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Research Article

Homeopathic Medicine Improves the Motility and Vigor of Semen in Rams

Abstract

The semen of rams suffers during cryopreservation, and the sperm plasma membrane breaks down during the process, creating the deleterious effect of reducing their fertilization capacity. The homeopathic medicines *Medicago sativa* and *Aloe vera* were tested to evaluate membrane protection during cryopreservation in diluent media established for the species. Semen samples from five rams were frozen in two different diluents and analyzed after thirty days of freezing. The Glycine-Yolk-Milk diluent supplemented with *Medicago sativa* in the twelfth Hahnemann's centesimal potency led to decreased loss of motility and vigor of semen in rams evaluated in farms.

Introduction

The word homeopathy is derived from the Greek words *homeo* (similar) and *pathos* (disease), and the practice was first discovered and employed by Samuel Hahnemann [1]. This therapy idealizes an innovative form of treatment, based on the Hippocratic philosophy *Similia Similibus Curentur*: like is cured by like [2]. Homeopathy was initially used for the treatment of diseases in humans, later becoming a therapy for diseases in various species [3]. In veterinary medicine, it is used mainly by small animal owners seeking solutions for chronic conditions such as dermatitis, ear infections, seizures, or behavioral disorders [3]. Mathie et al. [4], demonstrated that the highest demand for homeopathy comes from dog owners, followed by cat, horse, rabbit, guinea pig, bird, and goat owners, among others. Moreover, in the case of livestock, homeopathic medicine provides a great advantage over conventional treatments because it does not leave detectable residues in animal products, such as in the meat and milk; it is safe for keepers and does not cause environmental contamination [5].

Several studies have shown the benefits of homeopathy in the field of reproduction [6-8], and in association with the cryopreservation of germ cells, it can be an important tool to achieve higher levels of success in reproduction. The freezing of semen can cause irreversible damage to the sperm, thus compromising the final fertilization process [9]. The causes that contribute to the low fertility levels of frozen and thawed semen include the depression of the metabolic processes, which depletes the sperm over time [10]; serious damage to the plasma membrane and acrosome during cooling, freezing, and thawing [11]; and reduced viability of sperm in the female genital tract [12]. In addition, in sheep, certain peculiarities of the sperm membrane lead to decreased respiratory activity, large-scale cryo-capacitation, modification of the interaction with cells of the oviduct, and a small increase in rates of embryonic mortality, further decreasing the fertilization rate [13].

The medications *Medicago sativa* and *Aloe vera* promote the reduction of tiredness, fatigue and malnutrition, and weight and movement loss, arising from nervousness, insomnia, and indigestion and improvement with cold, respectively, enabling the improvement of these symptoms in humans [14].

Several studies have been conducted to improve the freezing techniques for semen, aiming to preserve the integrity of sperm cells during the process [15]. As the nature of diluents is important for preserving the biological properties of the sperm during freezing [16], this study aimed to evaluate the potency effect of two medications described in homeopathic medicine for maintaining sperm integrity after cryopreservation, namely, the addition of *Medicago sativa* and *Aloe vera* to the diluents TRIS-S (standard) and Glycine-Yolk-Milk (GGL) and evaluation of the parameters motility and vigor post-thaw.

Materials and Methods

Experimental location

The activities related to pre-freezing and freezing were performed from August to October 2012 at a farm located in the city of Campo Grande, Mato Grosso do Sul state, Brazil, near the Federal University of Mato Grosso do Sul - (Universidade Federal de Mato Grosso do Sul - UFMS). The processing of the GGL media and the post-thawing procedures were performed at the Laboratory of Reproduction Biotechnology of Small Ruminants (Laboratório de Biotecnologia da Reprodução de Pequenos Ruminantes - BIOCAPRI) of the UFMS in the city of Campo Grande, Mato Grosso do Sul, Brazil (latitude 20º 30' 38.59" S, longitude 54° 37' 18.04" W).

Animals

Five 12-month-old mongrel rams weighing 50 kg, previously subjected to general and specific clinical tests to determine soundness from a health and reproductive standpoint, were used. The animals were kept in confinement under natural light, feeding on corn silage

(5% weight) provided twice daily (early morning and late afternoon) with protein supplementation (18% crude protein) in the amount of 400 g/day/animal, mineral salts specific for the species, and water ad libitum. The experiment was conducted under natural conditions of temperature, rainfall, relative humidity, and solar radiation.

Semen harvest

Prior to semen collection, the animals underwent a period of adaptation and conditioning to mounting an artificial vagina and were subjected to clinical andrological examinations. The ejaculates were obtained weekly, with two collections/ram/week, from August to October 2012, using the short artificial vagina model [17] with a female mannequin. After collection, the semen tube was sent to the laboratory for sample analysis.

Semen analysis

After each harvest, the ejaculates were kept in a water bath at 36°C and then subjected to macroscopic evaluations [volume (mL), appearance (watery, milky, or creamy), and color] and to microscopic evaluations [motility (0-100%), vigor (0-5), and concentration (x10^6)] according to the methodology employed by Chemineau et al. [18].

Motility and vigor assessments were performed using bright field microscopy. After the determination of sperm concentration in a Neubauer chamber, the yield of the ejaculate doses was calculated, as well as the total volume, to establish the dilution, as each insemination dose should have a concentration of 100 x 10^6 sperm packaged in 0.25 mL straws. The calculation was based on the concentration, motility, ejaculate volume, desired concentration per dose of semen (100 x 10^6), and straw volume (0.25 mL), arriving at the following formula:

Number of doses = [ ] of ejaculate x motility x ejaculate volume / 100 x 10^6

Total volume = number of doses x 0.25 mL

Volume of diluent to be added = total volume - ejaculate volume

Dilution and freezing of semen

Two diluents were used as controls, Glycine-Yolk-Milk (GGL) and TRIS-Standard (TRIS-S), and their combinations with two homeopathic products, *Medicago sativa* 12C (HMS) and *Aloe vera* 12C (HAV), provided six experimental groups:

- TRIS-S
- TRIS-S+HAV
- TRIS-S+HMS
- GGL
- GGL+HAV
- GGL+HMS

The GGL diluent was processed at the BIOCAPRI, as proposed by Gonzalez [19]. The TRIS-S diluent was also processed at the same location, in accordance with Evans & Maxwell [20].

The homeopathic medicines (HAV and HMS) were made at the pharmacy Homeovitae Homeopathy and Manipulation (Homeovitae Homeopatia e Manipulação), with the medications starting at 11C and later diluted in saline solution to 12C. Subsequently, the other experimental groups were prepared. The GGL+HAV and GGL+HMS media were prepared with the addition of 20 mL of the respective homeopathic product to 80 mL of the 100 mL of distilled water required to make solution A. To prepare the TRIS-S+HAV and TRIS-S+HMS media, 20 mL of the respective homeopathic product was added to 64 mL of the 84 mL of distilled water required for their formulation.

After calculating the final volume, the semen *in natura* was diluted in the six experimental media. Subsequently, the semen was mechanically dispensed into 30 labeled 0.25 mL insemination straws for each experimental group, which were sealed with polyvinyl alcohol. During these steps, the samples and the diluent media were kept in a water bath at 36°C.

At the end of filling, the straws were cooled in a cold chamber at 4°C for three hours using a regular refrigerator. Immediately after cooling, the stabilization curve was initiated in liquid nitrogen vapor by placing the samples in a conventional 35 L Styrofoam box at a fixed distance of five cm above the nitrogen level for 20 minutes. After the stabilization period, the samples were frozen by direct immersion in liquid nitrogen. Then, the straws were stored in a cryogenic cylinder at -196°C until thawing.

Thawing, semen analysis and staining

The straws were thawed in a water bath at 36°C after 30 days of storage, with the semen placed in preheated 1.5 mL Eppendorf tubes and kept on a hot plate at 36°C. The thawed semen underwent microscopic analysis for vigor and motility, with the subsequent preparation of smears with eosin-nigrosin dye based on six samples from each diluent. The aim was to count 200 spermatozoa to determine the percentage of live and dead cells and the consequent viability of the material in relation to fertility, according to Garner et al. [21].

Statistical analysis

For statistical analysis, two-way ANOVA was performed to evaluate the effects of the medium, of the homeopathic compound, and of their interaction on the loss of motility and vigor. A Tukey's post-test was performed for multiple comparisons when the interaction was significant.

The linear correlation between the percentage of motile sperms post-thawing and the number of dead sperm post-thawing was evaluated using the Pearson correlation test. Statistical analysis was performed using the Sigma Stat software, version 3.5, with a 5% significance level.

Results

The results concerning sperm motility and vigor before and after thawing, as well as the loss of motility and vigor in the six media, with or without the addition of homeopathic compounds, are shown in Table 1.

There was a medium effect, a homeopathic compound effect, and an interaction between these factors, in relation to both the

Table 1: Motility and vigor of sperm before and after thawing, as well as the loss of motility and vigor in the media, 2012.

Media	Motility			Vigor		
	Before-thawing	After-thawing	Loss	Before-thawing	After-thawing	Loss
GGL	0.83±0.00	0.27±0.02	0.55±0.02Aa	3.50±0.06	2.50±0.09	1.00±0.10Aa
GGL+HMS	0.78±0.01	0.43±0.02	0.34±0.02Cb	3.00±0.00	2.87±0.09	0.13±0.09Bb
GGL+HAV	0.78±0.01	0.33±0.01	0.45±0.01Ba	2.78±0.05	2.55±0.08	0.23±0.09Ba
TRIS-S	0.78±0.01	0.24±0.03	0.54±0.03Ba	2.78±0.05	1.75±0.10	1.03±0.10Ba
TRIS-S+HMS	0.77±0.00	0.10±0.01	0.67±0.01Aa	2.60±0.06	1.23±0.07	1.37±0.11Aa
TRIS-S+HAV	0.77±0.01	0.33±0.02	0.43±0.02Ca	2.90±0.04	2.58±0.08	0.32±0.08Ca

Data are presented as the means ± standard error of the mean. Different uppercase letters in the column for each medium indicate significant differences between homeopathic compounds. Different lowercase letters in the column for each homeopathic compound indicate significant differences among the media (Tukey's post-test, $p<0.05$).

loss of motility (two-way ANOVA, $p<0.001$ for both the effects and interaction) and the loss of vigor ($p<0.001$ for both the effects and interaction) of the sperm after thawing. The loss of motility of sperm frozen with GGL+HMS was significantly lower than for the GGL alone and for GGL+HAV media (Tukey's post-test, $p<0.05$). In addition, the loss of motility of sperm frozen in GGL+HAV was significantly lower than for GGL media alone (Tukey's post-test, $p<0.05$).

The loss of motility of sperm frozen in TRIS-S+HAV was significantly lower than for TRIS-S alone and for TRIS-S+HMS media (Tukey's post-test, $p<0.05$). In addition, the loss of motility of sperm frozen in TRIS-S alone was significantly lower than for TRIS-S+HMS medium (Tukey's post-test, $p<0.05$). The loss of motility of sperm frozen in GGL+HMS was significantly lower than for TRIS-S+HMS medium (Tukey's post-test, $p<0.05$). In contrast, there was no difference between the GGL alone and TRIS-S alone media (Tukey's post-test, $p>0.05$) or between the GGL+HAV and TRIS-S+HAV media (Tukey's post-test, $p>0.05$) with regard to loss of motility.

The loss of vigor of sperm frozen in GGL+HMS and GGL+HAV was significantly lower than for the GGL medium alone (Tukey's post-test, $p<0.05$); however, there was no significant difference between GGL+HMS and GGL+HAV (Tukey's post-test, $p>0.05$). The loss of vigor of sperm frozen in TRIS-S+HAV was significantly lower than for TRIS-S alone and for TRIS-S+HMS media (Tukey's post-test, $p<0.05$). In addition, the loss of vigor of sperm frozen in TRIS-S alone was significantly lower than for TRIS-S+HMS medium (Tukey's post-test, $p<0.05$).

The loss of vigor of sperm frozen in GGL+HMS was significantly lower than for TRIS-S+HMS medium (Tukey's post-test, $p<0.05$). In contrast, there was no difference between the GGL alone and TRIS-S alone media (Tukey's post-test, $p>0.05$) or between the GGL+HAV and TRIS-S+HAV media (Tukey's post-test, $p>0.05$) with regard to the loss of vigor of sperm.

There was a moderate positive linear correlation between the percentage of motile sperm post-thaw and the number of dead sperm post-thaw (Pearson correlation, $p<0.001$). These results are shown in Figure 1.

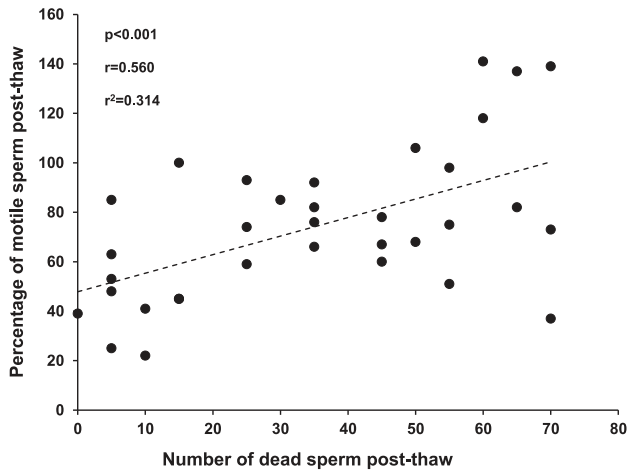

Figure 1: Linear correlation between the percentage of motile sperm post-thaw and the number of dead sperm post-thaw. Each point represents the measurement for both variables from a single animal. The dashed line represents the linear regression curve, 2012.

Discussion

In humans, *Aloe vera* homeopathic products are often prescribed for large intestine diseases, mainly in individuals presenting fatigue, sedentarism, and irritability, especially on cloudy days [1]. In this study, the *Aloe vera* homeopathic product (HAV®) added to the diluents GGL and TRIS-S was more effective in maintaining sperm motility and vigor than the standard media alone. Similar results were observed with the addition of *Medicago sativa* (HMS) to the diluents; however, in contrast to the results observed with HAV, TRIS-S+HMS showed a greater loss of sperm motility and vigor than TRIS-S alone. *Medicago sativa* is used as a mother tincture in conditions associated with malnutrition or severe weight loss, such as anorexia, cancer, nervous indigestion, or other symptoms such as headache [1]. *M. sativa* seems to be beneficial to germ cells, and the result was also consistent with sperm fertility post-thaw.

In humans, homeopathy has shown positive results in treating subfertility, as demonstrated by the work of Gerhárd & Wallis [22], in which the patients exhibited significant improvement in the parameters sperm density and percentage of spermatozoa with good progressive and propulsive motility after treatment, as well as an improvement in general health. However, in their study, the authors state that the improvement of the patients' overall quality of life

during the experiment may have contributed to the results, although this factor alone does not imply significant advances in the clinical condition, demonstrating the gains obtained from homeopathy. The direct use of homeopathic medicine in semen improvement good prospects on *in vitro* fertility after cryopreservation.

Often, the benefits obtained from homeopathic therapy are associated with the long-term relationship between doctor and patient, which raises questions regarding the effectiveness of this therapy. The results of homeopathic treatments in veterinary medicine eventually erased the doubt that the results in humans could often be attributed to the placebo effect [23]. Supporting this principle, Van Wassenhoven [24] states that studies in animals ultimately demonstrate the clinical efficacy of homeopathic treatment. Lobreiro [25] evaluated the effect of homeopathic treatment in infertility in a Nellore bull and found an increase in the number of doses of semen, an increase in sperm motility, and a decrease in total sperm defects, results similar to the study of Gerhárd & Walliss [22] in humans. In this study, homeopathic products administered directly in the processed ejaculate showed efficiency in maintaining the reproductive parameters of motility and vigor after cryopreservation compared with the use of diluents alone, and these parameters are in agreement with fertility analyzed based on the plasma membrane, demonstrating the favorable effect of homeopathy in reproduction and the variation of results depending on the medication and medium used. The benefits of homeopathy in reproduction have been studied by several researchers, including Souza et al. [7], who found that oral treatment in four bulls with reproductive disorders showed significant improvement in the seminal parameters evaluated, which, according to the authors, represents a breakthrough for livestock. Aziz et al. [8], observed an increase in sperm mitochondrial activity when homeopathic products based on ubiquinone and enzymatic complexes were added to fresh semen. The authors also observed that the use of the homeopathic products tested did not damage the viability, acrosome integrity, or chromatin structure of sperm [8]. Our findings reinforce the recommendation of the use of homeopathic therapy as an adjuvant in reproduction biotechnologies. More researches on ram semen must be investigate and others homeopathic medicines and its dinamizations evaluate because can work different from each animals, location, and nutrition, besides glycerol (10%) can be used to avoid thermal shock.

Conclusion

In conclusion, the use of the homeopathic medicine *Medicago sativa* at 12CH, added to GGL diluent, resulted in decreased loss of motility and vigor compared with *Aloe vera*, coinciding with fertility in ram semen manipulated and evaluated in the field at a farm.

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Highlights

- Homeopathic compounds were used to verify the maintenance of sperm quality.
- TRIS-S and GGL were diluent control, with or without homeopathic compounds.
- *Aloe vera* 12CH and *Medicago sativa* 12CH was added to control diluents.
- Sperm motility and vigor post thawing were evaluated parameters.
- *Medicago sativa* added to GGL was the best diluent in maintenance of sperm quality.

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