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PKA and NBC expression patterns in cryopreserved bovine spermatozoa after kaempferol treatment

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Abstract

Kaempferol (KAE) is a member of the flavonoid family, which exhibits antioxidant properties. The richest sources of KAE are green leafy vegetables and herbs like kale, spinach or dill. The goal of our study was to evaluate a possible cryoprotective effects of KAE through to gene expression of protein kinase A (PKA) and sodium bicarbonate cotransporter (NBC). For the experiments we used semen samples obtained from 12 adult Holstein bulls. Before cryopreservation, all samples were divided equally and supplemented by different concentrations of KAE (0, 12.5, 25 and 50 µmol/L), except for native control. After that, RNA was isolated, and the purity and quality of RNA was verified nanophotometrically at A260/A280 nm. Next step was a transcription of RNA into cDNA, which was used for two-step qPCR while expression of PKA and NBC was quantified under specific cycling conditions. Data was evaluated by the delta delta Ct method and One-way ANOVA using Tukey's range test. According to expression patterns, it was observed that 25 µmol/L of KAE significantly preserved ($P<0.0001$) the PKA compared to cryopreserved control without KAE treatment. In the case of NBC, there was slightly increase of expression in the group treated with 50 µmol/L of KAE but without significant changes. In conclusion, KAE treatment exhibit promising cryoprotective properties of PKA and NBC genes especially in higher concentration 25 and 50 µmol/L, which may successfully preserved proper expression after thawing of cryopreserved bovine spermatozoa.

1. Introduction

Flavonoids are the largest category of secondary plant-based metabolites. One of them is kaempferol (3,4',5,7-tetrahydroxyflavone) commonly found in green mass vegetable (spinach, broccoli, kale) and several medicinal plants or herbs. In general, antioxidant properties of flavonoids are well known but other positive effects like anti-microbial or anti-inflammatory properties have been studied **(Dabeek & Marra, 2019)**.

During freezing/thawing process, spermatozoa are sensitive to cryoinjury due to ultra-structural and sub-lethal damage accelerated by oxidative and osmotic stresses, which lead into decrease of motility and viability, acrosomal integrity, DNA fragmentation or cryo-induced capacitation **(Khan et al., 2021)**. Mammalian capacitation is triggered by the activation of transmembrane channels of spermatozoa including sodium bicarbonate cotransporter (NBC), which is responsible for bicarbonate (HCO_3^-) uptake and increase of intracellular pH. Synthesis of cyclic adenosine monophosphate (cAMP) is accompanied by the activation of protein kinase A (PKA), which indirectly regulates protein phosphorylation and sperm hyperactivation **(O'Flaherty et al., 2006)**.

On the contrary, cryo-induced capacitated spermatozoa are characterized by the low cAMP synthesis due to loss activity of crucial transmembrane channels such as NBC. Because of the higher membrane fatty acid content, spermatozoa are more vulnerable to cryopreservation-related damage and lipid peroxidation. A major part of the cryodamage is often detected in the plasmatic and acrosomal membrane because these parts are more exposed to cryo-environment during freezing/thawing cycle **(Khan et al., 2021)**.

Our recent research confirmed the potential of KAE as an extender supplement, which significantly decreased oxidative damage, stabilized membrane proteins and DNA as well as maintain sperm motility and functionality of mitochondria **(Ďuračka et al., 2019; Baňas et al., 2024)**. Hence, the purpose of our study was to assess the protective potential of KAE through monitoring of gene expression of two capacitation-associated genes (NBC and PKA) in cryopreserved bovine spermatozoa.

2. Material and methods

2.1 Cryopreservation procedure

Fresh semen samples were obtained from 12 adult Holstein bulls at local breeding station. Following cryopreservation each

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sample was divided into five equal parts. The first part (negative control) was native semen without KAE diluted in phosphate saline buffer (Sigma-Aldrich, St. Louis, MO, USA), while second part (positive control) was cryopreserved without KAE treatment. All experimental groups were treated by different KAE concentrations (12.5, 25 and 50 µmol/L) dissolved in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA). Before cryopreservation, samples were diluted in Triladyl-based extender (Minitüb GmbH, Tiefenbach, Germany), which contain 20% egg yolk and antibiotics. The diluted samples were loaded into French straws, cooled down to 4°C, frozen by digital freezing machine (Digitcool 5300 ZB 250; IMV, France) and stored in liquid nitrogen at -196°C for further analysis.

2.2 Genetic analysis

Total RNA from spermatozoa was isolated by using NucleoZol reagent (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) according to modified protocol. The purity and quantity of isolated RNA was measured with Implen NanoPhotometer (München, Germany) at A260/A280 nm. A total of 200 ng of RNA was used for the reverse transcription and cDNA synthesis by using commercially available kit Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase (ThermoFisher Scientific, Waltham, MA, USA). Specific PCR conditions for selected genes (PKA and NBC) were set according to our previous study **(Benko et al., 2022)**.

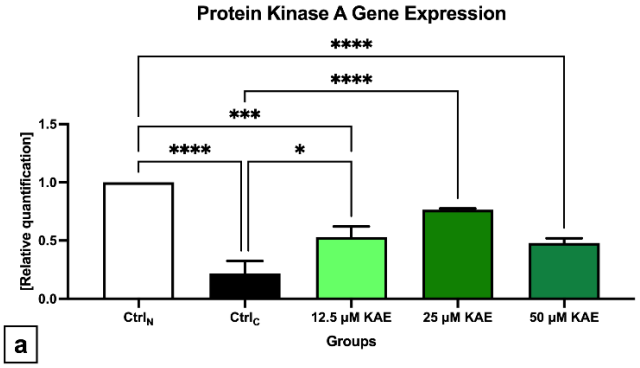
3. Results and discussion

Based on the collected data (Fig. 1), cryopreservation led to significant decline (P<0.0001; P<0.001) of expression in both PKA (Fig. 1a) as well as NBC (Fig. 1b) gene compared to fresh control. However, there was a significant improvement (P<0.0001) of PKA expression in the group treated with 25 µmol/L of KAE against un-treated cryopreserved control. In the case of NBC, there was no-significant changes between treated groups against un-treated control, but slightly higher preservation of NBC gene expression was observed in the group treated with the higher concentrations of KAE including 25 and 50 µmol/L of KAE.

Our hypothesis that KAE treatment could be effective against cryo-induced capacitation was previously validated by Western blotting, indicating that especially 25 µmol/L of KAE could prevent the inactivation of protein kinases A and C **(Baňas et al., 2024)**. This can be partially explained by the antioxidant ability of KAE to dispose of ROS before these can reach axoneme and outer membranes involved in the PKA induced sperm hyperactivation **(Baro Graf et al., 2020)**.

Data collected from previous research evaluated that KAE seems to be effective against hydroxyl radicals, which are the main cause of lipid peroxidation, oxidative DNA damage and apoptosis-inducers **(Tejero et al., 2007; Heo et al., 2020)**. It can be a straight link into the chemical structure of KAE and binding into already generated ROS by mitochondria as the main source of ROS in spermatozoa. It seems that KAE controls rather than eliminates the physiological levels of superoxide and hydrogen peroxide, which are needed for proper capacitation and later acrosome reaction. This opinion was already supported the studies on bovine and human spermatozoa, where KAE treatment stabilizes the enzymatic antioxidant capacity of superoxide dismutase **(Jamalan et al., 2018; Mohamed & Gouda, 2022)**.

We also confirmed that selected concentrations of KAE, especially 25 µmol/L successfully preserve the expression of target proteins (heat shock proteins 70/90, pro-apoptotic BAX and anti-apoptotic Bcl-2 protein) and prevent the loss of motility, DNA status and lipid profile in cryopreserved bovine samples **(Baňas et al., 2023)**. The preservation of PKA triggered by the KAE treatment could be associated with the higher level of protection to transmembrane channels crucial for the activation of adenylyl cyclase (ADCY) and cAMP. It is supported by the earlier findings observing that flavonoids may modulate AMP-activated protein kinase, which interfere with the cAMP-activated protein kinases, guanylate cyclase and ADCY sperm signaling **(Mansuri et al., 2014; Abdallah et al., 2020)**. According to **Hou & Kumamoto (2010)** flavonoids had the ability to bind directly to protein kinases and alter their phosphorylation state better than conventional hydrogen-donating antioxidants.



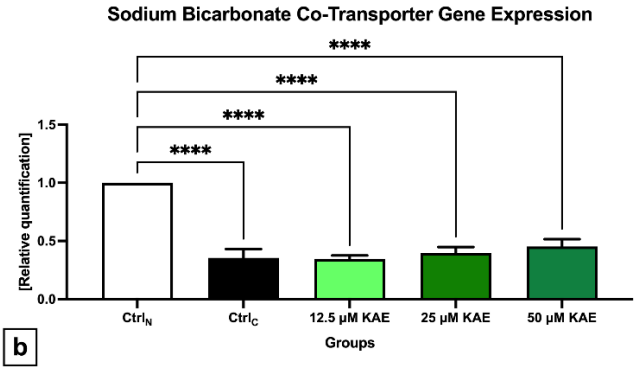


Figure 1 Expression patterns of two selected capacitation-associated genes: protein kinase A - PKA (a) and sodium bicarbonate cotransporter – NBC (b) in fresh control ($Ctrl_N$), cryopreserved control ($Ctrl_C$) without kaempferol (KAE) treatment and groups treated with 12.5, 25 and 50 µmol/L of KAE.

On the contrary, the impact of KAE treatment on the NBC expression was insignificant but a notable post-thaw improvement was recorded in the presence of 25 and 50 µmol/L of KAE. This could be a consequence of the lipophilic nature of KAE enabling the biomolecule to be incorporated into the plasma membrane and partially stabilize the network of transmembrane channels against oxidative cryodamage **(Mansuri et al., 2014)**. In a previous report, **Marunaka (2017)** suggested that flavonoids like quercetin elevate the activity of sodium-like channels by their absorption of both lipophilic and non-lipophilic flavonoid forms into the phospholipid bilayer of cells.

As we mentioned earlier, cryoinjury of sperm membrane may cause the loss of membrane channels but increased PKA expression alongside with a stabilization of the membrane bound ATP-ases suggest that KAE exhibits at least a partial protection on the NBC-initiated capacitation pathway responsible for a proper transport of Na+/HCO3-, cytoplasmic alkalization and membrane hyperpolarization.

A channel-opening ability of flavonoids was described by the **Bednarczyk et al. (2017)**, where the opening probability and activity of mitoK$_{ATP}$ and mitoBK$_{Ca}$ channels increased after supplementation with 10 µM of naringenin.
At last, we must remember that KAE treatment depend on the right dosage. Our study suggested that the most effective concentration is 25 µmol/L diluted with extender. However, we can see a similar phenomenon also in 50 µmol/L of KAE, but this dosage seems to be maximum at least in the case of spermatozoa because higher concentrations can cause self-oxidation **(Castaneda-Ariaga et al., 2018)**.

4. Conclusion

Based on the results, we may suggest that treatment of spermatozoa with 25 µmol/L of KAE effectively preserved the expression of PKA during cryopreservation. A certain degree of positive impact of KAE was also visible in the case of NBC gene expression but the exact mechanism of KAE protection on this selected ion channel must be verified in future experiments.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

1. Abdallah, H.M., Hassan, N.A., El-Halawany, A.M., Mohamed, G.A., Safo, M.K., El-Bassossy, H.M., 2020. Major flavonoids from Psiadia punctilata produce vasodilation via activation of endothelial dependent NO signaling. J. Adv. Res. 24, 273-279. https://doi.org/10.1016/j.jare.2020.01.002
2. Baňas, Š., Benko, F., Ďuračka, M., Lukáč, N., Tvrdá, E., 2023. Kaempferol Enhances Sperm Post-Thaw Survival by Its Cryoprotective and Antioxidant Behavior. Stresses 3, 687-700. https://doi.org/10.3390/stresses3040047
3. Baňas, Š., Tvrdá, E., Benko, F., Ďuračka, M., Čmiková, N., Lukáč, N., Kačániová, M., 2024. Kaempferol as an Alternative Cryosupplement for Bovine Spermatozoa: Cytoprotective and Membrane-Stabilizing Effects. Int. J. Mol. Sci. 25, 4129. https://doi.org/10.3390/ijms25074129
4. Baro Graf, C., Ritagliati, C., Stival C., Luque, G.M., Gentile, I., Buffone, M.G., Krapf, D., 2020. Everything you ever wanted to know about PKA regulation and its involvement in mammalian sperm capacitation. Mol. Cel. Endocrinol. 518, 110992. https://doi.org/10.1016/j.mce.2020.110992
5. Bednarczyk, P., Kicinska, A., Jarmuszkiewicz, W., Debowska, R., Szewczyk, A., 2017. Flavonoids as Natural Modulators of Mitochondrial Potassium Channel. Biophys. J. 112, https://doi.org/10.1016/j.bpj.2016.11.2195
6. Benko, F., Fialková, V., Žiarovská, J., Ďuračka, M., Lukáč, N., Tvrdá, E., 2022. In Vitro versus Cryo-Induced Capacitation of Bovine Spermatozoa, Part 2: Changes in the Expression Patterns of Selected Transmembrane Channels and Protein Kinase A. Int. J. Mol. Sci. 23, 14646. https://doi.org/10.3390/ijms232314646
7. Castaneda-Arriaga, R., Pérez-González, A., Reina, M., Alvarez-Idaboy, J.R., Galano, A., 2018. Comprehensive Investigation of the Antioxidant and Pro-oxidant Effects of Phenolic Compounds: A Double-Edged Sword in the Context of Oxidative Stress? J. Phys. Chem. B 122, 6198-6214. https://doi.org/10.1021/acs.jpcb.8b03500
8. Dabeek, W.M., Marra, M.V., 2019. Dietary Quercetin and Kaempferol: Bioavailability and Potential Cardiovascular-Related Bioactivity in Humans. Nutrients 11, 2288. https://doi.org/10.3390/nu11102288
9. Ďuračka, M., Debacker, M., Bučko, O., Lukáč, N., Tvrdá, E., 2019. The effect of kaempferol and naringenin may improve the in vitro quality of stored boar semen. J. Cent. Eur. Agric. 20, 1069-1075. https://doi.org/10.5513/jcea01/20.4.2294
10. Heo, S., Kim, S., Kang, D., 2020. The role of hydrogen peroxide and peroxiderins throughout the cell cycle. Antioxidants. 9, 280. https://doi.org/10.3390/antiox9040280
11. Hou, D., Kumamoto, T., 2010. Flavonoids as Protein Kinase Inhibitors for Cancer Chemoprevention: Direct Binding and Molecular Modeling. Antioxid. Redox Signal. 13. https://doi.org/10.1089/ars.2009.2816
12. Jamalan, M., Ghaffari, M.A., Hoseinzadeh, P., Hashemitabar, M., Zeinali, M., 2016. Human Sperm Quality and Metal Toxicants: Protective Effects of some Flavonoids on Male Reproductive Function. Int. J. Fertil. Steril. 10, 215-223. https://doi.org/10.22074/ijfs.2016.4912
13. Khan, I.M., Cao, Z., Liu, H., Khan, A., Rahman, S.U., Khan, M.Z., Sathanawongs, A., Zhang, Y., 2021. Impact of Cryopreservation on Spermatozoa Freeze-Thawed Traits and relevance OMICS to Assess Sperm Cryo-Tolerance in Farm Animals. Front. Vet. Sci. 25, 609180. https://doi.org/10.3389/fvets.2021.609180
14. Mansuri, M.L., Parihar, P., Solanki, I., Parihar, M.S., 2014. Flavonoids in modulation of cell survival signaling pathways. Genes Nutr. 9. https://doi.org/10.1007/s12263-014-0400-z
15. Marunaka, Y., 2017. Action of quercetin, a flavonoid, on ion transporters: its physiological roles. An. N.Y. Acad. Sci. 1398, 142-151. https://doi.org/10.1111/nyas.13361
16. Mohamed, M., Gouda, R.E., 2022. The Effect of Kaemferol on Buffalo Semen Freezability and Redox State. Benha Vet. Med. J. 42, 164-169. https://doi.org/10.21608/bvmj.2022.77459.1417
17. O'Flaherty, C., Beorlegui, N., Beconi, M.T., 2006. Heparin- and superoxide anion-dependent capacitation of cryopreserved bovine spermatozoa: Requirement of dehydrogenases and protein kinases. Free Radic. Res. 40, 427-432. https://doi.org/10.1080/10715760600577856
18. Tejero, I., González-Lafont, Á., Lluch, J.M., Eriksson, L.A., 2007. Theoretical Modeling of Hydroxyl-Radical-Induced Lipid Peroxidation Reactions. J. Phys. Chem. 111, 5684-5693. https://doi.org/10.1021/jp0650782