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In vitro Effects of Amygdalin on the Functional Competence of Rabbit Spermatozoa

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***Abstract*—**The present *in vitro* study was designed to reveal whether amygdalin (AMG) is able to cause changes to the motility, viability and mitochondrial activity of rabbit spermatozoa. New Zealand White rabbits (n = 10) aged four months were used in the study. Semen samples were collected from each animal and used for the *in vitro* incubation. The samples were divided into five equal parts and diluted with saline supplemented with 0, 0.5, 1, 2.5 and 5 mg/mL AMG. At times 0h, 3h and 5h spermatozoa motion parameters were assessed using the SpermVision™ computer-aided sperm analysis (CASA) system, cell viability was examined with the metabolic activity (MTT) assay, and the eosin-nigrosin staining technique was used to evaluate the viability of rabbit spermatozoa. All AMG concentrations exhibited stimulating effects on the spermatozoa activity, as shown by a significant preservation of the motility ($P<0.05$ with respect to 0.5 mg/mL and 1 mg/mL AMG; Time 5 h) and mitochondrial activity ($P<0.05$ in case of 0.5 mg/mL AMG; $P<0.01$ in case of 1 mg/mL AMG; $P<0.001$ with respect to 2.5 mg/mL and 5 mg/mL AMG; Time 5 h). None of the AMG doses supplemented had any significant impact of the spermatozoa viability. In conclusion, the data revealed that short-term co-incubation of spermatozoa with AMG may result in a higher preservation of the sperm structural integrity and functional activity.

***Keywords*—**Amygdalin, CASA, mitochondrial activity, motility, rabbits, spermatozoa, viability.

I. Introduction

DESPITE indisputable advancements in today's biomedicine disposing of a wide array of synthetic remedies, traditional medicine and pharmacology taking advantage of natural biomolecules isolated from plant resources are nowadays experiencing a scientific renaissance [1]. The beneficial effects of medicinal plants and their extracts lies in the presence of biologically active compounds which have the ability to prevent or counteract negative effects of a variety of endogenous or exogenous risk factors endangering human or animal health [1], [2]. Past decades of biological research come along with an increasing interest on the effects of diverse natural compounds on the reproductive system, its functions and manifestations. It is widely known that numerous natural biomolecules exhibit the ability to modulate the biological response of reproductive cells and/or tissues as well as to inhibit malignant cell growth by modulating the activity of specific enzymes and hormones [1]-[3]. AMG is considered to be one of such substances with interesting properties however with very sparse information about its behavior and mechanisms of action within a biological system.

AMG (D-mandelonitrile-β-D-gentiobioside) is a cyanogenic glycoside found in diverse plant species, principally in the seeds of apricots and bitter almonds. This substance is composed of two molecules of glucose, one benzaldehyde, which exhibits analgesic properties, and one hydrocyanic acid - an anti-neoplastic compound [4], [5]. Diverse studies have emphasized on the beneficial properties of AMG and its effective usage in the prevention or treatment of various diseases including cancers, migraine, chronic inflammation, fever and pain [6], [7]. However, AMG as a potential therapeutic agent has not yet received approval for its use in the majority of countries owing to insufficient clinical verification of its therapeutic efficiency hence the use of AMG remains controversial [8]. Moreover, there is not enough scientific evidence, which could provide information about the biological effects of AMG on healthy cells, as previous reports focused on this biomolecule have primarily studied its purification, antitumor mechanisms, as well as on its toxicity caused by the release of cyanide [4], [9]-[11].

Resuming the very sparse data available on the *in vitro* effects of AMG on mammalian tissues and/or cells it may be hypothesized that low doses of AMG may exhibit protective effects, yet higher AMG concentrations may be toxic to the biological system. Therefore, it is necessary to intensify the investigation on its positive or negative roles in animals and humans.

Specific questions related to the impact of AMG on male reproductive cells are yet to be answered. Therefore, the aim of this study was to evaluate the *in vitro* effects of AMG on the motility, mitochondrial activity and viability of rabbit spermatozoa.

II. Material and Methods

A. Sample Collection and Processing

Ten male rabbits (New Zealand white broiler line; approx. four months of age; 4.0±0.2 kg of weight) used in the experiment were obtained from the experimental farm of the Animal Production Research Centre Nitra (APRS Nitra), Slovak Republic. The animals were housed in individual cages in a partially air-conditioned rabbit house under a photoperiod

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of 16L:8D (minimum light intensity of 80 lux), air temperature of 20-24 °C and relative humidity of 65%. The rabbits were fed with a commercial diet and water was provided ad libidum.

Semen samples were collected on a single day (early in the morning) using an artificial vagina. Immediately after collection each sample was diluted in physiological saline solution (PS) (sodium chloride 0.9% w/v, Bieffe Medical, Italia) supplemented with 0 (Control group), 0.5 (group A), 1 (group B), 2.5 (group C) or 5 (group D) mg/mL AMG (>99% purity, from apricot kernels, Sigma-Aldrich, St. Louis, MO, USA) using a dilution ratio of 1:5. The samples were cultured at 37 °C. At cultivation times of 0 h, 3h and 5 h, further tests were performed.

B. Spermatozoa Motion Analysis

Spermatozoa motility was examined with the help of the CASA system using the SpermVision™ program (Minitube, Tiefenbach, Germany) and Olympus BX 51 phase contrast microscope (Olympus, Tokyo, Japan). The samples were placed into the Makler counting chamber (depth 10 μm, 37 °C; Sefi Medical Instruments, Haifa, Israel) and immediately assessed. At least 1000 cells were evaluated in each sample.

C. Mitochondrial Activity (MTT Test)

Viability of the cells exposed to AMG was evaluated by the metabolic activity (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT test. This colorimetric assay measures the conversion of a yellow tetrazolium salt (MTT) to blue formazan particles by mitochondrial succinate dehydrogenase of intact mitochondria within living cells. Formazan can be measured spectrophotometrically.

The MTT tetrazolium salt (Sigma-Aldrich) was dissolved in phosphate-buffered saline (Dulbecco's PBS; Sigma-Aldrich) at 5 mg/mL. Ten microliters of the solution were added to the cells (in 100 μL medium per well). After 2 h of incubation (shaker, 37 °C, 95% air atmosphere, 5% CO_2), the cells and the formazan crystals were dissolved in 150 μL of acidified (0.08 M HCl; Centralchem, Bratislava, Slovak Republic) isopropanol (Centralchem). The optical density was determined at a wavelength of 570 nm against 620 nm as reference by a microplate ELISA reader (Anthos MultiRead 400, Austria). The data were expressed as percentage of the control, set to 100% [12].

D. Spermatozoa Viability

Eosin-nigrosin staining method was used to evaluate the functional integrity of the sperm membrane, based on the ability of eosin to penetrate into non-viable cells [13]. Ten μL of each sample were placed on a tempered glass slide, mixed with 20 μL 5% eosin (Sigma-Aldrich), followed by 20 μL 10% nigrosin (Sigma-Aldrich). The mixture was smeared on a glass slide and let air dry at 37 °C. The slides were observed using bright filed microscopy at 1000 x magnification and with oil immersion. At least 200 spermatozoa per slide were evaluated and identified as either dead (with red heads) or live (with white heads) and expressed as a percentage rate. All slides were labeled and assessed by one observer.

E. Statistical Analysis

Statistical analysis was carried out using the GraphPad Prism program (version 3.02 for Windows; GraphPad Software, La Jolla California USA, www.graphpad.com). Descriptive statistical characteristics (mean, standard error) were evaluated at first. As we focused to study the impact of different AMG concentrations on the spermatozoa activity (experimental groups) in comparison to the control at a specific time frame, thus taking one factor into consideration, one-way ANOVA was used for specific statistical evaluations. Dunnett test was used as a follow-up test to ANOVA, based on a comparison of every mean to a control mean, and computing a confidence interval for the difference between the two means. The level of significance was set at $P < 0.001$, $P < 0.01$, and $P < 0.05$.

III. Results and Discussion

Over the past years, a significant attention has been driven towards the exploration of natural compounds and their impact on reproductive functions in animals as well as in humans [3], [14]-[18]. It is known now that numerous substances derived from plants have the ability to modify the physiological or pathological behavior of diverse cells by modulating the activity of specific biomacromolecules. AMG has captured the interest of numerous researchers [7], [19], [20] principally because its potential pharmacological properties. It has been reported that AMG has the ability to promote the efficacy of radiotherapy, block tumor promoters, regulate gene expression or activate specific pro-apoptotic signaling pathways. With respect to the male reproductive system, Chang et al. [5] have emphasized on the protective effects of AMG in the treatment of prostate cancer by regulating the expression of Bax, Bcl-2 and caspase-3.

At the same time, it was shown that AMG has the ability to exhibit a vast spectrum of positive, as well as negative biological effects, which is why it is necessary to explore its role in a biological system more specifically and thoroughly. Currently, very sparse data are available to at least partially explain its mechanism of action on male reproductive cells. This study was therefore focused to provide more specific information on the short-term in vitro effects of AMG on the functional activity of spermatozoa.

The CASA analysis showed a time-dependent decrease of the sperm motion in all assessed groups (Fig. 1). The highest motility was detected at time 0 h, ranging from 85.92±1.69% (Control group) to 92.42±0.88% (group C). No significant differences were recorded between the control and experimental groups at 0 h. After 3 h and 5 h of *in vitro* culture, a significantly higher motility was found in the experimental groups A ($P<0.001$ in case of 3 h and $P<0.05$ with respect to 5 h) as well as B ($P<0.01$ with respect to 3 h and $P<0.05$ in case 5 h; Fig. 1).

Spermatozoa motility is the single most important characteristic to allow their distribution in the female sexual system, followed by an effective fertilization of the ovum [21]. Numerous animal and human studies [13], [18], [21]-[23]

have highlighted the importance of sperm motility evaluation in order to assess or predict the fertilizing ability in males, to examine any possible effects of medical treatment or environmental factors on male fertility, as well as to study the reproductive function in subjects affected by specific pathologies [24].

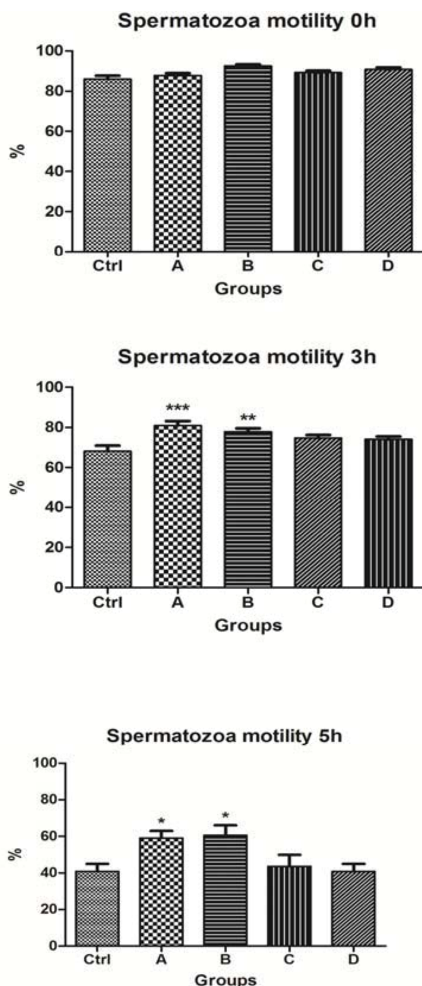

Fig. 1 The effect of AMG on the motility of rabbit spermatozoa; Mean ± SEM. * P<0.05; ** P<0.01; *** P<0.001. Ctrl – Control; A – 0.5 mg/mL AMG; B – 1 mg/mL AMG; C – 2.5 mg/mL AMG; D – 5 mg/mL AMG

The first, and to our knowledge the only study on the effects of AMG on spermatozoa was published by Tanyildizi and Bozkurt [16]. The report revealed that following *in vitro* incubation of bovine semen with 0.4, 0.8, 1 and 2 µmol/L AMG the spermatozoa motility decreased very significantly in a dose-dependent manner, and that all spermatozoa were immobile at 10 min. The authors suggested that bull spermatozoa heads contain a beta-type DNA polymerase [25] required for DNA replication, repair and cell-cycle checkpoint control, which may be inhibited by AMG [26], [27]. The contradictory motility data obtained in this study may be attributed to the biological material used as the AMG concentrations administered in our experiments were higher in comparison to those applied by [16] (approx. 1–10 mmol/L as opposed to 0.4–2 µmol/L). It has been previously shown that the dominant DNA polymerase enzyme in mammalian spermatozoa is type gamma present in mitochondria [28], [29] which might be more resilient to the potential toxicity by AMG. A different explanation to the increased sperm motility following exposure to AMG may be related to the actual metabolism of AMG. AMG is hydrolyzed by β-glucosidase, emulsin and amygdalase to gentiobiose and L-mandelonitrile. Gentiobiose is further hydrolyzed to glucose, whereas mandelonitrile is hydrolyzed to benzaldehyde and hydrogen cyanide [30]. It is interesting to find out that previous investigations on animal glycosidases have shown that certain enzymes from this group occur in a state of particularly high activity in male reproductive organs; for example, β-N-acetyl-glucosaminidase and α-mannosidase in the epididymis of the male rat [31]. Further, studies indicated high α-mannosidase, and relatively low β-N-acetylglucosaminidase activity in rat epididymal sperm, whereas a high activity of both enzymes in bull semen was largely confined to the seminal plasma [32]-[34]. We may therefore speculate that while AMG is being metabolized, glucose is the first component to be released and utilized as an energy substrate by the spermatozoon. The limited time of cultivation may also be an issue. As it seems that thanks to the presence of glucose in AMG, this molecule may exhibit stimulating effects as first. Inversely when cyanide is released from AMG, it may have toxic effects on the sperm activity hence it is highly important to perform further semen cultures of extended time periods.

Consistent with the motility data, a significant improvement of spermatozoa mitochondrial activity was recorded in groups supplemented with 1-5 mg/mL AMG (groups A–C), particularly at 3 h (P<0.001 with respect to group B and C, and P<0.05 in relation to group D; Fig. 2). The stimulating effect of all AMG concentrations on the spermatozoa viability remained notable for the rest of the *in vitro* culture, with a significantly higher mitochondrial activity in all experimental groups when compared to the control at time 5 h (P<0.05 with respect to group A; P<0.01 in case of groups B and C; P<0.001 in relation to group D; Fig. 2).

Mitochondrial activity is a vital factor supporting key spermatozoa functions, therefore their integrity and activity is crucial to ensure the fertilization success. Correspondingly, the presence of structural or functional alterations in sperm mitochondria identified in males with reproductive dysfunction confirms their pivotal roles in the maintenance of spermatozoa motility [33].

It is believed that cellular metabolism may fail because cyanide has the ability to inhibit the final step of the mitochondrial electron transport chain. On the other hand, the MTT assay is based on the ability of succinate dehydrogenase, complex II of the mitochondrial respiration chain to catalyze the conversion of tetrazolium to formazan. The abrupt increase of the MTT activity in this study may reflect on our earlier hypothesis related to the primary release of glucose from AMG, with may be subsequently used as a primary energetic

source for the glycolytic cycle followed by the mitochondrial respiration of spermatozoa. Moreover, the MTT assay reflects on the activity of the mitochondrial complex II exclusively [34], based on which we may speculate that the presence of AMG has no negative impact on the activity of NADH dehydrogenase or succinate dehydrogenase. It is questionable whether AMG has any effects on the subsequent complexes III or IV however the codependent increase of both motility and mitochondrial activity suggests that short-term administration of AMG to the sperm culture does not affect the mitochondrial activity in a negative manner. The mitochondrial behavior in response to the presence of AMG is however an important subject deserving further and detailed investigation.

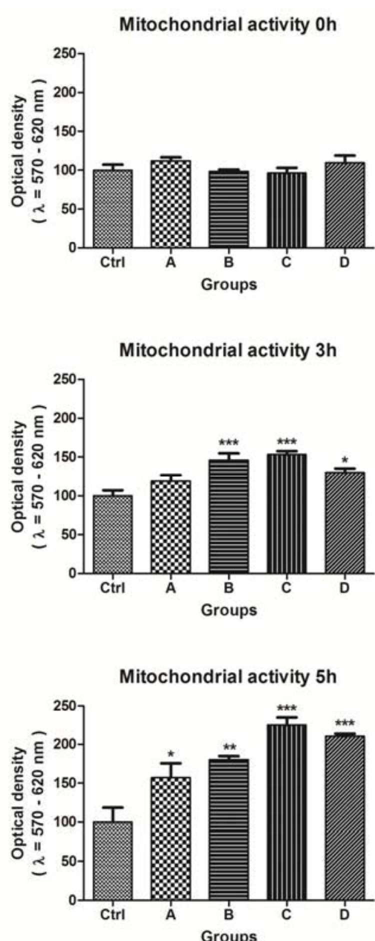

Fig. 2 The effect of AMG on the mitochondrial activity of rabbit spermatozoa. Each bar represents mean (±SEM) optical density as the percentage of the Control, which was set to 100% and the data are expressed as a percentage of the Control. Mean ± SEM. * P<0.05; ** P<0.01; *** P<0.001. Ctrl – Control; A – 0.5 mg/mL AMG; B – 1 mg/mL AMG; C – 2.5 mg/mL AMG; D – 5 mg/mL AMG

The viability staining showed that AMG supplementation to the sperm culture had no effects on the membrane integrity of rabbit spermatozoa (Fig. 3). The vitality of spermatozoa decreased in a time-dependent manner however no AMG concentration caused any significant positive or negative effect on the structural integrity of the rabbit sperm membranes (Fig. 3). This observation is in accordance with [16] who reported that *in vitro* AMG administration had no effect on the morphological abnormalities in bull spermatozoa. On the other hand, the same study emphasized that AMG supplementation led to a significant inhibition of the sperm hyaluronidase activity, leading to a possible drop in the fertilization ability of bull spermatozoa due to the prevention of the acrosome reaction [26].

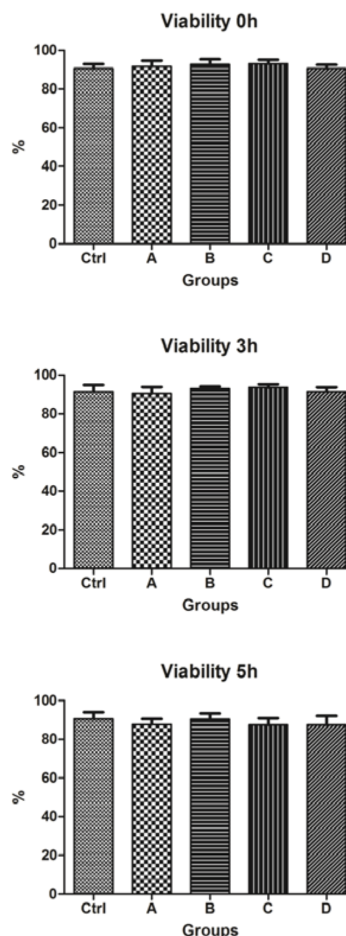

Fig. 3 The effect of AMG on the viability of rabbit spermatozoa Mean ± SEM. * P<0.05; ** P<0.01; *** P<0.001. Ctrl – Control; A – 0.5 mg/mL AMG; B – 1 mg/mL AMG; C – 2.5 mg/mL AMG; D – 5 mg/mL AMG

A current trend in bioeconomics and reproductive technology is to investigate the effects of different biologically active compounds on the reproductive cells of humans as well as animals. Nevertheless, in the case of AMG, the data are absent, sparse or highly controversial and contradictory.

The present study suggests that short-term AMG supplementation has no negative effects on the rabbit sperm survival *in vitro*. It may be suggested that glucose may be the first molecule to be released from AMG and to subsequently stimulate the mitochondrial metabolism followed by the motion activity of rabbit spermatozoa. Although being

preliminary, our data may provide more specific evidence to unravel the behavior of AMG in male reproduction. At the same time, it is essential to perform further experiments using a wider array of AMG doses and longer culture times in order to understand the possible toxic effects of AMG on the structural integrity and functional activity of male reproductive cells, and to offer a more comprehensive view of this biomolecule on the fertilization potential in males.

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