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Research Article

Effect of Nano Zinc Oxide on Post-Thaw Variables and Oxidative Status of Moghani Ram Semen

Jalil HEIDARI [1] Jamal SEIFDAVATI [1] Hossein MOHEBODINI [1]
Reza SEYED SHARIFI [1] Hossein ABDI BENEMAR [1]

[1] Department of Animal Science, University of Mohaghegh Ardabili, Ardabil, IRAN

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Abstract

The damages of freezing technique of sperm including motility, survival and membrane disorders involves the formation of ice crystals, oxidative stress, osmotic changes and the re-organization of lipid-protein in the cell membrane. Zinc plays basic role in membrane stability and physical properties of the attached fibers, sperm tail morphology and sperm motility. The aim of this study was to evaluate effect of Nano zinc oxide (Nano-znO) on post-thaw variables and oxidative status of Moghani ram semen. In order to collect semen of four Moghani rams, 3-4 years old with an average weight of 80-90 kg were used to twice a weekly. Sperm samples (in five replicates) were frozen in five groups: control group, 0.01, 0.1, 0.5 and 1 mg/mL of Nano-znO. The hypo-osmotic swelling test (HOST) was used to evaluate the integrity of the plasma membrane of the sperm. The results showed that the average of integrity of the plasma membrane of sperm and MDA production in the medium containing 0.1 mg/mL of Nano-znO was with the highest and lowest percentages, respectively ($P<0.05$). There was a significant difference in total motility for 0.1 and 1 mg/mL treatments compared to the control group and the use of 0.01 and 0.1 mg/mL treatments had a significant increase in the percentage of progressive motility compared to the control group but in 0.5 and 1 mg/mL treatments reduced progressive motility. Viability sperm in group 0.1 and group 0.01 was better than control group ($P<0.05$). The use of 0.1 mg/mL of Nano-znO in the ram semen diluted improved total motility, progressive motility and the percentage of survival. In conclusion using Nano-znO had a positive effect on qualitative properties of sperm and lead to a significant improvement in some antioxidant parameters of Moghani ram seminal plasma in the non-breeding season.

Keywords: *: Integrity, Motility, Nano zinc oxide, Peroxidation, Ram, Sperm, Viability*

Nano Çinko Oksitin Moghani Koç Semeninin Çözdürme Sonrası Değişkenler ve Oksidatif Durumuna Etkisi

Öz

Spermin dondurulma tekniğine bağlı olarak şekillenen mortalite, canlılık ve membran bozuklukları hasarları buz kristallerinin oluşmasını, oksidatif stresi, ozmotik değişiklikleri ve hücre zarında lipit-protein reorganizasyonunu içermektedir. Çinko membran stabilitesi, ataçlanmış fiberlerin fiziksel özellikleri, sperm kuyruk morfolojisi ve sperm motilitesi üzerinde önemli rol oynar. Bu çalışmanın amacı nano çinko oksitin (Nano-ZnO) Moghani koç semeninin çözdürme sonrası değişkenler ve oksidatif durumuna etkisini araştırmaktır. Ortalama ağırlıkları 80-90 kg olan 3-4 yaşlı dört Moghani koçundan haftada iki kere semen toplandı. Sperm örnekleri (beş tekrar olarak) kontrol, 0.01, 0.1, 0.5 ve 1 mg/mL Nano-ZnO ile dondurulan gruplara ayrıldı. Spermlerin plazma membran bütünlüklerini değerlendirmek amacıyla Hipo ozmotik şişme testi (HOST) kullanıldı. 0.1 mg/mL Nano-ZnO ile dondurulan spermanın sperm plazma membran bütünlüğü ortalaması en yüksek iken MDA üretimi ortalaması en düşük olarak belirlendi ($P<0.05$). Toplam motilite bakımından 0.1 ve 1 mg/mL uygulama grupları ile kontrol grubu arasında anlamlı fark tespit edildi. İlerleyici motilite yüzdesi 0.01 ve 0.1 mg/mL uygulama gruplarında kontrol grubu ile karşılaştırıldığında anlamlı derecede artma gösterirken 0.5 ve 1 mg/mL gruplarında düşme belirlendi. Sperm canlılığı kontrol grubuna göre 0.1 ve 0.01 gruplarında daha iyiydi ($P<0.05$). Koç semenin sulandırmada 0.1 mg/mL Nano-ZnO kullanmak total motiliteyi, ilerleyici motiliyeyi ve yüze hayatta kalma oranlarını iyileştirdi. Sonuç olarak, Nano-ZnO kullanmak sperm kalite özeliklerine pozitif etki gösterdi ve üreme sezonu dışında Moghani koç seminal plazmada bazı antioksidan parametrelerde anlamlı iyileşmelere neden oldu.

Anahtar sözcükler*: Bütünlük, Motilite, Nano Çinko oksit, Peroksidayon, Koç, Sperm, Canlılık*

İletişim (Correspondence)
+98 914 1538699
jseifdavati@uma.ac.ir

INTRODUCTION

Mammalian semen freezing has created a major development in the storage and protection of sperm cells and is a way of preserving the sex-cell protoplasm that can be used with biodiversity conservation [1]. Sperm banks can play an important role along with other reproductive technologies, in improving breeds and protecting endangered wild and native species. Freezing technique of sperm causes damage including motility, survival, membrane disorders and DNA abnormalities. The damage involves the formation of ice crystals, oxidative stress, osmotic changes, and the re-organization of lipid-protein in the cell membrane [2]. The formation of ice crystals in and around the cell in the freezing process is one of the most important destructive factors of the cell, which reduces sperm motility and fertility [3]. Rams sperm cells have high ratio of unsaturated fatty acids to saturated fatty acids, which makes membranes susceptible to peroxidation damage in the presence of oxygen free radicals. The sperm membrane is susceptible to lipid peroxidation because of containing a high amount of unsaturated fatty acids [4]. In this regard, the accumulation of free oxygen radicals due to metabolism leads to impairment in the membrane's strength, function, survival and fertility of the sperm. The most prominent effect of lipid peroxidation in cells is to disturb the order and function of cell membranes, so that the ion transfer process is changed and disrupted [4]. Zinc element plays basic role in membrane stability and physical properties of the attached fibers, sperm tail morphology and sperm motility. Lack of zinc may increase oxidative damage, resulting in poor sperm quality [5]. Also, zinc element in the sperm, reacts to tight dense fiber that reacts with sulfhydryl groups and prevents oxidation [6]. Some *in vivo* evidence suggests that zinc acts as *in vivo* as a superoxide cleaner produced by incomplete spermatozoids or leukocytes. Other tests have shown that zinc can clean up radicals induced by various factors, including ionizing radiation, and reduce MDA levels, so it is known as a highly antioxidant [7]. The most common combination of zinc element is its oxide form (ZnO) [8], which is preferred for two reasons: one that has the highest concentration of zinc [9], and the other is that it is absorbed high in the body and is also better tolerated by the target tissues [10]. Recently, Nano-zinc oxide has attracted much attention in animal studies [11,12]. Different nanoparticles are new forms of materials with high biologic properties and low toxicity, which seem to have high potential for passing through physiological barriers and access to specific target tissues [13]. The use of antioxidants, such as Nano-zinc oxide, can be important in reducing the production of free radicals and increasing sperm survival [4,14]. Therefore, the aim of this study was to evaluate effect of Nano zinc oxide on post-thaw variables and oxidative status of Moghani ram semen.

MATERIAL and METHODS

Animals, Semen Collection and Freeze-thawing Process

This experiment was performed at University of Mohaghegh Ardabili, Iran Ardabil (Latitude: 38.253736°; Longitude: 48.299990°; Elevation: 4423 ft) and lasted from May to June. The animals (3-4 year old rams) were maintained in open front barns; under uniform nutritional conditions, so that levels of nutrition remained equal as each ram was fed a daily with *ad libitum* diet according to the National Research Council (NRC) containing a 20% concentrate (75% barley, 25% corn, soya and bran) and 80% alfalfa hay. Semen was collected, twice a week for 4 weeks, from four mature Moghani breed rams during the non-breeding season (from mid-January to mid-February 2017) that had been trained to serve an artificial vagina (42-43°C). This study has been approved by local ethical committee of Mohaghegh Ardabili University (Permission No: 51-1842 date: 2016-11/08). The training period lasted four to six weeks. An ovariectomized ewe was used as a teaser and rams were placed in a pen next to the collection area for semen collection of a ram using an artificial vagina. The semen collection procedure could be visualized by each ram [15]. Immediately after collection, the ejaculates were immersed in a warm water bath at 37°C until freezing process. Before the freezing process, macroscopic and microscopic examination was performed for all samples, which included amount of the volume of ejaculate, apparent colors, sperm concentration, and native sperm activity. The native sperm activity was evaluated by subjective method according to the motile sperms percentage in the native ejaculate (Student Microscope Model SM 5 with phase contrast by INTRACTO MICRO, spol. s r. o., lens 10 × /0, 25 PHD). In this experiment, the semen samples showed natural color (color cream), and semen samples with more than 70% of progressive motility and a more than 2.5×10^9 of concentration were used for freezing process.

The basic extender consisted of 3.07 g Tris (hydroxymethyl-aminoethane, Merck 64271, Germany), 1.64 g citric acid (BHD 1081, England), and 1.26 g fructose (BDH 28433, England) in 100 mL distilled water, containing 5.0% (v/v) glycerol (Merck, 2400, Germany) and 20% (v/v) egg yolk with 150 ppm α- tocopherol [16]. The experimental treatments were supplementation of the base extender with 0, 0.01, 0.1, 0.5 or 1 mg/mL of Nano zinc oxide. Nano Zinc Oxide was purchased from Iranian agent of US Research Nanomaterial, Inc. Port Co., Ltd., USA. The sizes of elemental ZnO particles ranged from 10 to 30 nm, stock: US3590, in the form of white powder and Purity: 99%, APS: 10-30 nm, Color: white, Crystal Phase: single crystal, Morphology: nearly spherical, SSA: 20-60 m^2/g, True Density: 5.606 g/cm^3.

Extenders were centrifuged at 15.000×g for 30 min. The supernatants were aspirated and filtrated through a 0.45 mm membrane filter (Millipore, S.A., Molsheim, France). At

each semen collection time, eight straws per extender (16 replicates) were then stored in liquid nitrogen. Two straws per extender per semen collection were thawed at 37°C for 30 sec after 24 h, for evaluation of the post-thawing sperm characteristics. The mean values of the sperm characteristics obtained for the two straws were used in statistical analysis of the data (16 replicates for each cryopreservation extender).

The semen diluent was prepared and kept in a water bath at 37°C, on the day of semen collection. Semen samples were pooled and diluted (1:4) before freezing. Diluted semen samples were cooled to 5°C over 120 min, transferred into 0.25 mL straws, equilibrated for 2 h at 5°C and frozen in liquid nitrogen vapor (4-6 cm from the liquid nitrogen surface level) for 10 min. They were then stored in liquid nitrogen for 24 h. The straws were thawed in water bath at 37°C for 30 sec and the frozen-thawed semen assessed to semen characteristics.

Evaluation of Sperm Motility Characteristics

To evaluate the sperm motility characteristics, after the freezing-thawing process, a CASA computer analysis system equipped with an x-ray contrast microscope (CKX41; Olympus, Tokyo, Japan) was used, with 5 µL. The semen samples were preheated (37°C) and analyzed by Computer computer-assisted analysis sperm (CASA) after coating with lamina. The evaluation was based on a count of at least 100 sperm [16]. The sperm motility, and sperm motion variables that were estimated by these systems included total motile, progressively motile, straight line velocity (VSL), curvilinear velocity (VCL), average path velocity (VAP), linearity (LIN = VSL/VCL), and straightness (STR = VSL/VAP), lateral head displacement, beat cross frequency (BCF) *(Fig. 1)*. Eosin-nigrosin was used to evaluate the number of live and dead sperm. The basis of this staining is that the eosin color penetrates into the dead sperm, while the sperm do not color *(Fig. 2)*. From each sample 200 sperm were counted and the percentage of colored sperm (dead) and sperm (no color) were calculated.

Evaluation of the Integrity of the Plasma Membrane of the Sperm

Hypo-osmotic swelling test (HOST) was used to assess the integrity of the plasma membrane of the sperm [17]. For this purpose, 30 µL of semen samples were incubated with 100 µL of hypo osmotic medium. The hypo-osmotic solution was prepared by dissolving 0.735 g of Tri-sodium citrate dehydrate and 1.351 g of fructose in 100 mL of double-distilled water. First, 500 µL of hypoosmotic solution was mixed with 50 µL of each specimen and incubated at 37°C for 30 min, then 0.2 mL of the mixture was poured onto the slurry and was spread out [18]. The resultant lens was evaluated under a contrast phase microscope (CKX41; Olympus, Tokyo, Japan) and magnified at ×400. From each slide, 200 sperm were counted and the percentage of tangled sperm (healthy membrane sperm) were calculated *(Fig. 3)* [18]. In this experiment, kinky sperm were identified as healthy sperm because the main function of the tail is motility, since there is no fertility without motility [15]. MDA was used to evaluate oxidative stress as major bioactive marker of lipid peroxidation.

Evaluation of Lipid Peroxidation

MDA concentration was measured as a lipid peroxidation

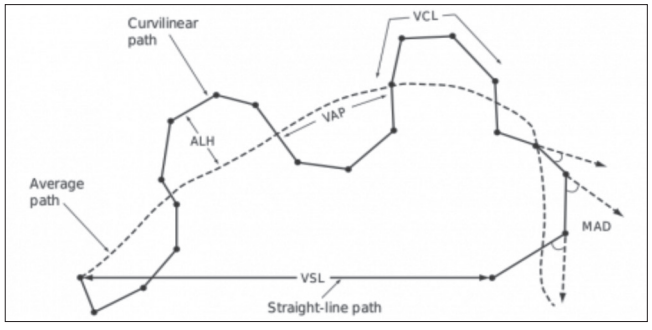

Fig 1. The characteristics of sperm motility

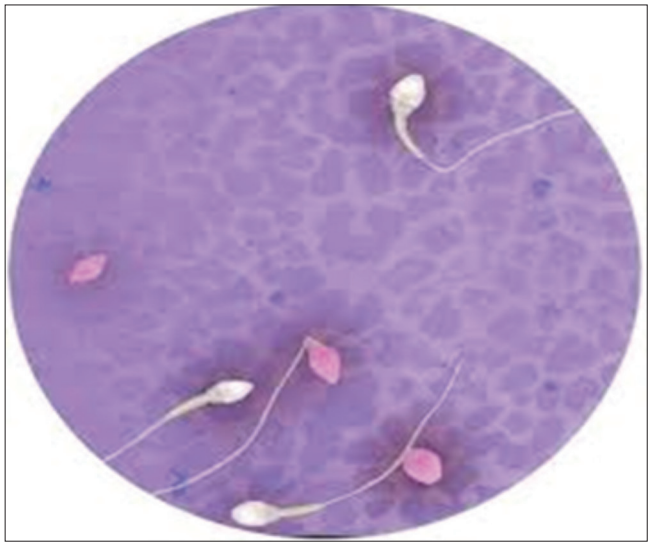

Fig 2. Eosin test: Pink stained sperms are non-viable sperms as compared to white ones with intact membranes

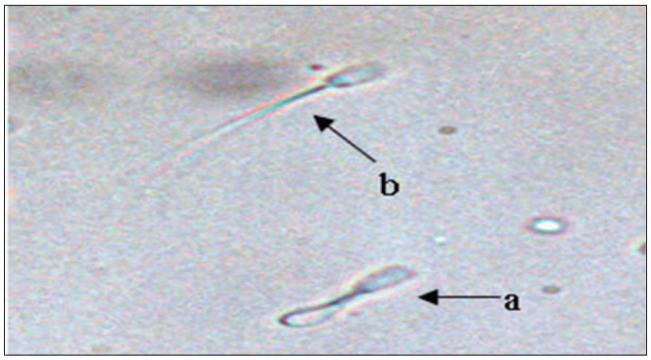

Fig 3. Plasma membrane integrity of the sperm. **a:** healthy sperm and **b:** dead sperm

index in semen samples using the TBARs method and by spectrophotometry [19]. At 95°C, the MDA molecule reacts with two molecules of TBB (TBA) and forms a pink complex. To measure the concentration of MDA, 200 µL of each sample was mixed with 1 mL of EDTA, 1 mL of TCA, and centrifuged at 3.000 rpm, then mixed with 1 mL of butylated hydroxy toluene (BHT), and heated at 95°C for 40 min. The samples were centrifuged for 10 min at 7.000 rpm after cooling in ice. Samples were recorded at 532 nm with spectrophotometry and MDA concentration in nmol/dL.

Methods of Data Analysis and Statistical Model

The normalization of the data was evaluated using SAS software [20]. Values are reported as mean ± SD. Statistical significance between groups was computed by analysis of variance and Duncan's test was used to compare the meanings. Data was analyzed using one way ANOVA test with SAS software [20] and using the GLM procedure in a completely randomized analysis and $P<0.05$ was considered significant. Statistical model research design was as:

$$Y_{ij} = \mu + A_i + e_{ij}$$

where: Y_{ij} is the observation, μ is the population mean, A_i is the effects of experimental treatments and e_{ij} is the residual error.

RESULTS

The effects of different levels of Nano zinc oxide on the motility parameters are presented in *Table 1*. The total motility percentage for the 0.01 and 0.1 mg/mL treatments was different compared with the control group and treatment with 1 mg/mL caused a significant decrease in total motility. The use of 0.01 and 0.1 mg/mL treatments was resulted in a significant increase in progressive motility than the control group. The use of the 0.1 mg/mL Nano zinc oxide level had the greatest impact compared to other levels in VCL (Curvilinear Velocity), VSL (Straight line velocity) and VAP (Average path velocity). The effects of different levels of Nano zinc oxide on survival of sperm are presented in *Table 2*. The use of 0.1 and 0.01 mg/mL Nano zinc oxide levels resulted in the survival of sperm at the highest level compared to other levels and also the use of 1 mg/mL lead to in the most significant reduction compared to the control group.

The effects of different levels of Nano zinc oxide on the average percentage of integrity of the plasma membrane of sperm and the amount of MDA production as lipid peroxidation indicator are presented in *Table 3*. The amount of MDA production was lower at 0.1 mg/mL Nano zinc oxide and was significantly different from the control group ($P<0.05$). The average percentage of integrity of the plasma membrane of sperm was highest in the medium containing 0.1 mg/mL Nano zinc oxide, which was significantly different from other groups ($P<0.05$).

DISCUSSION

The resulting papers reported that some protection and distribution to spermatozoa for high fertility and genetic value suggest a freeze–thawing process or cryo-

Table 1. *Effect of Nano zinc oxide on ram sperm motility parameters (mean ± SD)*

Treatments (mg/mL)	Total Motile (%)	Progressively Motile (%)	VCL (µ/s)	VSL (µ/s)	VAP (µ/s)
0	44.4±2.68 ^{b}	17.6±1.35 ^{b}	102.6±3.28 ^{b}	43.9±1.51 ^{b}	53.6±1.83 ^{b}
0.01	54.2±2.68 ^{a}	23.4±1.35 ^{a}	114.9±3.28 ^{a}	48.6±1.51 ^{ab}	58.6±1.83 ^{ab}
0.1	58.4±2.68 ^{a}	26.0±1.35 ^{a}	117.6±3.28 ^{a}	52.3±1.51 ^{a}	60.8±1.83 ^{a}
0.5	42.2±2.68 ^{bc}	18.8±1.35 ^{b}	111.3±3.28 ^{ab}	46.2±1.51 ^{b}	55.7±1.83 ^{ab}
1	35.0±2.68 ^{c}	17.2±1.35 ^{b}	101.1±3.28 ^{a}	43.8±1.51 ^{b}	55.1±1.83 ^{ab}

The same letters in each column represent significantly different at 5% level
VCL (Curvilinear Velocity), VSL (Straight line velocity) and VAP (Average path velocity)

Table 2. *Effect of Nano zinc oxide on survival of ram sperm (mean ± SD)*

Treatments (mg/mL)	Survival of Ram Sperm (%)
0	51.0±2.18^{b}
0.01	64.3±2.18^{a}
0.1	66.4±2.18^{a}
0.5	52.6±2.18^{b}
1	46.1±2.20^{b}

The same letters in each column represent significantly different at 5% level

Table 3. *The effect of Nano zinc oxide on lipid peroxidation and plasma membrane integrity*

Treatments (mg/mL)	Malondialdehyde (MDA)	Plasma Membrane Integrity
0	3.6±0.41^{ab}	33.5±1.89^{cd}
0.01	3.0±0.41^{bc}	46.7 ±1.89^{b}
0.1	2.1±0.41^{c}	56.9 ±1.89^{a}
0.5	3.5±0.41^{ab}	38.8± 1.89^{c}
1	4.6±0.41^{a}	28.7 ±1.89^{d}

The same letters in each column represent significantly different at 5% level

preservation [21-23]. In the study conducted by Dissanayake et al.[24] on human sperm, it was shown that adding 1.2 µmol per mL of zinc to the culture medium significantly reduced the percentage of human sperm counts between zero to four hours, this means that with time declined the percentage of motile spermatozoa. However, this researcher added the amount of 0.6 µmol/mL to the culture medium was observed that sperm motility increased compared to the control group. Also, the percentage of sperm recovery was higher in group 0.6 µmol/mL, and there was a significant difference with the control group [24]. This is likely to be related to the function of the element in Zn such that Zinc is associated with ATP, plays a role in shrinking and regulating the energy of its phospholipids, and therefore has a direct impact on sperm motility [25]. In a research on buffalo sperm, it has been shown that zinc in the semen plasma has a direct relationship with the survival and progressively motile of sperm [26]. In another study on buffalo sperm, it has been shown that the presence of semen in the plasma directly affects the survival and progressively motile of sperm [26]. In the present experiment, the levels above 0.1 mg/mL (administration at high concentration) have not a positive effect on the parameters of sperm motility and viability, which it can be expressed as a toxic level for the sperm dilution environment *(see Tables)*. Our results indicated that any Zn imbalance in seminal plasma or spermatozoa may have a negative impact on seminal abnormalities and/or oxidative stress development and therefore may be considered as a risk cause for ram fertility problems [26].

Animal studies have shown that [27] zinc effect on oxidative damage in rat testicular was investigated and the results showed that iron concentration in testes in zinc deficient male diets was higher and there was a significant difference in diets with zinc. Observed oxidative damage may occur due to the increased free radicals associated with the accumulation of iron in the tissue or the reduction of zinc-dependent antioxidant processes [27]. The addition of zinc element in human sperm samples produced a significant difference in lipid peroxidation and hypo-osmotic swelling test (HOST) with the control group [28]. Although the evidence for the antioxidant properties of zinc is compelling, the mechanisms are still unclear. But the important effects are generally thought to involve two mechanisms: protection of protein sulfhydryls or reduction in the formation of ·OH from H_2O_2 through the antagonism of redox-active transition metals, such as iron and copper [29]. At the our study, the level of 0.1 mg/mL Nano zinc oxide showed that the production level of malondialdehyde was in minimum which showed that there was less peroxidation in Moghani ram sperm after freeze-thawing process, and indicated that there was not stress oxidative or peroxidation in sperm cryopreservation medium containing Nano zinc oxide. Lipid peroxidation is the oxidative degradation of lipids. It is the process in which reactive oxygen species (ROS) or free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage due to higher production of the oxidative cryo-injury resulting from sperm cooling, freezing and thawing. Furthermore, the cryopreservation process induces ROS such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) [30] and when spermatozoa are attacked by ROS, a loss in polyunsaturated fatty acids (PUFAs) from the plasma membrane is occurred and their survival and fertilizing ability is declined [31]. These results are consistent with previous data showing that Zinc can clean up radicals induced by various factors, including ionizing radiation, and reduce the amount of lipid peroxidation, and therefore it is known as a high-protection antioxidant [7]. Also, in other study found that zinc oxide nanoparticles could maintain the integrity of the cell membrane against oxidative damage, increase the amount of antioxidant enzymes, reduce the amount of malondialdehyde, improve the anti-oxidant activity, and reduce the amount of free radicals [12]. In this study, the antioxidant properties of zinc oxide nanoparticles were helpful in reducing the production of malondialdehyde, and the addition of zinc oxide to the environment in diluent improved the integrity of the membrane. According to the results, the use of 0.01 and 0.1 mg/mL Nano zinc oxide improved sperm survival and sperm motility characteristics. As a result, the use of appropriate levels of zinc supplementation in ram semen diluent can be useful in maintaining the quality of the sperm during the freeze-thawing process.

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