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The effect of kaempferol and naringenin may improve the *in vitro* quality of stored boar semen

Účinok kaempferolu a naringenínu môže zlepšiť *in vitro* kvalitu uchovávaných kančích spermií

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ABSTRACT

Determination of the effect of pure bioactive substances on male reproductive cells is a current theme amongst veterinary andrologists. The aim of this study was to investigate the *in vitro* effect of various concentrations of kaempferol and naringenin on boar male gametes in different time periods (0, 24, 48 and 72h). The computer-assisted semen analysis was used to measure the sperm motility. Mitochondrial activity was determined by the mitochondrial toxicity test (MTT). The formation of the superoxide radical, as a marker of oxidative stress, was analysed by the nitroblue-tetrazolium (NBT) test. The results showed that every selected concentration of both substances came along with benefitial effect on the structure and function of spermatozoa. Particularly, naringenin at concentrations of 10-50 µmol/L and kaempferol at concentrations of 5-25 µmol/L may provide benefitial effects on spermatozoa, thus prolong the storage period and improve the quality of preserved boar spermatozoa.

Keywords: antioxidants, bioactive compounds, kaempferol, naringenin, sperm, spermatozoa, oxidative stress, reactive oxygen species, mitochondrial activity

ABSTRAKT

Determinácia účinku čistých bioaktívnych látok na samčie reprodukčné bunky je aktuálnou témou medzi veterinárnymi andrológmi. Cieľom tejto štúdie bolo vyšetriť *in vitro* účinok rôznych koncentrácií kaempferolu a naringenínu na samčie gaméty kancov v rozličných časových periódach (0, 24, 48 a 72h). Počítačom-asistovaná analýza spermií bola použitá na meranie motility spermií. Mitochondriálnu aktivitu sme určili mitochondriálnym testom toxicity (MTT). Tvorenie superoxidového radikálu, ako markera oxidatívneho stresu, sme analyzovali nitroblue-tetrazolium (NBT) testom. Výsledky ukázali, že každá vybratá koncentrácia oboch substancií má prospešný účinok na štruktúru a funkciu spermií. Obzvlášť naringenín o koncentráciách 10-50 µmol.l^{-1} a kaempferol o koncentráciách 5-25 µmol*l^{-1} môžu poskytnúť prospešné účinky na spermie, čím sa predĺži čas uchovávania a zlepší sa kvalita prezervovaných kančích spermií.

Kľúčové slová: antioxidanty, kaempferol, naringenín, spermie, oxidatívny stres

INTRODUCTION

The preservation of boar semen comes along with various issues. Oxidative stress (OS) is a major factor of infertility, caused by an imbalance between the concentration of reactive oxygen species (ROS) and antioxidant capacity. Although, controlled concentrations of ROS are needed due to the physiological processes in sperm such as capacitation or acrosome reaction, increased concentration of ROS leads to irreversible damage of sperm structures (Bansal and Bilaspuri, 2011). Firstly, sperm membrane is the most sensitive structure to ROS, because of high content of polyunsaturated fatty acids. Moreover, cytoplasm lacks antioxidant protection (Buettner, 1993). In accordance to Sabeti et al. (2016), major structures producing ROS in sperm are plasma membrane and mitochondria. Moreover, leukocytes and macrophages may generate a wide spectrum of oxygen and nitrogen reactive species.

The use of plants in relation to male reproduction has been tremendous in recent years. Herbal extracts rich in flavonoids may not only improve overall health of pigs, but also could effectively stimulate an animal growth (Valchev et al., 2009).

Flavonoids, a widely distributed group of phytochemicals, possess many benefitial effects on human and animal health (Lee et al., 2007). Thousands of natural flavonoids have been determined in different parts of plants (Harborne and Williams, 2000). Naringenin (NAR) is a natural flavonoid commonly available in tomatoes skin and citrus fruits (Patel et al., 2018). Similarly, kaempferol (KAE) belongs to a class of flavonoid commonly present in citrus fruits. Moreover, it was found in wide spectrum of herbs and vegetables, especially tomatoes, broccoli or ginkgo biloba leaves and tea (Shields, 2017). NAR and KAE in particular was identified also in botanical products commonly used in traditional medicine. Both NAR and KAE provide an abundant amount of antioxidant acitivity (Calderon-Montano et al., 2011; Patel et al., 2018). Application of antioxidants to cell cultures is an effective way to manage OS. Chemical multiplicity, availability, intrinsic biological activity and lack of toxic effects allows the use of bioactive compounds to cell cultures (Tvrdá et al., 2015). Several studies confirmed beneficial effects of bioactive substances, taxonomically classified as flavonoids, on spermatozoa of various species. Especially, ROS-scavenging activity of flavonoids was attributed to the maintenance of plasma membrane and acrosome integrity, mitochondrial membrane potential (Silva et al. 2012) and DNA fragmentation (Majzoub et al., 2017). Moreover, they have potential to protect spermatozoa in cryopreservation and post-thaw processes (Silva et al., 2012; Ardeshirnia et al., 2017).

The aim of this study was to evaluate the *in vitro* effect of selected pure bioactive substances on several structural and functional parameters: motility, mitochondrial activity and superoxide formation. Until now, KAE and NAR have recieved little attention unlike others.

MATERIALS AND METHODS

Semen samples were obtained from 15 adult Duroc boars (Farm Terezov, Hlohovec, Slovak republic). After collection, the samples were transported to the laboratory within 1 hour and held at laboratory temperature (20-22 °C). Each sample was preserved in the Androhep Plus™ extender (Minitüb GmbH, Tiefenbach, Germany) using a dilution ratio of 1:20. The control group contained no treatment (except the extender). The experimental groups were supplemented with different concentrations (100, 50, 25, 10 and 5 µmol/L) of NAR or KAE dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA). The analyses were carried out at times of 0, 24, 48 and 72 hours. All samples were held at the laboratory temperature during the entire lenght of the experiment.

Computer-assisted semen analysis (CASA)

Ten µL of each sample was placed into a Makler counting chamber (depth 10 µm, 37 °C; Sefi Medical Instruments, Haifa, Israel) and subjected to motility (MOT; %) examination using the Computer-assisted semen analysis (CASA; Version 14.0 TOX IVOS II; Hamilton-Thorne Biosciences, Beverly, USA). A minimum of 300 cells in 10 fields were subjected to motility analysis.

Mitochondrial toxicity test (MTT test)

The viability of sperm cells were measured by mitochondrial toxicity test (MTT test). The principle of this method is the conversion of yellow colored insoluble tetrazolium salt (3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to purpe colored formazan by mitochondrial succinate dehydrogenase. The tetrazolium salt was dissolved in Dulbecco's Phosphate Buffer Saline (PBS; Sigma-Aldrich, USA) to a concentration of 5 mg/mL. Ten µL of the tetrazolium-PBS solution was added to each sample (200 µL) and incubated (1 hour, 37 °C, 5% CO_2). The formazan salts were dissolved in 80 µL of isopropanol (Centralchem, Slovakia). The optical density was analysed using the Glomax Multi⁺ Combined Spectro-Fluoro-Luminometer (Promega, Madison, WI, USA) at a wavelength of 570 nm against 620 nm as a reference. The obtained data were expressed as percentage of the control, which was set to 100%.

The quantification of superoxide radicals (NBT test)

The intracellular production of the superoxide radical was measured by the nitroblue-tetrazolium (NBT) test. The principle of this method is based on counting the cells which contain blue NBT formazan solids. Formazan solids are generated by reduction of the yellow-colored, permeable, water-soluble nitroblue tetrazolium chloride (2,2'-bis(4-nitrophenyl)-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'diphenylene) ditetrazolium chloride; Sigma-Aldrich) and superoxide radical. The NBT salt was dissolved using PBS and containing 1.5% DMSO to a concentration of 1 mg/mL and subsequently added to the cell suspension. The samples were subjected to incubation (1 h, 37 °C, 5% CO_2), washed twice with PBS and centrifuged (300 × g, 10 min.). Finally, the sperm cells and formazan solids were dissolved in 2M KOH (Potassium Hydroxide; Centralchem) in DMSO. The optical density was measured using the Glomax Multi⁺ Combined Spectro-Fluoro- Luminometer (Promega, Madison, WI, USA) at a wavelength of 570 nm against 620 nm as a reference. The obtained data were expressed as percentage of the control, which was set to 100% (Tvrdá, 2018).

Statistical analysis

Statistical evaluation was performed by the GraphPad Prism program (version 5.0 for Windows; GraphPad Software incorporated, San Diego, California, USA, www.graphpad.com). The results are expressed as the arithmetic mean (AM) ± standard error of mean (SEM). The comparative analysis was performed by a one-way ANOVA with Dunnett's test. The significance level for the analysis was set to *P<0.05; **P<0.01; ***P<0.001.

RESULTS

The aim of present study was to investigate boar spermatozoa exposed to different concentrations (100, 50, 25, 10 and 5 µmol/L) of bioactive molecules – kaempferol (KAE) and naringenin (NAR) at different time periods (0, 24, 48 and 72 h). From the results of this research, it is clear that both substances had a dose-dependent effect on motility (Figure 1). Every single selected concentration of both substances significantly improved the motion properties of spermatozoa with elapsed time; at time of 0h there were no differences due to a short interaction between the sperm cells and supplements. In the next 3 time periods were observed the same significance levels with respect to every concentration of the treatment. The most favorable results were achieved using concentrations varying from 5 to 25 µmol/L of KAE (P<0.001). In case of NAR, concentrations varying from 50 to 10 µmol/L were the most benefitial to the sperm motility (P<0.01).

Mitochondrial activity is straight-connected with spermatozoa motility, as mitochondria are the source of energy for movement of the sperm. Thereby, results of mitochondrial activity should reflect the results of motility analysis. Unlike the motility analysis, in the MTT analysis revealed significant differences (P<0.001 in case of 10 µmol/L KAE; P<0.05 in case of 10-25 µmol/L NAR and 25 µmol/L KAE) already within the initial measurement, which may be caused due to the length of cultivation, until the plate is spectrophotometrically

measured. In the next time periods (24, 48 and 72 h), the results of the mitochondrial activity (Figure 2) correlated with the results of the sperm motility. Concentrations of 5-25 µmol/L KAE increased the mitochondrial activity with a significance level of P<0.001. On the other hand, concentrations of 10-50 NAR increased the mitochondrial activity with a significance level of P<0.01. These results emphasized on the benefitial effects of the KAE/NAR treatment on the sperm mitochondrial activity.

The superoxide quantification (Figure 3) as well as mitochondrial activity showed significant differences already at the initial time; P<0.001 in case of 10 µmol/L KAE, P<0.05 in case of 5 µmol/L KAE and 25-50 µmol/L NAR. Similarly, the explanation for such results may be the length of incubation (1 h) during which the bioactive substances probably interacted with the spermatozoa. In the next time periods (24, 48 and 72 h), concentrations of 5-25 µmol/L KAE significantly decreased the superoxide production (P<0.001), when compared with Control. In case of NAR was observed a significant decrease (P<0.01) in the groups treated with 10-25 µmol/L. The experimental groups treated with KAE/NAR concentrations of 50-100 µmol/L decresed the formation of superoxide with a significance of P<0.05. All the concentrations of selected bioactive substances demonstrated superoxide-scavenging activity during the storage.

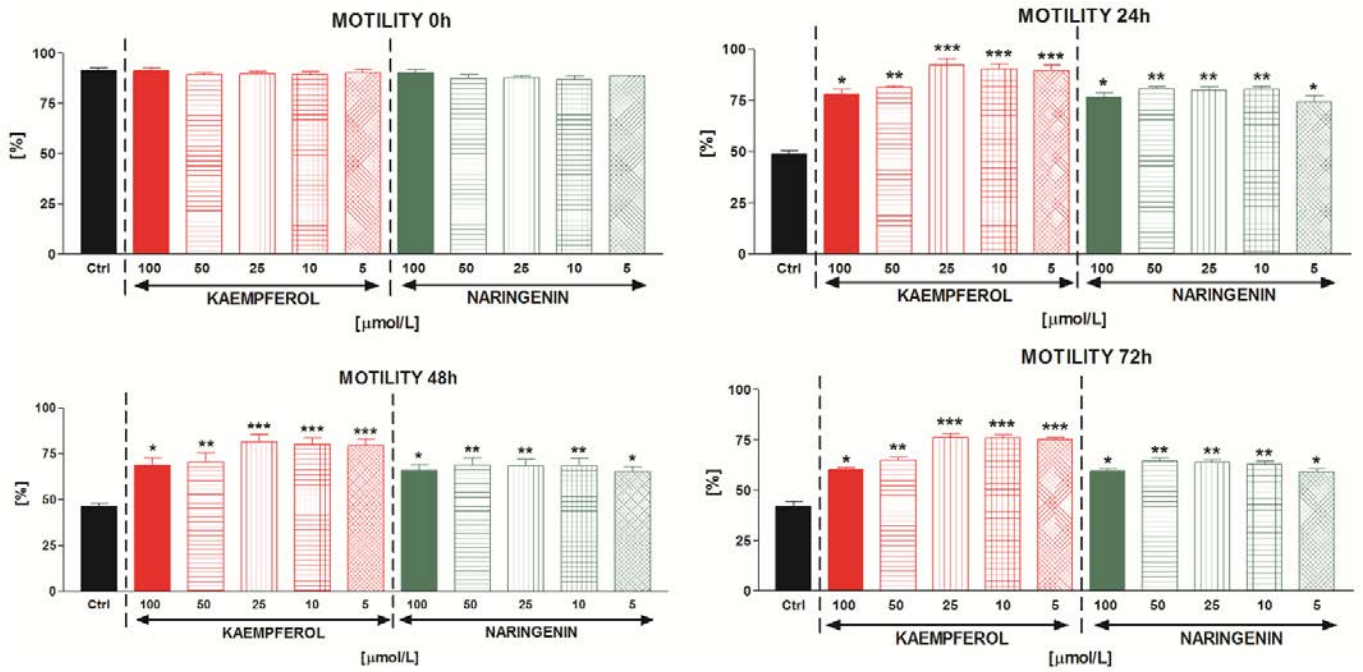

Figure 1. Development of sperm motility during 72 h treatment with the various concentrations of kaempferol/ naringenin (the results are expressed as AM±SEM; *P<0.05; **P<0.01; ***P<0.001)

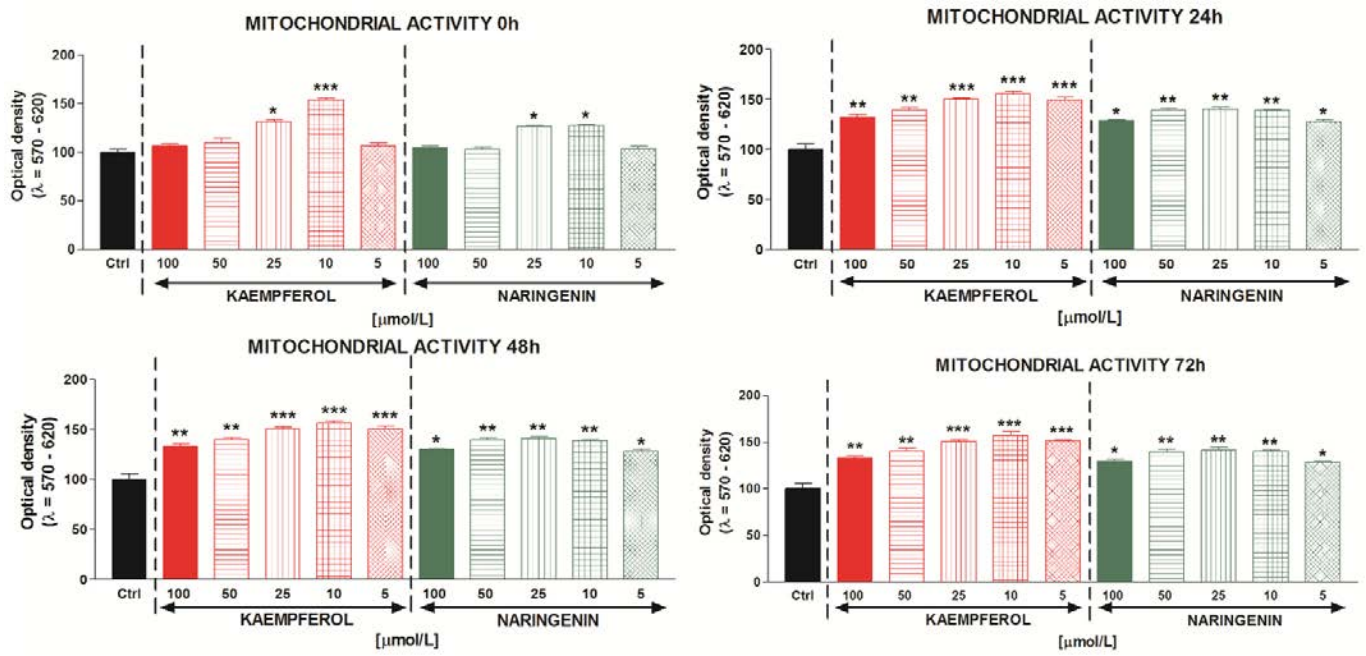

Figure 2. Development of mitochondrial activity during 72 h treatment with the various concentrations of kaempferol/ naringenin (the results are expressed as AM±SEM; *P<0.05; **P<0.01; ***P<0.001

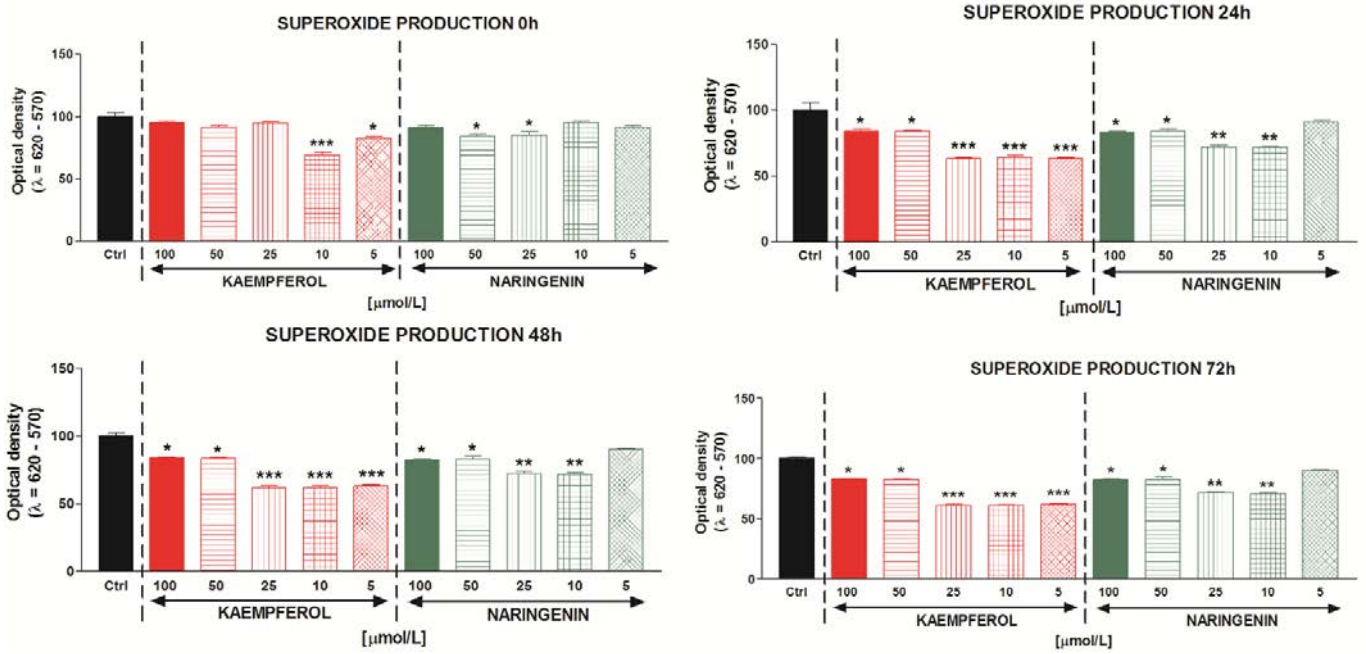

Figure 3. Development of the superoxide production during 72 h treatment with the various concentrations of kaempferol/ naringenin (the results are expressed as AM±SEM; *P<0.05; **P<0.01; ***P<0.001)

DISCUSSION

In this study was evaluated the possible protective effect of NAR and KAE on boar semen during a 72 h *in vitro* storage. Boar semen subjects to OS with elapsed time, therefore there should be good reason for antioxidant supplementation. Pilane et al. (2016) assessed the susceptiblity of boar semen to OS using hydrogen peroxide. Hydrogen peroxide induced oxidative damage to spermatozoa related to alterations in the semen velocity parameters, motility and viability. Moreover, there was a high positive correlation with apoptosis. The authors concluded that the use of antioxidant therapy is necessary throughout any boar semen processing, especially during cryopreservation. Zapryanova et al. (2017) noticed that the quality of stored boar ejaculate is dependent on various factors, such as age, season, individuality and obviously time of storage. With increasing boars age and time of storage, quality of semen has decreased. Therefore, identification and description of new antioxidant substances such as NAR and KAE are desirable.

In accordance to Jamalan et al. (2016), KAE and NAR, especially at concentrations varying between 25-500 µmol/L were effective in reducing MDA concentration in metal-exposed ($AlCl_3$, $CdCl_2$ and $PbCl_4$) semen samples, leading to an increased sperm motility. In accordance to this study, the use of concentrations higher than 100 µmol/L of KAE/NAR could act counterproductively. Other studies also demonstrated that bioactive substances could act as a double-edged sword – low concentrations may improve the structural and functional characteristics of spermatozoa, on the other hand, high concentrations may have deleterious effects (Bouayed and Bohn, 2010; Castañeda-Arriaga et al., 2018).

Mitochondria play an important role in the motion properties of spermatozoa. Strict regulation of ions by ion channels is necessary to maintain appropriate motion-promoting properties of spermatozoa. Montero et al. (2004) demonstrated that flavonoids, including kaempferol, could modulate the mitochondrial Ca^{2+} uniporter in the plasma membrane without requiring ATP, thus phosphorylation. The possible engagement of antioxidant properties was refuted by the investigation of Ca^{2+} uptake of classical lipid- and water-soluble antioxidants. This mechanism offers an alternative way of flavonoid-mediated stimulation of mitochondrial activity.

Recent studies devoted to the effect of NAR on male reproductive system present debatable results. Ranawat and Bakshi (2017) reported that intra-peritoneall treatment of NAR (2, 8 and 20 mg/kg b.w.) during 2 weeks increased the ROS generation and lipid peroxidation in the mice testes. Activities of antioxidant enzymes, such as catalase, superoxide dismutase, glutathione peroxidase or reduced glutathione decreased with the increasing dose of NAR. Moreover, expression of pro-apoptotic markers (c-jun, c-fos and NF-κB) increased proportionally with the dose of NAR. On the other hand, Ganaie (2015) claimed that NAR may protect testes in rats prior to cisplatin treatment, which is a highly effective chemotherapeutic agent resulting in gonadotoxicity and obviously, OS. Concentration of malondialdehyde (a product of lipid peroxidation) in the testes of cisplatin-exposed rats was clearly decreased using NAR (50 mg/kg b.w.). Furthermore, the glutathione level was elevated in comparison to the positive control. A different *in vivo* study revealed an improvement of diabetes-induced testicular dysfunction in diabetic rats following NAR treatment. NAR contributed to an increased activity of the superoxid dismutase, catalase and glutathione peroxidase in the testicular tissue, leading to a more balanced oxidant-antioxidant status (Roy et al., 2013).

CONCLUSIONS

Based on the presented results, a rational use of kaempferol and naringenin during the semen storage may improve not only the motion properties, but also maintain mitochondrial activity and reduce the formation of superoxide radical by boar spermatozoa. The concentrations varying from 5-25 µmol/L of kaempferol and 10-50 µmol/L of naringenin could be used as a radical-scavenging and metabolic promoting supplement, especially convetional andrological techniques such as cryopreservation, artificial insemination or *in vitro*

fertilization. Higher concentrations could disturb the oxidative-antioxidant status of male gametes. It is necessary to use the above studied bioactive compounds in suggested concentrations, so that the quality and time period of the semen storage may be improved. Further research of naringenin and kaempferol in relation to male reproduction is however needed.

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