Research Article

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Evaluating the spermicidal activity of an antimicrobial peptide from the *Bufo kavirensis*, MaximinBk: in vitro study

[Bufo kavirensis’ten elde edilen MaximinBk’in in vitro çalışmalarda antimikrobiyal peptidinin sperm öldürücü aktivitesinin değerlendirilmesi]

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Abstract

Objective: The aim of this study was to evaluate the spermicidal activity of this peptide to introduce a new potent agent for prevention of sexually transmitted infections and unplanned pregnancies.

Methods: The purified MaximinBk (with amino acid sequence: ILGPVLGLVGRLAGGLIKRE) was diluted with Ham’s F10 solution in 100, 200, 400, 600, 800 and 900 μg/mL. One milliliter from peptide solution with different dosage was mixed with 200 μL prepared sperm solution in microtube. Sperm motility, viability and morphology were assessed at different time intervals (0.3, 5, 10, 15 min). Eosin–Nigrosin staining and Giemsa staining methods were applied for sperm viability and morphology detection, respectively.

Results: Total spermicidal activity was shown after addition of 900 μg/mL for 0.3 min without any morphological change in the sperm head, midpiece or tail. Also, Eosin–Nigrosin staining indicated MaximinBk can disturb membrane integrity of normal sperm that is in dose-dependent manner.

Conclusion: MaximinBk has spermicidal activity in addition to antimicrobial activities (especially on vaginal infections such as candidal vulvovaginitis). It seems this peptide might be a potential candidate in order to use in male contraception, although, this preliminary study needs more studies to elucidate final conclusion.

Keywords: Antimicrobial; Candidal vulvovaginitis; MaximinBk; Sexually transmitted infections; Spermicidal.

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Özet

Amaç: Bu çalışmaların amacı, cinsel yolla bulaşan enfeksiyonların ve planlanmamış gebeliklerin önlenmesi için yeni bir potensiyel ajan ile bu peptitin sperm öldürücü aktivitesini değerlendirilmektir.

Yöntemler: Sfaşırlımsız MaximinBk (amino asit dizisi: ILGPVLGLVGRLAGGLIKRE ile), 100, 200, 400, 600, 800 ve 900 μg/mL’de Ham’s F10 çözeltisi ile seyreltildi. Farklı dozajlı peptit çözeltisinden 1 mL, 200 μL mikrotüp içinde hazırlanmış sperm çözeltisi ile karıştırıldı. Sperm
motilitiesi, canlılığı ve morfolojisi, farklı zaman aralıklarında (0.3, 5, 10, 15 dakika) değerlendirildi. Sırıstıla, sperm canlılığı ve morfolojisi için Eosin-Nigrosin boyama ve Giemsa boyama yöntemleri uygulandı.

Sonuçlar: Toplam sperm öldürücü aktivite, sperm başı, orta parça veya kuyruktu herhangi bir morfolojik değişiklik yapılmaksızın 0.3 dakika boyunca 900 μg/mL ilave edildiken sonra gösterildi. Ayrıca, Eozin-Nigrosin boyama, MaximinBk’ın normal spermlerin membran bütünliğinin doz bağımlı biçimde bozabileceğini gösterdi.

Sonuç: MaximinBk, antimikrobiyel aktiviteleri ek olarak (özellikle de kandidal vulvovaginitis gibi vajinal enfeksiyonlarda) sperm öldürücü aktiviteye sahiptir. Bu peptid, erkek kontrasepsiyonu kullanılmak için potansiyel bir aday olabilir gibi görünse de, bu ön araştırmanın nihai sonucu aydınlatmak için daha fazla çalışma ihtiyacı vardır.

Anahtar Kelimeler: Antimikrobiyal; Candidal vulvovaginitis; MaximinBk; Cinsel yolla bulaşan enfeksiyon; Spermisidal.

Introduction

Sexually transmitted infections and unplanned pregnancies are two great concerns in the reproductive health. Many agents such as octoxynol, nonoxynol, chlorhexidine and benzalconium chlorides are used for prevention of pregnancy [1, 2]. Most of these agents are detergent such as nonoxynol-9 (N-9) and benzalconium chlorides [2, 3]. Different studies have showed that detergent spermicides are not able to inhibit transmigration of infection in addition to have side effects such as detergent-like action on epithelial cells and normal vaginal flora [4–6]. So, it is necessary to develop other agents with an antimicrobial activity in order to reduce the risk of sexually transmitted diseases. In between, some of antimicrobial peptides (AMPs) have the suitable properties, e.g. spermicidal and antimicrobial activities. AMPs are one of the important components in innate immune system in all of living organisms from bacteria to humans [7]. These components have broad antimicrobial activities against different pathogens such as gram positive and gram negative bacteria, fungi, viruses, protozoa [7–11]. These natural compounds also show other biological activities such as anticancer, insecticidal and chemotactic activity [12, 13]. A few peptides have been found to be cytotoxic to sperm cell. Until now, nine AMPs with spermicidal activity were recorded in AMP database (http://aps.unmc.edu/AP/database/antiS.php). Five peptides from these nine peptides were isolated from different amphibians. Amphibian skin has glands that are rich resources for AMPs [14, 15]. Different AMPs such as bereninis, temporins, magainin and dermaseptins have been purified from different amphibians [16–22].

*Bufokavirensis* is one of toad that lives in Kavir desert in Iran. To the best of our knowledge, no studies have examined the skin secretions of this species [20]. MaximinBk is new cationic AMPs that have been identified in the skin secretion of *Bufokavirensis* that introduced in our previous study. We showed that this peptide can have antimicrobial activity and according to this study, collection of MaximinBk was done by electrical stimulation of dorsal part of toad. Peptide purification was done by according to our previous study [23]. The MaximinBk has considerable antimicrobial activity against gram positive and gram negative bacteria as well as fungi with minimum inhibitory concentrations (MIC) values in range of 8.1–20.78 μg/mL and 25.7–35.6 μg/mL, respectively [23]. Due to suitable biological activities of MaximinBk, especially vaginal pathogen, the aim of this study was to investigate spermicidal activity of this peptide.

Materials and methods

Semen collection, preparation and analysis

Ten normal fresh normal semen samples were obtained from men aged between 28 and 34 who were referred to Yazd research and clinical center for infertility work-up. Indeed, the normal semen samples among the original samples of the patients referred to our clinic for infertility work-up were met inclusion criteria. Normal semen was defined as follow: sperm concentration (10^6 per mL) > 15, progressive motility (%) > 32, total motility (progressive + non-progressive, %) > 40, sperm vitality (live spermatozoa, %) > 58, sperm morphology (normal forms, %) > 4. Written informed consent was obtained from all individuals. Semen samples were collected by masturbation in sterile containers after 2–7 sexual abstinence days. The samples were liquefied at room temperature (22–24°C) for 30 min. Semen analysis was performed according to WHO guidelines [24]. Sperm preparation was done by direct swim-up which was previously described [25]. Makler chamber and phase contrast microscopy were used for sperm count and motility. Motility was reported as progressive percentage. Eosin–Nigrosin method was applied for evaluation of sperm viability. The dead spermatozoa were stained in red while viable spermatozoa remained unstained (white). At least 200 sperm cells were evaluated using light microscope (1000×) and viability rate was reported as percentage. Giemsa staining was
used in order to detect any types of sperm abnormal morphology in head, mid piece and tail. The 200 μL from prepared sperm solution was mixed with peptide solution. The main goal is to evaluate the spermicidal activity of peptide. So, the sperm number was not adjusted accordingly. This 200 μL from prepared sperm solution was similar for any concentration of peptide.

**Preparation of peptide**

Serial dilutions of new peptide were obtained by Ham’s F10 solution in 0–900 μg/mL. The spermicidal action was determined on spermatozoa separated from seminal plasma by studying the effect of various concentrations of MaximinBk on sperm motility evaluated by the Sander and Cramer test [26]. Each sample was examined using different MaximinBk concentrations at different time intervals. Briefly, 1 mL from peptide solution with different dosage was mixed with 200 μL from prepared sperm solution in microtube. Sperm motility, viability and morphology were assessed 20 s, 5, 10 and 15 min later. The weakest concentration (or highest dilution) that completely immobilized all the spermatozoa within 20 s was recorded as EC100. Commercial N-9 (100 μg/mL) was used as positive control.

**Statistical analysis**

Data were expressed as means±SD. For statistical analysis of data, repeated measure was employed. Differences were considered statistically significant if p < 0.05.

**Result**

**The assay of spermicidal activity**

**Sperm viability and morphology**

At first we tested the Sphericity assumption using Mauchly’Sphericity test. The Sphericity assumption was not satisfied (p < 0.001) so we used the results of Greenhouse-Geisser test to evaluate the effect of Within-Subject Factor. It showed that the interaction effect of time and MaximinBk on sperm viability was significant (p < 0.001). So the changes in dependent variable would be affected by time variations. The treatment of human sperm with peptide at EC100 had a potent spermicidal effect. As it was shown in Figure 1, the effect of peptide on sperm motility and viability showed dose dependent manner which

![Figure 1](image-url)
Sperm motility analysis

As mentioned before, we tested the Sphericity assumption and it was significant \((p < 0.001)\). The Greenhouse-Geisser test for evaluating the Within-Subject Factor effect showed a significant effect of both time and MaximinBk on sperm motility. The inhibitory effect of this peptide on sperm motility is shown in Figure 2. This compound immobilized sperm with its effective dose only within 20 s (EC100) at a concentration of 900 μg/mL. It was also observed that with increasing in concentration or increasing in time at a specific concentration, there was significant suppression of motility. It was found that the effect of peptide on sperm motility and viability was dose dependent \((p\text{-value} = 0.000)\).

Discussion

Today, rapid growth of population is one the major problem in the least developed countries [27]. So, various agents have been developed to prevent unplanned pregnancies. In addition, sexually transmitted infections are the other important concerns in reproductive health. Thus, none of developed agents can be considered as ideal [28]. Discover of new products that have both activities e.g. antimicrobial properties to reduce sexually transmitted infections and spermicidal activity to prevent pregnancies, is an important challenge [29]. There were many efforts to develop and discover new agents for these purposes. In between, natural products such as different plant extract, natural peptides, essential oil are suitable candidate [29–35].

In the current study, we investigated the spermicidal activity of natural AMPs (MaximinBk) that was purified from the *Bufo kavirensis*. Using Eosin–Nigrosin method showed MaximinBk can affect membrane integrity irreversibly that was dose-dependent so that in dose 900 μg/mL for 0.3 min it can kill the sperm completely.

The results in previous study showed that MaximinBk has potent antimicrobial activities against the tested microorganisms including Gram-positive and Gram-negative bacteria and fungi. This peptide also showed considerable antimicrobial activity against microbes that participate on sexually transmitted infections, e.g. *Escherichia coli* and *candida albicans* at low concentrations (≤ 21 μg/mL, ≤ 36 μg/mL, respectively) [23]. But, this concentrations of antimicrobial peptide inhibits the bacterial or fungal growth after 24 h at 37°C may not have negative

Figure 2: Effect of different concentrations of MaximinBk on spermatozoa motility. Each sample was examined using different MaximinBk concentrations. Data represent means of motility of 10 samples at different time intervals.
effect on normal vaginal flora in a short time applied as a spermicidal agent. However, this is an in vitro study and it is recommended to assess further in vivo evaluations on the effects of different doses of MaximinBk on normal vaginal flora.

Vaginal infections are the most common gynecological complaint in all of world and many women suffer this problem. There are several types of vaginal infection. Some vaginal infections are caused by bacteria. By studying activity of this peptide in in vitro, results showed that this peptide has especially potent antimicrobial activity on *candida* [32]. This fungus is the one important pathogen that induces the vaginal infections. Candidal vulvovaginitis or vaginal thrush is the one of infection of the vagina’s mucous membranes by *Candida albicans* [36]. This fungus is second factor that cause of vaginal infection. MaximinBk peptide can inhibit completely growth of *candida* at 32.1 μg/mL in in vitro according to previous study [32].

The mechanism of spermicidal activity of MaximinBk is not known yet; but, according to previous studies [29, 37, 38], since this peptide has positive net charge, perhaps this positively status cause this peptide preferentially interact with anionic phospholipids. The structure of MaximinBk was predicted based on the structure of Maximin 7 (this peptide has the highest similarity to MaximinBk) and helical wheel was obtained. According to predicted structure (Figure 3), its side chains are oriented in an amphiphilic arrangement, with all charged residues separated to one face of the helix (The total hydrophobic ratio = 50% and the total net charge = +2). This new peptide may form α-helices with at least 10 residues on the same hydrophobic surface. The sperm membrane has high concentration of phosphatidyl glycerol (a strong anionic phospholipid). So, MaximinBk maybe interact with spermatozoa with high affinity which leads to the loss of permeability of the plasma membrane. Most of spermicidal agents disturb the natural vaginal milieu and so, they could be toxic for vagina [2]. Thus, development and discover of agents that have both effective antimicrobial and spermicidal activity without causing vaginal toxicity would be necessary. MaximinBk inhibited motility and viability of spermatozoa at concentration of 900 μg/mL, however, this concentration is high in comparison with other agents, but low level of cytotoxicity of MaximinBk on vagina would be worthwhile. On the other hand, researchers showed that increasing the number of positive charges of the peptide resulted in a reduced cytotoxicity without affecting the spermicidal effect [29]. The spermicidal activity of MaximinBk is lower than different agents such as *Azadirachta indica* (neem) leaf extract [33], seaweeds [39]. Since MaximinBk has a positive net charge, its may be that this peptide has low cytotoxicity. Zaïri et al. showed that the change of amino acid sequence of antimicrobial peptides (especially in positive charge and hydrophobicity of peptide) was affected on spermicidal and antimicrobial activities as well as cell toxicity. They optimized the biological activity of Dermaseptin S₄ by change of peptide sequence [29, 40].

The results in this study showed that MaximinBk has potent and irreversible spermicidal activity against human sperm (EC100 at a concentration of 900 μg/mL). This peptide showed antimicrobial activity at low concentration and its spermicidal activity occurs at very higher concentration. So, for acquisition of both purposes (microbicidal and spermicidal actions), it is necessary to use high concentration of peptide (more than 900 μg/mL). This is one challenge for use of MaximinBk, because this peptide could also effects on normal vaginal flora and mucosa.

Based on to this challenge and Zaïri et al. study, the biological effects of MaximinBk (antimicrobial and spermicidal activity) could be enhanced by changing the amino acid sequence and artificially synthesizing those derivatives. This changes maybe be especially useful for reduction of required concentration for spermicidal activity because the concentration required for spermicidal activity are high in comparison with concentration required for antimicrobial activity. So we suggested that synthesize and examine such derivatives from MaximinBk.
in order to improve its biological activity in future studies. The future studies in this way can be improve the positive attributes and reduce the negative activities of MaximinBk.

It is noteworthy that the identified peptide with wide-spectrum antimicrobial and spermicidal activity may be beneficial in the contraception, but this preliminary study needs more detailed researches assay such as evaluating cytotoxicity of MaximinBk, preserve their activity and lack of toxicity when tested in vivo or in clinical trials and modify it in order to improve spermatozoa immobilization in minimum concentration and time. Also, investigation of its ability to be utilized in combination with currently available contraceptives, probably having side effects seem be required.

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