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Research Article

In Vitro Effects of Linamarin, Amygdalin and Gossypol Acetic Acid on Hyaluronidase Activity, Sperm Motility and Morphological Abnormality in Bull Sperm

Sadettin TANYILDIZI
Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Fırat University, 23119, Elazığ - TURKEY
Tanzer BOZKURT
Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Fırat University, 23119, Elazığ - TURKEY

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Abstract: : In vitro effects of various doses of linamarin, amygdalin and gossypol acetic acid on sperm hyaluronidase activity and the percentages of motile spermatozoa and morphology were investigated in bull sperm. Holstein bulls (n = 12) aged between 2 and 3 were used in the study. Semen samples were collected from each animal 3 times and used for incubation. The samples were divided into 5 equal parts and mixed (v/v) with linamarin at doses of 0.5, 0.75, 1 and 2 µM, with amygdalin at doses of 0.4, 0.8, 1 and 2 µM and with gossypol acetic acid at doses of 2.5, 5, 10 and 20 µM. The colorimetric method based on the liberation of saccharides with N-acetylglucosamine end – groups from hyaluronic acid was used for the determination of semen hyaluronidase activity in this study. Sperm motility and morphology were assessed under a phase-contrast microscope.

The incubation of the 3 compounds with sperm caused significant decreases in spermatozoa motility ($P < 0.001$) and hyaluronidase activity ($P < 0.01$) compared with the control group. Additionally, gossypol acetic acid produced a significant ($P < 0.001$) increase in the rate of abnormal spermatozoa during the incubation period. These results showed that linamarin, amygdalin and gossypol acetic acid have deleterious effects on bull spermatozoa in a dose - dependent manner.

Key Words: Cyanogenic glycosides, hyaluronidase, motility, bull, semen.

Linamarin, Amigdalin ve Gossipol Asetik Asit'in Boğa Spermasının Hiyaluronidaz Aktivitesi, Sperm Motilitesi ve Morfolojik Anormallik Üzerine Olan İn Vitro Etkileri

Özet: Bu çalışmada, farklı dozlardaki linamarin, amigdalin ve gossipol asetik asit'in in vitro olarak boğa spermi hyaluronidaz aktivitesi, motilitesi ve anormal spermatozoa yüzdeleri üzerine olan etkileri araştırıldı. Çalışmada 2 ile 3 yaşları arasında 12 adet Holştayn ırkı boğa kullanıldı. İnkubasyonlar için kullanılacak olan sperma örnekleri her hayvandan üç kez alındı. Numuneler 5 eşit kısma ayrıldı ve 1:1 (v/v) oranında, 0,5, 0,75, 1 ve 2 µM linamarin, 0,4, 0,8, 1 ve 2 µM amigdalin ve 2,5, 5, 10 ve 20 µM gossipol asetik asit solusyonlarıyla karıştırıldı. Bu çalışmada hyaluronik asitten son ürün olarak sakkaritler ve N-asetilglukozamin ayrışması esasına dayanan kolorimetrik metot kullanıldı. Sperm motilitesi ve morfolojisi ise faz-kontrast mikroskop yardımıyla tayin edildi.

Üç bileşiğin boğa spermi ile inkübasyonu, hyaluronidaz aktiviteleri ($P < 0,01$) ile sperm motilitelerinde ($P < 0,001$) kontrol grubuna göre önemli oranda azalmalara neden oldu. Ayrıca, gossipol asetik asit, inkübasyon periyodunda anormal spermatozoa oranını da önemli ($P < 0,001$) oranda artırdı. Bu sonuçlar, linamarin, amigdalin ve gossipol asetik asit'in boğa spermi üzerinde doza bağımlı olarak zararlı etkilere neden olduğunu göstermektedir.

Anahtar Sözcükler: Siyanojenik glikozitler, hyaluronidaz, motilite, boğa, sperma

Introduction

Linamarin is a cyanogenic glycoside found in a variety of plants including cassava (*Manihot esculenta*), lima beans (*Phaseolus lunatus*), bird's foot trefoil (*Lotus corniculatus*), white clover (*Trifolium repens*) and alfalfa (*Medicago sativa*) (1,2). These plants and their preparations are commonly consumed by ruminants. Additionally, amygdalin is a major component of apricot kernels, bitter almonds and peach, plum, pear and apple seeds (3,4). These cyanogenic plants are not normally

used for food but have caused accidental poisoning of animals. The amount of cyanogenic glycosides in plants varies with plant species and environmental effects (5). For example, apricot seeds and bitter almond contain approximately 20-80 µmol/g and 100 µmol/g of amygdalin, respectively. Additionally, air-dried lima bean seeds contain 70-100 µmol/g of linamarin (3). This glycoside is absorbed unmetabolized in the jejunum of the rat via the transport system of glucose to the blood and is then concentrated in the spleen, liver, kidney, stomach and intestines (6,7). After oral administration of amygdalin and linamarin, both glycosides are hydrolyzed by ruminal microorganisms and released benzaldehyde, glucose and cyanide. Both glycosides and released cyanide have toxic effects on animals (1,4). There is no published record about the effects of amygdalin and linamarin on hyaluronidase activity, spermatozoa motility and morphological abnormality.

Gossypol is a polyphenolic coloring agent present in the seed of the cotton plant (*Gossypium hirsutum*) (8). Ciereszko and Dabrowski (9) revealed that gossypol acetate inhibits the sperm motility and sperm fertilizing ability of yellow perch when used in vitro. Hyaluronidase enzyme is released from the head of the sperm during acrosomal reaction and assists the penetration of sperm through the cumulus oophorus matrix during fertilization (10,11). Hirayama et al. (12) claimed that low hyaluronidase activity in the acrosome may cause a decrease in the fertilizing capability of sperm. For these reasons, in vitro effects of 3 toxins on sperm motility, morphological abnormality and hyaluronidase activity in bull sperm were investigated.

Materials and Methods

Chemicals

Linamarin (95% pure, mol. wt. 247.2), D-amygdalin (99% pure, mol. wt. 457.4), gossypol acetic acid (95% pure, mol. wt. 578.6) and other chemicals were purchased from Sigma Co. (St. Louis, MO, USA).

Animals and Semen Collection

Twelve, healthy Holstein bulls, aged between 2 and 3 years, were used. The bulls were fed on grass and corn silage. Water and food were provided ad libitum. Semen samples were collected from all bulls using an artificial vagina (13). For all incubations, 1 ml of semen + 1 ml of glycoside solution were used. The bull semen had a sperm concentration of $1.3 \pm 0.2 \times 10^9$ spermatozoa/ml.

The determination of sperm motility and morphological abnormality

Sperm motility was assessed subjectively under a phase-contrast microscope, in a 20 µl aliquot diluted with sodium citrate (3%). This requires a dilution rate of about 1 to 100 for bull semen, at x400, together with a heated stage adjusted to 37 ºC. Motility estimations were performed from 3 different fields in each sample. The mean value from 3 successive estimations was used as the final motility score. Sperm counts were taken using a hemocytometer. Additionally, the rate of abnormal spermatozoa was determined from slides prepared with Indian ink, and a total of 300 sperm cells were counted on each slide under phase-contrast microscope at x100 magnification (13).

The incubations of linamarin and amygdalin with semen

Linamarin solutions at concentrations of 0.5, 0.75, 1 and 2 µM and amygdalin solutions at doses of 0.4, 0.8, 1 and 2 µM were prepared in isotonic saline. Low concentrations of linamarin and amygdalin were used for these experiments, as these glycosides are found in low concentrations in plant tissues (between 1 and 100 µM/g) (3). Linamarin and amygdalin solutions were mixed (v/v) with semen samples and incubated at 37 °C in air. Then spermatozoa motilities were determined at 1, 2, 5 and 10 min.

The incubation of gossypol acetic acid with semen

Gossypol acetic acid was dissolved in 10 mM ethanol prepared with isotonic saline and kept at 0 °C (9). Before use, it was prepared at concentrations of 2.5, 5, 10 and 20 µM. Control sperm also contained a solution of 1% ethanol. Both control and gossypol solutions were mixed with sperm at a rate of 1:1 and incubated at 37 °C in air and then spermatozoa motilities were determined at 5, 10, 20 and 40 min.

Semen samples were mixed (v/v) with the same doses of linamarin and amygdalin solutions at 37 °C in air. Control samples of both compounds contained only saline solution. In addition, the solutions of gossypol acetic acid were dissolved in 10 mM ethanol prepared with saline solution at the same concentrations and incubated with sperm samples at 37 °C in air. Control sperm of the

gossypol groups contained 1% ethanol solution. To evaluate sperm morphology Indian ink was used according to the methods described by Bearden and Fuquay (13).

Enzyme Assay

Hyaluronidase activity was performed according to the colorimetric technique of Wilkinson et al. (14) and Joyce et al. (15). Semen samples were centrifuged at 600 g for 5 min to separate sperm cells, which were washed 3 times with saline solution, and enzyme from spermatozoa was extracted in Triton X-100 (0.1%, v/v in 0.15 M saline solution). The methods are based on the liberation of glucuronic acid and N-acetylglucosamine from hyaluronic acid by hyaluronidase enzyme. The N-acetylglucosamine is quantitated by heating with alkaline tetraborate to form an intermediate that reacts with p-dimethylaminobenzaldehyde in acidic medium to form a colored product. The absorbance of the colored product was read at 582 nm within 30 min using a spectrophotometer. Hyaluronidase activity was expressed as the mean µmol NAG/min/l. After treatments of semen samples with linamarin, amygdalin and gossypol acetic acid, hyaluronidase activity of sperm samples was measured at 5 min.

Statistical Analyses

Results are expressed as mean ± SEM. Chi-square analysis was used to determine differences in the percentages of spermatozoa motilities and morphological abnormality between the control and treatment groups. Additionally, the non-parametric Mann-Whitney U-test was applied to determine statistically significant differences between the control and treatment groups. All analyses were carried out using SPSS (Windows 6.0).

Results

Semen characteristics were determined prior to initiation of treatment. We determined an average (mean ± SEM) sperm concentration of 1.3 ± 0.2 x 10^9 spermatozoa/ml; the mean spermatozoa motility was 75 ± 2.11% and the ejaculates had a volume of 6.5 ± 0.4 ml. The mean value of sperm pH was 6.9 ± 0.2. During the incubation period, significant decreases were detected in the percentage of sperm motility and the hyaluronidase activity in the experimental groups.

The effects of linamarin and amygdalin on hyaluronidase activity of sperm, sperm motility and morphology

The treatment of semen samples with linamarin and amygdalin significantly ($P < 0.01$) inhibited hyaluronidase activity of sperm when compared with the control group (Figures 1 and 2). After the incubation of linamarin and amygdalin, the spermatozoa motility decreased very significantly ($P < 0.001$) in a dose-dependent manner, and all spermatozoa were immobile at 10 min (Figures 3 and 4). In addition, the percentages of morphological abnormality did not change in comparison with the control group. The control values were between 4.21% and 6.87%.

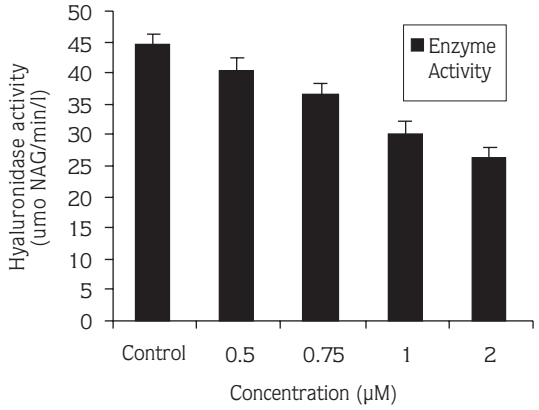

Figure 1. Hyaluronidase activity of sperm samples at 5 min, after incubation of linamarin.

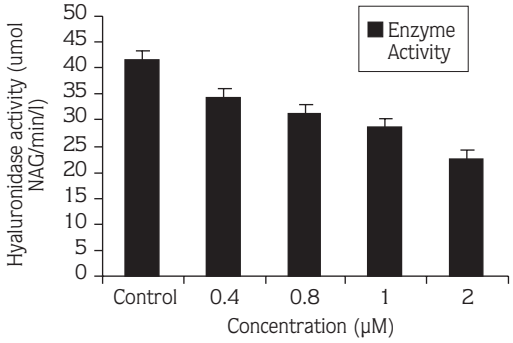

Figure 2. Hyaluronidase activity of sperm samples at 5 min, after incubation of amygdalin.

The effects of gossypol acetic acid on hyaluronidase activity of sperm, motility and morphology

The sperm hyaluronidase activities decreased rather significantly ($P < 0.01$) when compared with the control

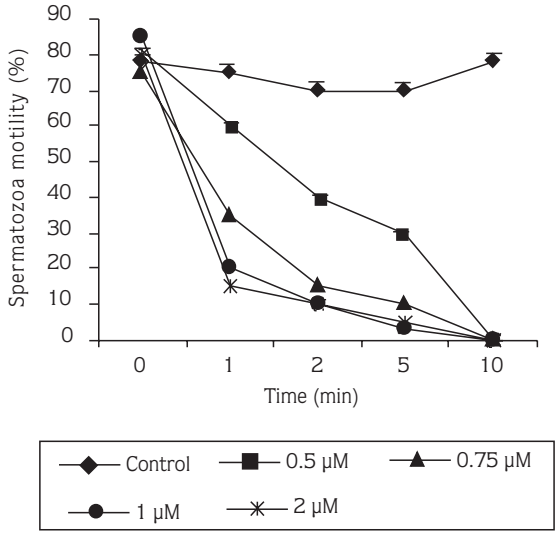

Figure 3. The percentages of motile spermatozoa of Holstein bulls, after treatment with linamarin.

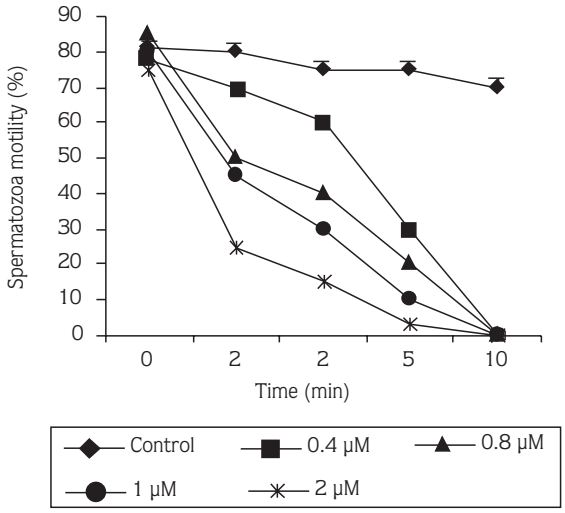

Figure 4. The percentages of motile spermatozoa of Holstein bulls, after treatment with amygdalin.

samples at all doses except for 2.5 µM (Figure 5). Additionally, significant ($P < 0.001$) decreases were observed in the percentages of sperm motility incubated with gossypol acetic acid (Figure 6). At microscopic examination, significant ($P < 0.001$) increases were observed in the percentages of morphological abnormality in the tails of spermatozoa (from 4.65 ± 0.18% to 48.82 ± 3.41%) in the gossypol groups when

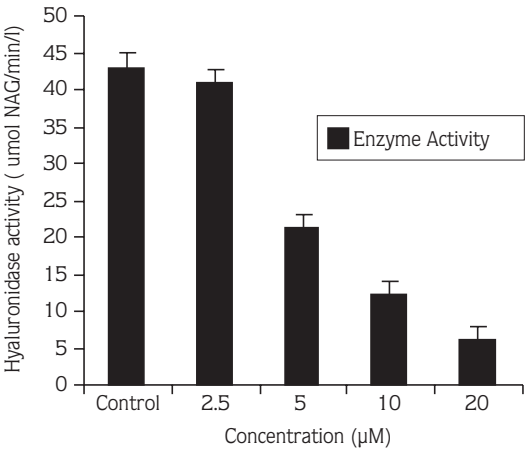

Figure 5. After incubation of gossypol acetic acid at different doses, hyaluronidase activities of semen samples at 5 min.

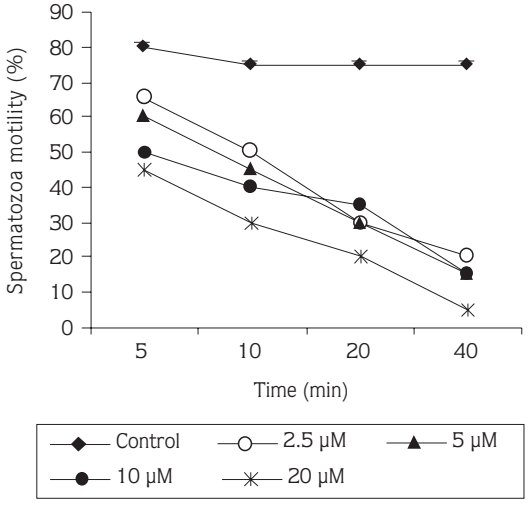

Figure 6. The percentages of motile spermatozoa in Holstein bulls, after treatment with gossypol acetic acid.

compared with the control group. All spermatozoa treated with gossypol acetic acid showed deflagellation, coiling of the tail and shoehook tail.

Discussion

A number of cultivated forages, including white clover, cassava, bird's foot trefoil and roseaceous species, consumed by ruminants contain the cyanogenic glycosides linamarin and amygdalin (1,3). This is the first study on the effects of linamarin and amygdalin on spermatozoa. It

has been reported that gossypol crosses the blood–testes barrier (16). However, it is not known whether amygdalin and linamarin cross this barrier. It has been reported that hyaluronidase enzyme plays an important role in supporting sperm penetration into the cumulus oophorus matrix (17). As shown in this study, hyaluronidase activities were inhibited significantly by low concentrations of linamarin ($P < 0.001$) and amygdalin ($P < 0.01$) (0.4 to 2 µM). Additionally, gossypol at concentrations of 5, 10 and 20 µM caused significant ($P < 0.01$) decreases in hyaluronidase activity. However, linamarin and amygdalin did not produce any morphological abnormality in bull spermatozoa. The inhibition of sperm hyaluronidase activity and spermatozoa motility showed that these compounds have deleterious effects on bull sperm in vitro. Nevertheless, more detailed studies are required to understand the modes of action of linamarin, amygdalin and gossypol on bull spermatozoa as in vitro and in vivo.

It has been reported that bull spermatozoa heads contain a beta-type DNA polymerase enzyme (18) and that this enzyme is a required DNA replication, repair and cell-cycle checkpoint control in eukaryotic cells (19). Additionally, Mizushina et al. (20) noted that amygdalin glycoside dose-dependently inhibited the activity of rat DNA polymerase beta. The present findings suggest that spermatozoa motilities were inhibited significantly at low concentrations by linamarin ($P < 0.001$) and amygdalin ($P < 0.01$) (0.4 to 2 µM) and that all spermatozoa lost their motilities and were immobile at 10 min in a dose-dependent manner. The decrease in spermatozoa motilities may indicate that the activity of DNA polymerase beta enzyme in bull spermatozoa was inhibited by amygdalin and linamarin. This conclusion is supported by Fujisawa et al. (21) who suggested that the activities of DNA polymerase alpha, beta and gamma were significantly lower in infertile men than in normal controls.

The inhibitory effects of gossypol acetic acid on the motility of mouse, rat, human, dog and monkey spermatozoa are known (22,23). It has been reported that gossypol inhibits sperm motility by blocking ATP production and utilization (24). Additionally, the activity of Mg-ATPase and Ca-Mg-ATPase and calcium uptake by plasma membranes in ram and bull spermatozoa were inhibited by 10 µmol of gossypol (25). These findings indicate that gossypol acetic acid inhibits bull spermatozoa motility in a dose- and time-dependent manner depending on the elevation of percentages of morphological abnormalities in the tail region. Additionally, the inhibition of spermatozoa motility after gossypol treatment may be due to the inhibition of ATP utilization, ATPase activity and calcium uptake by plasma membranes in the spermatozoa. Haffer (26) suggested that abnormal spermatozoa were due to ultrastructural defects, mainly in the mitochondrial sheath, in rats fed with gossypol. High concentrations of long-term exposure to gossypol cause morphological abnormalities in spermatozoa, including mitochondrial damage to the flagella and damage to the sperm acrosome (27). Significant ($P < 0.01$) increases were noted in the percentage of abnormal spermatozoa after incubation of gossypol acetic acid. Morphological abnormalities in the tail (from 4.65 ± 0.18% to 48.82 ± 3.41%) may cause a decrease in male fertility. Ciereszko and Dabrowski (9) reported that the spermatozoa motility of yellow perch decreased significantly at gossypol concentrations of 50 µM and higher. The results of this study indicated that bull spermatozoa motilities were reduced significantly at concentrations of 2.5 µM and higher. This variation may be due to the greater susceptibility of bovine spermatozoa to gossypol than that of fish sperm.

The acrosomal reaction is a vital factor in fertilization due to the release of hyaluronidase and other acrosomal enzymes (11,28). Hyaluronidase enzyme has an important role in the penetration of spermatozoa into the cumulus matrix (9). As shown in this study, treatment with linamarin, amygdalin and gossypol acetic acid significantly inhibited sperm hyaluronidase activity. The inhibition of hyaluronidase activity can cause a drop in the fertilization ability of bull spermatozoa due to the prevention of acrosomal reaction. For this reason, the measurement of hyaluronidase activity may be used for assessing the fertilizing ability of males.

In conclusion, linamarin, amygdalin and gossypol have toxic effects on bull sperm. The deleterious effects of linamarin, amygdalin and gossypol on hyaluronidase activity, spermatozoa motility and sperm morphology indicate that the fertilizing ability of bull spermatozoa can be inhibited by the excessive consumption by bulls of diets containing cyanogenic plants and cotton seed.

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