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# Effects of Taurine and Vitamin E on Sperm Characteristics and Oxidative Stress in the Freezing Semen of Pigs

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## ABSTRACT

**Background:** We evaluated the effects of taurine and vitamin E on sperm characteristics (motility and viability) and oxidative stress (reactive oxygen species and nitric oxide) in the freezing semen of pigs. We determined sperm motility in fresh and freezing boar semen.

**Methods:** Viability, reactive oxygen species and nitric oxide determination in freezing sperm were determined by SYBR-14/PI kit, DCF-DA and DAF-2.

**Result:** The motility of freezing sperm was not significantly different between taurine- and vitamin E-treated samples. We found that taurine and vitamin E increased the viability of sperm, and decreased reactive oxygen species and nitric oxide in the freezing semen of pigs. In conclusion, the use of taurine and vitamin E in boar semen cryopreservation can enhance sperm quality *via* protection from reactive oxygen species and nitric oxide damage.


**Key words:** Nitric oxide, Reactive oxygen species, Sperm, Taurine, Vitamin E.

## INTRODUCTION

Sperm cryopreservation in reproduction is important in cell viability and fertility preservation for extended periods and improves the efficiency of artificial insemination in swine production. Cryoprotectant plays a crucial role in preventing ice crystal formation within sperm cells. The permeation of cryoprotectants through the sperm membrane safeguards cellular structures, enhancing the viability of boar sperm on thawing (Yeste, 2016; Tamburrino *et al*., 2023). Although many studies have tried to minimize the risk of ice crystal damage and maintain the structural and functional integrity of sperm cells for sperm viability and fertility preservation during freezing and thawing, sperm cells have been damaged in the long-term viability of cryopreserved boar semen (de Mercado *et al*., 2020; Oldenhof *et al*., 2021). Since the freezing and thawing of sperm processes can induce DNA damage by oxidative stress in sperm cells, sperm viability also decreases in boar semen (Kadlec *et al*., 2022). Sperm damage is regulated with free radicals, reactive oxygen species and nitric oxide (Espinosa-Garcia *et al*., 2023; Patil *et al*., 2023; Kumar *et al*., 2024).

Reactive oxygen species play dual roles in sperm physiology. In physiological concentrations, reactive oxygen species act as signalling molecules involved in sperm capacitation, a process necessary for fertilization (Pezo *et al*., 2020; Zhang *et al*., 2021). However, excessive reactive oxygen species levels can lead to detrimental effects on sperm membrane integrity, DNA integrity, motility, and viability. Mitochondrial respiration during sperm metabolism is a primary source of reactive oxygen species (Zhu *et al*., 2019). During cryopreservation, the formation of ice crystals and changes in temperature can trigger the release of reactive oxygen species (Grotter *et al*., 2019). As sperm undergo the freeze-thaw process, temperature fluctuations and cryoprotectants can increase reactive oxygen species. Also, the activation of spermatozoa during freezing and thawing contributes to reactive oxygen species generation, as the sudden changes in temperature and osmotic conditions induce oxidative stress.

Nitric oxide is involved in various physiological processes and has been implicated in reproductive functions. Understanding the dynamics of nitric oxide generation during the freezing of boar semen is critical for optimizing cryopreservation techniques (Restrepo *et al*., 2023; Upadhyay *et al*., 2023). During cryopreservation, changes in temperature and exposure to cryoprotectants may influence nitric oxide synthesis activity, leading to altered nitric oxide production. In physiological conditions, nitric oxide is a signaling molecule with essential roles in vasodilation, neurotransmission and immune responses (Tsopka *et al*., 2021). In reproduction, nitric oxide has been implicated in sperm capacitation, acrosome reaction, fertilization and viability (Staicu *et al*., 2019). Temperature fluctuations during the freezing process can impact the activity of nitric oxide synthesis enzymes, influencing nitric oxide production. Also, the interaction between cryoprotectants

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and sperm membranes can affect nitric oxide generation (Maciel *et al*., 2018). The balance of nitric oxide levels is critical for maintaining sperm viability and motility. Both deficiency and excess of nitric oxide can have adverse effects on sperm function, emphasizing the need for precise regulation during cryopreservation.

However, the functions of taurine and vitamin E in reactive oxygen species and nitric oxide have not been reported in freezing boar semen. Moreover, it is unknown whether taurine and vitamin E maintain sperm viability in semen cryopreservation with a combined antioxidant supplement. Thus, we studied the roles of taurine and vitamin E on sperm motility and viability and reactive oxygen species and nitric oxide in the freezing sperm of pigs.

## Materials and Methods

### Animal and reagents

Five crossbred pigs (Duroc × Landrace × Yorkshire, around 28 months) were used (Artificial Insemination Center, Wonju, Republic of Korea, Research period: 2022-2023). Taurine, vitamin E (Table 1) and other chemical reagents were purchased (Sigma-Aldrich, St. Louis, MO, USA).

### Experimental design

We designed experiments as the following:

**Experiment 1:** Does-dependent experiments of taurin and vitamin E on sperm viability in the fresh and freezing semen of pigs.

**Experiment 2:** Experiments of taurine and vitamin E on sperm motility in the freezing semen of pigs.

**Experiment 3:** Experiments of taurin and vitamin E on sperm viability, reactive oxygen species, and nitric oxide in the freezing semen of pigs.

### Semen preparation

We collected semen from ten crossbred pigs. The fresh samples were diluted in the Modena extender with bovine serum albumin (BSA, 3.0 g/L) and gentamicin sulfate (0.3 g/L). Transported semen at 18.0°C was used for sperm viability, reactive oxygen species and nitric oxide production in the freezing experiment and fresh semen was used for sperm viability. The samples were diluted until sperm concentration was $1.0 \times 10^9$ sperm/mL for the analysis of sperm characteristics.

### Freezing and thawing

A freezing extender was carried out according to previous research (Kim *et al*., 2015; Lee *et al*., 2019). Briefly, the first and second freezing extenders were composed of 20.0% egg yolk, 11.0% α lactose, 9% glycerol and 1.5% Orvus Es Paste (OEP; Nova Chemical Sales Inc., Pittsburgh, PA, USA), respectively. We added 0, 5.0, 10.0 and 20.0 mM taurine or 0, 50.0, 100.0 and 200.0 μM vitamin E to the freezing extender. The diluted semen was cooled at 5°C for 120 min and cooled to -120°C for 10 min in liquid nitrogen. The sample was thawed in a water bath (37.5°C, 45 sec). Then, the freezing extender was carefully removed by a centrifuge (400 × g, 5 min). Finally, samples were re-suspended to Modena extender (final sperm concentration $1.0 \times 10^7$ sperm/mL). Then, samples were used for experiments.

### Sperm motility

We evaluated the motility of the sperm. A 20.0 μL sperm was re-placed on the slide glass. At least 500 sperms were examined at × 400 magnification with a phase-contrast microscope (Olympus, Kyoto, Japan).

### Sperm viability assay

A freezing extender was carried out according to previous research (Lee *et al*., 2023). Sperm viability was measured using 40 nM SYBR-14 and 2.0 μM propidium iodide reagents (L-7011, Invitrogen, New York, NY, USA). The treated samples (10,000 sperms) were evaluated by flow cytometry (488 nm, FACSCaliber, BD Biosciences, Franklin Lakes, NJ, USA). The dot plot data was analyzed by CELLQuest software (Version 6.0, Becton Dickinson, San Jose, CA, USA).

### Reactive oxygen species and nitric oxide determination

Reactive oxygen species and nitric oxide were assessed as described previously (Lee *et al*., 2023). Briefly, reactive oxygen species using a 2‘,7‘-dichloro-fluorescein diacetate (DCF-CA, Invitrogen). The Produced reactive oxygen species was quantified by the DCF standard curve. The reactive oxygen species production was analyzed using a histogram (CELLQuest softwar, Becton Dickinson) and was normalized using the control treatment values. The production of nitric oxide was detected using a nitric oxide 4,5-diaminofluorescein diacetate (DAF-2 DA) reagent (Sigma). We mearsured the incubated samples for nictic oxide production of sperm using flow cytometry (FACSCaliber, BD Biosciences).

### Statistical analysis

All statistical data analyses were performed using one-way ANOVA followed by Fisher-protected least significant difference analysis (SDA) using Stat View (SAS Institute, Cary, NC, USA). Experiment data were presented as mean±standard error mean (SEM).

## Results and Discussion

### Does-dependent experiments of taurin and vitamin E on sperm viability in fresh semen of pigs

We determined the sperm viability on different concentrations of taurine (Table 2) and vitamin E (Table 3)

**Table 1:** Used dose-dependent concentration and chemical information.

| | Taurine | Vitamin E |
|---|---|---|
| Concentration | 5.0, 10.0, 20.0 mM | 50, 100, 200 μM |
| MW[1] | 125.15 | 430.71 |
| Formula | $NH_2CH_2CH_2SO_3H$ | $C_{29}H_{50}O_2$ |

[1]MW: Molecular weight.

in the fresh semen. Tables 2 and 3 show sperm viability on fresh semen was significantly ($p<0.05$) increased in a dose-dependent 10.0 and 20.0 mM taurine and 100.0 and 200.0 µM vitamin E. We also determined sperm viability in the freezing semen, such as Fig 1 and 2.

**Dose-dependent experiments of taurine and vitamin E on sperm viability in freezing semen of pigs**

We determined the sperm viability on different concentrations of taurine (Fig 1) and vitamin E (Fig 2) in the freezing boar semen. Sperm viability in a 20.0 mM taurine ($p<0.05$) and a 200.0 µM vitamin E ($p<0.05$) treated freezing samples were increased. But, sperm viability was not increased in 10.0 mM taurine and 100.0 µM vitamin E. Therefore, we used a concentration of 20.0 mM taurine and 200.0 µM vitamin E to determine ROS and NO and sperm viability and motility in freezing semen in pigs.

Taurine has been studied for its beneficial effects on sperm quality and function (Li *et al.*, 2023). We suggest that taurine supplementation may enhance the viability of fresh sperm by improving sperm motility and morphology. Taurine is believed to exert antioxidant properties, protecting sperm cells from oxidative stress-induced damage, which can improve their viability and fertilization potential in pigs. We also found that sperm viability in fresh semen of pigs was significantly increased on taurine-treated sperm. The mechanisms through which taurine exerts its effects on sperm viability are multifaceted. Taurine is known to regulate ion channels and intracellular calcium levels in sperm cells, which are crucial for sperm motility and function (Boni *et al.*, 2017; Baliou *et al.*, 2021). Additionally, taurine acts as an osmolyte, helping to maintain cell volume and integrity, particularly during exposure to environmental stresses such as freezing and thawing (Dayang *et al.*, 2018). Thus, we have to determine sperm viability in the freezing boar semen. As a result, sperm viability is also increased by taurine. Although taurine is an important antioxidant in improving sperm viability, it is essential to consider individual variability in response to supplementation and potential side effects. Nevertheless, taurine supplementation holds potential as a strategy for enhancing sperm viability in both fresh and frozen states, offering promising avenues for improving fertility outcomes and advancing the reproductive system. Moreover, sperm research and clinical research are warranted to fully understand the therapeutic influence of taurine and its applications in the field of male infertility and assisted reproduction.

**Effects of taurine and vitamin E on sperm motility in freezing semen of pigs**

We evaluated sperm motility on the effects of taurine and vitamin E in freezing semen of pigs. As Table 4, sperm motility was significantly decreased in frozen-thawed semen compared with fresh semen ($p<0.05$). However,

**Table 2:** The dose-dependent experiment of taurine on the sperm viability in fresh semen in pigs.

| | Taurine (mM) | | | |
|---|---|---|---|---|
| Viability, % | 0.0 | 5.0 | 10.0 | 20.0 |
| | 77.84±0.65 | 78.40±0.54 | 81.93±0.90* | 82.68±0.72* |

*Significantly different values, n = 10.

**Table 3:** The dose-dependent experiment of vitamin E on the sperm viability in fresh semen in pigs.

| | Vitamin E (µM) | | | |
|---|---|---|---|---|
| Viability, % | 0.0 | 50.0 | 100.0 | 200.0 |
| | 76.62±0.54 | 77.15±0.60 | 82.41±0.84* | 82.11±0.66* |

*Significantly different values, n = 10.

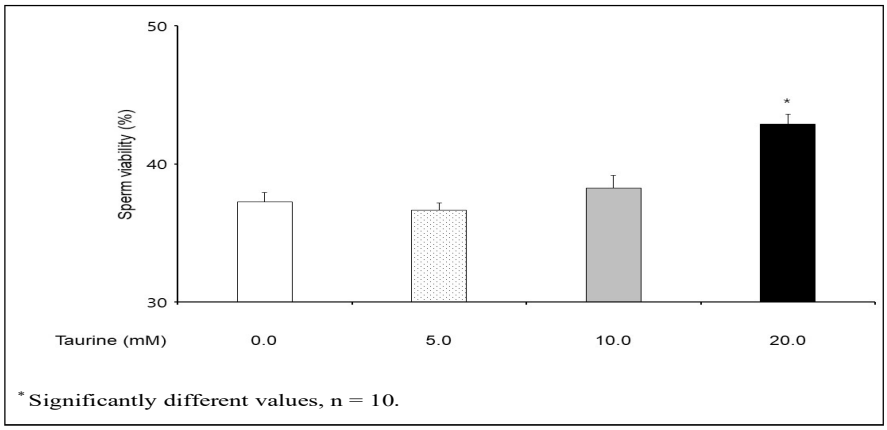


**Fig 1:** Does-dependent experiment of taurine on the sperm viability in freezing semen of pigs.

sperm motility was not changed in 20.0 mM taurine and 200.0 µM vitamin E samples and also both treated samples. There was no difference in the taurine- and vitamin E-treated freezing boar semen.

Our studies have suggested that vitamin E supplementation may enhance the quality and function of fresh and freezing boar sperm. By scavenging free radicals and reducing oxidative stress, vitamin E can help preserve sperm membrane integrity, maintain sperm motility, and protect DNA integrity, ultimately improving sperm viability (Ghafarizadeh *et al*., 2021; Espina-Avila *et al*., 2021). Vitamin E affects sperm viability through several mechanisms. As an antioxidant, vitamin E neutralizes free radicals and lipid peroxidation products, which are known to impair sperm function and viability (Zakosek Pipan *et al*., 2017; Losano *et al*., 2018). Vitamin E also interacts with other antioxidants, such as vitamin C, glutathione and selenium, synergistically enhancing their protective effects on sperm cells (Moghbeli *et al*., 2016; Yuan *et al*., 2023). Cryopreservation can induce oxidative stress and membrane damage in sperm cells, leading to reduced viability and motility. Moreover, vitamin E can help counteract these effects by stabilizing cell membranes, preventing lipid peroxidation and preserving mitochondrial function, thus improving sperm viability in freezing semen. However, further research is needed to optimize vitamin E supplementation protocols, determine the optimal dose and duration of supplementation and evaluate its effect on different mammalian sperm.

**Effects of taurine and vitamin E on Reactive oxygen species and nitric oxide in freezing semen of pigs**

As shown in Fig 3 and 4, reactive oxygen species and nitric oxide in 20.0 mM taurine and 200.0 µM vitamin E treated samples were significantly decreased ($p<0.05$). And taurine and vitamin E treated samples were lower than the alone treated groups. However, the samples were not significantly different. Also, sperm viability in 20.0 mM taurine- and 200.0 µM vitamin E-treated samples was significantly different (Fig 5, $p<0.05$). The co-treated samples were higher than the non-treated samples ($p<0.05$). However, it was not significantly different.

Reactive oxygen species- and nitric oxide-induced damage during boar semen cryopreservation needs to involve implementing targeted strategies to minimize oxidative stress (de Andrade *et al*., 2018; Zhang *et al*., 2021). Thus, optimizing freezing protocols and investigating antioxidant supplements are important in freezing sperm cells. Antioxidants play a crucial role in scavenging Reactive oxygen species and nitric oxide and preventing oxidative damage. Selecting appropriate antioxidants and determining optimal concentrations for supplementation are key considerations in developing effective antioxidant strategies for boar semen cryopreservation. Taurine is a sulfur-containing amino acid widely distributed in mammalian tissues, with high concentrations in the heart, brain and skeletal muscles (El Idrissi *et al*., 2019; Jakaria *et al*., 2019; Wen *et al*., 2019; Jong *et al*., 2021). It plays diverse roles, including osmoregulation, bile salt formation and modulation of cellular calcium levels. Additionally, taurine exhibits antioxidant properties, scavenging free radicals and protecting cells from oxidative damage. Taurine's antioxidant activity involves multiple mechanisms (Baliou *et al*., 2021). It acts as a free radical scavenger, particularly targeting reactive species like singlet oxygen and hydroxyl radicals. Taurine also enhances endogenous antioxidant defenses by upregulating enzymes such as superoxide dismutase (SOD) and catalase.

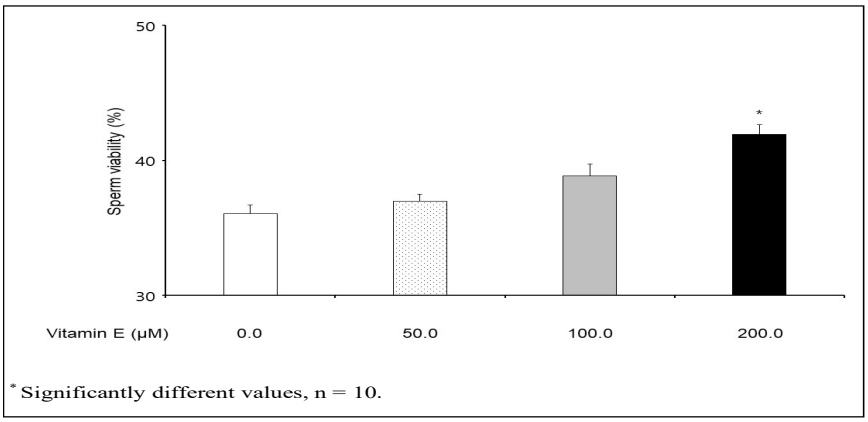


**Fig 2:** Does-dependent experiment of vitamin E on the sperm viability in freezing semen of pigs.

**Table 4:** Effects of taurine and vitamin E on the sperm motility in freezing semen of pigs.

| | Fresh | | Frozen-thawed | | |
|---|---|---|---|---|---|
| 20 mM Taurine | – | – | + | – | + |
| 200 µM Vitamin E | – | – | – | + | + |
| Motility(%) | 84.04±1.38 * | 38.36±0.82 | 39.07±1.06 | 38.70±1.09 | 38.49±1.01 |

*Significantly different values, n= 10.

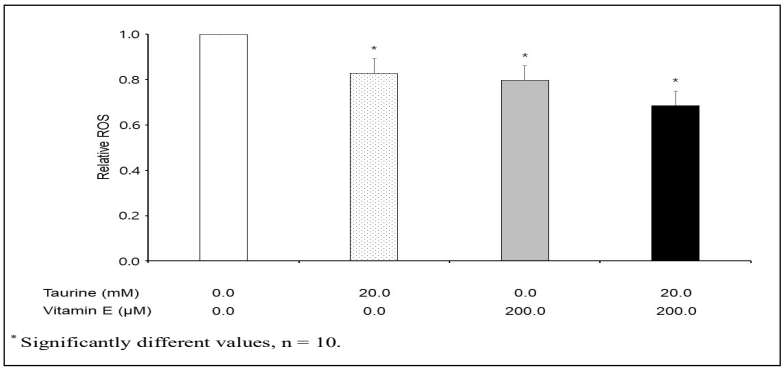


**Fig 3:** Effects of taurine and vitamin E on reactive oxygen species in freezing semen of pigs.

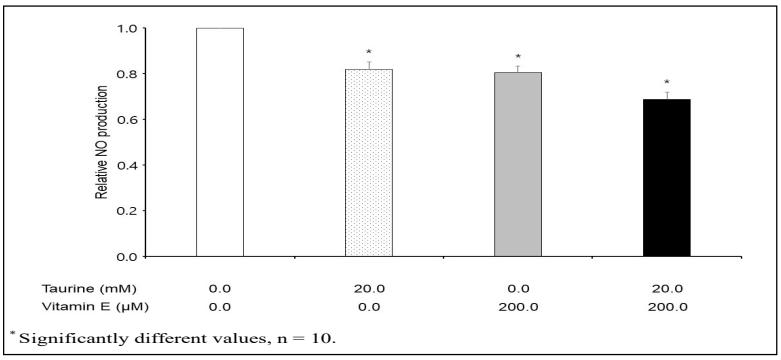


**Fig 4:** Effects of taurine and vitamin E on nitric oxide in freezing semen of pigs.

The antioxidant action of vitamin E involves scavenging lipid peroxyl radicals and breaking the lipid peroxidation chain reaction (Baj *et al*., 2019). The primary role of vitamin E is to protect cell membranes from oxidative damage (Ehsan *et al*., 2018). As a lipophilic antioxidant, it resides in the hydrophobic regions of cell membranes, shielding unsaturated fatty acids from peroxidation. The importance of vitamin E in maintaining cellular integrity is evident in its ability to prevent oxidative stress-induced cell membrane damage. Taurine, being water-soluble, can scavenge reactive oxygen species in the aqueous environment, including the cytoplasm and cellular organelles (Shimada *et al*., 2015). Vitamin E, lipid-soluble, concentrates in cell membranes, protecting against lipid peroxidation (Traber *et al*., 2021). The synergy between these antioxidants spans both hydrophilic and lipophilic cellular compartments, providing comprehensive protection against oxidative stress. Both taurine and vitamin E have been reported to enhance the activity of endogenous antioxidant enzymes. Taurine modulation of SOD and catalase, combined with the ability of vitamin E to support these enzymatic defenses, creates a synergistic effect that reinforces the cellular antioxidant defense system. Also, mitochondria are a major source of reactive oxygen species and their efficient functioning is essential for cellular energy production. Taurine, by preserving mitochondrial function and reducing reactive oxygen species generation, complements the role of vitamin E in protecting mitochondrial membranes from lipid peroxidation. Overall, we suggest that the role of taurine and vitamin E is different in freezing semen. Thus, the combined extender will effectively protect sperm damage from oxidative stress.

## CONCLUSION

In conclusion, the synergy between taurine and vitamin E in cellular protection represents a promising avenue in the field of antioxidant research. Their complementary mechanisms, spanning hydrophilic and lipophilic cellular compartments, offer comprehensive protection against oxidative stress. As our understanding of their combined effects deepens, the potential applications of taurine and vitamin E in disease prevention and therapeutic interventions continue to expand, paving the way for innovative strategies to combat oxidative stress-related pathologies.

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### Disclaimers

The views and conclusions expressed in this paper are those of the author and do not necessarily reflect those of their affiliated institutions.

**Informed consent**

The animal study protocol was approved by the Institutional Animal Care and Use Committee of Kangwon National University (KIACUC-09-0139).

**Conflict of interest**

The author declares that there are no conflicts of interest regarding the publication of this paper.

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