RESEARCH ARTICLE

Supplementation of Freezing Medium with Alpha-Mangostin Improves Human Sperm Quality after Cryopreservation

Wanlapa Kokmas[1], Hathairat Kruevaisayawan[2], Patcharada Amatyakul[3], Onrawee Khongsombat[1]

[1] *Department of Physiology, Faculty of Medical Science, Naresuan University, Phitsanulok, Thailand*
[2] *Department of Anatomy, Faculty of Medical Science, Naresuan University, Phitsanulok, Thailand*
[3] *Department of Obstetrics & Gynecology, Faculty of Medicine, Naresuan University, Phitsanulok, Thailand*

Abstract

Sperm cryopreservation, a routine technique in assisted reproductive technology (ART), is an efficient procedure to preserve sperm viability. Even though cryo-preservation has benefits on fertility preservation, it has a negative effect on sperm due to oxidative stress, causing damage and affecting sperm quality. The addition of antioxidants to the freezing media may protect the sperm quality after cryopreservation. Alpha-mangostin (α-MG) has been confirmed to have high potential antioxidant effects in several cell types. However, the protective effect of α-MG on sperm quality after cryopreservation has not yet been explored. This study aimed to investigate the effect of α-MG supplementation to a freezing media on motility, viability, malondialdehyde (MDA) level and catalase (CAT) activity in human sperm. Semen samples were obtained from 7 healthy men and divided into following groups (n=7 per group): control, freezing medium supplemented with 25 and 50 µM α-MG. Sperm samples were frozen by vitrification technique and stored at -196°C. After 2 weeks, the sperm samples were thawed and all parameters were evaluated. The results showed that supplementation with 25 and 50 µM α-MG in the freezing medium tended to increase the total and progressive sperm motility. Furthermore, supplementation with 50 µM α-MG significantly increased sperm viability and CAT activity as well as decreased MDA level ($p<0.05$). Alpha-mangostin possesses an antioxidant effect that could scavenge the reactive oxygen species (ROS) thus achieving a balance between ROS and the antioxidant activity. Therefore, α-MG may protect human sperm damage from cryopreservation.

Keywords: Human sperm, sperm cryopreservation, alpha-mangostin, oxidative stress, antioxidants

Address correspondence and reprint request to: Onrawee Khongsombat, Department of Physiology, Faculty of Medical Sciences, Naresuan University, Phitsanulok 65000, Thailand.
E-mail address: onraweek@nu.ac.th.

การเติมสารอัลฟ่าแมงโกสทินลงในน้ำยาแช่แข็งอสุจิสามารถเพิ่มคุณภาพอสุจิภายหลังจากกระบวนการแช่แข็ง

วัลลภา ก๊กมาศ[1], หทัยรัตน์ เครือไวศยวรรณ[2], พัชรดา อมาตยกุล[3], อรระวี คงสมบัติ[1]

[1] ภาควิชาสรีรวิทยา คณะวิทยาศาสตร์การแพทย์ มหาวิทยาลัยนเรศวร จังหวัดพิษณุโลก ประเทศไทย
[2] ภาควิชากายวิภาคศาสตร์ คณะวิทยาศาสตร์การแพทย์ มหาวิทยาลัยนเรศวร จังหวัดพิษณุโลก ประเทศไทย
[3] ภาควิชาสูติ-นรีเวช คณะแพทยศาสตร์ มหาวิทยาลัยนเรศวร จังหวัดพิษณุโลก ประเทศไทย

บทคัดย่อ

การแช่แข็งอสุจิเป็นกระบวนการของเทคโนโลยีช่วยการเจริญพันธุ์ ซึ่งมีประสิทธิภาพในการคงสภาพการมีชีวิตของอสุจิ ถึงแม้ว่ากระบวนการแช่แข็งอสุจิจะมีประโยชน์ในการรักษาสภาพการมีชีวิตของอสุจิได้ แต่ก็พบการเกิดภาวะเครียดจากปฏิกิริยาออกซิเดชั่นในกระบวนการแช่แข็งอสุจิซึ่งส่งผลเสียต่อคุณภาพของอสุจิ การเติมสารต้านอนุมูลอิสระลงในน้ำยาแช่แข็งอสุจิ อาจช่วยป้องกันผลเสียจากภาวะเครียดจากปฏิกิริยาออกซิเดชั่นได้ สารอัลฟ่าแมงโกสทินเป็นสารที่มีคุณสมบัติเป็นสารต้านอนุมูลอิสระ ซึ่งได้มีการศึกษาในเซลล์ชนิดต่าง ๆ แต่ยังไม่มีการศึกษาผลของสารอัลฟ่าแมงโกสทินต่อคุณภาพอสุจิภายหลังจากการกระบวนการแช่แข็งมาก่อน วัตถุประสงค์ของการวิจัยนี้เพื่อศึกษาผลของสารอัลฟ่าแมงโกสทินที่เติมลงในน้ำยาแช่แข็งต่อการเคลื่อนที่ การมีชีวิตรอด ระดับมาลอนไดอัลดีไฮด์ (MDA) และการทำงานของเอนไซม์คาตาเลส (CAT) ในอสุจิมนุษย์ น้ำเชื้อตัวอย่างได้รับจากอาสาสมัครจำนวน 7 คน และนำมาแบ่งออกเป็นกลุ่มต่าง ๆ ได้แก่ กลุ่มควบคุม กลุ่มที่น้ำยาแช่แข็งอสุจิเติมสารอัลฟ่าแมงโกสทินที่ความเข้มข้น 25 และ 50 ไมโคร-โมลาร์ อสุจิตัวอย่างถูกแช่แข็งด้วยวิธีวิทริฟิเคชั่น (vitrification) และแช่แข็งที่อุณหภูมิ -196 องศาเซลเซียส ภายหลังจากการแช่แข็งเป็นเวลา 2 สัปดาห์ อสุจิจะถูกทำละลายและนำไปตรวจสอบคุณภาพของอสุจิ ผลการศึกษาพบว่าการเติมสารอัลฟ่าแมงโกสทินที่ความเข้มข้น 25 และ 50 ไมโครโมลาร์ มีแนวโน้มในการเพิ่มอัตราการเคลื่อนที่และอัตราการเคลื่อนที่ไปข้างหน้าของอสุจิได้ นอกจากนี้พบว่าการเติมสารอัลฟ่าแมงโกสทินที่ความเข้มข้น 50 ไมโครโมลาร์ในน้ำยาแช่แข็งอสุจิสามารถเพิ่มอัตราการมีชีวิตรอด เพิ่มการทำงานของเอนไซม์คาตาเลส และลดระดับมาลอนได-อัลดีไฮด์ได้เมื่อเปรียบเทียบกับกลุ่มควบคุม ($p<0.05$) สารอัลฟ่าแมงโกสทินมีคุณสมบัติในการจับกับสารอนุมูลอิสระซึ่งสามารถรักษาสมดุลระหว่างสารอนุมูลอิสระและสารต้านอนุมูลอิสระได้ ดังนั้นสารอัลฟ่าแมงโกสทินอาจจะช่วยป้องกันความเสียหายที่เกิดจากกระบวนการแช่แข็งอสุจิได้

คำสำคัญ: อสุจิมนุษย์, กระบวนการแช่แข็งอสุจิ, อัลฟ่าแมงโกสทิน, ความเครียดจากปฏิกิริยาออกซิเดชั่น, สารต้านอนุมูลอิสระ

Introduction

Cryopreservation is a necessary procedure to preserve sperm viability at sub-zero temperature in assisted reproductive technology (ART), which becomes particularly important for men who undergo treatments such as chemotherapy and radiation. Even though sperm cryopreservation has been developed since 1950, sperm quality after cryopreservation remains lower than that of fresh sperm. Human sperm exposure to sub-zero temperatures and re-warming can result in the loss of structural integrity and functional capabilities in around 50% of the sperm.[1,2] Spermatozoon injury due to cryopreservation causes reduction of post-thaw motility and viability by disrupting the integrity of the acrosomal plasma membranes, inducing biochemical alterations and decreasing DNA stability.[3,4] Oxidative stress, which involves lipid peroxidation (LPO) has been reported to be one of the most important factors that induce poor sperm quality.[5-7] During cryopreservation, reactive oxygen species (ROS) are produced from aerobic metabolism in human sperm and cryopreserved media.[8,9] This causes imbalance between ROS production and antioxidant activity, leading to oxidative stress.

Human sperm contains a high amount of polyunsaturated fatty acids which are sensitive to oxidation by ROS.[10] Excessive amounts of ROS can induce oxidative stress in human sperm, which leads to membrane integrity disruption.[11] Losing membrane fluidity, along with impaired sperm motility and the induction of sperm apoptosis subsequently decrease sperm motility and viability.[12] An antioxidant system in human spermatozoa is comprised of antioxidant enzymes including glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT), which serve as a defense mechanism against the LPO of human sperm. However, the antioxidant defense system is insufficient in preventing LPO during the cryopreservation process.[13] Many studies have examined the effect of antioxidants supplementation on the cryopreservation-induced damage to sperm quality such as sperm motility and viability.[14-16] Therefore, supplementation with antioxidants to the freezing medium has been developed to counteract the negative effect of oxidative stress in human sperm during cryopreservation.

Mangosteen (*Garcinia mangostana* L.) is a tropical tree native to Southeast Asia and is extensively distributed across Thailand, Malaysia, and India. Xanthone α-MG is a major bioactive compound found in mangosteen[17,18] which has been confirmed to have antioxidant[19,20] and anti-inflammatory properties[21-23] in several cell types. The previous study by Nelli et al. reported the positive effects of α-MG on rat sperm, as evidenced by significantly increased the percentage of progressive spermatozoa, sperm viability and antioxidant enzyme levels. It also decreased the malondialdehyde (MDA) level, a second metabolite of the LPO process.[24] Therefore, α-MG might have a positive antioxidant effect on sperm quality. However, the effect of α-MG on human sperm has not been explored. The objective of this study was to investigate the effects of α-MG supplementation on the quality of sperm including sperm motility and viability, MDA level and CAT activity in cryopreserved human sperm.

Materials and Methods

Collection of semen samples

Semen samples were collected from healthy volunteers (N=7) who presented at the Infertility Centre of the Naresuan University Hospital for semen analysis. The protocol was approved by the Naresuan University Institutional Review Board (IRB No. 532/59). All participants signed an informed consent form before participating in this study. The semen samples were collected by masturbation into a sterile container and allowed to liquefy for 30 min at 37°C. Only semen samples exhibiting parameters within normal range according to World Health Organization (WHO) guideline[25] were used in the study.

Preparation of α-MG stock solution and α-MG supplemented freezing media

Stock solution (5 mM) of α-MG (Aktin chemicals, Chengdu, China) was initially prepared in 1% dimethyl sulfoxide (DMSO) in phosphate buffer saline (PBS). On the day before starting the experiment, sperm freezing medium (Coopersurgical, Malov, Denmark) was incubated at 37°C for at least 30 min and subsequently supplemented with stock solution of α-MG to give the final concentrations of 25 and 50 µM.

Semen sample preparation

Liquefied semen samples were placed on top of two-layer gradient (45% and 90% fractions) of Sil-Selected Plus (FertiPro NV, Beernem, Belgium) and centrifuged at 360×g for 15 min to separate immotile and motile sperm from seminal plasma. The separated motile sperm were washed twice with Ferticult flushing medium (FertiPro NV, Beernem, Belgium) by centrifuging at 300×g for 5 min. Motile sperm were divided into three equal aliquots (10×10^6 cells each).

Sperm cryopreservation and thawing

Each aliquot was frozen in the freezing media as follows: (1) freezing medium supplemented with 0.01% DMSO (control), (2) freezing media supplemented with 25 µM α-MG, and (3) freezing media supplemented with 50 µM α-MG. In brief, sperm aliquots were gently mixed, drop by drop, with an equal volume of sperm freezing media at room temperature. The sperm samples were placed in liquid nitrogen vapor at -80°C for 30 min and subsequently plunged into liquid nitrogen. Sperm were frozen for at least 2 weeks and then thawed at room temperature for 30 min. After that, the sperm pellets were resuspended in PBS and then processed to evaluate sperm motility and viability. The remaining samples were stored at -80°C for subsequent study.

Assessment of sperm motility and viability

The evaluation of sperm motility was performed immediately after thawing by using Makler counting chamber under a light microscope. Sperm motility was graded according to WHO guideline[25] and reported as the percentages of total sperm motility and progressive sperm motility. Progressive sperm motility refers to the sperm movement with straight line or in a large circle without regarding the speed of the sperms.

Sperm viability was examined by eosin-nigrosin staining. Briefly, 10 μL of sperm sample was mixed with 20 μL of 1% eosin Y for 15 s followed by 20 μL of 10% nigrosin for 15 s. After that, the sample was smeared on a glass slide and observed under a 40X objective lens in bright-field mode. Sperms were classified as live (unstained heads) or dead sperm (stained red or dark pink heads) and reported as the percentage of live sperm.

Assessment of LPO

LPO was assessed by measuring MDA level, an end product of LPO process. A sperm suspension containing 1×10^6 sperm cells was lysed by sonication and centrifuged (4,000×g) at 4°C for 15 min, and the supernatant was collected. One hundred microliters of supernatant was added to a mixture containing 1.5 mL of 35% acetic acid, 200 μL of 8.1% sodium dodecyl sulfate and 1.5 mL of 0.8% thiobarbituric acid, and incubated at 90°C for 1 h. The MDA level was measured by a spectrophotometer at a wavelength of 532 nm. The results were expressed as nmol/10^6 cells.

Measurement of CAT activity

A sperm suspension containing 1×10^6 sperm cells was sonicated and centrifuged at 4,000×g at 4°C for 15 min, and the supernatant was mixed with CAT buffer (pH 7.4). CAT activity in sperm was quantified using the method described by Beer and Sizer.[26] Briefly, a reduction of hydrogen peroxide (H_2O_2), a substrate of CAT, was determined using a spectrophotometer at a wavelength of 240 nm. CAT activity was measured immediately after adding of 30 mM H_2O_2 solution at 1 min intervals for a period of 5 min. The results were expressed as nmol H_2O_2/min/10^6 cells.

Statistical analysis

Data was expressed as the mean ± standard error of the mean (SEM). The difference in data between fresh and frozen semen (control group) samples were compared by Student's t-test and significance was determined by $p<0.05$. One-way analysis of variance (ANOVA) with Tukey's post hoc test for multiple comparisons was employed to identify individual differences between means of samples in the freezing media with or without α-MG with statistical significance at $p<0.01$ or $p<0.05$. All analyses were performed using GraphPad Prism 5.0 software (La Jolla, CA, USA).

Results

Effects of α-MG on sperm motility

After cryopreservation, the percentages (mean±SEM) of total and progressive motility of cryopreserved sperm significantly decreased compared to those of fresh sperm (fresh *vs.* cryopreserved sperm: 88.52±3.05 *vs.* 56.38±4.93 and 79.42±5.08 *vs.* 43.08±8.08, respectively; $p<0.05$). The results showed that supplementation with either 25 or 50 μM α-MG in the freezing medium tended to increase the progressive and total sperm motility but the increases were not statistically significant when compared to control group (Figure 1).

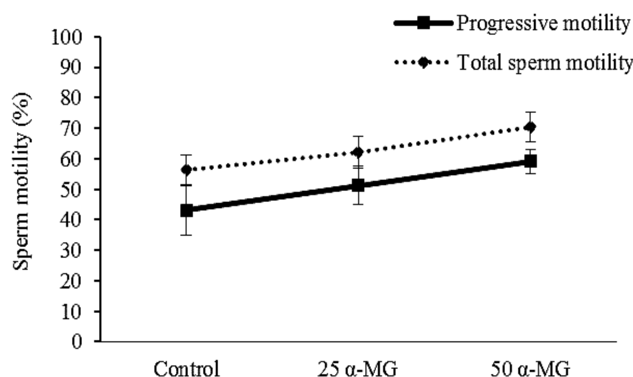

Figure 1. Effect of alpha-mangostin on total and progressive sperm motility. Data were presented as mean±SEM. 25 α-MG=25 μM α-MG; 50 α-MG=50 μM α-MG.

Effects of α-MG on sperm viability

The sperm viability (mean±SEM) in fresh sperm (92.14±2.11%) significantly decreased after cryopreservation (74.57±1.78%) ($p<0.05$). Supplementation with 25 μM and 50 μM α-MG in the freezing medium significantly increased the percentage of viable sperm when compared to control group (81.07±1.73%; $p<0.05$ and 83.14±1.06%; $p<0.01$) (Figure 2).

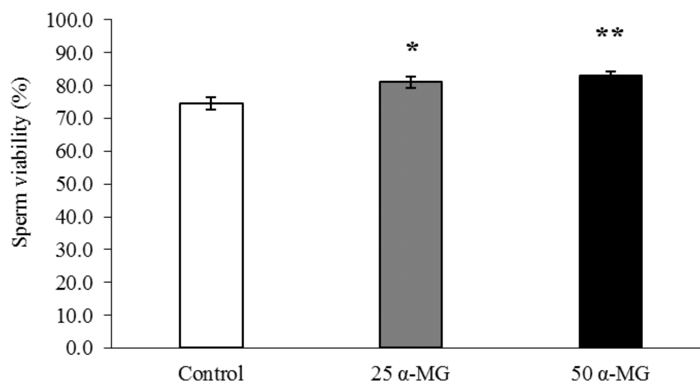

Figure 2. Effect of alpha-mangostin on sperm viability. Data were presented as mean±SEM. 25 α-MG=25 μM α-MG; 50 α-MG=50 μM α-MG. *$p< 0.05$, **$p< 0.01$ compared to control.

Effects of α-MG on MDA level

MDA level (mean±SEM) significantly decreased in the group supplemented with 50 μM α-MG compared to the control group (1.15±0.36 *vs.* 3.53±0.43 nmol/10^6 cells) ($p<0.05$). However, there was no statistical difference in MDA level between the group supplemented with 25 μM α-MG (1.61±0.39 nmol/10^6 cells) and the control as shown in Figure 3.

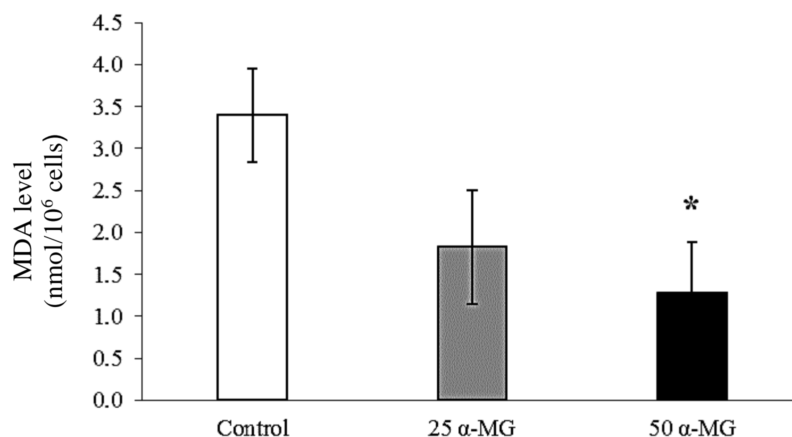

Figure 3. Effect of alpha-mangostin on MDA level in cryopreserved human sperm. Data were presented as mean±SEM. 25 α-MG=25 µM α-MG; 50 α-MG=50 µM α-MG. *$p < 0.05$ compared to the control.

Effects of α-MG on CAT activity

Supplementation with 50 µM α-MG in the freezing medium significantly increased CAT activity (mean±SEM) when compared to the control group (13.84±2.82 *vs.* 5.36±0.57 nmol H_2O_2/min/10^6 cells; $p<0.05$). An increase in CAT activity was also observed in 25 µM α-MG supplemented group, but it was not statistically significant (11.48±1.70 nmol H_2O_2/min/10^6 cells) (Figure 4).

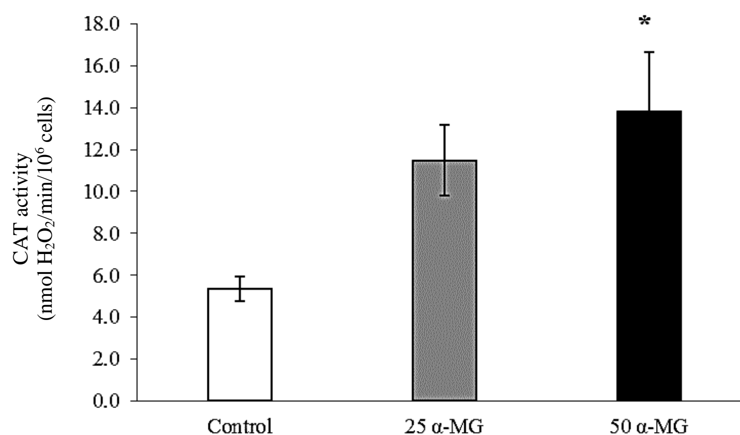

Figure 4. Effect of alpha-mangostin on CAT activity in cryopreserved human sperm. Data were presented as mean±SEM. 25 α-MG=25 µM α-MG; 50 α-MG=50 µM α-MG. *$p < 0.05$ compared to the control.

Discussion

This current study examined the antioxidant effect of α-MG on human sperm quality after cryopreservation. The results demonstrated that supplementation with α-MG to the freezing medium protected human sperm damage from cryopreservation, as shown by increased sperm viability and motility. Moreover, supplementation with α-MG to the freezing medium also decreased MDA level and increased CAT activity.

During cryopreservation, exposure to the sub-zero temperature might induce sperm damage including the decreases in motility and viability. Several studies confirmed that cryopreservation induced sperm damage was related to the imbalance between ROS level and antioxidant activity.[27,28] It has been known that ROS production negatively influenced the quality of cryopreserved sperm and this effect can be protected by the addition of antioxidants.[29-31]

Previous study showed the α-MG, as a scavenge of $^1O_2$ and $O_2^{\cdot}$, might protect the conversion of $O_2^{\cdot}$ to H_2O_2.[32,33] This activity may reduce the production of OH^- and H_2O_2 from the Fenton and Haber-Weiss reactions.[34] Hydroxyl radicals are the most toxic radicals on sperm quality via the reaction of LPO.[35] Our study revealed that α-MG supplementation to the freezing medium improved the sperm viability and motility. This might be particularly attributed to protective effect of α-MG on sperm cells against damage induced by LPO, as evidenced by significant decrease in MDA level in α-MG supplemented cryopreserved human sperm. This finding is in concordance with the previous published results showing that α-MG significantly inhibited the accumulation of LPO products in various cell types.[36-38] The decrease in MDA level might prevent the disruption of membrane integrity, resulting in increases in sperm viability and motility after the cryopreservation process. Furthermore, α-MG also increases CAT activity, an important antioxidant enzyme that converts H_2O_2 to H_2O and O_2, which subsequently reduce the negative effect of ROS on sperm quality. The previous study also confirmed that α-MG increased the activity of CAT in several cell types.[36,23,39] Therefore, supplementation with α-MG to the freezing medium might improve human sperm quality from damage during cryopreservation by reducing the oxidative stress and increasing CAT activity.

Conclusions

Our results demonstrated that α-MG supplementation to the freezing medium improved human sperm quality after cryopreservation by protecting sperm cells against ROS and increasing sperm viability and motility. These findings may be an importance in fertility treatment by improving sperm cryopreservation. However, well-controlled clinical trials are required to validate these results in male infertility.

Acknowledgements

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