Studies on safety aspects of contraceptive Magainin-A in rabbits

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We have previously reported the contraceptive potential of Magainin-A in rats and rabbits under in vitro and in vivo condition. In this report we evaluated the effect of Magainin-A on the structural organisation of vaginal epithelium in rabbits. The effect of this compound on the erythrocytes and its rate of absorption and clearance from systemic circulation was also studied. The effective contraceptive dose of Magainin-A (1 mg) when administered intra-vaginally for five consecutive days did not induce any structural or morphological abnormalities in vaginal epithelial cells. No adverse effect was observed on the erythrocytes. The rate of Magainin-A absorption and clearance from the circulation was found to be rapid. These results suggest that Magainin-A may be used as a safe intra-vaginal contraceptive compound in future.

Materials and Methods

Material — Magainin-A (M 7152) was obtained from Sigma (St. Louis, MO, USA). The compound was dissolved in 0.9% saline solution just prior to use.

Animals — Twelve New Zealand white rabbits (8-10 months old, 2.4 ± 0.2 kg body weight) were used in the present study. The rabbits were housed in individual metal cages and fed high fiber rabbit formula and water ad libitum. A constant room temperature of 23 ± 1°C, 60 ± 10% RH and a 12 hr dark light cycle was maintained throughout the study.

Effect of Magainin-A on vaginal epithelium — In the first set of experiments, structural abnormalities in the vaginal epithelium was studied after repeated application of Magainin-A. Contraceptive dose of Magainin-A (1 mg) was applied to a group of six animals for five consecutive days as described earlier. The vaginal lavages were aspirated and the effect on vaginal cell morphology was observed. The does were sacrificed on day 6th after the fifth and final treatment. One ml of sterile saline was applied to the control rabbits, instead of Magainin-A. At autopsy, the vagina was slit open ventrally between urethral orifice and fornices and representative samples of proximal portion (near urethral orifice), middle portion and distal portion were taken and fixed in Bouins solution for histological evaluation. After
fixation, the specimens were embedded in paraffin, sectioned at 5 μm and stained with hematoxyline-eosin. A piece of uterine horn and/or ovary was similarly processed to exclude the presence of pseudo-pregnancy.

The vaginal sections were evaluated semiquantitatively. Four basic criteria, viz. epithelial ulceration, leucocyte infiltration, oedema and vascular congestion were considered. The magnitude of vaginal changes were rated according to the method suggested by Eckstein et al. A score of 0-11 (absent to severe) was allotted to each of them and compared with controls. A total score between 0 and 4 is considered as acceptable, 5 to 8 as marginal (either acceptable or unacceptable) and over 9 unacceptable.

In vitro effect of Magainin-A on erythrocyte hemolysis — The cytotoxicity of the Magainin-A peptide was tested on erythrocytes obtained from three animals. The peptide was found to be toxic above 200 μg/ml of blood. Therefore the effect of different concentrations (10-320 μg/ml) of Magainin-A on erythrocyte hemolysis was tested. Briefly to 75×12 mm borosilicate test tubes containing a predetermined amount of dried peptide in duplicate, 2.5 ml of diluted rabbit erythrocyte suspension (10% v/v in isotonic PBS) was added. After gentle mixing and incubation for 10 min at 37°C the tubes were centrifuged at 300 g for 10 min. The supernatant was separated from cells and debris, diluted (if necessary) and OD350 measured in spectrophotometer (UV-160A, Schimadzu, Japan). Different concentration of triton X-100 (1-4 μl/ml) was also tested under similar experimental conditions. 95% of hemolysis was observed with 4 μl of triton X-100.

Quantification of circulatory levels of Magainin-A by ELISA — In the second set of experiments after intravaginal application of 1 mg of Magainin-A, 3-4 ml of blood was collected at 0, 30, 60, 180, 360 and 1440 min. Serum was separated by centrifugation and kept at -70°C until use.

Preparation of Magainin-A conjugate and raising of anti-peptide antibodies — Magainin-A was coupled via the NH2-terminal cysteine residue to Keyhole Limpet Hemocyanin (KLH) using the water soluble heterobifunctional cross-linking reagent, maleimido-benzoyl sulfosuccinimide ester, according to the manufacturer's instructions (Pierce Chemical Co, Rockford, IL, USA). Rabbit antisera to the peptide-KLH conjugate was raised in New Zealand white rabbits. After confirming the sensitivity and specificity of the antibodies, they were utilized to measure the rate of absorption, time of retention and clearance of Magainin-A from the circulation at definite intervals of time by ELISA.

Statistical analysis — Statistical analysis of variance between control and experimental values was done by Student's t test.

Results and Discussion

Our studies show that application of Magainin-A did not cause any abnormalities on the morphology of the vaginal epithelial cells when compared to controls as observed by light microscopy. The cytological pictures observed on the fifth day after the fifth and final application of Magainin-A is presented in Fig. 1. The compound did not induce inflammatory reactions in subendothelial connective tissue of the vagina. There was no edematous thickening of the submucosal layer or infiltration of polymorphonuclear leucocytes into the mucosa (Figs. 2b, 2d, 2f) when compared to controls (Figs. 2a, 2c, 2e). The overall mean semiquantitative score of the microscopic analysis showed that these changes did not exceed the definition for acceptability (Table 1). These results suggests that Magainin-A did not cause any noticeable abnormalities in the structure of vaginal epithelium. However studies over a longer period of
time with higher doses of Magainin-A are mandatory before reaching a definite conclusion.

Further our studies indicated that at any point of time the total amount of Magainin-A absorbed and retained in the blood circulation did not exceed 2 μg/ml of blood which indicate that Magainin-A had no significant effect on erythrocyte hemolysis when tested in vitro up to 200 μg/ml of blood (Table 2).

In our earlier studies we have reported that Magainin-A is surface active and is thought to interact with sperm plasma membrane. Because of this property we have hypothesized that Magainin-A may kill the sperm by permeabilizing the plasma membrane. On the other hand our present results

Fig. 2 — Photomicrographs showing cross sections of vaginal tissue in control (a,c,e) and magainin-A treated rabbits (b, d) proximal (a,b), middle portion (c,d) and distal portion (e,f). LP = Lamina propria, SE = Squamous epithelium (magnification x 40)
indicate that Magainin-A does not have any detrimental effect on the vaginal epithelial cells. This shows the selective action of Magainin-A on different cells.

Recently, Chen and his colleagues have compared the membrane lytic activity of Magainin-A with hemolytic capacity of mellittin\(^4\). These authors further reported that the activity of Magainin-A on zwitterionic phospholipids seems to be necessary for effective action. The presence of high level of phospholipids in sperm possibly increases the susceptibility of the membrane to lysis by Magainin-A\(^\text{15}\). Tytler \textit{et al.} have reported that eukaryotic cells are resistant to lysis by Magainin-A because of peptide-cholesterol interactions in their membranes that inhibit the formation of peptide structures capable of lysis, perhaps by hydrogen bonding between glutamine and cholesterol\(^\text{16}\). It has also been reported that bacterial membranes lacking cholesterol are susceptible to lysis by Magainin-A\(^\text{17,18}\). Hence we presume that low phospholipid content on the outer cell membrane and relatively high levels of cholesterol, combined with the high transmembrane potential, contribute to the protection of RBCs and vaginal cells from Magainin-A attack. However, the precise mechanism of action of Magainin-A on these cells is not known at present. Attempts are in progress to elucidate the mode of action of Magainin-A at molecular level using various cell lines.

For the evaluation of safety of a new contraceptive formulation, it is important to examine the concentrations achieved in serum after vaginal application and the potential systemic effects in addition to any local reaction. Hence, an ELISA method was standardized to measure the systemic Magainin-A levels. The results indicated that maximum amount of Magainin-A was detected in the blood sample drawn 60 minutes after the treatment.

| Table 1 — \textit{in vivo} effect of Magainin-A on rabbit vaginal epithelium |
| Group | No. of animals | Calculation of score in sub mucosal layer epithelial line |
|       |                | Ulceration | Oedema | Leucocyte infiltration | Vascular congestion | Total score |
| Control (saline) | 2 | — | — | — | — | — |
| Treated (1 mg) | 2 | — | — | — | — | — |
|           | 2 | — | — | — | — | 1 |

Individual scoring 0-11 = Absent to severe
Total score 0-4 = Acceptable, 5-8 = Above marginal
Above 9 = Unacceptable.

<p>| Table 2 — Effect of Magainin-A on erythrocyte hemolysis |</p>
<table>
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<tr>
<th>Parameter ((\mu g/ml))</th>
<th>Trito X-100 conc ((\mu g/ml))</th>
<th>Magainin-A conc</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
<td>10</td>
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<tr>
<td>Erythrocyte Hemolysis (%)</td>
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<td>(\pm 2.30)</td>
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| Table 3 — Circulatory levels of Magainin-A in rabbits |
| Magainin-A Conc. (ng/ml) | 30 | 60 | 180 | 360 | 1440 |
| Group | No. of Animals | Time (min) | ND | ND | Time (min) | ND | ND | Time (min) | ND | ND |
| Control (Saline) | 3 | ND | ND | ND | ND | ND | ND |
| Treated (1 mg) | 2 | 342 \(\pm 21.67\) | 1267 \(\pm 46.39\) | 439 \(\pm 20.67\) | 263 \(\pm 19.97\) | ND |
|           | 2 | 297 \(\pm 19.14\) | 1094 \(\pm 51.45\) | 515 \(\pm 23.94\) | 307 \(\pm 23.26\) | ND |
|           | 2 | 384 \(\pm 20.66\) | 1026 \(\pm 59.08\) | 466 \(\pm 32.73\) | 256 \(\pm 19.87\) | ND |

ND = Not detected
There was still a detectable concentration at 6 hr which thereafter declined rapidly and reached a baseline level by 24 hr. This rapid absorption of Magainin-A may be attributed to its direct entry into the systemic circulation without passing through the liver. In general these results attributed that there are no apparent systemic effects following the application of Magainin-A.

It is well documented that frog skin is the richest source of Magainin-A and has potent anti-bacterial activity\textsuperscript{10}. It is also known to facilitate wound closure and reduce inflammation\textsuperscript{10, 19}. Magainin-A thus has a unique advantage that may greatly enhance its prophylactic capability against conventional sexually transmitted diseases.

From the above study, it may be concluded that Magainin-A is safe to use intravaginally. It seems to be a highly promising and safe vaginal contraceptive compound for future use in human.

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References