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Usefulness of rifabutin loaded floating beads for the treatment of Helicobacter pylori infection in experimental Mongolian gerbils

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Abstract

The objective of the present study was to develop stomach-specific drug delivery system of rifabutin and to evaluate their effectiveness in eradication of Helicobacter pylori in experimental Mongolian gerbil model.

Rifabutin loaded floating gellan gum beads prepared by calcium induced ionotropic gelation in acidic medium. The prepared beads were evaluated for in vitro characterization and in vivo Helicobacter pylori clearance efficiency following repeated oral administration to Helicobacter pylori infected Mongolian gerbils. Our results showed that the rifabutin loaded floating beads possess significant anti- Helicobacter pylori effect in the in vivo gerbil model.

The findings support that rifabutin floating beads could be a promising gastrointestinal drug delivery system in treatment of Helicobacter pylori.

Keywords: Rifabutin, floating beads, gellan gum, Helicobacter pylori.

Introduction

It is estimated that more than half-human population has infected with Helicobacter pylori [1]. Most people are unaware that they are infected because they remain asymptomatic throughout life and survive without any harmful infection-related clinical consequences. However, some develop duodenal or gastric ulcers and a small proportion are diagnosed with lymphoma or gastric malignancy [2]. Helicobacter pylori are specifically suited to the colonization of the human stomach, in which it causes an inflammatory reaction and epithelial damage with cellular swelling and cytoplasmic Vacuolation, both in vivo and in vitro. The two main bacterial factors involved in this cellular damage are urease and vacuolating toxin A [3]. Ammonia produced by urease action potentiates the action of vacuolating toxin A to Vacuolation in stomach. Ammonia crosses cell membranes in an uncharged state, is trapped by protonation within acidic intracellular compartments, and thereafter induces osmotic swelling of these compartments, which in turn causes cell Vacuolation.

Rifabutin, a spiropiperidyl derivative of Rifamycin, has been shown to exhibit high in vitro activity against Helicobacter pylori [4, 5]. It inhibits Helicobacter pylori growth in vitro at very low concentrations [6]; therefore, it may be indicated in addition to second line therapy as well as in first line therapy in H. pylori infection. In the present study, we investigated the efficacy of rifabutin loaded floating gellan beads in the treatment of Helicobacter pylori infected experimental Mongolian gerbils.

Material and methods

Materials

Rifabutin was obtained from Henan Kangtai Pharm Group, China. Deacetylated gellan gum (Kelcogel) was obtained as a gift sample from CP Kelco UK Ltd, Surrey, U.K. De-ionized water (HPLC grade) purchased from Quilagens, India. All other chemicals purchased were of analytical or microbiological grade. Helicobacter pylori strain NCTC 11637 was obtained from IVRI, Bareilly, India.

Methods of Preparation of Floating Beads

The beads, whose composition is shown in Table 1, were prepared by ionotropic gelation technique [7] with some modification. Gellan solution (1--3%w/v) was prepared by dissolving the gellan in deionized water by at 90 °C. The drug, rifabutin, and sodium bicarbonate were dissolved/dispersed uniformly in gellan solution at or just below 40 °C under continuous stirring. Stirring was continued until a uniform dispersion was obtained. The bubble free slurry (dispersion) was added drop wise into gelation medium consisting of varying concentration of Ca^{2+} in 10%v/v acetic acid using 25ml hypodermic syringe through a 20G needle. This medium was continuously stirred during bead formation to enhance the mechanical strength of the beads and also to prevent their aggregation. The beads were cured for 5 min, separated by filtration, washed thrice with deionized and dried at 40 °C in a hot air oven (DR 101, Universal, India).

In vitro buoyancy of the beads

In-vitro study of bead buoyancy was performed using a USP dissolution apparatus type II (paddle type, Electrolab, Mumbai, India). The beads were dispersed in 500ml of 0.01M HCl (pH 2.0) at 37 ± 1^0C with continuous agitation at 50 rpm for 24 h. The floating beads were separated from submerged beads and their proportion was determined (%) as described earlier [7, 8].

Determination of drug contents and entrapment efficiency of floating beads

The drug contents and entrapment efficiency of each formulation was determined by first extracting rifabutin from an accurately weighed quantity (100 mg) of the beads using 0.01M HCl (pH 2.0) and determining the amount of the drug by high performance liquid chromatography (HPLC). For HPLC determination of drug, the mobile phase consisted of 0.05M potassium dihydrogen phosphate and 0.05M sodium acetate (pH adjusted to 4.0 with acetic acid)/acetonitrile (53:47, v/v) at a flow rate of 1 ml/min at 25 °C. The HPLC system (Shimadzu, SPD-20A detector, LC-20AT pump) consisted of a UV detector set to 275 nm. The analytical column was A Pursuit XRs C-8 column (USP-L7).

Tab .1. Formulation variables of Floating beads of Rifabutin

Formulation Code	Weight % polymer	Weight % drug	Con. Of $NaHCO_3$ (% w/v)	Con. Of $CaCl_2$ (% w/v)
A1	1	1	0.4	3
A2	2	1	0.4	3
A3	3	1	0.4	3
A4	1	1	0.4	4
A5	2	1	0.4	4
A6	3	1	0.4	4
A7	1	1	0.4	5
A8	2	1	0.4	5
A9	3	1	0.4	5

A standard solution of rifabutin (100µg/ml) was prepared by weighing the appropriate amount of bulk rifabutin and dissolving it in the mobile phase. Further stock solutions were made by diluting the initial stock standard solution with mobile phase. A seven-point calibration curve ranging from 1 to 7 µg /ml were used for the quantification of rifabutin. A stock solution of 1.0 µg/ml was stored at −30 °C and a sample of this stock solution was always injected together with the analyzed samples to verify the precision of the obtained concentrations of rifabutin in samples and controls from their peak area-concentration response.

Cultivation of Helicobacter pylori

Solid media were used for growth of strains before inoculation of liquid media. H pylori isolates and strain NCTC 11637 were subcultured on chocolate agar plates with 7% sheep blood. The medium was supplemented with 2,500 U/ml of Polymyxin B, 5 mg/l of Trimetoprim and 5 mg/l of Amphotericin B (Fluka, Switzerland). The agar plates were incubated at 37^{o}C for 3-5 days under microaerophillic conditions (4% Oxygen, 5% Hydrogen, 5% Carbon-di-oxide, and 86% Nitrogen) in a Carbon Dioxide Incubator (Khera Instruments, India). The identification of Helicobacter pylori was done on the basis of colonial morphology (1–2 mm, small and translucent colonies), microscopically (the presence of curved Gram-negative cells) and by positive urease reaction. Gram stain with carbol-fuchsin was used for microscopic examination. Liquid media used for a series of studies were based on brain heart infusion broth (with 5% horse serum and 0 25% yeast extract (HIMEDIA Laboratories, India). Cultures from chocolate agar plates were inoculated into 5 ml aliquots of nutrient broth to achieve graded concentrations of bacterial cells, determined by comparator turbidity tubes (McFarland No 0-5-No 5). Each suspension (1 ml) was transferred to a 200 milliliter tissue culture flask containing 100 ml prewarmed liquid medium. The flasks were incubated at 37^{o}C for 3-5 days under microaerophillic conditions (4% Oxygen, 5% Hydrogen, 5% Carbon-di-oxide, and 86% Nitrogen) in a Carbon Dioxide Incubator. The cultures were examined daily for viable cell counts, and the urease test and Gram stain were also used. Oxidase and catalase tests were also used to out rule contamination. Dilutions of 10^{-1} , $10^{-3,}$ and 10^{-4} were made from the tissue culture flasks by suspending the culture in nutrient broth. The suspensions were inoculated with a 1 µl calibrated loop on to chocolate agar plates in triplicate. The plates were incubated as described above and colonies counted after five days of incubation. The mean value of three readings was calculated and expressed as colony forming units per milliliter (CFU/ml).

Animals and Groupings

Male 5-wk-old male Mongolian gerbils weighing 30-40 g (Central Drug Research Institute, Lucknow, India) were acclimated to the housing facilities for 5 days before initiation of the study. Free access to standard pellet chow was allowed throughout the experimental protocol, with the exception of overnight fasting before induction of Helicobacter pylori infection. The Animal Care and Use Committee of College of Pharmacy, IFTM, Moradabad India where the study was conducted approved all protocols. Infection was induced by giving the Helicobacter pylori inoculums of 2 ml (1 x 10^{8} H. Pylori/ ml) orally every alternate for seven days. Two month after confirming the infection, a total 30 Mongolian gerbils were divided into five equal groups (n=6 each group). Group I (control group) animals were given floating gellan gum beads (without drug), Group II, Group III and Group IV (treated) animals were given floating beads containing rifabutin equivalent to 2 mg/k.g, p.o, 4 m.g/k.g, p.o and 6mg/k.g. p.o respectively for 7 days whereas Group V were given rifabutin suspension equivalent 6mg /kg in 0.4%w/v gellan gum.

Helicobacter pylori Clearance study

One day after administration of the final dose, the Mongolian gerbils were sacrificed and the stomachs were removed. Each stomach was

homogenized with Brucella broth (3 ml/stomach), and serial dilutions were plated on modified Skirrow's medium. The agar plates were incubated for 4 days at 37 °C under microaerophillic environment in a carbon dioxide incubator. The viable cell counts for each stomach determined by counting the number of colonies on the agar plates. The colonies were identified as Helicobacter pylori by morphology and urease activity. The number of colonies per plate was counted and expressed as log CFU (Colony Forming Units) per gastric wall. The advantage of this evaluation method is that errors caused by sampling site variation can be avoided because the whole stomach is used to determine the bacterial cell count.

Morphological Evaluation and Histopathology

Gastric tissues collected from Mongolian gerbils in each group, stained with Giemsa Stain followed by serial slide from Mongolian gerbils in each group with hematoxilin-eosin (HE) and then observed under light microscope at 200x or 400-x magnification.

SYTO 9 Dye Staining

This method allows visualization of cells with intact ('live') cell membrane [9]. The fluorescent stain is a green SYTO 9 dye (Bio Source, USA). SYTO 9 dyes bind to DNA and penetrate the cell membrane. Green SYTO 9 passes readily through the intact membrane and under the fluorescent microscope the intact ('live') cells thus emit green light.

Statistics

All the data were analyzed by Student's t test and one-way ANOVA to determine statistical difference in the results. A probability value $p < 0.05$ was considered statistically significant. The software used was SigmaPlot® 11 (Systat Software Inc).

Results

In vitro floating properties:

As Table 2 shows, the floating properties of the formulation depended largely on sodium bicarbonate. While the control beads (without sodium bicarbonate) sank completely in the medium, the beads containing 0.4 % $NaHCO_3$ demonstrated good floating characteristics. There was no lag time as the beads floated immediately when placed in 0.01M hydrochloric acid and remained so for up to 14 h.

Tab .2. Physiochemical characteristics of Floating beads of Rifabutin

Formulation Code	Diameter (mm [abc])	% Entrapment Efficiency[a, c]	Floating ability[a, c] (%)	Drug Content per (100 mg beads) [a, c]
A1	1.26±0.04	41.12±1.32	98.77±1.10	20.51±1.24
A2	1.48±0.06	45.14±1.17	95.71±1.32	15.22±1.11
A3	1.54±0.05	48.22±1.50	91.67±1.47	12.08±1.08
A4	1.22±0.02	44.33±1.37	96.13±1.39	22.16±1.17
A5	1.40±0.05	48.71±1.28	92.34±2.13	16.34±1.04
A6	1.47±0.05	52.67±1.41	90.41±1.48	13.62±1.07
A7	1.10±0.03	47.89±1.18	93.17±1.56	23.37±1.28
A8	1.25±0.03	53.89±1.35	90.44±1.66	18.01±1.14
A9	1.33±0.02	59.78±1.44	88.24±1.51	14.67±1.02

[a]Mean± SD, [b]n= 20, [c]n= 3

Entrapment efficiency of floating beads

Rifabutin entrapment ranged from 41.12% to 59.78% (Table 2). Drug entrapment efficiency varied with polymer content and the calcium concentration of the gelation medium. Increase in calcium concentration enhanced drug entrapment efficiency ($p < 0.05$) (compare batches A1and A3, A4 and A6 and A7 and A9). Similarly, as the concentration of gellan gum rose, entrapment efficiency also increased (compare A1 and A7; and A2 and A8). Overall, however, the rifabutin entrapment efficiency of the beads was low.

Table 3: Effect of Rifabutin on clearance of Helicobacter pylori in Mongolian gerbils.

Animal Groups	Dose (mg/kg)	After treatment Clearence rate		Bacterial recovery Log CFU/Stomach
		No. of Animals	% Clearence	
Control				
(Gr. I)	0	0/6	0	9.55±0.58
Treated				
(Gr. II)	2	2/6	33	7.11±0.31
(Gr. III)	4	3/6	50	2.4±0.27
(Gr. IV)	6	6/6	100	ND

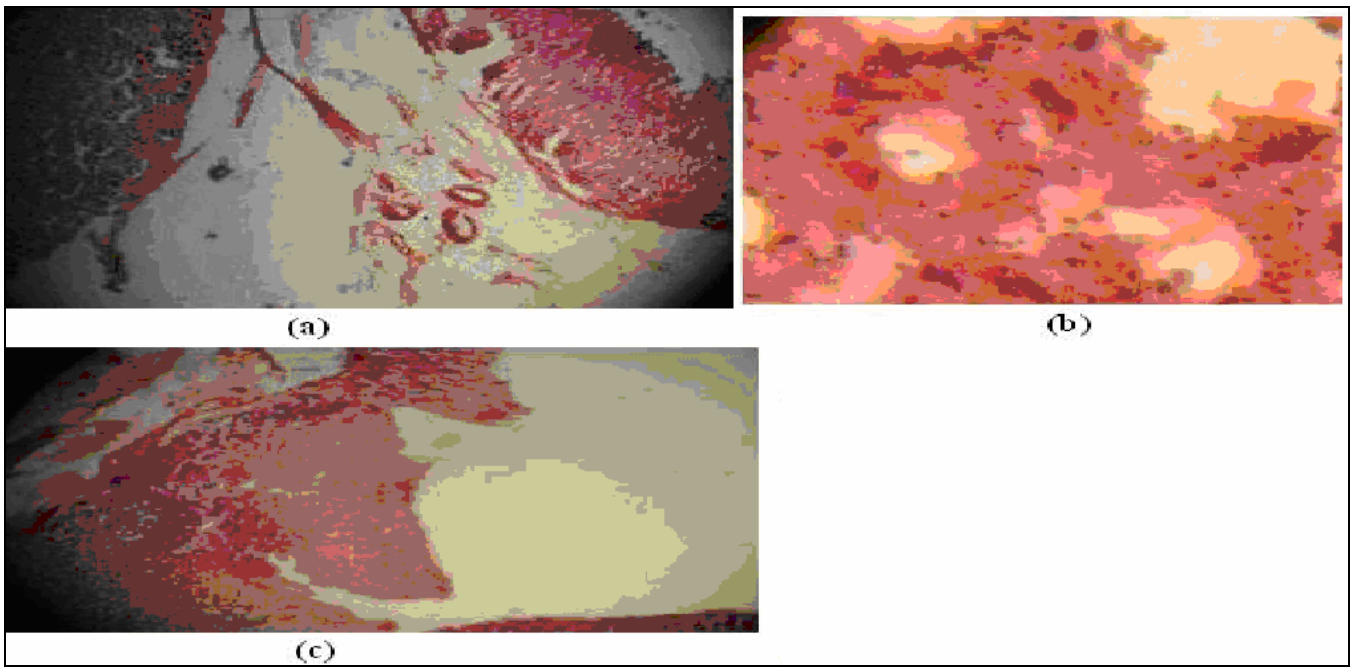

Figure 1. (a) Shows the normal histopathology of stomach of M. gerbils without any sign of ulcer without infection of H. Pylori (H.E x 100). **(b)** Shows the distorted glandular architecture with overlying the fibrous tissue with numerous H. pylori after 4 months infected M. gerbils (H.E. x 400) (control Group). **(c)** Shows the glandular architecture with marginal vein slightly inflamed but no sign of bleeding after treatment with rifabutin 6 mg/kg for 7 days of 2 months infected of M. Gerbils by H. Pylori. (H.E. x 100) (Group IV).

Helicobacter clearance study

Activities of Helicobacter pylori clearance were 7.11± 0.316 and 2.40± 0.271 at a dose of 2 and 4 mg /kg rifabutin from floating beads (formulation A1) respectively ($p < 0.05$ 2mg/kg compared to 4mg/kg)), whereas placebo group showed maximum count of Helicobacter pylori ($p<0.05$ compared to 2mg/kg and 4mg/kg treated groups). Complete eradication of Helicobacter pylori was observed at a dose of rifabutin 6 mg /kg body weight whereas rifabutin suspension of 6 mg /kg body weight did not show complete eradication of Helicobacter pylori (Table 3 and 4 and figure 1).

Table 4: Histopathological changes observed in Helicobacter pylori infected Mongolian gerbils with rifabutin therapy.

Rifabutin loaded floating beads					**Rifabutin suspension**
	Gr. I	**Gr. II**	**Gr. III**	**Gr. IV**	**Gr. V**
Dose (mg/kg)	0	2	4	6	6
Ulcer Suface area (mm2) (Mean ± SD)	**4.11±0.34**	**3.28±0.29**	**2.90±0.36**	**1.03±0.20**	**1.88±0.22**
M.Gerbils with persistant ulcers (before dosing)	6/6	6/6	6/6	6/6	6/6
M.Gerbils with healed ulcers (after treatment)	0	2/6	3/6	6/6	3/6
Microscopic presence of Helicobacter Pylori (NCTC 11637)	+++	++	+	—	+

SYTO 9 Dye Staining

In order to strengthen our findings regarding the clearance of Helicobacter pylori from infected Mongolian gerbils, we have carried out SYTO 9 staining to get information about the live intact cells before and after the treatment with rifabutin loaded floating beads. The results are depicted in figure 2.

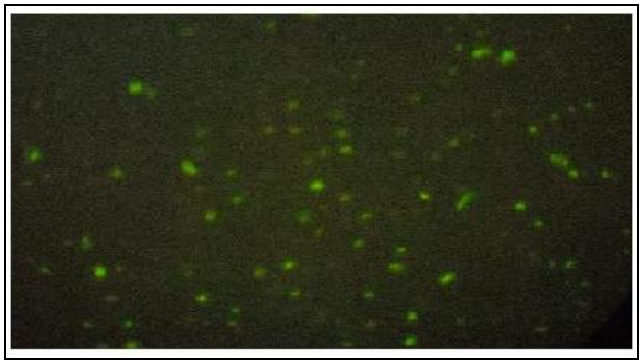

Figure 2. (a) Live staining of H. Pylori of control group shows green-stained viable cells (200x).

Figure 2. (b) Shows decreases viable of H. Pylori after treatment with rifabutin beads 6 mg/kg body weight (Group III) (200x).

Discussion

Rifabutin loaded floating gellan gum beads

The numerous pores in the bead interior may be directly related to the presence of sodium bicarbonate (figure was and is in agreement with the findings of Choi et al [8]. The increase in bead size with increasing gellan concentration could be attributed to increase in microviscosity of the polymeric dispersion, leading eventually to the formation of bigger beads [10].

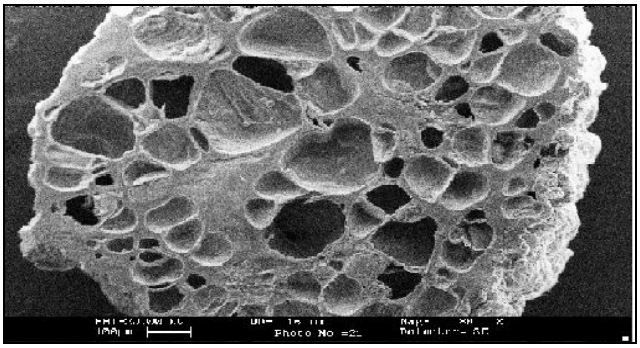

Figure.3: Scanning electron microscope images transverse section, of rifabutin- loaded bead.

The low drug entrapment efficiency of the beads may be due to the use of sodium bicarbonate as gas-generating agent, which reacting with acetic acid releases carbon dioxide. The released carbon dioxide gets entrapped in the gel network, thus increasing bead porosity and decreasing the strength of the bead wall. As a result, the drug readily diffuses out into the gelation fluid. Stockwell and Davis [11] also reported the low entrapment efficiency of alginate beads due to violent gas generation.

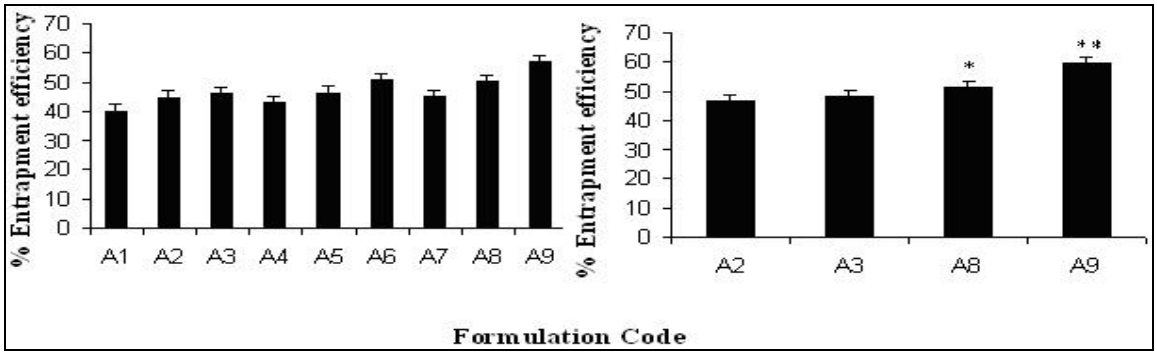

Figure.4. (a) Entrapment efficiency of floating beads. (b) Effect of increasing polymer concentration on Entrapment efficiency.A2& A8 and A3&A9 were significantly different from each other ($p< 0.05$).

The increase in the drug entrapment efficiency of beads with increasing polymer concentration may be because increase in the gellan concentration resulted in the formation of larger beads which entrapped more drug (figure 4). Furthermore, higher polymer concentration affords higher viscosity, which would hinder easy escape of the drug from the polymer matrix to the gelation medium.

Helicobacter clearance studies

Helicobacter pylori are gram-negative rods found in the human gastric mucosa and associated with digestive disease and causative bacteria of active chronic gastritis, a main factor in the peptic ulcer and a risk factor in the development of gastric cancer [12]. Since 1994 it has been considered, a class 1 carcinogen by IARC (World Health Organization). The organism is also involved in the developments of Mucosa Associated Lymphoid Tissue (MALT) and several other published reports exist which associate it with extra-digestive diseases [13, 14]. Therapeutic efficacy in Helicobacter pylori infections always has been problematic. Helicobacter pylori are microorganisms, capable of surviving in highly acidic condition. As a result, chronic infection generally develops, thus requiring prolonged therapy [15].

In the present investigation, the effects of rifabutin floating beads were investigated in Helicobacter pylori infection in Mongolian gerbils. The reason for choosing gellan gum as carrier for rifabutin was its excellent biocompatibility. Although PLGA polymers were also considered during the process of selection of polymer, as these are also biocompatible but were rejected because their biodegradation resulted in acidic components viz. lactic acid and glycolic acid and Helicobacter pylori are reported to be susceptible in acidic medium, therefore PLGA polymers were not considered for formulation development.

Our results showed that ulcer produced by Helicobacter pylori was significantly reduced in size ($p< 0.05$) after treatment of rifabutin 6 mg/ kg and bacterial count reached non detectable levels after treatment of one week. The reduction in ulcer size could be attributed to the inhibition of Helicobacter pylori due to rifabutin therapy which might have resulted in the absence of causative factors for ulcer, that is, urease and vacuolating toxin A [3]. The results obtained were well supported by findings from SYTO 9 staining which showed almost absence live Helicobacter pylori after treatment. Earlier study showed that long-term therapies are required for eradication of Helicobacter pylori but failed to reach the baseline level even after 4 weeks after treatment [15]. Therefore, the rifabutin beads showed effective treatment strategy for Helicobacter pylori infection in short- time.

Authors' contributions

All the work was carried out by the corresponding author under the supervision of the second author.

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