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ORIGINAL PAPER

Stimulation of bovine sperm mitochondrial activity by homeopathic dilutions of monensin

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Mitochondrial activity is an important viability parameter of spermatozoa and is linked to sperm motility. Monensin is commonly used as an inhibitor for sperm mitochondrial activity in the laboratory. This study was conducted to evaluate the influence of some homeopathic dilutions of monensin on sperm mitochondrial activity. Fresh ejaculates from 6 mature bulls were used in the study. Samples of the semen were tested using a flow cytometer for mitochondrial activity and sperm viability using Rhodamine 123 and SYBR-14, respectively. The 9× dilution of monensin resulted in very highly significant (P<0.001) stimulation of mitochondrial activity. Monensin 5×, 7×, 8× and 13× caused highly significant (P<0.01) stimulation of the sperm mitochondrial activity. Other homeopathic dilutions of monensin (6×, 10×, 11×, 12× and 14×) also had a significant (P<0.05) stimulatory effect. The use of monensin did not have any negative effect on sperm viability. We conclude that some homeopathic dilutions of monensin increase mitochondrial activity of bovine spermatozoa without negative effect on sperm viability, the 9× dilution was the most effective. Further *in vivo* studies are required to estimate the effect of homeopathic dilutions of monensin on semen quality. *Homeopathy* (2005) **94,** 229–232.

Keywords: sperm; mitochondrial activity; monensin; homeopathy

Introduction

Mitochondrial activity is an important viability parameter of spermatozoa. It is correlated significantly with sperm motility.[1]

Homeopathy is a form of medicine developed approximately 200 years ago by Samuel Hahnemann. Homeopathic medicines made from over 2000 substances currently exist and the number is growing.[2] Most are derived from natural substances of plants, minerals, or animal origins.[3,4] The principles of homeopathy indicate that the toxic or inhibiting agents become stimulatory or protective, if used in high or ultra-high dilutions.[5,6]

Monensin (MON) is one of the ionophore substances derived from *Streptomyces cinnamonensis* is used successfully as anticoccidial agents in preventing coccidiosis in all classes of poultry.[7] MON is also efficacious in the control of coccidiosis in lambs and calves.[8,9] In beef cattle, it improves the feed efficiency by 10–15% due to an increase in the production of more propionic acid which yields more energy than acetic or butyric acids following ruminant fermentation.[10] As with other ionophores, MON forms lipophilic complexes with monovalent cations, which easily cross cellular and subcellular membranes; their high affinity for Na^+ results in an influx of this ion with a corresponding efflux of H^+ and K^+, leading to a secondary increase in intracellular Ca^{2+}.[11] These cation imbalances are thought to be the cause of a number of severe biochemical and histological changes, including mitochondrial swelling, decrease in ATP production, lipid peroxidation, and eventual loss of the integrity of cell membranes.[12] MON is characterized by a narrow safety margin, and accidental poisoning has been described in horses, pigs, cattle, chickens, turkeys, sheep, goats, rabbits,[13] and dogs.[14]

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The toxicity and inhibitory characterhistic of MON especially on the mitochondrial activity of the cells have led to its use as an inhibitor for sperm mitochondrial activity in the laboratory.[15,16]

In this study, different homeopathic dilutions of MON were prepared and tested to evaluate their stimulatory effects on bovine sperm mitochondrial activity.

Material and methods

Preparation of homeopathic dilution of monensin: MON 1 mM in 1% ethanol was used as the testing substance to prepare the homeopathic dilutions. Dilutions from 1 to 14× were prepared in normal saline. The first dilution (1×) was prepared by mixing 1 g with 9 g of normal in a 15 ml glass bottle, the second dilution was prepared by mixing 1 g of the first dilution with 9 g of normal saline. Other dilutions were prepared by the same method. Each dilution was prepared in a separate bottle. After each dilution, the dilution was shaken 10 times.

Experiments

To evaluate the effect of the MON diluents on sperm mitochondrial activity and viability, 14 groups of semen diluents were prepared by the addition of 1 ml of each above MON dilutions to 9 ml of Hepes-BSA. Also, three control groups were prepared: Hepes-BSA alone, Normal saline + Hepes-BSA (1 ml + 9 ml), and MON 1 mM + Hepes-BSA (1 ml + 9 ml).

Fresh semen was collected from 6 bulls (Rinder Union West, Borken, Germany). Following the determination of the ejaculate volume, and concentration of spermatozoa in each sample, semen samples from each bull was divided to 17 aliquots and diluted with the above 17 diluents to obtain a concentration of 30×10^6 sperm/ml. All groups of the diluted semen were incubated at 37 °C. After 30 min the samples were analyzed by flow cytometry to evaluate the mitochondrial activity and viability of sperm.

Semen analysis

To evaluate the sperm mitochondrial activity, stock solutions of 0.53 mM rhodamine 123 (R-302, Molecular Probes, Eugene, OR, USA) were prepared in DMSO and 2.99 mM propidium iodide (PI) (P-4170, Sigma, Deisenhofen, Germany) in Tyrode's salt solution (T-2397, Sigma, Deisenhofen, Germany). The final staining solution contained 3 µl of R123 stock solution and 12 µl PI stock solution/ml Hepes-BSA.[17] Diluted semen (150 µl) was stained with 300 µl of the final staining solution of R123. The samples were incubated at 37 °C for 30 min before examination. The changes in the mitochondrial activity were measured depending on the variations of the fluorescence intensity of R-123.[18,19]

To evaluate the sperm viability, LIVE/DEAD sperm viability kit (L-7011, Molecular Probes, Eugene, OR, USA) was used. A staining solution was prepared that contained 0.8 µl SYBR-14 and 9 µl PI/ml Hepes–0.1% BSA. Diluted semen (100 µl) was stained with 300 µl of the staining solution of SYBR-14. The samples were incubated at 37 °C for 15 min, prior to flow cytometry examination.[17]

Flow cytometry (FACScan, Becton Dickinson, Heidelberg, Germany) was performed to determine sperm viability, mitochondrial activity and acrosomal integrity. A total of 10,000 events was analyzed for each sample.

Statistical analysis

Samples from each bull were repeated three times. The data were tested for distribution using Normality Test (Kolmogorov-Smirnov). Subsequently the data were compared by ANOVA. Significant differences were determined by the Duncan's multiple range test. The data sets that did not fulfill the assumption of normal distribution were analyzed using AVOVA on Ranks. Significant differences were analyzed by Dunn's test. Data were analyzed using SigmaStat (Jandel scientific software V2.0), and $P<0.05$ was considered as statistically significant.

Results

Representative examples of the outcome from the flow cytometer are shown in Figures 1 and 2.

The effects of homeopathic dilutions of MON on sperm mitochondrial activity are shown in Figure 3. There is no variation between the mitochondrial

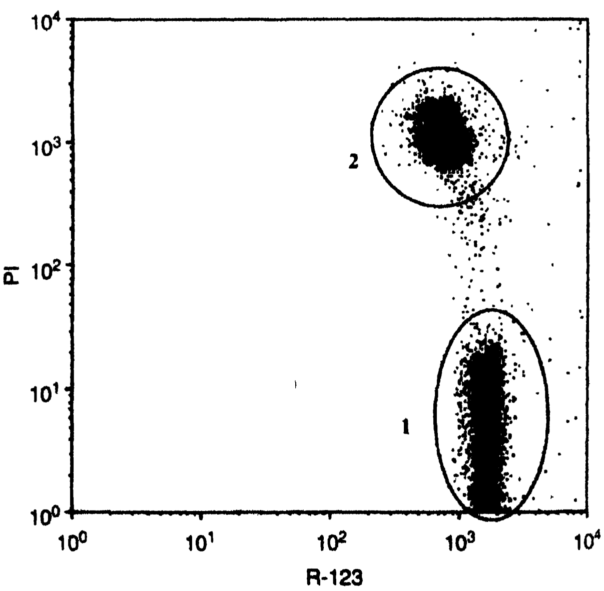

Fig. 1. Dot plots representing R-123/PI-stained spermatozoa. (1) Spermatozoa with active mitochondria stained with R-123 (green fluorescence). (2) Dead spermatozoa stained with PI (red fluorescence).

activity of semen samples in the first control group (Hepes–BSA only) and those which were treated with normal saline; however, sperm mitochondrial activity of the samples treated with MON 1 mM (negative control), was decreased significantly ($P<0.001$), compared with those in the first two control groups.

Mitochondrial activity was decreased significantly in the groups, which were treated with MON 1× ($P<0.001$) and 2× ($P<0.01$). MON 3× also had a negative effect on mitochondrial activity, but this effect was statistically not significant.

With an increasing dilution of MON from 4× to 14×, mitochondrial activity increased, but no correlation between the mitochondrial activity and the grade of MON dilution was observed. Mitochondrial activity of samples which were treated with 4× was increased relatively but not significantly. Stimulation of mitochondrial activity was very highly significant ($P<0.001$) in the samples which were treated with 9× of MON; however, addition of 5×, 7×, 8× and 13× of MON cause a high significant ($P<0.01$) stimulation of the sperm mitochondrial activity. The remaining MON homeopathic dilutions (6×, 10×, 11×, 12× and 14×) have also a significant ($P<0.05$) stimulatory effect on mitochondrial activity.

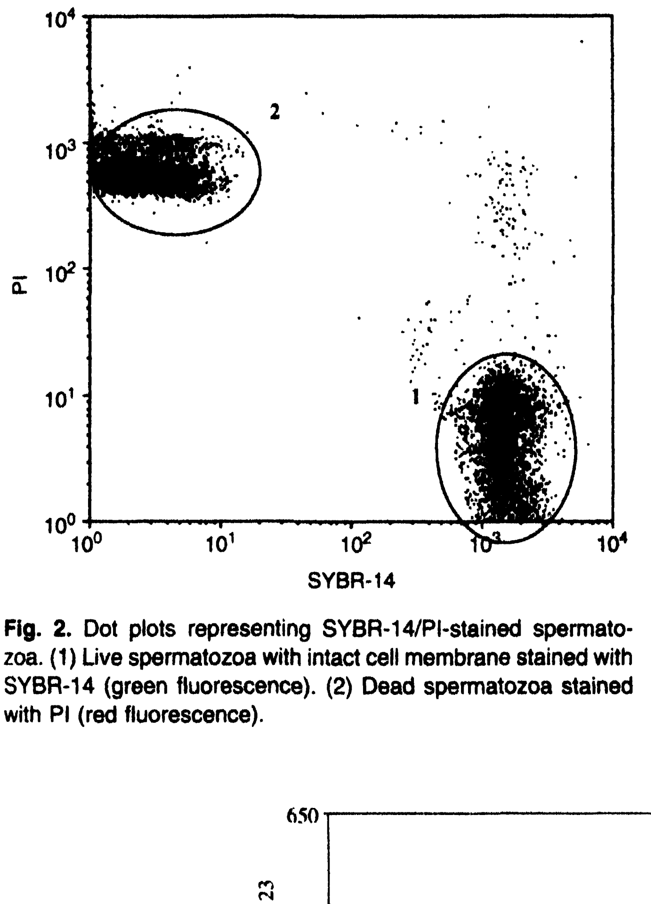

Fig. 2. Dot plots representing SYBR-14/PI-stained spermatozoa. (1) Live spermatozoa with intact cell membrane stained with SYBR-14 (green fluorescence). (2) Dead spermatozoa stained with PI (red fluorescence).

In control and treated groups, there was a significant variation in mitochondrial activity between; also, there was some variation in the effect of each dilution of MON on mitochondrial activity between the bulls, but statistical analysis for the data showed no significant variation between the bulls.

Addition of the MON homeopathic dilutions to bovine semen samples and their effects on viability of sperm are presented in Table 1. Percentage of sperm with active mitochondria and percentage of viable sperm were decreased significantly ($P<0.001$) in semen samples treated with MON 1 mM. The results showed some variation in these two sperm parameters between the control groups and those treated by homeopathic dilutions of MON, but this variation was not important and statistically not significant.

Discussion

Because MON is a strong mitochondrial inhibitor and used to produce a negative control group in the studies of sperm mitochondrial activity,[11,16] this study was conducted to evaluate the influence of homeopathic dilutions of MON on bovine sperm mitochondrial activity.

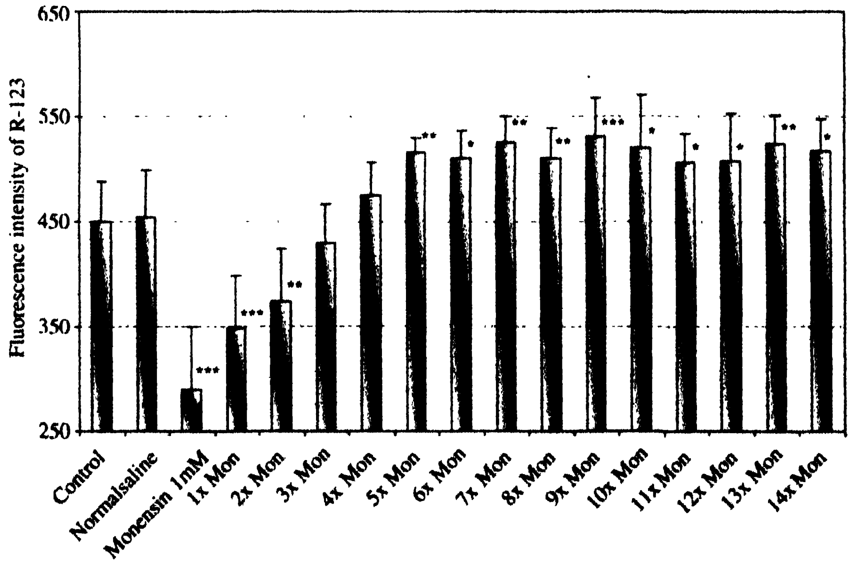

Fig. 3. The effect of different homeopathic dilutions of monensin on mitochondrial activity of bovine spermatozoa, $n = 6$.

Table 1 Percentage of sperm with active mitochondria and percentage of viable sperms of semen samples in the control and treated groups, $n = 6$.

Groups	% of sperms with active mitochondria (R-123)	% of viable sperms (SYBR-14)
Control	72.29 ± 9.05	73.87 ± 11.22
Normal saline	71.30 ± 11.07	72.80 ± 10.56
Monensin 1 mM	28.89*** ± 9.45	22.11*** ± 10.18
1 × of monensin	65.74 ± 9.23	72.38 ± 11.13
2 × of monensin	68.67 ± 9.27	72.73 ± 10.96
3 × of monensin	71.53 ± 9.54	72.80 ± 10.02
4 × of monensin	71.30 ± 10.50	70.55 ± 10.48
5 × of monensin	68.79 ± 9.88	69.40 ± 10.19
6 × of monensin	72.01 ± 10.15	72.38 ± 10.49
7 × of monensin	71.96 ± 11.63	71.95 ± 12.09
8 × of monensin	72.52 ± 9.76	72.62 ± 10.54
9 × of monensin	72.31 ± 9.76	72.29 ± 10.35
10 × of monensin	71.17 ± 10.39	72.94 ± 12.39
11 × of monensin	69.68 ± 11.90	70.33 ± 13.75
12 × of monensin	71.09 ± 10.62	72.67 ± 12.67
13 × of monensin	70.21 ± 11.69	71.07 ± 15.03
14 × of monensin	70.85 ± 10.05	71.05 ± 10.93

The results indicate that mitochondrial activity was decreased in the negative control group and in the first three dilutions of MON, due to the known inhibitory effect of MON,[16] while the mitochondrial activity was increased in all samples treated with 5× to 14× of MON. These stimulatory effects of the homeopathic dilutions of MON on sperm mitochondria activity agree well with the principles of homeopathy.[5,6]

In clinical use, homeopathic medicines, prescribed by trained professionals, are safe and unlikely to provoke severe adverse reactions.[20] Results of this study show that the tested homeopathic dilutions 4× to 14× of MON have a stimulatory effect on mitochondrial activity, but no detectable negative effects on sperm viability. This agrees with the meta-analysis or systematic reviews of controlled clinical trials, which showed that homeopathic drugs were more efficacious than corresponding placebos, but do not have adverse effects.[21] The reason for the absence of the negative effects of the tested homeopathic dilutions of MON may be due to the highly diluted nature of the medicine.[3,4,22]

Conclusion

The results of this study indicate that the tested homeopathic dilutions of MON stimulate the mitochondrial activity of bovine spermatozoa without negative effect on the viability of the sperms, 9× was the most effective dilution of MON. Further *in vivo* studies are required to estimate the effect of 9× of MON on semen quality and fertility.

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