals were deprived of water for 23 h, and then a water bottle was introduced with a shielded spout so that only one animal of a pair can drink at a time. Duration and frequency of spout possession and water consumption of dominant rat were recorded for 5 min (day 1 of experiment) and the aggressive animal of the pairs was marked for identification (test 1). Animals were then allowed another 55 min for water consumption and again deprived of water for next 23 h. The same procedure was repeated on next day (test 2). At the end of this, aggressive rat was administered with either drugs or vehicle for seven consecutive days. Frequency and time in seconds of spout possession of same rat (dominant) were again recorded for 5 min as mentioned above on the sixth and seventh day (test 3 and 4, respectively). Treatment effects were assessed by comparing the values before drug treatment with the values obtained after the drug treatments. Duration of water consumption in this test is considered to be a more specific parameter for evaluating effects of agents on aggressiveness of more dominant rats (25,27).

2.5. Statistical analysis

All values are expressed as mean ± SEM. Statistical significance between control and treatment groups was analysed by one way analysis of variance (ANOVA) followed by Students Neuman-Keuls post hoc analyses. Statistical analysis was performed using the software Graphpad prism version 5. Statistical significance between same treatment groups was analysed by Student’s t-test and p values less than 0.05 were considered statistically significant.

3. Results

3.1. Foot shock induced aggression

Hyperforin significantly reduced the rearing and leaping behavior (p < 0.001). Running behavior in response to foot-shock was also inhibited by hyperforin treatment (p < 0.01). Tendency to face each other and number of fights were also significantly reduced by hyperforin (p < 0.01). Effect of lorazepam in this model was qualitatively similar to those of hyperforin (Figure 1).

3.2. Isolation-induced aggression

Hyperforin extended latency period to first attack and the number of fighting episodes (p < 0.001). Number of aggressive postures, number of screamings and tail rattle frequency were also significantly reduced by hyperforin (p < 0.001). Qualitatively, these effects of hyperforin (10 mg/kg) were identical to that of the lorazepam (2.5 mg/kg) (Figure 2).

3.3. Resident-intruder aggression

Hyperforin treatment prolonged the latency period of first attack (p < 0.001) and reduced the total duration and mean number of fights (p < 0.001). Mean numbers of lateral threats and aggressive grooming were also lowered in the hyperforin treated group as compared to control group (p < 0.001). The observed effects of lorazepam in this model were qualitatively similar to those of hyperforin (Figure 3).

3.4. Water competition test

In control group rats, duration of water consumption of the animals did not change significantly over the four different test days (Table 1), indicating that a stable relationship of water consumption and aggressive behavior.
behavior had been established in each pair. Hyperforin treatment significantly \((p < 0.001)\) reduced the duration of water intake by the dominant rats. Similar relationship was obtained during frequency of water spout possession (Table 2).

### 4. Discussion

The behavioral data reported here presents significant antiaggressive activity of hyperforin (10 mg/kg) in rodents, and the effect was qualitatively comparable to that of lorazepam (2.5 mg/kg). Various antidepressant drugs have been reported to be effective in the treatment of aggression \((28,29)\). Hyperforin has been identified as one of the main components of SJW extracts responsible for its antidepressant effects.

![Figure 2. Effect of hyperforin on isolation induced aggressive behavior.](image)

![Figure 3. Effect of hyperforin on resident intruder aggressive behavior.](image)

### Table 1. Effect of hyperforin on duration of water consumption

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Test 1</th>
<th>Test 2</th>
<th>Average 1</th>
<th>Test 3</th>
<th>Test 4</th>
<th>Average 2</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>138.16 ± 2.18</td>
<td>139.83 ± 2.1</td>
<td>139.0 ± 2.12</td>
<td>146.0 ± 2.01</td>
<td>146.33 ± 2.75</td>
<td>146.16 ± 2.32</td>
<td>7.16 ± 1.28</td>
</tr>
<tr>
<td>Hyperforin (10 mg/kg)</td>
<td>140.0 ± 2.20</td>
<td>141.66 ± 2.28</td>
<td>140.13 ± 2.64</td>
<td>129.16 ± 2.33</td>
<td>129.66 ± 2.41</td>
<td>129.41 ± 2.27</td>
<td>-12.91 ± 1.59</td>
</tr>
<tr>
<td>Lorazepam (2.5 mg/kg)</td>
<td>139.66 ± 2.16</td>
<td>142.16 ± 3.16</td>
<td>141.0 ± 3.09</td>
<td>123.16 ± 1.99</td>
<td>119.16 ± 2.16</td>
<td>121.16 ± 2.05</td>
<td>19.83 ± 1.24</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM (\(n = 6\)). Average 1 indicates mean of tests 1 and 2; Average 2 indicates mean of tests 3 and 4; Difference indicates Average 2 – Average 1. * \(p < 0.001\), compared to control.

### Table 2. Effect of hyperforin on frequency of water spout possession

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Test 1</th>
<th>Test 2</th>
<th>Average 1</th>
<th>Test 3</th>
<th>Test 4</th>
<th>Average 2</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.33 ± 0.42</td>
<td>13.16 ± 0.70</td>
<td>13.25 ± 0.35</td>
<td>13.66 ± 0.55</td>
<td>14.66 ± 0.55</td>
<td>14.16 ± 0.16</td>
<td>0.91 ± 0.23</td>
</tr>
<tr>
<td>Hyperforin (10 mg/kg)</td>
<td>15.66 ± 1.22</td>
<td>15.33 ± 1.14</td>
<td>15.5 ± 1.16</td>
<td>10.33 ± 0.66</td>
<td>9.33 ± 0.61</td>
<td>9.83 ± 0.60</td>
<td>-5.66 ± 0.71</td>
</tr>
<tr>
<td>Lorazepam (2.5 mg/kg)</td>
<td>15.33 ± 1.02</td>
<td>16.0 ± 1.06</td>
<td>15.66 ± 0.92</td>
<td>9.33 ± 0.55</td>
<td>9.0 ± 0.73</td>
<td>9.16 ± 0.61</td>
<td>-6.5 ± 0.69</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM (\(n = 6\)). Average 1 indicates mean of tests 1 and 2; Average 2 indicates mean of tests 3 and 4; Difference indicates Average 2 – Average 1. * \(p < 0.001\), compared to control.
providing gift sample of hyperforin. Schwabe, Gmbh & Co. KG, Karlsruhe, Germany for Senior Scientist, Pharmacological Division, Dr. Willmar

The authors are thankful to Dr. Michael Noeldner, Acknowledgement

observed antiaggressive activity. hyperforin may be potentially responsible for the
earlier investigation. Likewise, increased GABAergic transmission is therapeutically beneficial in aggression (e.g. benzodiazepines) (42). Hyperforin is a neurotransmitter reuptake inhibitor, affecting the synaptosomal uptake of serotonin, dopamine, noradrenalin, glutamate and GABA with similar efficiencies (34). Therefore, increased serotonergic transmission due to reuptake inhibition may be responsible for observed antiaggressive activity of hyperforin.

The findings of this study are consonant with our earlier investigation (23) and further elucidate that hyperforin may be potentially responsible for the observed antiaggressive activity.

Acknowledgement

The authors are thankful to Dr. Michael Noeldner, Senior Scientist, Pharmacological Division, Dr. Willmar Schwabe, Gmbh & Co. KG, Karlsruhe, Germany for providing gift sample of hyperforin.

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ABSTRACT: An effervescent floating liquid formulation with \textit{in situ} gelling properties has been assessed for its potential for sustaining drug delivery and targeting. The formulation consisted of sodium alginate and glyceryl monooleate (GMO). The developed formulation met all pre-requisites to become an \textit{in situ} gelling floating system and it gelled and floated instantaneously in the pH conditions of the stomach. Moreover, the gels formed in situ remained intact for more than 48 h to facilitate sustained release of drugs. Increasing the mannuronic acid ratio of sodium alginate and the GMO concentration significantly retarded the release rate and extent. The \textit{in vitro} release of both hydrophilic and hydrophobic drugs from the prepared formulations followed root-time kinetics during the sustained release period. Replacing the free drug with drug encapsulated microspheres enabled tailoring of the release profile and achieved zero-order release kinetics. The system retained its appearance and rheological properties for 12 months at ambient conditions. The values of the similarity factor $S_d$ proved the absence of any significant difference in the release profile upon storage.

\textbf{Keywords:} Sodium alginate, glyceryl monooleate, floating, bioadhesive, \textit{in situ} gelation

1. Introduction

Variable and short gastric emptying times can result in incomplete drug release from the drug delivery system above the absorption zone (stomach or upper part of small intestine) leading to diminished efficacy (1). Gastric retention solves the problems connected with gastric emptying time. Several approaches have been proposed to control the residence of drug delivery systems (DDS) in the upper part of the gastrointestinal tract (2), namely: high density DDS, mucoadhesive DDS, magnetic DDS, swelling/expanding DDS and floating DDS. The floating systems offer the most effective and rational protection against early and random times of gastric emptying (3). A floating DDS basically floats in the gastric fluid because of its lower bulk density compared to that of the aqueous medium. Drugs should have an absorption window in the stomach or in the upper small intestine (4), drugs that act locally in the proximal part of gastrointestinal tract and drugs that are poorly soluble or unstable in the intestinal fluid (5). Two different technologies to achieve buoyancy have been proposed, namely, non-effervescent and effervescent systems.

Alginate in a form of free acid or sodium salt is a collective term for a family of natural anionic polysaccharide obtained by extraction from marine brown algae. Alginate is a linear binary copolymer consisting of $(1\rightarrow4)$-linked $\beta$-d-mannuronic acid (M) and $\alpha$-l-guluronic acid (G) residues. The relative amount of the two uronic acid monomers and their sequential arrangement along the polymer chain vary widely, depending on the origin of the alginate. The uronic acid residues are distributed along the polymer chain in a pattern of blocks, where homopolymeric blocks of G residues (G-blocks), homopolymeric blocks of M residues (M-blocks) and blocks with alternating sequence of M and G units (MG-blocks) coexist (6). Aliginic acid polymers form interchain associations in the presence of di- and trivalent cations produce hydrated gels. There have been many investigations of the use of alginate gels for the sustained release of drugs in the form of tablets, matrices, solid beads, and capsules. There have been very few reports on the use of alginates in liquid sustained release preparations for oral administration. A liquid sustained release formulation containing sodium alginate intended for the eradication of \textit{Helicobacter pylori} has been reported (7). The formulation depends for its action on in situ gelling induced by the separate oral administration of a solution of a calcium salt immediately following that of the sodium alginate solution. Others included...
a source of \( \text{Ca}^{2+} \) ions in the formulation, in addition to sodium citrate, which complexes the free \( \text{Ca}^{2+} \) ions and releases them only in the highly acidic environment of the stomach. The formulation thus remains in liquid form until it reaches the stomach, when gelation is instantaneous (8).

Glycerol monooleate (GMO) is a fatty acid ester with a low molecular weight. It forms liquid crystals at body temperature in the presence of water. In the presence of excess water (35%, w/w water), GMO forms viscous gels known as the cubic phase. The cubic phase has a transparent, stiff, gel-like appearance and constitutes a three-dimensional network of curved lipid bilayers separated by a network of congruent water channels (9). This property of GMO (monoolein) has been used to sustain the delivery of various water-soluble and water-insoluble drugs (10). GMO possess bioadhesive properties and thus can be used to enhance the therapeutic efficacy of the dosage form by increasing the contact time at the site of action. The exact mechanism for this mucoadhesion is still unknown and possibly involves dehydration of mucosa (11). GMO has been approved by the FDA for human consumption.

The objective of this study was to develop a liquid effervescent floating drug delivery system with the advantage of ease of administration and patient compliance. The system depends on the in situ gelation of sodium alginate in the presence of \( \text{H}^+ \) of the stomach and formation of the insoluble alginic acid, rather than the crosslinking with \( \text{Ca}^{2+} \) ions. The \( \text{CO}_2 \) generated from the reaction between sodium bicarbonate and the stomach \( \text{H}^+ \) will be entrapped in the formed insoluble alginic acid gel keeping it buoyant on the gastric contents. Incorporating GMO is supposed to control the release of different drugs, to aid the gastroretentive properties of the proposed drug delivery system, and to enhance the solubility of hydrophobic drugs in the formulation due to its cubic phase properties, its bioadhesiveness, and its self-emulsifying property, respectively.

The work also studied the effect of the sodium alginate chemical composition and GMO concentration on the rheological and gelling properties and buoyancy as well as the in vitro release profile.

2. Materials and Methods

2.1. Materials

Two types of sodium alginate (Na alg) with different mannuronic-to-guluronic-acid ratios (M/G ratio of 1.5 and 0.67) were purchased from CDH Labs., New Delhi, India, glyceryl mono-oleate (GMO) was from Fluka, USA, sodium bicarbonate (NaHCO\(_3\)) was from Carlo Erba, Milano, Italy, ethyl cellulose was from Sigma-Aldrich, St Louis, MO, USA, and polyvinyl alcohol (m.wt 14.000 RL) was from Laboratory Rasayan, Boisar, India. Pamabrom-US was obtained as a free sample from Granules India Limited, Hyderabad, India, and carbimazole was obtained as a gift sample from International Drug industries (Egypt). All other reagents were of analytical grade.

2.2. Preliminary experiments

For the feasibility of the study to detect the effect of the selected parameters, namely, the sodium alginate type and the GMO concentration, the other factors were kept constant during the experiments. Preliminary trials were done to determine the optimum concentration of the gelling agent (sodium alginate) and the gas generating element (sodium bicarbonate). Two grades of sodium alginate (M/G ratio of 1.5 and 0.67) were used to prepare aqueous solutions in different gradual concentrations and the obtained solutions were evaluated for the in situ gel formation and the acceptable consistency for oral drug delivery. The tested sodium alginate concentration range was (0.25-2%). Sodium bicarbonate was added in concentrations ranging from 1 to 5% and the obtained solutions were evaluated for the time required for buoyancy, the integrity of the formed gel, and the buoyancy time in 0.1 N HCl. The trials also included the determination of the acceptable GMO concentration range.

2.3. Formulation of the delivery system

Aqueous sodium alginate solutions (1%) were prepared in distilled water by maceration overnight. GMO (3, 6, and 9%, w/v) was melted at 45°C and added to the sodium alginate solution with sonication for 50 min. Two percent of sodium bicarbonate was added and magnetically stirred for 5 min. Depending on their solubilities, pamabrom was added to the alginate solution while carbimazole was added to the melted GMO. Drug loaded microspheres were dispersed in the finished system with magnetic stirring for 5 min.

2.4. Preparation of pamabrom microspheres

The microspheres were prepared by the emulsion-solvent diffusion technique (12). Ethyl cellulose (1 g) and pamabrom (1 g) were dissolved in an organic solvent blend consisting of methanol, acetone and dichloromethane (7:10:10, mL). The resulting solution was emulsified with 100 mL aqueous medium containing 0.1 g polyvinyl alcohol under stirring at 500 rpm. The emulsion was first stirred at room temperature for 0.5 h and later at 35-40°C for about 2-3 h. The dispersion was filtered and the microspheres were washed with water (about 500 mL). The microspheres were dried at room temperature.
2.5. Evaluation of the delivery system

2.5.1. Rheological and gelling properties

The viscosity determination of alginate-GMO solutions (drug-free) were carried out using a cone and plate Brookfield viscometer (model HBDV-I, Brookfield Engineering Laboratories, Inc., Middleboro, MA, USA) at 20°C using 1 mL aliquots of the sample. Sample viscosity was measured at different angular velocities (10-100 rpm) with a similar wait at each speed. Viscosity measurement for each sample was done in triplicate. For comparison, the exponential formula was used and the exponent N (Farrow’s constant) was calculated. Gelation was observed visually.

2.5.2. In vitro buoyancy

In vitro buoyancy was determined using a USP dissolution apparatus II with 500 mL of simulated gastric fluid (pH 1.2). The medium temperature was kept at 37°C. Five milliliters of each prepared formulation were placed into a Petri dish and kept in the dissolution vessel with little disturbance. The time the formulation took to emerge on the medium surface (floating lag time) and the time the formulation constantly floated on the dissolution medium surface (duration of buoyancy) were noted.

2.5.3. In vitro drug release

In vitro drug release was determined using a USP dissolution test apparatus (USP 24) with a paddle stirrer at 50 rpm as described previously (13). This speed was slow enough to avoid disruption of the gelled formulation and was maintaining with the mild agitation conditions believed to exist in vivo. The dissolution medium used was 500 mL of 0.1 N HCl (pH 1.2), and temperature was maintained at 37°C. Drug loadings were calculated to meet sink conditions where the amount of drug to be dissolved does not exceed 5% of the drug solubility in the dissolution medium (14). A 5 milliliter sodium alginate/GMO solution was collected in a disposable syringe and placed into a Petri dish and the Petri dish containing formulation was then kept in the dissolution vessel with little disturbance. At each time interval, a precisely measured sample of the dissolution medium was removed and replenished with fresh medium. Absorbance of pamabrom and carbimazole was measured at 272 and 229 nm, respectively, using the UV Spectrophotometer (Shimadzu, UV-1601, Kyoto, Japan). Each study was conducted in triplicate for up to 6 h.

The release rate was estimated by calculating the mean dissolution time (MDT) from the following equation:

$$\text{MDT} = \frac{\sum_{i=1}^{n} t_{\text{mid}} \times \Delta M}{\sum_{i=1}^{n} \Delta M}$$

where, $i$ is the number of released samples, $n$ is the sample release time, $t_{\text{mid}}$ is the time at the midpoint between $i$ and $i-1$, and $\Delta M$ is the additional amount of drug dissolved between $i$ and $i-1$. The higher the MDT, the slower the release rate (15).

The release extent was determined by measuring the release efficiency after 6 h (RE$_{6h}$) as well as the percentage of drug released after 6 h (RP$_{6h}$) (16).

The release kinetics from the prepared systems were investigated by fitting the release data into both Higuchi (17) and Hixson-Crowell (18) models and applying linear regression analysis.

2.6. Stability study

Formulation number 7 (high G alginate-6% GMO solution) containing pamabrom was selected, as an example, to evaluate the physical stability of the prepared systems. The solution was stored at ambient conditions for 12 months. Samples of 5 mL were withdrawn at time intervals of 2, 4, 6, and 12 months and evaluated for their appearance, rheological properties, and in vitro release profile. The similarity factor $S_d$ (19) was calculated from the mean release data and used to evaluate the effect of storage on the release profile. The $S_d$ is defined as:

$$S_d = \frac{\sum_{i=1}^{n-1} \log \left( \frac{\text{AUC}_{\text{st}}}{\text{AUC}_{\text{fr}}} \right) \times \Delta t}{n-1}$$

where $n$ is the number of data points collected during the in vitro test and $\text{AUC}_{\text{st}}$ and $\text{AUC}_{\text{fr}}$ are the areas under the release curves of the fresh and stored solutions, respectively, at time $t$. The percentage difference between two release profiles increases with an increase in $S_d$. The following equation was used to calculate the percentage difference between release profiles:

$$\text{Percentage difference} = \frac{(S_d - 0.0022)}{0.0038}$$

3. Results and Discussion

3.1. Preliminary experiments

Alginate gels have been successfully produced by using a low pH of 2.8-4.0 (20), where sodium alginate is converted to alginic acid. Many researchers reported that the release rate from the hydrated insoluble gel of alginic acid did not depend upon the viscosity grade of the sodium alginate used. Thus the chemical composition, rather than the viscosity, was considered here in the current study. Two types of sodium alginate with different M/G ratios were investigated (M/G ratio
of 1.5, high M alginate and M/G ratio of 0.67, high G alginate). In the selection of the concentration of the gelling polymer, a compromise is sought between a sufficiently high concentration for the formation of gels of satisfactory gel strength for use as a delivery vehicle and a sufficiently low concentration to maintain an acceptable viscosity for ease of swallowing. The sodium alginate concentration was fixed at 1% for both grades; above this concentration, the viscosity of the solutions was inappropriate for oral delivery. At lower concentrations, there was improper gelation and gelation took too long. The apparent viscosity, \( \eta \), of a 1% (w/w) solution was measured at 20°C using a Brookfield digital rotational viscometer with a spindle rotation set at 10 rpm. The viscosity values were 2,740 and 1,440 c.p for the high M and the high G alginate, respectively.

Sodium bicarbonate was selected as the gas generating agent rather than calcium carbonate to avoid any internal ionotropist gelation effect of calcium on alginites (21). After the addition of the different concentrations of sodium bicarbonate to each alginate solution, all the solutions were found to gel spontaneously on contact with the 0.1 N HCl. For NaHCO3 concentrations less than 2%, the formed alginic acid gels had partial buoyancy after a lag time of 2-4 min. The solutions prepared at 2%, the formed gels had partial buoyancy after a lag time of 2-4 min. The solutions prepared at 2-3% NaHCO3 showed immediate buoyancy and the formed gels remained intact, leaving a clear test medium. At NaHCO3 concentrations above 3%, the formed gels floated immediately but they were divided due to the high concentration of the CO2 produced, leaving turbid solutions below. Such weak gels are not suitable as oral liquid formulations, as they will be removed earlier from the stomach by peristaltic movements. Thus, 2% was selected as the optimum NaHCO3 concentration for both sodium alginate types.

GMO was then added to each sodium alginate solution in different concentrations. For GMO at > 9% (w/v), a highly viscous solution was formed. GMO concentrations lower than 3% (w/v) did not retard drug release (data not shown). Thus 3, 6, and 9% (w/v) GMO were used in the drug delivery system. The obtained systems were evaluated for their rheological properties and in vitro buoyancy.

Two different model drugs were incorporated alone and in encapsulated form and their in vitro release was evaluated.

### 3.2. Evaluation of the delivery system

#### 3.2.1. Evaluation of the delivery system

Figures 1 and 2 show the shear dependency of the viscosity of the alginate-GMO solutions. All systems exhibited pseudoplastic rheology. The solutions showed a marked increase in viscosity with increasing concentration of GMO. Solutions containing the high M alginate showed higher viscosity values and a lower degree of pseudoplasticity (lower N values) (Table 1).

![Figure 1. Rheological properties of high M alginate-GMO solutions at various concentrations of GMO.](image1)

![Figure 2. Rheological properties of high G alginate-GMO solutions at various concentrations of GMO.](image2)

#### Table 1. Composition and evaluation of the prepared sodium alginate-GMO solutions

<table>
<thead>
<tr>
<th>Formulation number</th>
<th>Na alg type (M/G ratio)</th>
<th>GMO conc. (%)</th>
<th>Floating lag time (sec)</th>
<th>Duration of floating (h)</th>
<th>Apparent viscosity at rpm of 10 (c.p)</th>
<th>Farrow’s constant (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.5</td>
<td>0</td>
<td>4.93 ± 0.31</td>
<td>&gt; 48</td>
<td>2,740 ± 4.22</td>
<td>1.974 ± 0.22</td>
</tr>
<tr>
<td>2</td>
<td>1.5</td>
<td>3</td>
<td>5.20 ± 1.31</td>
<td>&gt; 48</td>
<td>3,340 ± 3.21</td>
<td>1.982 ± 0.12</td>
</tr>
<tr>
<td>3</td>
<td>1.5</td>
<td>6</td>
<td>6.33 ± 0.58</td>
<td>&gt; 48</td>
<td>4,000 ± 2.98</td>
<td>1.998 ± 0.21</td>
</tr>
<tr>
<td>4</td>
<td>1.5</td>
<td>9</td>
<td>7.37 ± 0.71</td>
<td>&gt; 48</td>
<td>4,000 ± 2.98</td>
<td>1.976 ± 0.14</td>
</tr>
<tr>
<td>5</td>
<td>0.67</td>
<td>0</td>
<td>2.04 ± 0.23</td>
<td>&gt; 48</td>
<td>1,440 ± 3.91</td>
<td>2.880 ± 0.24</td>
</tr>
<tr>
<td>6</td>
<td>0.67</td>
<td>3</td>
<td>2.33 ± 0.58</td>
<td>&gt; 48</td>
<td>2,260 ± 3.33</td>
<td>2.969 ± 0.25</td>
</tr>
<tr>
<td>7</td>
<td>0.67</td>
<td>6</td>
<td>3.83 ± 0.29</td>
<td>&gt; 48</td>
<td>2,500 ± 3.40</td>
<td>2.889 ± 0.26</td>
</tr>
<tr>
<td>8</td>
<td>0.67</td>
<td>9</td>
<td>5.50 ± 0.50</td>
<td>&gt; 48</td>
<td>3,320 ± 1.67</td>
<td>2.492 ± 0.15</td>
</tr>
</tbody>
</table>

M/G ratio of 1.5 = High M alginate, M/G ratio of 0.67 = High G alginate, All values are mean of three readings ± S.D. Farrow’s constant is calculated from the exponential formula (\( F = \eta' G \)).
3.2.2. In vitro buoyancy

Upon contact with the acidic medium, *in situ* gelation and reaction with H⁺ ions occurred immediately to provide a gel barrier at the surface of the formulation. The sodium bicarbonate effervesced, releasing carbon dioxide. The dissolved carbon dioxide is entrapped in the gel network, producing a buoyant formulation. In addition, the three-dimensional network of GMO further restricted the diffusion of carbon dioxide and resulted in prolonged buoyancy.

All the formulations demonstrated excellent buoyancy, regardless of the change in the alginate type or GMO concentration. The floating lag time values were less than 10 sec and the formulations retained their integrity without dissolving or eroding and remained buoyant for more than 48 h (Table 1). Various drug loadings did not produce any significant change in buoyancy.

3.2.3. In vitro drug release

*In vitro* release of the hydrophilic drug (pamabrom) was rapid from alginate solutions alone, with almost 100% of the drug released within 60 min for both the high M and the high G sodium alginate solutions (Figures 3 and 4). In acidic medium, sodium alginate converts rapidly to insoluble alginic acid, which swells upon hydration. Some dissolution of the polymer did occur due to a temporary rise in pH within the hydrating matrix as a result of the intrinsic buffering capacity of sodium alginate. This results in an intact but relatively porous, rubbery texture (22). This porous structure enables solute egress and explains the rapid release of the drug. The drug release was linear with the square root of time.

Table 2. Release and release kinetics data

<table>
<thead>
<tr>
<th>Formulation number</th>
<th>MDT* (min)</th>
<th>RP&lt;sub&gt;6h&lt;/sub&gt;* (%)</th>
<th>RE&lt;sub&gt;6h&lt;/sub&gt;* (%)</th>
<th>R&lt;sup&gt;2&lt;/sup&gt; Higuchi's model</th>
<th>Hixson-Crowell</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pamabrom</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5.44 ± 0.091</td>
<td>100.0 ± 0.00</td>
<td>96.61 ± 1.23</td>
<td>0.855 ± 0.021</td>
<td>0.696 ± 0.041</td>
</tr>
<tr>
<td>2</td>
<td>12.30 ± 0.31</td>
<td>87.44 ± 2.99</td>
<td>68.53 ± 0.76</td>
<td>0.984 ± 0.012</td>
<td>0.983 ± 0.042</td>
</tr>
<tr>
<td>3</td>
<td>15.67 ± 1.58</td>
<td>67.74 ± 0.36</td>
<td>46.57 ± 0.69</td>
<td>0.940 ± 0.031</td>
<td>0.908 ± 0.026</td>
</tr>
<tr>
<td>4</td>
<td>14.88 ± 1.86</td>
<td>73.8 ± 0.27</td>
<td>51.79 ± 0.11</td>
<td>0.959 ± 0.024</td>
<td>0.946 ± 0.025</td>
</tr>
<tr>
<td>5</td>
<td>4.26 ± 0.11</td>
<td>100.0 ± 0.00</td>
<td>97.64 ± 1.39</td>
<td>0.926 ± 0.015</td>
<td>0.839 ± 0.013</td>
</tr>
<tr>
<td>6</td>
<td>10.05 ± 0.13</td>
<td>100.0 ± 0.00</td>
<td>88.11 ± 0.56</td>
<td>0.990 ± 0.021</td>
<td>0.876 ± 0.014</td>
</tr>
<tr>
<td>7</td>
<td>13.87 ± 0.31</td>
<td>93.01 ± 1.39</td>
<td>70.73 ± 0.82</td>
<td>0.964 ± 0.033</td>
<td>0.964 ± 0.022</td>
</tr>
<tr>
<td>8</td>
<td>15.57 ± 0.72</td>
<td>94.55 ± 0.77</td>
<td>67.45 ± 1.55</td>
<td>0.994 ± 0.024</td>
<td>0.923 ± 0.012</td>
</tr>
<tr>
<td><strong>Carbimazole</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>11.91 ± 0.53</td>
<td>100.0 ± 0.00</td>
<td>89.74 ± 0.99</td>
<td>0.995 ± 0.015</td>
<td>0.929 ± 0.032</td>
</tr>
<tr>
<td>2</td>
<td>17.54 ± 0.91</td>
<td>77.17 ± 0.25</td>
<td>57.58 ± 0.11</td>
<td>0.995 ± 0.023</td>
<td>0.919 ± 0.015</td>
</tr>
<tr>
<td>3</td>
<td>19.13 ± 1.04</td>
<td>52.43 ± 2.03</td>
<td>34.00 ± 0.53</td>
<td>0.999 ± 0.013</td>
<td>0.981 ± 0.016</td>
</tr>
<tr>
<td>4</td>
<td>18.44 ± 0.77</td>
<td>55.62 ± 0.53</td>
<td>32.85 ± 0.15</td>
<td>0.995 ± 0.021</td>
<td>0.946 ± 0.021</td>
</tr>
<tr>
<td>5</td>
<td>8.28 ± 1.78</td>
<td>100.0 ± 0.00</td>
<td>93.96 ± 1.54</td>
<td>0.988 ± 0.011</td>
<td>0.981 ± 0.023</td>
</tr>
<tr>
<td>6</td>
<td>17.80 ± 0.24</td>
<td>92.08 ± 1.30</td>
<td>65.45 ± 0.22</td>
<td>0.994 ± 0.023</td>
<td>0.963 ± 0.014</td>
</tr>
<tr>
<td>7</td>
<td>19.55 ± 0.57</td>
<td>71.03 ± 2.79</td>
<td>49.53 ± 1.88</td>
<td>0.992 ± 0.021</td>
<td>0.941 ± 0.011</td>
</tr>
<tr>
<td>8</td>
<td>20.00 ± 1.21</td>
<td>67.15 ± 1.20</td>
<td>45.03 ± 0.51</td>
<td>0.995 ± 0.021</td>
<td>0.940 ± 0.012</td>
</tr>
</tbody>
</table>

* MDT = mean dissolution time, RP<sub>6h</sub> = % drug released at 6 h; RE<sub>6h</sub> = release efficiency at 6 h, all values are mean of 3 readings ± SD, composition of the different Na alg-GMO formulations are shown in Table 1.

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time up to 95% of drug release, proving that the release mechanism is predominantly diffusion-controlled (Table 2). This result was supported by visual observation, which revealed a strong rubbery matrix that was not eroded at the end of the dissolution testing.

Similarly, Tabata and Ikada (23) stated that sustained release over a long period cannot be expected from hydrogels because the release from hydrogels is generally diffusion-controlled, with rapid passage through hydrogels due to their loose network structure. In addition, water insoluble drugs are not readily incorporated into hydrogels because of their incompatibility and phase separation. Thus, GMO was suggested here for these purposes.

The incorporation of 3% GMO significantly retarded this release (increased MDT and decreased RE6h) from both the high M and the high G sodium alginates (Table 2). This extended drug release is probably due to the restriction of the diffusion of drug molecules in the three-dimensional network of GMO. This release was characterized by an initial phase of high release during the first hour (burst effect) followed by a second phase of moderate release. This bi-phasic pattern of release is a characteristic feature of matrix diffusion kinetics (24). Increasing GMO from 3 to 6% significantly slowed the pamabrom release rate (MDT) and decreased the percentage drug dissolved after 6 h (RP6h) from both alginate solutions. This reduction may be due to formation of cubic phases with higher viscosities. However, the further increase in GMO concentration (9%) insignificantly influenced the drug release characteristics and resulted in almost similar MDT and RP6h values. A plot of the % released, as a function of the square root of time, revealed that the Higuchi’s model adequately described the release of pamabrom during the sustained release period (1-6 h), $R^2$ ranged from 0.959-0.999.

The same release pattern was observed with carbimazole (a hydrophobic drug) for both alginate solutions, but the initial burst effect was considerably reduced (15 min). Figures 5 and 6 and Table 2 show that increasing the concentration of GMO decreased both the rate and extent of release up to 6% GMO. The drug release from sodium alginate-GMO solutions followed the Higuchi’s model ($R^2$ ranged from 0.926-0.994) for the sustained release phase.

The effect of the sodium alginate chemical composition on the release rate (MDT) and the release extent (RE6h) of both drugs from the alginate-GMO solutions was statistically analyzed at each GMO concentration using simple analysis of variance (one-way ANOVA) or independent sample t-test. The significance of the difference was determined at 95% confident limit ($\alpha = 0.05$). Results showed a significant retardation in the release rate and extent from solutions containing high M alginate than from high G solutions in all cases (Figure 7). One possibility is that high M alginate hydrated faster and built up the diffusion barrier more rapidly, resulting in slower release. These results are in good agreement with previous reports (25-27) that investigated the advantages of high M alginate in sustaining drug release from matrix tablets.

To further sustain the release of the drug from alginate-GMO solutions, pamabrom was incorporated in the delivery system as pamabrom loaded ethylcellulose...
microspheres and as a 50:50 (w/w) physical mixture of free pamabrom and pamabrom microspheres. In vitro release profiles are depicted in Figure 8. The use of drug loaded microspheres reduced the burst effect, increased the MDT value by 3.26-fold and decreasing the RE6h by 2.25-fold in relation to the free drug. This may be explained by the additional barrier to drug diffusion in the microspheres. The release profile can be tailored by mixing the free and encapsulated drug as well as by changing their ratios. The overall curve fitting showed that the drug release from the microspheres followed the zero-order model ($R^2 = 0.992$).

3.3. Stability study

The stored solution retained its colour and appearance during the whole study. No marked changes were recorded in its rheological properties (Figure 9). The similarity factor is a realistic means of comparing the release behavior as it takes into account the release profile as a whole. The similarity factor $S_d$ was selected over the similarity factor $f_2$ (28) due to its simplicity, flexibility, and ability to quantitatively express the difference in release profile. The $S_d$ values were 0.0156, 0.0064, 0.0035, and 0.0068 after storage periods of 2, 4, 6, and 12 months, respectively. All the values are considerably close to zero, indicating relatively similar release profiles (Figure 10). Percentage differences from the fresh solution were calculated from $S_d$ and ranged from 0.342 to 3.527%.

4. Conclusions

This study has demonstrated the feasibility of forming floating gels in the stomach by the oral administration of aqueous solutions of alginate-GMO. Furthermore, sustained release of the model drugs was achieved from the gel vehicles over a period of at least 6 h. The release profile and kinetics can be tailored by changing the M/G ratio of sodium alginate, changing the GMO amount, or by encapsulating the drug. The system was physically stable for 12 months at ambient conditions.

References


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Microspheres of tramadol hydrochloride compressed along with a loading dose: A modified approach for sustaining release


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ABSTRACT: The purpose of this research was to study mucoadhesive microspheres of tramadol hydrochloride compressed into tablet along with a loading dose. Microspheres containing tramadol hydrochloride were prepared by employing sodium alginate in combination with a mucoadhesive polymer, i.e., Carbopol 971P. An orifice-ionic gelation method was used to prepare the microspheres. A 3² factorial design was used to investigate the combined effect of two independent formulation variables in the preparation of microspheres. The concentration of sodium alginate (X₁) and Carbopol 971P (X₂) were selected as independent variables. Nine batches were used in the experimental design and evaluated for swelling index, mucoadhesion, and drug entrapment efficiency. A surface plot is presented to graphically represent the effect of the independent variables on the evaluation parameters. The best batch exhibited drug entrapment efficiency of 70.12%, swelling index of 2.3 and mucoadhesion of 95.42%. Microspheres showing maximum drug entrapment were compressed with the loading dose and subjected to in vitro dissolution studies. Drug release from tablets was found to follow a matrix model. Initial burst release from these tablets indicated the release of the loading dose and then a sustained effect over the time. This modified approach to formulation of tablets was found to be effective in sustaining drug release.

Keywords: Carbopol 971P, factorial design, mucoadhesive microspheres, tramadol hydrochloride

1. Introduction

A sustained release dosage form is mainly designed for maintaining therapeutic blood or tissue levels of the drug for an extended period of time with minimized local or systemic adverse effects. Economy and greater patient compliance are other advantages (1). In recent years, clinical studies on tramadol hydrochloride have demonstrated that this drug is an effective agent for moderate to severe chronic pain (2-5). The half-life of the drug is about 5.5 h and the usual oral dosage regimen is 50 to 100 mg every 4 to 6 h with a maximum dosage of 400 mg/day (6). To reduce frequent administration of dosage form and to improve patient compliance, a sustained-release formulation of tramadol is desirable. The drug is freely water soluble and hence judicious selection of release retarding excipients is necessary to achieve a constant in vivo input rate of the drug. Various approaches have been used by researchers to sustain drug release in the form of tablets (7-9).

Microsphere carrier systems made from naturally occurring biodegradable polymers have attracted considerable attention for several years in sustained drug delivery. Microspheres form an important part of novel drug delivery systems (10-12). They have varied applications and are prepared using various polymers. However, the success of these microspheres is limited due to their short residence time at the site of absorption. It would, therefore, be advantageous to have a means for providing an intimate contact of the drug delivery system with the absorbing membranes (13-17). This can be achieved by coupling mucoadhesion characteristics to microspheres and developing mucoadhesive microspheres. Mucoadhesive microspheres have advantages including: efficient absorption and enhanced bioavailability of the drugs due to a high surface to volume ratio, a much more intimate contact with the mucus layer and specific targeting of drugs to the absorption site (18-21).

A modified approach of compressing the mucoadhesive microspheres into tablets with loading dose will provide efficient delivery by causing burst release of tablet with loading dose into the stomach and then attachment of mucoadhesive microspheres to gastric mucosa will provide sustained release of the drug.
2. Materials and Methods

2.1. Materials

Tramadol was a gift from Panacea Biotec (New Delhi, India). Avicel PH-102 was a gift from Okasa Pharmaceuticals (Maharashtra, India), Carbopol 971P was supplied by Noveon Asia Pacific Ltd. (Hong Kong, China), Sodium starch glycolate was a gift from Okasa Pharmaceuticals (India). Sodium alginate was purchased from Merck India (Mumbai, India). All other reagents and chemicals were of analytical grade.

2.2. Preparation of microspheres

Batches of microspheres were developed using a $3^2$ factorial design (22,23). The advantages of a factorial design include greater precision. By using a factorial design, it is possible to examine the effect of one variable when other factors are changed, which is not possible using traditional methods of investigation. The independent formulation variables were taken at three different levels: low, medium and high (-1, 0, and 1, respectively; Table 1). The factors selected were $X_1$ – weight of sodium alginate and $X_2$ – weight of Carbopol 971P. The Orifice-Ionic Gelation Method was used for preparation of microspheres as follows:

Sodium alginate and Carbopol 971P were dispersed in purified water (50 mL) to form a homogeneous polymer mixture (Table 2). Tramadol (1,000 mg) was added to the polymer premix and mixed thoroughly to form a smooth viscous dispersion. The resulting dispersion was then sprayed through a glass nozzle with a 1.61 mm inner diameter and 4 mm outer diameter into calcium chloride (10%, w/v) solution. The addition was done with continuous stirring. Produced droplets were retained in the calcium chloride solution for 15 min to complete the curing reaction and to produce rigid spherical microspheres. The resulting microspheres were collected using decantation, and the separated product was washed repeatedly with purified water to remove excess calcium impurities deposited on the surface of microspheres and then dried at 45°C for 12 h.

2.3. Characterization and evaluation of microspheres

2.3.1. Surface characterization, particle size and swelling index of microspheres

Prepared microspheres were morphologically characterized. The particle size of the microspheres was determined using optical microscopy. Approximately 100 microspheres were counted for particle size using a calibrated optical microscope (Labomed CX RIII, Ambala, India). For estimating the swelling index, the microspheres (~100) were suspended in 5 mL simulated gastric fluid USP (pH 1.2). The particle size was monitored by microscopy every 1 h using an optical microscope (Labomed CX RIII). The increase in particle size of the microspheres was noted for up to 8 h and the swelling index was calculated as described by Ibrahim (24).

\[
\text{Swelling index} = \frac{\text{Volume after 12 h}}{\text{Original volume}} \quad (1)
\]

2.3.2. In vitro mucoadhesion evaluations

The mucoadhesive force of all the batches was determined by the method of Choi, et al. (25). A section of gastric mucosa was cut from sheep's stomach and instantly attached with mucosal side out onto each glass vial using a rubber band. The vials with mucosa were stored at 37°C for 5 min. The next vial with a section of mucosa was connected to the balance in inverted position while the first vial was placed on a height adjustable pan. A fixed weight of mucoadhesive microspheres was placed onto the gastric mucosa of the first vial. Then the height of the second vial was adjusted so that the mucosal surfaces of both vials came in intimate contact. Ten minutes of contact was used. The weight was increased in the pan until vials were detached. Mucoadhesive force

---

Table 1. Coded levels and their translation into actual units

<table>
<thead>
<tr>
<th>Coded level</th>
<th>$X_1$ (weight of sodium alginate) (mg)</th>
<th>$X_2$ (weight of carbopol 971P) (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−1</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>0</td>
<td>750</td>
<td>750</td>
</tr>
<tr>
<td>+1</td>
<td>1,000</td>
<td>1,000</td>
</tr>
</tbody>
</table>

Table 2. Formulation design of mucoadhesive microspheres

<table>
<thead>
<tr>
<th>Batch No.</th>
<th>$X_1$</th>
<th>$X_2$</th>
<th>Sodium alginate (mg)</th>
<th>Carbopol 971P (mg)</th>
<th>Tramadol HCl (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>−1</td>
<td>−1</td>
<td>500</td>
<td>500</td>
<td>1,000</td>
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<td>0</td>
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<td>0</td>
<td>1</td>
<td>750</td>
<td>1,000</td>
<td>1,000</td>
</tr>
<tr>
<td>F9</td>
<td>1</td>
<td>1</td>
<td>1,000</td>
<td>1,000</td>
<td>1,000</td>
</tr>
</tbody>
</table>
was the minimum weight required to detach two vials. The mucosa was changed for each measurement.

\[
\text{% Mucoadhesion} = \left(\frac{\text{Wt. of sample} - \text{Wt. of detached particles}}{\text{Wt. of sample}}\right) \times 100 \quad \text{(2)}
\]

2.3.3. Drug entrapment efficiency

Microspheres (50 mg) were crushed in a glass mortar-pestle and the powdered microspheres were suspended in 10 mL phosphate buffer (pH 7.4). After 24 h, the solution was filtered and the filtrate was analyzed for drug content. The drug entrapment efficiency was calculated using the following formula.

\[
\text{Microencapsulation efficiency} = \frac{\text{Practical drug content}}{\text{Theoretical drug content}} \times 100 \quad \text{(3)}
\]

2.3.4. Compression of microspheres with loading dose

The batch showing the maximum percentage of entrapment was considered for preparation of tablet. The microspheres equivalent to 100 mg were accurately weighed and compressed with 50 mg of tramadol as loading dose with 200 mg of Avicel PH-102 and sodium starch glycolate (7% of the total weight of mix) using a single punch tablet compression machine.

2.3.5. In vitro dissolution studies

Tablets made up by compressing microspheres showing greater entrapment efficiency are studied for dissolution testing with USP II apparatus at 50 rpm and at temperature 37 ± 1°C. Samples were withdrawn at different time intervals and were assayed at 271.5 nm for tramadol content using Shimadzu UV-Vis Spectrophotometer (UV 1700; Shimadzu Corporation, Kyoto, Japan).

3. Results

3.1. Surface characterization, particle size and swelling studies

Mucoadhesive microspheres of tramadol prepared were well-rounded spheres with ridges of shrinkage due to presence of Carbopol 971P coat. Particle size was in between range of 217 μm to 368 μm. The swelling index (Table 3) varied from 1.4 to 2.3 (Figure 1) and showed correlation coefficient of 0.9488.

3.2. In vitro mucoadhesion evaluation

The percentage mucoadhesion varied from 82.34 to 95.42% (Table 3; Figure 2) and showed good correlation coefficient (0.9543).

3.3. Drug entrapment efficiency

Drug entrapment efficiency was found to be 61.83% to 70.12% (Table 3). It was found that increasing Carbopol 971P concentration was found to increase in entrapment efficiency. Correlation coefficient was found to be 0.9377. The surface response showing effect of variables on drug entrapment efficiency is shown in Figure 3.

3.4. In vitro dissolution studies

Comparative analysis of dissolution profiles (Figure

![Surface response showing effect of variables on swelling index.](image1.png)

**Table 3. Results of various evaluation parameters of mucoadhesive microspheres**

<table>
<thead>
<tr>
<th>Batch No.</th>
<th>Swelling index</th>
<th>Percentage mucoadhesion</th>
<th>Drug entrapment efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>1.4</td>
<td>82.34</td>
<td>61.83</td>
</tr>
<tr>
<td>F2</td>
<td>1.78</td>
<td>86.23</td>
<td>63.23</td>
</tr>
<tr>
<td>F3</td>
<td>2.1</td>
<td>89.54</td>
<td>65.42</td>
</tr>
<tr>
<td>F4</td>
<td>1.68</td>
<td>86.65</td>
<td>62.56</td>
</tr>
<tr>
<td>F5</td>
<td>1.97</td>
<td>88.64</td>
<td>65.45</td>
</tr>
<tr>
<td>F6</td>
<td>2.1</td>
<td>90.12</td>
<td>66.21</td>
</tr>
<tr>
<td>F7</td>
<td>1.87</td>
<td>87.31</td>
<td>64.32</td>
</tr>
<tr>
<td>F8</td>
<td>2.1</td>
<td>92.12</td>
<td>67.41</td>
</tr>
<tr>
<td>F9</td>
<td>2.3</td>
<td>95.42</td>
<td>70.12</td>
</tr>
</tbody>
</table>
4) between compressed tablets with loading dose and marketed sustained release tablets showed initial burst release in case of prepared formulation which is 31.922 ± 1.12. This release is equivalent to 47.88 mg. The $f_2$ value (Similarity factor) was found to be 66.12.

The Korsmeyer-Peppas release exponent of prepared formulation was found to be 0.2701.

4. Discussion

4.1. Surface characterization, particle size and swelling studies

The drug-loaded microspheres were spherical and yellowish white in appearance and whiteness gradually increases with increase in Carbopol 971P concentration. Microspheres with a coat of mucoadhesive polymer were found to be discrete, spherical, free flowing and of monolithic matrix type. The microspheres were uniform in size for each batch. Micromeritic property such as particle size is mainly governed by the polymer concentration. Particle size increases with increasing Carbopol 971P concentration. This may be due to increased viscosity of the dispersion, which affects the performance of spraying the mixture, causing formation of larger droplets.

The amount of polymer directly affected the solvent transfer rate and thus as the Carbopol 971P concentration increases the swelling index also increases. This may be due to higher water sorption capacity of Carbopol 971P than sodium alginate. Good correlation coefficient of 0.9488 indicates that amount of Carbopol 971P and sodium alginate directly affects the swelling index.

4.2. In vitro mucoadhesion evaluation

The percentage mucoadhesion varied from 82.34 to 95.42% (Table 3) and showed a good correlation coefficient (0.9543). At higher concentrations of both variables mucoadhesion increased, this may be attributed to an increase in particle size that causes an increase in mucoadhesion. Thus we can conclude that the amount of Carbopol 971P and sodium alginate directly affects the percentage of mucoadhesion.

4.3. Drug entrapment efficiency

The drug entrapment efficiency is an important variable for assessing the drug loading capacity of microspheres and their drug release profiles, thus suggesting the amount of drug availability at the site. These parameters are dependent on the process of preparation, physicochemical properties of drug and formulation variables. Batch F9 (70.12%) which showed maximum percentage entrapment was considered for preparation of tablet.

4.4. In vitro dissolution studies

Dissolution testing is a critical parameter for pharmaceutical dosage forms (26). Initial burst release in
the case of prepared formulation is equivalent to 47.88 mg. This indicates that prepared formulation releases the loading dose at 15 min. Subsequent release occurs from microspheres representing sustained release. The dissolution profile of marketed formulation follows a Matrix model. The model independent method such as similarity factor (f2) provides a simple way to compare dissolution data. US FDA guidance proposes that f2 values of 50-100 indicate equivalence in dissolution profiles. The f2 value was found to be 66.12 indicating similarity in dissolution profiles. The Korsmeyer – Peppas release exponent of prepared formulation indicates non-Fickian diffusion, i.e., initially there is rapid release, which is followed by tailing off over time.

5. Conclusion

The results of a 3^2 full factorial design revealed that the concentration of Carbopol 971P and sodium alginate significantly affected the dependent variables percentage mucoadhesion, swelling index, and drug entrapment efficiency. The mucoadhesive microspheres exhibited good mucoadhesive properties in an in vitro test. The entrapment efficiency increased as the concentration of Carbopol 971P increased. The microsphere batch showing maximum entrapment was compressed into tablets which showed an initial burst release of loading dose. This novel approach indicates a sustained release tablet with a loading dose can be helpful to achieve an immediate therapeutic level followed by sustained release over time.

References


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Buccal mucoadhesive tablets of flurbiprofen: Characterization and optimization

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ABSTRACT: The aim of this work was to develop and optimize sustained-release mucoadhesive tablets of flurbiprofen. Mucoadhesive polymers used were chitosan as primary polymer and hydroxypropylmethyl cellulose, hydroxypropyl cellulose, or sodium carboxymethyl cellulose as secondary polymer. Tablets were evaluated in terms of weight variation, thickness, hardness, friability, swelling, surface pH, in vitro mucoadhesive force, and in vitro release. The compatibility between flurbiprofen and the tablet excipients was confirmed by Fourier transfer infrared studies. Both the primary and secondary polymers were found to have synergistic effects on tablet swelling, bioadhesion, and in vitro drug release. Formulations containing sodium carboxymethyl cellulose (F1) showed a maximum swelling index of 4.144 after 8 h, maximum mucoadhesive force (0.27 N), and convenient in vitro release over 8 h. D-optimal design was employed to evaluate the effect of the ratio of the primary polymer (X1) and the type of secondary polymer (X2) on the swelling index after 8 h (Y1), drug release after 8 h (Y2) and time taken for 30% drug release (Y3).

Keywords: Flurbiprofen, buccal delivery, mucoadhesive tablets, chitosan, D-optimal design

1. Introduction

The buccal region of the oral cavity is an attractive target for drug administration. Buccal delivery involves the administration of the desired drug through the buccal mucosal membrane lining the oral cavity which offers some distinct advantages. It is richly vascularized and more accessible for the administration and removal of a dosage form. Avoiding acid hydrolysis in the gastrointestinal tract and bypassing the "first-pass" effect are some of the advantages of this route of drug delivery. Moreover, the oral cavity is easily accessible for self-medication and can be promptly terminated in case of toxicity just by removing the dosage form from the buccal cavity. In addition, the buccal route enables the administration of drugs to comatose patients (1,2).

Chitosan is a natural polyaminosaccharide obtained by N-deacetylation of chitin. This material is non-toxic, biocompatible, and biodegradable. Chitosan has a suitable mucoadhesive profile for combating the flushing effect of saliva and mastication (3). Chitosan interacts with mucin, the basic component of mucous, by multiple modes, mainly due to molecular attractive forces formed by electrostatic interaction between positively charged chitosan and negatively charged mucosal surfaces. These properties may be attributed to strong hydrogen bonding groups like -OH, -COOH, strong charges, high molecular weight (MW), sufficient chain flexibility, and to surface energy properties favoring spreading into mucus (4).

Attempts have been made to formulate various buccal mucoadhesive dosage forms, including tablets (5), films (6), patches (7), disks (8), and gels (9). A suitable buccal drug delivery system should possess good bioadhesive properties, so that it can be retained in the oral cavity for the desired duration. In addition, it should release the drug in a unidirectional way toward the mucosa, in a controlled and predictable manner, to elicit the required therapeutic response (10).

Flurbiprofen (FP) is a nonsteroidal anti-inflammatory agent indicated for the acute or long-term treatment of the signs and symptoms of rheumatoid arthritis and osteoarthritis. FP is extensively metabolized in the liver. Serious gastrointestinal toxicity, such as bleeding, ulceration, and perforation, can occur at any time, with or without warning symptoms, in patients treated chronically with FP (11).

In the present study, the mucoadhesive buccal...
tablets of FP were developed using chitosan as the primary mucoadhesive polymer and a secondary polymer either hydroxypropylmethylcellulose (HPMC), hydroxypropylcellulose (HPC), or sodium carboxymethylcellulose (SCMC). The effect of the secondary polymer on drug release from mucoadhesive tablets was studied. The buccal tablets were evaluated in terms of weight variation, thickness, hardness, friability, surface pH, swelling index, mucoadhesive strength, and in vitro drug release. The compatibility between FP and the tablet excipients was studied using Fourier transfer infrared (FTIR) spectroscopy.

2. Materials and Methods

2.1. Materials

Flurbiprofen (FP) was kindly supplied by the Egyptian International Pharmaceutical Company (EIPICO), Egypt; chitosan, highly viscous [2-amino-2-deoxy (1-4)-β-D-glucopyranan], agar, and HPC-NF (low viscosity) were purchased from Fluka Chemica, Switzerland; HPMC K4M was from Dow Chemical Company, NJ, USA; SCMC (low viscosity) was from Hercules Incorporation, DE, USA; D-mannitol was from Merck, Germany; magnesium (Mg) stearate was from Belike Chemical Co., China; disodium hydrogen phosphate and potassium dihydrogen phosphate were from El-Nasr Pharmaceutical Chemicals Co., Cairo, Egypt.

2.2. Preparation of buccal tablets

Mucoadhesive buccal tablets were prepared by a direct compression procedure. Various batches were prepared by varying the chitosan:drug ratio to identify the most effective formulation. The mucoadhesive drug/polymer mixture was prepared by homogeneous mixing of the drug with chitosan, secondary polymer, and D-mannitol, in a glass mortar for 15 min. Then, Mg stearate was added and mixed for 5 min (Table 1). The mixture was compressed using a tablet machine (Type EK: O.Erweka apparatur, Frankfurt, Germany) using flat-tip punches and dies with 8-mm-diameter. Each tablet weighed 212 mg with a thickness of 3.1 mm.

2.3. Physicochemical parameters of tablets

The tablets were checked for weight variation. Tablet thickness was measured using a micrometer (Mitutoyo, 103-260, Japan). Hardness of tablets was determined using a hardness tester (model: TH-16, China). Friability was determined using a Roche friabilator (Erweka Apparatebau GmbH, Germany). Drug content uniformity was determined by dissolving the crushed tablets in ethyl alcohol and filtered through 0.45-μm PTFE filter (Millipore Co., Bedford, MA, USA). The filtrate was diluted with phosphate buffer (pH 6.8) and analyzed at 248 nm (15) using a UV spectrophotometer (Shimadzu, model UV-1601 PC, Japan) using a reference to a standard calibration curve of the drug ($r^2 = 0.998$). The experiments were performed in triplicate and the average values ± standard deviation (SD) were reported.

2.4. Swelling study

The swelling index for each tablet was determined in triplicate and the mean ± SD was calculated. Each buccal tablet was weighed individually ($W_1$), placed separately in 2% agar gel plates, and incubated at 37 ± 1°C. At regular 1-h time intervals for 8 h, the tablet was removed from the petri dish and excess surface water was removed carefully using filter paper. The swollen tablet was reweighed ($W_2$), and the swelling index (SI) was calculated using equation 1 (16):

$$SI = \frac{(W_2 - W_1)}{W_1}$$  

--- (1)

2.5. Surface pH study

The surface pH of the buccal tablets was determined using the method adopted by Bottenberg, et al. (17). As an acidic or alkaline pH may irritate the buccal mucosa, we sought to keep the surface pH as close to neutral as possible. The tablet was allowed to swell by keeping it in contact with 2 mL simulated saliva fluid (pH 6.8) for 2 h at room temperature and pH was noted by bringing the electrode of the pH-meter (Jenway 8510, Baroworld Scientific Ltd., Essex, UK) in contact with tablet surface.

Table 1. Formulation of flurbiprofen buccal tablets

<table>
<thead>
<tr>
<th>Codes</th>
<th>FP (mg)</th>
<th>Chitosan (mg)</th>
<th>SCMC (mg)</th>
<th>HPMC (mg)</th>
<th>HPC (mg)</th>
<th>Mannitol (mg)</th>
<th>Mg Stearate (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F_1</td>
<td>50</td>
<td>50</td>
<td>100</td>
<td>–</td>
<td>–</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>F_2</td>
<td>50</td>
<td>100</td>
<td>50</td>
<td>–</td>
<td>–</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>F_3</td>
<td>50</td>
<td>25</td>
<td>125</td>
<td>–</td>
<td>–</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>F_4</td>
<td>50</td>
<td>50</td>
<td>–</td>
<td>100</td>
<td>–</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>F_5</td>
<td>50</td>
<td>100</td>
<td>–</td>
<td>50</td>
<td>–</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>F_6</td>
<td>50</td>
<td>25</td>
<td>–</td>
<td>125</td>
<td>–</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>F_7</td>
<td>50</td>
<td>50</td>
<td>–</td>
<td>–</td>
<td>100</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>F_8</td>
<td>50</td>
<td>100</td>
<td>–</td>
<td>–</td>
<td>50</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>F_9</td>
<td>50</td>
<td>25</td>
<td>–</td>
<td>–</td>
<td>125</td>
<td>10</td>
<td>2</td>
</tr>
</tbody>
</table>
and allowing the surface to equilibrate for 1 min. The surface pH for each tablet was determined in triplicate and the mean ± SD was calculated.

2.6. In vitro mucoadhesive force

The two-armed balance method reported by Parodi, et al. (18) with minor modifications was used for studying the bioadhesive force of the prepared tablets using fresh eggshell membrane (19) as illustrated in Figure 1. Briefly, the eggshell membrane was fixed on the bottom of a smaller beaker attached to a larger beaker. Fresh phosphate buffer (pH 6.8) was added to the beaker up to the upper surface of the membrane. A tablet was attached to the upper clamp and the platform was slowly raised until the tablet surface came in contact with membrane. After a preload time of 5 min, water was added with a polypropylene bottle until the tablet was detached from the membrane. The mass of water, in grams, required to detach the tablet from the membrane surface gives the measure of mucoadhesive strength. The force of adhesion was deduced using the following equation (20):

\[
\text{Force of adhesion (N)} = \frac{\text{bioadhesive strength} \times 9.81}{1,000} \quad (2)
\]

2.7. In vitro drug release study

The USP dissolution tester (Vankel Industries 750D, Weston Parkway, USA) with rotating paddle was used to study the drug release from the mucoadhesive tablets. The dissolution medium consisted of 250 mL of phosphate buffer, pH 6.8. The release study was performed at 37 ± 0.5°C with a rotation speed of 50 rpm. The buccal tablet was attached to a glass disk (by the use of rubber band) and was placed at the bottom of the dissolution vessel, thereby allowing drug release only from the upper side of the tablet. Samples of 5 mL were withdrawn at predetermined time intervals and replaced with fresh medium. Samples were filtered through 0.45-μm PTFE filter (Millipore Co., Bedford, MA, USA) and analyzed after appropriate dilution by UV spectrophotometry at 248 nm. Dissolution tests were performed at least three times for each sample.

2.8. Fourier transfer infrared spectroscopy (FTIR)

Samples (2-3 mg) of the ground tablets were each mixed with about 100 mg of dry potassium bromide, and were compressed into discs under pressure of 10-15 pounds/ inch². The FTIR spectra were recorded using a Mattson FTIR spectrophotometer (Model Genesis II, UK).

2.9. Data analysis

A twelve run, two factor, three variable D-optimal design was employed to evaluate the effect of primary polymer conc (X₁) and the type of secondary polymer (X₂) on the responses studied for the drug; the swelling index after 8 h (Y₁), drug release after 8 h (Y₂) and time taken for 30% drug release (Y₃) (Table 2).

The following cubic model was built to describe the response:

\[
Y_i = b_0 + b_1X_1 + b_2X_2 + b_{11}X_1^2 + b_{12}X_1X_2 + b_{13}X_1X_2^2
\]

where \(Y\) is the response, \(X\) the factors and \(b\) the coefficients of each term calculated by multiple regression analysis.

3. Results and Discussion

3.1. Physicochemical parameters

The results of the physical characteristics of the prepared mucoadhesive tablets of FP are shown in Table 3. Physical evaluation of the compressed matrix tablets showed that all physical parameters were within specifications. Tablet weights varied between 208 and 213 mg with SD of ± 1.81; thickness, between 2.94 and 3.36 mm with SD of ± 0.19; hardness, between 3.0 and 6.0 kg/cm² with SD of ± 1.44, and friability ranged from 0.2 to 0.4.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>X₁ primary polymer conc.</td>
<td>1:1 1:2 1:4</td>
</tr>
<tr>
<td>X₂ type of secondary polymer</td>
<td>SCMC HPC HPMC</td>
</tr>
</tbody>
</table>

Table 2. Experimental domains and coding of the variables

Responses:
- \(Y_1\) swelling after 8 h
- \(Y_2\) drug release after 8 h
- \(Y_3\) time taken for 30% drug release

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3.2. Swelling study

Appropriate swelling behavior of a buccal adhesive tablet is essential for uniform and prolonged release of the drug and effective mucoadhesion (21). The swelling index was dependent on the type of secondary polymer and its ratio to primary polymer in each formulation as shown in Figure 2. Tablets containing SCMC as secondary polymer showed maximum swelling index ($F_1 = 4.14$) as illustrated in Figure 3. This finding may be due to the fast-swelling properties of SCMC compared to either HPMC or HPC. Results also show that increasing the amount of chitosan led to an increase in the swelling behavior in formulations containing either of the two hydrophilic polymers HPMC or HPC ($F_5 = 2.838$ and $F_8 = 0.668$, respectively). This may be attributed to the increase in the ionized NH$_2$ group of chitosan, which results in loosening of the tablet matrix as a result of electrostatic repulsion between the polymers and decreased hydrogen bonding possibilities caused by charged NH$_3$$^+$ species. Moreover, protonation favors hydration and hence a higher water absorption capacity is observed.

3.3. Surface pH study

Surface pH of all tablets was found to range from 5.91 to 6.5 with SD of ± 0.21 (Table 3). These results reveal that all formulations provide an acceptable pH in the range of salivary pH (5.5 to 7.0) and that they will not produce any local irritation to the mucosal surface.

3.4. In vitro mucoadhesive force

As already indicated by several authors, the bioadhesive properties of polymeric materials are significantly affected by the model mucous membrane employed as a substrate for in vitro bioadhesion measurements and due to the use of either tissues or mucous membrane of various animals or different regions of the gastrointestinal tract of these animals, a wide variability was noted due to variation in the thickness of the layer covering the epithelium of these organs or tissues (22). In this study, egg shell membrane was employed as a natural substrate. The outer surface of the shell is covered with mucin protein which acts as a soluble plug for the pores in the shell (23,24). Therefore, egg shell membrane possesses an intricate lattice network of stable and water-insoluble fibers and has high surface area resulting in various applications such as adsorbent (25).

The force of adhesion was calculated from the bioadhesive strength as indicated in equation 2 and the results are shown in Figure 4. Chitosan is a cationic polymer and its mucoadhesion is mainly based on ionic interactions with anionic substructures of the mucus layer. The type of secondary polymer affected the mucoadhesion force significantly. SCMC, which is a polyanionic polymer, had a faster hydration rate and achieved maximum swelling more quickly. That is why $F_1$ showed a maximum bioadhesive force (0.27 N). HPMC and HPC tablets, which hydrated at a slower

![Figure 2. In vitro swelling studies of flurbiprofen buccal tablets.](image)

![Figure 3. Mucoadhesive buccal tablet F3 in 2% agar at zero time (A) and after 8 h (B).](image)

Table 3. Physical evaluation of prepared flurbiprofen tablets

<table>
<thead>
<tr>
<th>Codes</th>
<th>Weight (mg)</th>
<th>Hardness (kg/cm)</th>
<th>Friability (%)</th>
<th>Thickness (mm)</th>
<th>% Drug content</th>
<th>Surface pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>$F_1$</td>
<td>209 ± 2.5</td>
<td>6.9 ± 0.3</td>
<td>0.01 ± 0.00</td>
<td>2.94 ± 0.06</td>
<td>99.0 ± 0.6</td>
<td>5.91 ± 0.2</td>
</tr>
<tr>
<td>$F_2$</td>
<td>210 ± 2.7</td>
<td>3.5 ± 0.7</td>
<td>0.02 ± 0.00</td>
<td>3.02 ± 0.03</td>
<td>98.4 ± 0.5</td>
<td>6.0 ± 0.1</td>
</tr>
<tr>
<td>$F_3$</td>
<td>213 ± 2.4</td>
<td>5.5 ± 0.2</td>
<td>0.01 ± 0.01</td>
<td>3.06 ± 0.02</td>
<td>100.7 ± 0.3</td>
<td>6.05 ± 0.1</td>
</tr>
<tr>
<td>$F_4$</td>
<td>212 ± 4.1</td>
<td>3.2 ± 0.5</td>
<td>0.02 ± 0.01</td>
<td>3.36 ± 0.10</td>
<td>98.4 ± 0.8</td>
<td>6.5 ± 0.3</td>
</tr>
<tr>
<td>$F_5$</td>
<td>208 ± 1.6</td>
<td>3.0 ± 0.5</td>
<td>0.04 ± 0.02</td>
<td>3.36 ± 0.12</td>
<td>98.1 ± 0.7</td>
<td>5.95 ± 0.2</td>
</tr>
<tr>
<td>$F_6$</td>
<td>213 ± 3.3</td>
<td>3.7 ± 0.6</td>
<td>0.01 ± 0.00</td>
<td>3.36 ± 0.10</td>
<td>100.2 ± 0.3</td>
<td>5.97 ± 0.2</td>
</tr>
<tr>
<td>$F_7$</td>
<td>210 ± 1.5</td>
<td>3.5 ± 0.6</td>
<td>0.03 ± 0.01</td>
<td>2.95 ± 0.04</td>
<td>100.4 ± 0.3</td>
<td>6.42 ± 0.1</td>
</tr>
<tr>
<td>$F_8$</td>
<td>209 ± 1.6</td>
<td>3.3 ± 0.7</td>
<td>0.03 ± 0.01</td>
<td>2.97 ± 0.03</td>
<td>99.5 ± 0.1</td>
<td>6.09 ± 0.1</td>
</tr>
<tr>
<td>$F_9$</td>
<td>210 ± 2.4</td>
<td>6.0 ± 0.2</td>
<td>0.01 ± 0.00</td>
<td>2.99 ± 0.02</td>
<td>99.9 ± 0.0</td>
<td>6.07 ± 0.2</td>
</tr>
</tbody>
</table>

Values are mean ± SD ($n = 3$).
rate than SCMC, showed a smaller bioadhesive force. The rank order for bioadhesive force can be represented as F₁ > F₂, F₆ > F₃ > F₄, and F₇ > F₉ > F₅.

3.5. In vitro drug release study

The most significant factor affecting the rate of drug release from buccal tablets is the drug to polymer ratio. Figure 5 shows that complete drug release occurred within 4 h from tablets containing drug alone, while tablets prepared with chitosan alone, as primary polymer, showed complete drug release within 8 h. The reason for this delay in drug release is that chitosan, being a hydrophilic polymer, retains water in its structure forming gel spontaneously which is swellable and erodible, thus retarding drug release (26).

An increase in the concentration of the secondary polymer not only causes an increase in the viscosity of the gel structure surrounding the tablet upon hydration, but also leads to the formation of a gel layer with a longer diffusional path. This leads to a decrease in the diffusion of the drug and therefore a reduction in the rate of drug release (27). In the present study, the rate of drug release from formulations prepared with HPMC followed this predictable pattern. As seen in Figure 6, the percent drug released after 8 h from formulations containing least HPMC ratio (F₅) was 46.328%, while tablets containing highest HPMC concentration showed least drug release (F₆ = 22.086%). This is probably due to high gelling properties of HPMC. Formulations containing HPC showed the highest drug release among all prepared tablets (Figure 7). This is because HPC swells and partly dissolves, thus enabling chitosan to swell to its maximum size. On the other hand an explanation for slower drug release in formulations containing HPMC than those with HPC can be explained by the fact that HPMC has a higher swelling ability than HPC.

Results also demonstrate that the incorporation of SCMC results in a delay in FP release compared with tablets containing chitosan alone (Figure 5). This may be attributed to the possible ionic interaction between chitosan (a cationic polymer) and anionic SCMC within the tablet. In fact, it is already known that the cationic nature of chitosan permits the formation of complexes with oppositely charged drugs and polymers (28). There was a direct relationship between the SCMC ratio in tablet and the percent of drug released (F₁ = 66.783% while F₃ = 92.54%) as increasing the polymer ratio was accompanied by a decrease in chitosan amount within the tablet which resulted in the formation of the mentioned complex.

3.6. Fourier transfer infrared spectroscopy (FTIR)

Figure 8 demonstrates the FTIR spectra of FP, chitosan, SCMC, HPMC, HPC alone and F₃, F₆ and F₉ formulations. In the FTIR spectrum of FP powder,
3.7. Data analysis

The concentration of primary polymer ($X_1$) and the type of secondary polymer ($X_2$) were chosen as formulation variables and the swelling index after 8 h. Drug release after 8 h and the time taken for 30% drug release were selected as response variables ($Y_1$-$Y_3$), as shown in Table 2. The causal factors and response variables were related using a polynomial equation with statistical analysis through Design-Expert® software (32).

Tables 4-6 summarize the experimentally observed yields, swelling indices, drug release after 8 h and time taken for 30% drug release. The relationship between the dependent and independent variables was further elucidated using contour and response surface plots. The effects of $X_1$ and $X_2$ and their interaction on $Y_1$-$Y_3$ are given in Figures 9-11. At low and high levels of...
$X_2$, $Y_1$ increases compared with the middle value. The same observation was found for $Y_2$, while the reverse was found for $Y_3$, that at low and high levels of $X_2$, $Y_2$ decreases.

Also, contour plots were used to illustrate the simultaneous effect of the casual factors on individual and combined response variable. This expression gives an insight into the effect of the different independent variables (response). A positive sign of coefficient indicates a synergistic effect while a negative term indicates an antagonistic effect upon the response. The larger coefficient means the causal factor has a more potent influence on the response. As shown in Table 7, the coefficient of $X_1X_2$ and $X_1^2X_2$ were largest, showed that the effect of combination of the two independent factors, polymer type and concentration was the main influence on the responses, swelling and drug release. The value of coefficients of $X_2$ was less than that of $X_1$, indicated that the influence of the polymer type is less than that of polymer concentration.

We can also conclude from Table 7 that there was a high and significant $R^2$ between independent variables

| Table 5. Actual, predicted, residual values for drug release after 8 h as a function of primary polymer concentration ($X_1$) and type of secondary polymer ($X_2$) |
|---------------------------------|-------------|-------------|-------------|-------------|-------------|
| Standard order | $X_1$ | $X_2$ | Actual value | Predicted value | Residual | Run order |
| 1 | 1 | –1 | 92.54 | 91.88 | –0.66 | 10 |
| 2 | –1 | 1 | 30.13 | 29.80 | –0.33 | 6 |
| 3 | 0 | –1 | 52.33 | 53.66 | 1.33 | 5 |
| 4 | 1 | 1 | 22.08 | 21.75 | –0.33 | 3 |
| 5 | –1 | –1 | 66.78 | 66.45 | –0.33 | 8 |
| 6 | 1 | 0 | 80.30 | 81.63 | 1.33 | 1 |
| 7 | –1 | –1 | 66.78 | 66.45 | –0.33 | 7 |
| 8 | –1 | 1 | 30.13 | 29.26 | –0.78 | 4 |
| 9 | 1 | 1 | 22.08 | 21.75 | –0.33 | 9 |
| 10 | 0 | 0 | 91.92 | 89.26 | –2.66 | 12 |
| 11 | –1 | 0 | 99.18 | 100.5 | 1.32 | 11 |
| 12 | 0 | 1 | 46.33 | 47.77 | 1.33 | 2 |

| Table 6. Actual, predicted, residual values for time taken for 30% drug release as a function of primary polymer concentration ($X_1$) and type of secondary polymer ($X_2$) |
|---------------------------------|-------------|-------------|-------------|-------------|-------------|
| Standard order | $X_1$ | $X_2$ | Actual value | Predicted value | Residual | Run order |
| 1 | 1 | –1 | 4.0 | 3.94 | –0.06 | 10 |
| 2 | –1 | 1 | 8.0 | 7.97 | –0.03 | 6 |
| 3 | 0 | –1 | 6.0 | 6.12 | 0.12 | 5 |
| 4 | 1 | 1 | 9.0 | 8.97 | –0.03 | 3 |
| 5 | –1 | –1 | 5.0 | 4.97 | –0.03 | 8 |
| 6 | 1 | 0 | 2.5 | 2.62 | 0.12 | 1 |
| 7 | –1 | –1 | 5.0 | 5.12 | 0.12 | 7 |
| 8 | –1 | 1 | 8.0 | 7.97 | –0.03 | 4 |
| 9 | 1 | 1 | 9.0 | 8.97 | –0.03 | 9 |
| 10 | 0 | 0 | 1.0 | 1.0 | –0.23 | 12 |
| 11 | –1 | 0 | 0.5 | 0.62 | 0.12 | 11 |
| 12 | 0 | 1 | 5.0 | 4.97 | –0.03 | 2 |

| Table 7. Optimal regression equation (cubic model) for each response variable as a function of primary polymer conc ($X_1$) and type of secondary polymer ($X_2$) |
|----------------|-------------|-------------|-------------|
| Model | Coefficient | $Y_1$ | $Y_2$ |
| $b_0$ | 0.5 | 89.26 | 0.77 |
| $b_1(X_1)$ | –0.1 | –9.44 | 1.0 |
| $b_2(X_2)$ | 0.49 | –3.0 | –0.5 |
| $b_3(X_1^2)$ | 0.087 | 1.81 | 0.85 |
| $b_4(X_2^2)$ | 1.86 | –38.6 | 4.85 |
| $b_5(X_1X_2)$ | 3.75 | 3.75 | –11.75 |
| $b_6(X_1^2X_2)$ | –1.2 | –23.69 | 2.51 |
| $b_7(X_1X_2^2)$ | –0.53 | 13.78 | –1.01 |
| Quadratic | CV | 2.12 | 3.34 |
| $R^2$ | 0.9996 | 0.9983 | 0.9988 |
| Adjusted $R^2$ | 0.9988 | 0.9953 | 0.9966 |
| PRESS* | 0.50 | 1.0870.1 | 8.27 |

* Predicted residual error sum of squares.
(polymer type and concentration) and dependent variables ($Y_1$-$Y_3$). $R^2$ value of 0.99 and above for all the models in this study suggested adequate modeling. The $R^2$ values for $Y_1$-$Y_3$ were 0.9996, 0.9983, and 0.9988, respectively, which are in reasonable agreement with the adjusted $R^2$ of 0.9988, 0.9953, and 0.9966.

4. Conclusions

The prepared mucoadhesive buccal tablets of flurbiprofen...
can help bypass extensive hepatic first-pass metabolism and improve drug bioavailability. The in vitro release studies showed that 66.78% of drug was released from F₂ by the end of 8 h, which can be used in a twice-a-day tablet, thus allows for reduction in daily drug dosage and subsequent side effects. Moreover, it adheres well to the mucous membrane and is simple to apply.

References


Pathological gambling associated with cabergoline in a case of recurrent depression

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ABSTRACT: Pathological gambling has been described frequently in patients with Parkinson disease or other movement disorders who were treated with dopamine agonists. Here, we report a patient with recurrent depression who developed pathological gambling after administration of the dopamine agonist cabergoline. A 36-year-old male Japanese patient presented with his third episode of depression. His depressive symptoms responded minimally to fluvoxamine. Cabergoline was then added to augment the antidepressant's efficacy. Although this regimen resulted in dramatic improvement, he started to spend considerable money and time every day in pachinko parlors and go to the horse racing track every weekend. He spent more than twenty thousand US dollars in total. He tried to stop gambling many times but failed to control his urge. His gambling behavior did not stop even though he was experiencing a marital crisis. He had not displayed any manic symptoms during this entire period. This complication fulfilled the criteria for pathological gambling according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision edition. The patient's perplexing behavior did not end until cabergoline was discontinued. Thus far, pathological gambling associated with cabergoline has rarely been reported while gambling associated with pramipexole and ropinirole, dopamine agonists, has frequently been documented. In conclusion, clinicians should be aware of the potential for pathological gambling when prescribing cabergoline to patients with depression.

Keywords: Pathological gambling, cabergoline, dopamine agonist, depression

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1. Introduction

Pathological gambling has been described frequently in patients with Parkinson disease (1-6) or other movement disorders (7). This phenomenon is attributed to stimulation of dopamine receptors by dopamine replacement therapy and/or dopamine agonist administration. Dopamine agonists are occasionally used to treat antidepressant-resistant depression (8-10). However, pathological gambling has not been described in patients with depression treated with dopamine agonists. Reported here is the first case of recurrent depression in a patient who exhibited pathological gambling after administration of cabergoline.

2. Case Report

Mr. X is a 36-year-old government official. His younger brother has suffered from social anxiety disorder for several years. Mr. X has had no serious physical illness thus far. After graduating from university, he started to work in a government office and was transferred to this area at the age of 31. He had occasionally enjoyed pachinko (11), the most popular form of gambling in Japan, and horse racing earlier in his life but never had any problems. Three months after the transfer, he started to experience impaired concentration, decreased motivation, anxiety, depressive moods, malaise, and insomnia. He consulted a psychiatrist near his office and was treated for six months. At the age of 32, he started to experience similar symptoms and consulted another psychiatrist. His symptoms were treated successfully by administration of fluvoxamine 150 mg/d plus amoxapine 30 mg/d. At the age of 33, he had a recurrence of similar symptoms and consulted another psychiatrist. His depressive symptoms were not alleviated by combined treatment of paroxetine 30 mg/d and amoxapine 100 mg/d, so he was referred to this clinic for a consultation.

Fluvoxamine was initiated and was gradually increased to 150 mg/d. It was partially effective in that allowed the patient to work for brief periods of four hours a day. Since he lacked energy, fluvoxamine was increased to 200 mg/d and cabergoline 1 mg/d was
added. The course of the drug regimen and clinical response after introduction of cabergoline are shown in Figure 1. Six weeks after the addition of cabergoline, the dose was increased to 2 mg/d because lack of energy persisted. The patient’s moods gradually returned to normal but his difficulty in sleeping was exacerbated. Quetiapine 25 mg was initiated for insomnia and was effective. Six months later, his sense of fatigue had almost disappeared, allowing him to being exercising at a local gym. At around the same time, he started to go to pachinko parlors and the horse racing track, although he lacked sufficient energy to work full-time. He spent considerable money and time every day in pachinko parlors and went to the horse racing track every weekend. Neither the patient nor his family was aware that his behavior was problematic, so he did not report it to his psychiatrist. Eight months later, his depressive symptoms had almost disappeared, so he was able to work for up to six hours. Nine months later, cabergoline was decreased to 1.5 mg/day because the patient exhibited no depressive symptoms except insomnia. However, his gambling behavior persisted. In the meantime, he exhibited no manic or hypomanic symptoms.

One year later, his wife finally noticed that his gambling behavior was excessive and that he had spent more than twenty thousand US dollars in total. He subsequently often quarreled with his wife over his gambling and always pledged to stop. Nevertheless, he could not refrain from going to pachinko parlors. He spent more money and time gambling. He sometimes went to a pachinko parlor to recover his losses on the same day he lost substantial money betting on horse racing. He and his wife visited this clinic together to discuss his gambling problem. His gambling behavior fulfilled criteria for pathological gambling according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision edition (DSM-IV-TR). Since pathological gambling might have been triggered by cabergoline administration, the dose was gradually decreased with close monitoring of the patient's gambling behavior and moods. His gambling behavior continued until one week after cabergoline was discontinued, but depressive symptoms subsequently recurred.

3. Discussion

The current case involved a patient developing pathological gambling after the introduction of cabergoline, a dopamine agonist. This phenomenon did not disappear until the drug was discontinued. Thus far, four cases of pathological gambling associated with cabergoline have been reported (1,3,4,12). Three involved patients suffering from Parkinson disease who were taking cabergoline with L-dopa. Two were taking 4 mg/d of cabergoline. The other patient was taking cabergoline 0.25 mg weekly to treat pituitary prolactinoma. Like in the current case, gambling behavior stopped with the discontinuation of cabergoline except in one case where gambling stopped after reduction of L-dopa instead (1).

Other dopamine agonists which have been associated with pathological gambling include pramipexole, ropinirole, and pergolide (2,13-16). Two case series have implicated pramipexole as the agent most likely to cause pathological gambling in patients with Parkinson disease (2,16). However, more recent and larger-scale studies have not supported a differential association between specific dopamine agonists and pathological gambling (13-15). Instead, these recent studies have consistently indicated a higher prevalence of pathological gambling among patients treated with a dopamine agonist than those without (13-15). The lifetime prevalence of pathological gambling was 7.2% among patients with Parkinson disease on a dopamine agonist and 3.4% among those on any medication (14).

The mechanism by which dopamine agonists induce pathological gambling is not clear, but stimulation of dopamine D3 receptors has been repeatedly suggested (4). D3 receptors are distributed most abundantly in the limbic system, where the reward system is supposedly

![Figure 1. Schematic time-course of the cabergoline dose and severity of pathological gambling and depressive symptoms after introduction of cabergoline.](www.ddtjournal.com)
regulated (17). Pramipexole, ropinirole, and pergolide all show 10 to 100-fold greater selectivity for D3 receptors than for D2 receptors (18). Although cabergoline is not as selective, it is still slightly more selective for and fairly potent against D3 rather than D2 receptors. In contrast, bromocriptine, which is more selective for D2 receptors than for D3 receptors (18), has been linked with pathological gambling in only two cases (5,6).

In the current case, pathological gambling began after depression was partially alleviated and it persisted even after depression abated. Mood disorder itself has been associated with pathological gambling in many reports (19). Depression is prevalent in over half of the pathological gambling population, according to some studies. Depressive patients may start gambling to relieve their depressive mood while other patients may exhibit depression secondary to gambling behavior. Since the current patient did not exhibit pathological gambling in his preceding depressive episodes, his pathological gambling should not be attributed primarily to his depression. He did not show any manic symptoms, which must be ruled out according to the DSM-IV-TR criteria because clinical symptoms of pathological gambling and gambling as a symptom of mania share similarities.

Pathological gambling in the current case finally disappeared one week after cabergoline was discontinued. Most other reports have noted that pathological gambling had been successfully resolved by reducing or discontinuing the dopamine agonist prescribed. Some authors have suggested the efficacy of selective serotonin reuptake inhibitors (SSRIs) at curbing pathological gambling (3,19). Although the mechanism is not clear, research has suggested that the efficacy of SSRIs may be related to their anti-obsessional effect (19). However, the current patient developed pathological gambling while taking fluvoxamine, an SSRI, indicating that the prophylactic effect against pathological gambling by fluvoxamine is questionable.

In conclusion, physicians should be aware of the potential for the development of pathological gambling when prescribing cabergoline as well as pramipexole, ropinirole, and pergolide.

References


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