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Correlation between in vitro sperm kinetic, oxidative stress assessments and field fertility of cryopreserved bull semen

Correlação entre cinética espermática in vitro, avaliações de estresse oxidativo e fertilidade à campo em sêmen criopreservado de touros

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Highlights

ROS showed correlation with fertility after sperm selection.
The model TBARS+ROS+VSL+VAP had high correlations with fertility.
High fertility bulls seem to be more susceptible to lipid peroxidation.
Lipid peroxidation is an important indicator of fertility in bulls.

Abstract

This study assessed kinetic parameters and oxidative stress in bull sperm after post-thaw (PT) or after sperm selection by Percoll™ gradient, and thermo resistance test (SS + TRT) to identify useful indicators of field fertility. For the experiment, commercial doses of frozen semen were obtained from six Aberdeen Angus bulls. Three of the bulls were classified as high fertility and three as low fertility according to the IFert™ index provided by the international breeding company CRV Lagoa. Pooled semen samples were distributed between two treatment groups for analysis: post-thaw (PT) or sperm selection (SS) (Percoll™) and thermal resistance test (SS + TRT). The samples were evaluated using sperm kinetics (CASA) (motility %, progressive motility %, VCL µm/s, VSL µm/s, VAP µm/s, LIN %, STR % and WOB%), production of reactive oxygen species (ROS), lipid peroxidation, superoxide dismutase (SOD) enzyme activity and total antioxidant capacity. Data were analyzed using Two-Way ANOVA, considering the fertility index, the treatment used in the samples as effects, and the interaction between these factors. When a significant effect was observed, the values were compared using the Bonferroni test. A Pearson Correlation analysis was performed between the fertility indices and the sperm parameters analyzed in vitro, to evaluate the relationship between sperm quality and the fecundity rate obtained by the bulls. Sperm kinetic parameters,

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including total motility, progressive motile, and beat cross-frequency, were higher in low fertility compared to high fertility bulls (P < 0.05). However, curvilinear velocity was greater in high fertility bulls followed by SS + TRT. Straight-line velocity, average path velocity, linearity, and beat cross-frequency beat were higher in high fertility bulls after SS + TRT. Reactive oxygen species was correlated with fertility after SS. In addition, there was a decrease in lipid peroxidation was observed only in high fertility bulls. However, lipid peroxidation and high fertility were correlated after PT and SS + TRT. The combination of in vitro sperm kinetic parameters predicted in vivo fertility more accurately than individual kinetic parameters. The lipid peroxidation of sperm is an important indicator of fertility in bulls. High fertility bulls appeared to be more susceptible to lipid peroxidation, which was only reduced in high fertility bulls, suggesting that their sperm can repair the damage induced by oxidative stress.
Key words: Bovine sperm. Percoll™ gradient. Oxigen-reactive species. CASA.

Resumo

Este estudo avaliou parâmetros cinéticos e estresse oxidativo em espermatozoides de touros após pós-descongelamento (PT) ou após seleção de espermatozoides por gradiente de Percoll™ e teste de termorresistência (SS + TRT) para identificar indicadores úteis de fertilidade em campo. Para o experimento foram obtidas doses comerciais de sêmen congelado de seis touros Aberdeen Angus. Três dos touros foram classificados como de alta fertilidade e três como de baixa fertilidade de acordo com o índice IFert™ fornecido pela empresa internacional de criação CRV Lagoa. Amostras de sêmen agrupadas foram distribuídas entre dois grupos de tratamento para análise: pós-descongelamento (PT) ou seleção espermática (SS) (Percoll™) e teste de resistência térmica (SS + TRT). As amostras foram avaliadas através da cinética espermática (CASA) (% de motilidade, % de motilidade progressiva, VCL µm/s, VSL µm/s, VAP µm/s, LIN %, STR % e WOB%), produção de espécies reativas de oxigênio (ROS), peroxidação lipídica, atividade da enzima superóxido dismutase (SOD) e capacidade antioxidante total. Os dados foram analisados por meio de ANOVA de duas vias, considerando como efeitos o índice de fertilidade, o tratamento utilizado nas amostras e a interação entre esses fatores. Quando foi observado efeito significativo, os valores foram comparados pelo teste de Bonferroni. Foi realizada uma análise de Correlação de Pearson entre os índices de fertilidade e os parâmetros espermáticos analisados in vitro, para avaliar a relação entre a qualidade espermática e a taxa de fecundidade obtida pelos touros. Os parâmetros cinéticos de sêmen, incluindo motilidade total, motilidade progressiva e frequência cruzada de batimentos, foram maiores em touros de baixa fertilidade em comparação com touros de alta fertilidade (P < 0,05). No entanto, a velocidade curvilínea foi maior em touros de alta fertilidade seguidos de SS + TRT. A velocidade em linha reta, a velocidade média de trajeto, a linearidade e a frequência cruzada de batimento foram maiores em touros de alta fertilidade após SS + TRT. As espécies reativas de oxigênio foram correlacionadas com a fertilidade após SS. Além disso, houve diminuição da peroxidação lipídica observada apenas em touros de alta fertilidade. No entanto, peroxidação lipídica e alta fertilidade foram correlacionadas após PT e SS + TRT. A combinação de parâmetros cinéticos espermáticos in vitro previu a fertilidade in vivo com mais precisão do que os parâmetros cinéticos individuais. A peroxidação lipídica em espermatozoides é um importante indicador de fertilidade em touros. Os touros de alta fertilidade parecem ser mais suscetíveis à peroxidação lipídica, que só foi reduzida em touros de alta fertilidade, sugerindo que seus espermatozoides podem reparar os danos induzidos pelo estresse oxidativo.
Palavras-chave: CASA. Espécies reativas de oxigênio. Gradiente PercollTM. Sêmen bovino.

Introduction

Predicting male fertility offers a substantial benefit for predicting the economic success of livestock from improvements to herd conception and pregnancy rates. Consequently, there is a great interest in finding a suite of sperm features to field fertility that can be used to predict the performance of a bull or its frozen semen in the field. Thus, bulls with potentially low fertility can be identified and removed from a breeding program. Many laboratory tests have been developed to assess sperm quality; however, no method has yet been able to explore all sperm attributes that guarantee genuine fertility (Utt, 2016). In addition, the results of such sperm analyses performed in vitro do not always correlate with the field fertility of an insemination dose (Simonik et al., 2015). However, some studies already indicate that evaluating the quality of thawed bull sperm using CASA and considering kinetic parameters can provide a reasonable prediction of bovine semen fertility (Sellem et al., 2015; Gliozzi et al., 2017). Furthermore, studies already relate kinetic and biochemical parameters of fresh and cryopreserved bull semen with fertilizing capacity (Vigolo et al., 2022).

The relationship between oxidative stress in sperm and fertility has been studied in vitro and in vivo. Increased levels of reactive oxygen species (ROS) have been correlated with decreased motility parameters in bulls (Bilodeau et al., 2002; Castro et al., 2016), and it is widely known that an excess of ROS contributes to sperm DNA damage and lipid peroxidation, which lower male fertility (Kasimanickam et al., 2007; Oliveira et al., 2012; Walczak-Jedrzejowska et al., 2013). Seminal cryopreservation can lead to a large production of reactive oxygen species, keeping the sample under oxidative stress. Cryoprotectants have a deleterious effect on sperm when heated for long periods of time (Sanchez et al., 2012). In addition, during sperm migration, only viable sperm reach the oviduct. Therefore, to mimic what occurs during sperm migration and minimize the effects of the cryoprotectant, we performed a thermoresistance test after sperm selection. Sperm selection (SS) is a term used to describe techniques that separate seminal plasma, diluents, and cryoprotectants from sperm. Sperm selection also separates live and dead sperm, avoiding deleterious effects and improving the chances of in vitro fertilization success (Morrell et al., 2018).

In addition, the removal of seminal plasma from sperm selection allows the removal of plasma proteins. The proteins present in seminal plasma are different between high and low fertility bulls (Vianna et al., 2008). Thus, to evaluate the characteristic of the sperm, simulating what occurs in the reproductive tract during sperm migration, we performed the sperm selection followed by the modified thermoresistance test by incubating the sperm for 3 hours at 36 ºC.

Studies have assessed the relationship between post-thaw sperm parameters and fertility (Prakash et al., 1998; Hallap et al., 2004; Oliveira et al., 2013; Singh et al., 2016; Morell et al., 2018); however, few studies have investigated the relationship between field fertility and semen parameters after of removal of seminal plasma, similar to what occurs in vivo in the female's reproductive tract (Bailey et al., 2000; Hallap et al., 2004; García-Álvarez et al., 2010). The

use of the bull selection methodology based on the IFert™ index as a classification of fertility in bulls to separate the experimental groups used in the present work has already been tested and its reliability assessed by some authors (Leite et al., 2022; Silva et al., 2023). In this study, we assessed sperm kinetic parameters and oxidative stress after post-thaw (PT) or SS + TTR using a discontinuous Percoll™ gradient in bulls of known fertility to identify valuable indicators of in vivo fertility.

Material and Methods

All chemical reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise stated. The experiments were performed in accordance with the National Council of Control in Animal Experimentation of Brazil (CONCEA, CEUA Unipampa).

Experimental Design

Commercial doses of frozen semen were obtained from six Aberdeen Angus bulls (CRV Lagoa, São Paulo, Brazil). Three of the bulls were classified as high fertility and three as low (three replicates each) according to the IFert™ index provided by the international breeding company CRV Lagoa. Ifert™ is a fertility index that predicts the fertility potential of beef cattle used in fixed-time artificial insemination (FTAI) programs. Currently, it contemplates the use of 814 breeding animals. It's a wide model of statistical evaluation that has careful filters to verify the "bull effect" in a more adjusted way. The IFert™ determines in percentage points the potential breeder design rate in relation to the average design rate obtained by the database of the FTAI's evaluated. High fertility bulls had a significantly higher pregnancy rate (59.08 ± 7.10) than low fertility bulls (35.61 ± 6.36; $P < 0.01$) in IFert™ index. For this study, straws (0.25 mL) from three different batches of each bull were thawed for 30 s at 35 °C in a water bath and pooled to reduce the batch variability. Pooled semen samples were distributed between two treatment groups for analysis: post-thaw (PT) or sperm selection (SS) (Percoll™) and thermal-resistance test (SS + TRT). Samples were evaluated using sperm kinetics (CASA), reactive oxygen species (ROS) production, lipid peroxidation, superoxide dismutase enzyme (SOD) activity, and total antioxidant capacity. The experimental design can be seen in Figure 1.

Percoll™ gradient

An isotonic Percoll™ solution was used to prepare 90, 60, and 30 % solutions with modified Talp-Fert media (Parrish et al., 1986). The Percoll™ density gradient was performed by layering 300 µL of each solution into a 1.5 mL microtube, starting with the 90 % Percoll™ solution at the bottom. At the top of the tube, there was a layer of 300 µL of thawed semen. The tubes were centrifuged for 5 min at 2200 × g (Guimarães et al., 2014). Then, the resultant pellet was re-suspended using 300 mL of sp-TALP and centrifuged again for 1 min at 2200 × g. After centrifugation, the sperm samples were analyzed as described above.

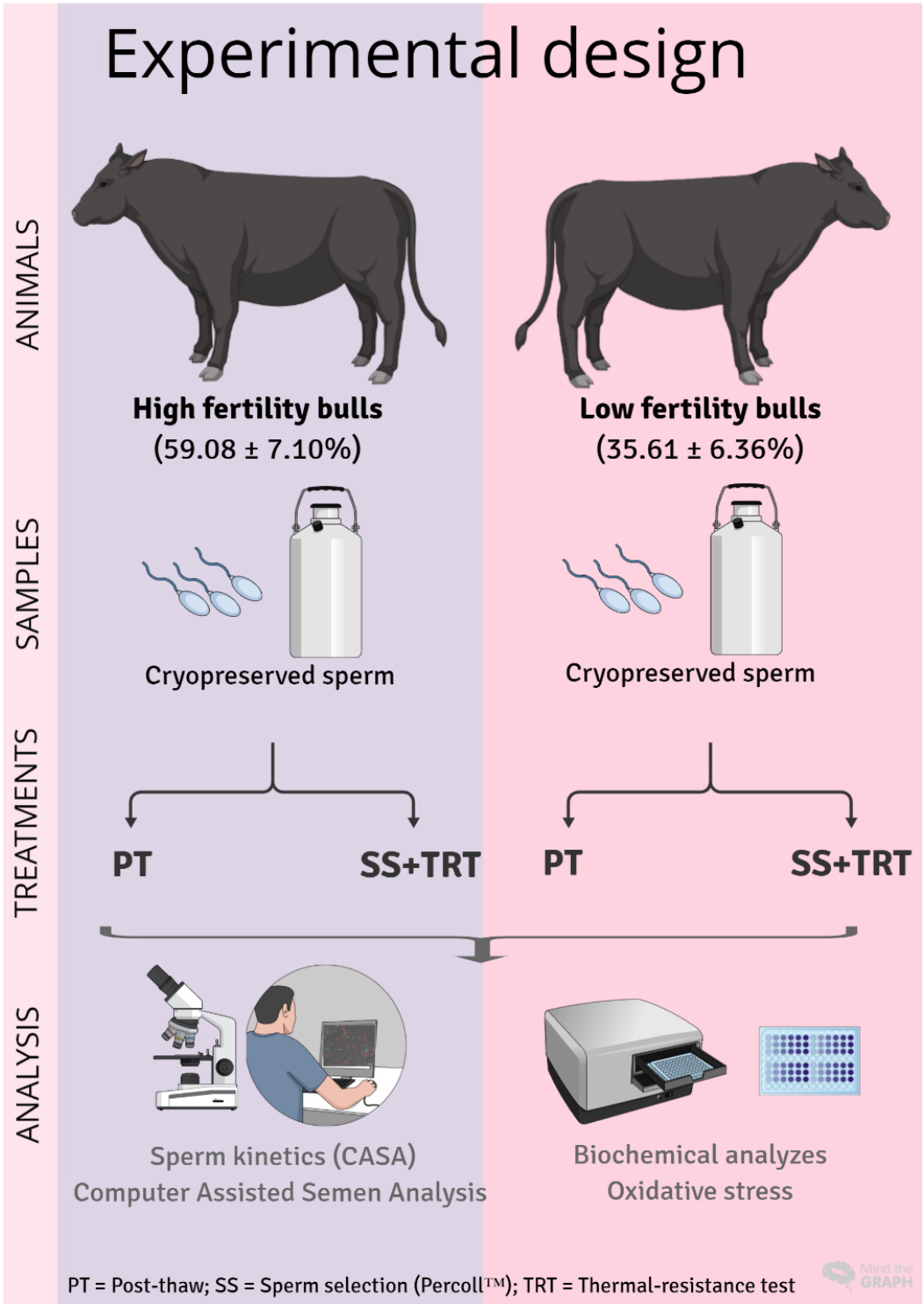

Figure 1. Experimental Design.

Sperm thermal-resistance test (TRT)

The thermal-resistance test (TRT) was used to verify the longevity of sperm from bulls of low or high fertility. After sperm selections using the Percoll™ gradient, an aliquot of 100 µL of semen was put into a warmed microcentrifuge tube, and incubated at 36 °C. After 3 h, the sperm samples were analyzed as described above.

Sperm quality parameters assessment

Assessment of sperm kinetics

Sperm kinetics was evaluated using a Computer Assisted Semen Analysis (CASA) system fitted with Sperm Class Analyzer (SCA) software (Version 5.1; Microptic, Barcelona, Spain). The following parameters were analyzed: the percentage of total motile sperm (MTOT, %), the percentage of progressive motile sperm (MP, %), curvilinear velocity (VCL, µm/s; the average velocity measured over the actual point-to-point track followed by the cell), straight-line velocity (VSL, µm/s; the average velocity measured in a straight line from the beginning to the end of the track), average path velocity (VAP, µm/s; the average velocity of the smoothed cell path), linearity (LIN, %; the average value of the ratio VSL/VCL), straightness (STR), wobble (WOB = VAP/VCL × 100, %; a measure of the oscillation of the actual trajectory about its spatial average path), amplitude of lateral head displacement (ALH, µm; the mean width of the head oscillation as the sperm cells swim), beat cross-frequency (BCF, Hz; the frequency of sperm heads crossing the average path in either direction), and hyperactivity (HYP, % of sperm with VCL > 35 µm/s, ALH > 2.5 µm, and STR > 85 µm/s).

Assessment of reactive oxygen species production

Reactive oxygen species (ROS) levels were measured using the spectrofluorimetric method (Loetchutinat et al., 2005), where sperm were incubated in Tris–HCl in the presence of 2′,7′-dichloro dihydrofluorescein diacetate (DCHFDA) for 60 min at 37 °C in the dark. This dye is a fluorogenic probe commonly used to detect cellular ROS production. DCHF-DA is a stable, cell-permeable non-fluorescent probe. It is de-esterified intracellularly and becomes highly fluorescent 2′,7′-dichlorofluorescein (DCF) upon oxidation. The oxidation of DCHF-DA to DCF was used to detect and measure the intracellular ROS. The DCF fluorescence intensity emission was recorded at 520 nm (with 480 nm excitation) using a Shimadzu spectrofluorometer (model RF5301PC, Kyoto, Japan). ROS levels were expressed as arbitrary units of fluorescence (UF).

Assessment of lipid peroxidation

Lipid peroxidation was performed using the formation of thiobarbituric-acid-reactive substances (TBARS) during an acid-heating reaction, as previously described by Ohkawa et al. (1979). An aliquot of sperm was incubated at 95 °C for 2 h. The absorbance was read at 532 nm (Hidex Plate Chameleon V Multitechnology Platereader, model 425–156). The data were expressed as nmol malondialdehyde (MDA)/mg protein.

Determination of superoxide dismutase (SOD) activity

Superoxide dismutase activity was measured as described by Misra and Fridovich (1972). This method is based on the ability of SOD to inhibit the auto-oxidation of epinephrine to adrenochrome. The color reaction can be monitored at 480 nm. One enzymatic unit (1 IU) is defined as the amount of enzyme necessary to inhibit the epinephrine auto-oxidation rate by 50 % at 26 °C.

Ferric reducing antioxidant potential (FRAP)

Total antioxidant capacity in sperm was measured using the FRAP assay with slight modifications (Benzie & Strain, 1996). Antioxidant capacity was determined as the "ferric reducing antioxidant potential" at the maximal absorption at 593 nm, where the antioxidants present in a sample were evaluated as reducers of Fe^{+3} to Fe^{+2}, which is chelated by 2,4,6-Tri-(2-pyridyl)-s-triazine (TPTZ) to form the complex Fe^{+2}-TPTZ. An ascorbic acid standard curve was plotted, and the results were expressed as nmol Fe^{+2}PTZ/g.

Statistical analysis

Data were analyzed using two-way analysis of variance (Two-Way ANOVA), considering the fertility index (high and low fertility bulls), the treatment used in the samples (PT or SS + TRT) as effects, and the interaction between these factors. When a significant effect was observed, the values were compared using the Bonferroni test. For the analysis of variance and for the mean test, $P < 0.05$ was considered significant. Bar charts are shown with mean and standard deviation.

A Pearson Correlation analysis was performed between the fertility indices and the sperm parameters analyzed *in vitro,* to evaluate the relationship between sperm quality and the fecundity rate obtained by the bulls. $P < 0.10$ was considered significant. Analyzes were performed using GraphPad Prism 7.0 software.

Results and Discussion

The results of the analysis of sperm kinetics of cryopreserved semen from high and low fertility bulls can be seen in Figures 2 and 3.

There was a significant effect of the fertility index and treatment for the percentage of total motility (Figure 2a), with the highest values (65.5 ± 12.4 % of high fertility bulls; and 73.56 ± 8.4 % of low fertility bulls) observed in the treatment in which the samples were only thawed (PT), when compared to samples from the SS + TRT group (28.1 ± 8.7 % of high fertility bulls; and 51.3 ± 9.7 % of low fertility bulls). In the samples that were submitted to the SS + TRT treatment, the semen from the low fertility bulls showed greater total motility in relation to the samples from the high fertility bulls. The percentage of progressive motility (Figure 2b) was affected only by the treatments, with samples from the PT group showing higher progressive motility (47.6 ± 5.1 % of high fertility bulls; and 53.9 ± 3.4 % of low fertility bulls) than those from the SS + TRT group (8.8 ± 5.1 % of high fertility bulls; and 9.7 ± 5.1 % of low fertility bulls). The

hyperactivity variable (Figure 2f) was affected by the treatments, where we observed greater hyperactivity in the low fertility bulls of the PT group samples (13.8 ± 7.9%), when compared to the SS + TRT group samples (2.3 ± 2.5 %). In high fertility bulls, there was no difference between treatments. We did not observe significant effects of fertility index and treatments for linearity, STR, and WOB variables (Figure 2c-e).

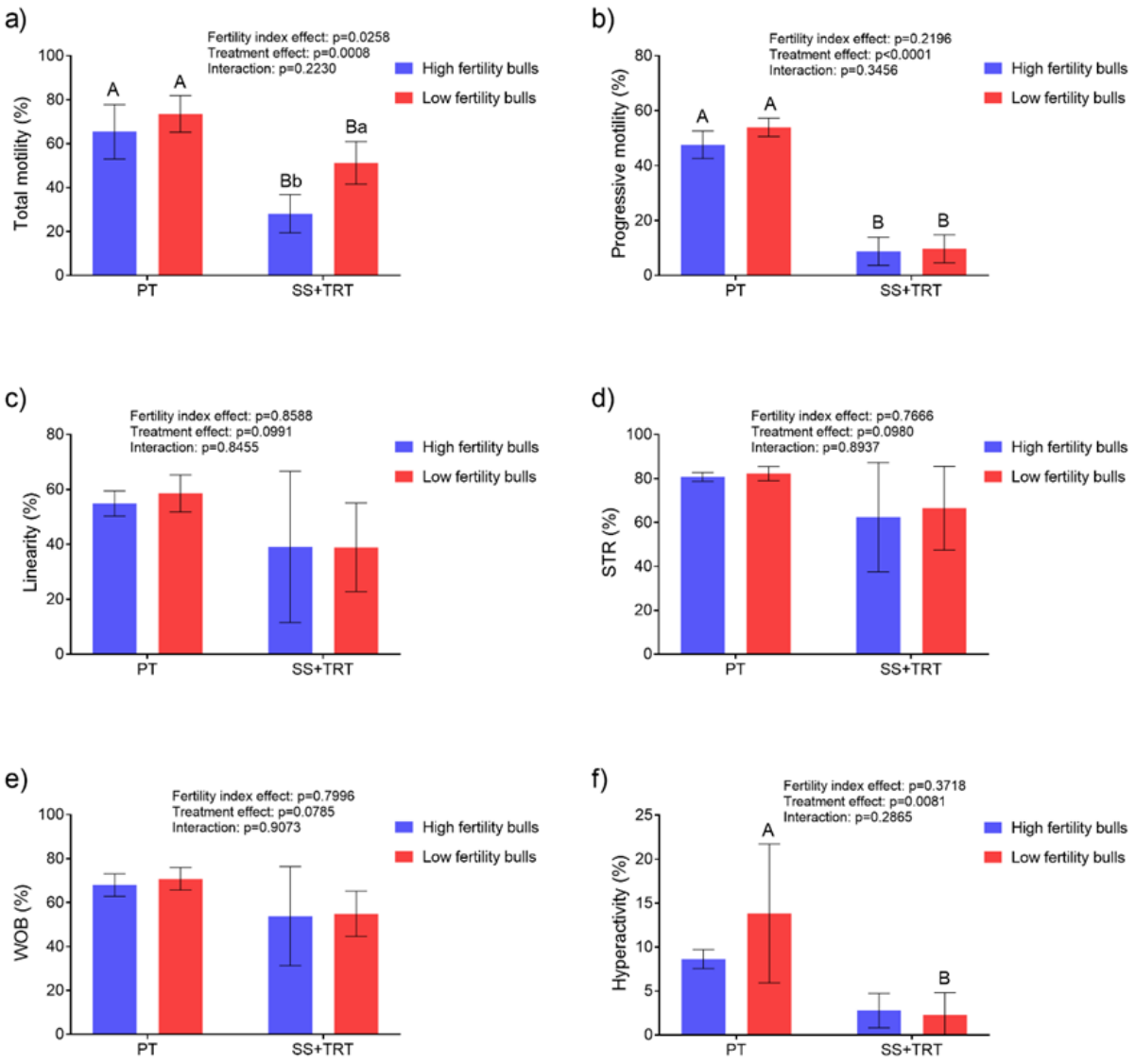

Figure 2. Sperm kinetics evaluated by the Computer Assisted Semen Analysis (CASA). Variables analyzed by two-way ANOVA, considering the effect of the fertility index of bulls, treatments, and the interaction between these factors. When a significant effect ($P < 0.05$) of one of the factors was observed, the means were compared using the Bonferroni test ($P < 0.05$). Capital letters indicate differences between treatments within bulls of the same fertility rate, and lowercase letters indicate differences between fertility rates within the same treatment. PT: Post-thaw; SS: Sperm selection; TRT: Thermo resistance test. n = 3.

The VCL, VSL, VAP (Figure 3 a-c), and BCF (Figure 3e) were affected by the treatments. We observed greater VCL, VSL and VAP in the low fertility bulls of the PT group samples (62.5 ± 23.1 μm.s^{-1}, 37.6 ± 18.5 μm.s^{-1} and 45.1 ± 20.1 μm.s^{-1}, respectively), when compared to the SS + TRT group samples (22.8 ± 9.5 μm.s^{-1}, 10.1 ± 5.5 μm.s^{-1} and 13.4 ± 6.2 μm.s^{-1}, respectively). In high fertility bulls, there was no difference between treatments for velocity variables. On the other hand, we observed higher BCF in bulls of high fertility in samples from the PT group (11.1 ± 0.9 Hz) in relation to the SS + TRT group (7.6 ± 3.1Hz). We did not observe significant effects of fertility index and treatments for ALH (Figure 3d).

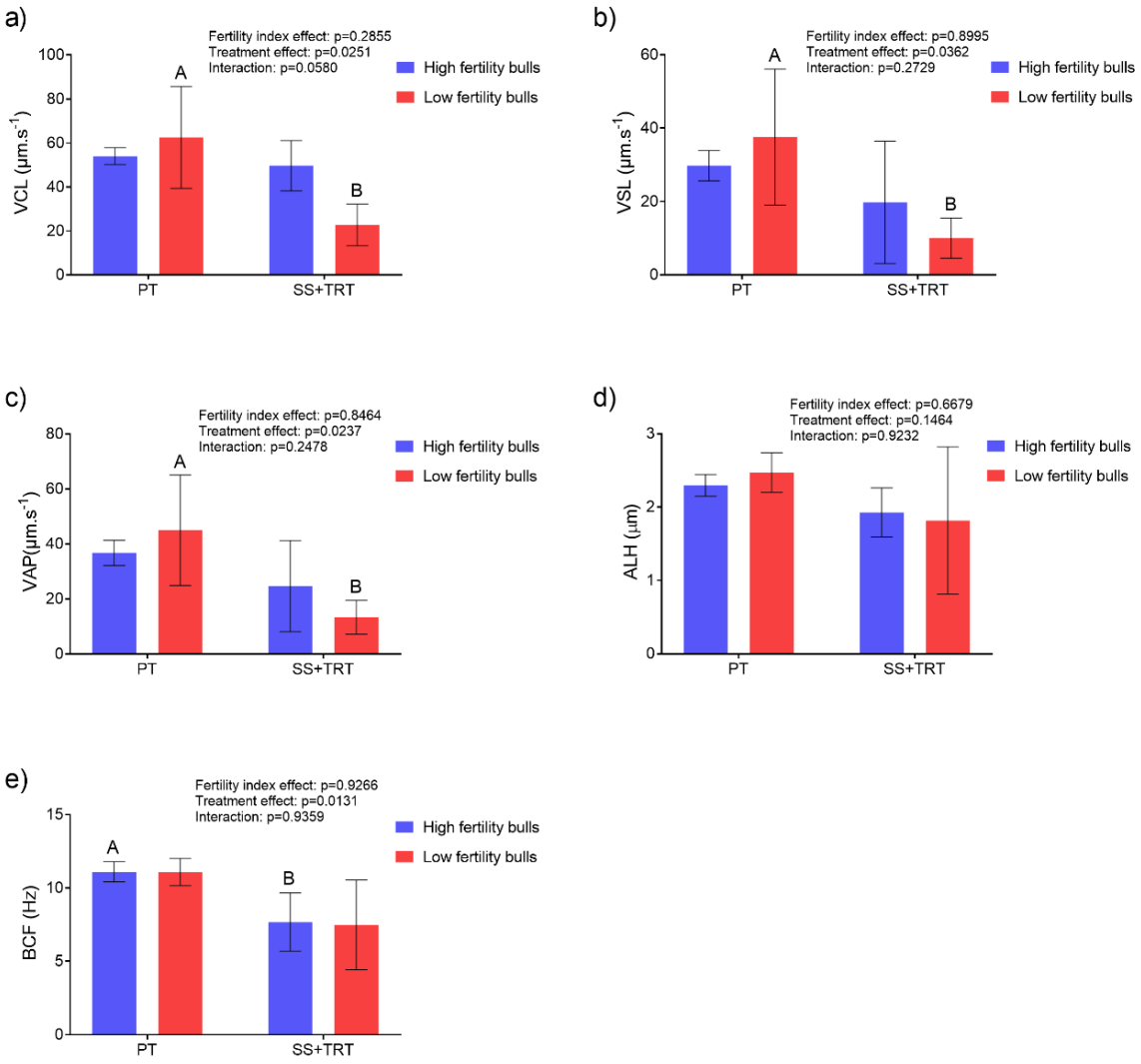

Figure 3. Sperm kinetics evaluated by the Computer Assisted Semen Analysis (CASA). Variables analyzed by two-way ANOVA, considering the effect of the fertility index of bulls, treatments, and the interaction between these factors. When a significant effect ($P < 0.05$) of one of the factors was observed, the means were compared using the Bonferroni test ($P < 0.05$). Capital letters indicate differences between treatments within bulls of the same fertility rate. PT: Post-thaw; SS: Sperm selection; TRT: Thermo resistance test. n = 3.

The results of the biochemical analysis in the semen of bulls with high or low fertility, submitted to two experimental groups (PT and SS + TRT), can be seen in Figure 4. We observed a significant effect of fertility index on SOD activity (Figure 4a), where semen samples from low fertility bulls showed higher SOD activity (62.6 ± 9.4 UI) than from high fertility bulls (35.6 ± 14.3 UI), in the PT group. In the SS + TRT group, no difference was observed between high and low fertility bulls.

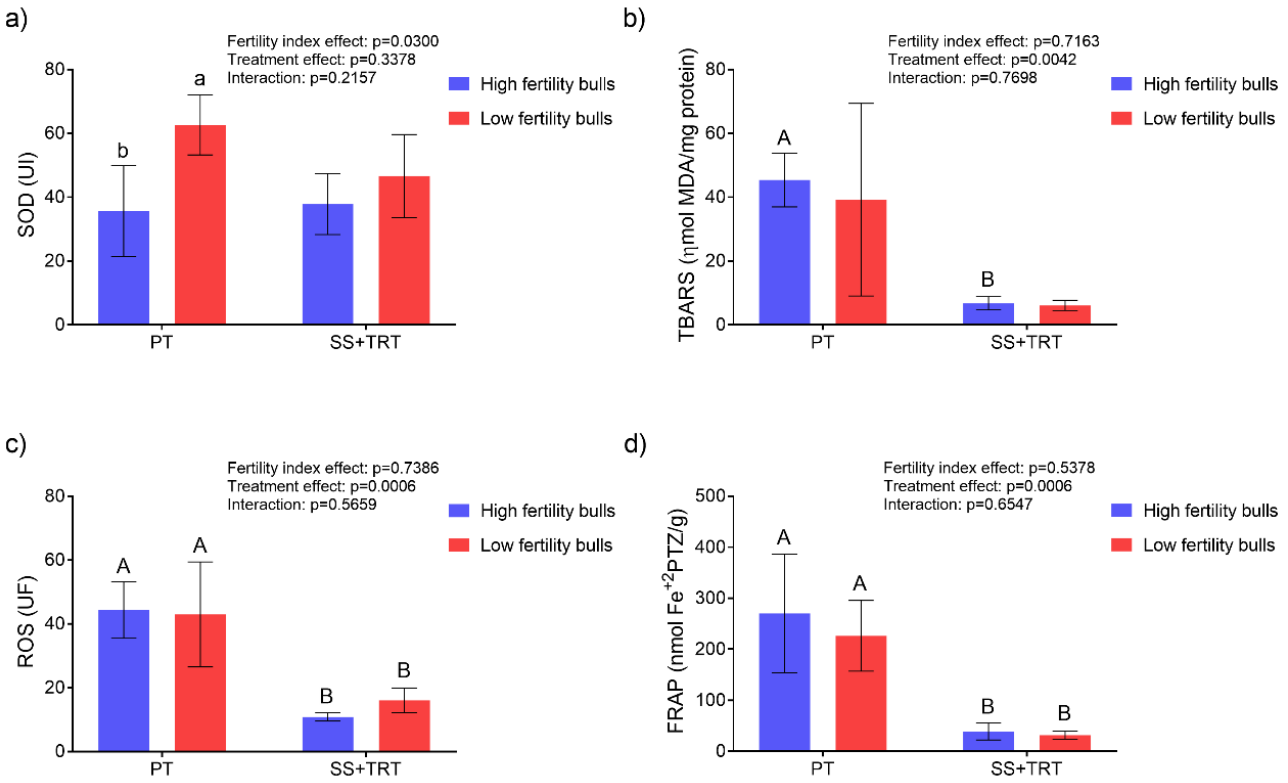

Figure 4. Sperm oxidative stress variables. Variables analyzed by two-way ANOVA, considering the effect of the fertility index of bulls, treatments, and the interaction between these factors. When a significant effect ($P < 0.05$) of one of the factors was observed, the means were compared using the Bonferroni test ($P < 0.05$). Capital letters indicate differences between treatments within bulls of the same fertility rate, and lowercase letters indicate differences between fertility rates within the same treatment. PT: Post-thaw; SS: Sperm selection; TRT: Thermo resistance test. n = 3.

The lipid peroxidation, ROS production, and FRAP (Figure 4b-d) were affected by the treatments. In the TBARS test, higher lipid peroxidation was observed in samples from high fertility bulls for the PT group (45.4 ± 8.4 nmol MDA/mg protein), compared to the SS + TRT group (6.8 ± 2.1 nmol MDA/mg protein), while in samples from low fertility bulls, there was no difference between treatments. For ROS production, a higher generation of reactive species was observed in samples from high and low fertility bulls, for the PT treatment (44.4 ± 8.9 UF of high fertility bulls; and 42.9 ± 16.4 UF of low fertility bulls), when compared to the SS + TRT treatment (10.9 ± 1.3 UF of high fertility bulls; and 16.1 ± 3.9 UF of low fertility bulls). The same behavior was observed for the FRAP analysis, where higher values were observed in samples from high and low fertility bulls, for the PT treatment (270.1 ± 116.1 nmol Fe^{+2}PTZ/g of high fertility bulls; and 226.4 ± 69.2 nmol Fe^{+2}PTZ/g of low fertility bulls), when compared to the SS + TRT treatment (38.9 ± 16.5 nmol Fe^{+2}PTZ/g of high fertility bulls; and 31.9 ± 7.9 nmol Fe^{+2}PTZ/g of low fertility bulls).

Sperm are capable of generating and degrading ROS, which at low and controlled concentrations are necessary for specific sperm functions, such as hyperactivation, capacitation, and acrosome reactions (Bailey et al., 2000). However, oxidative stress is known to be an important factor in male infertility (Bansal & Bilaspuri, 2011). In the present study, four parameters associated with oxidative stress were evaluated (ROS, SOD, FRAP, and lipid peroxidation) after PT and SS + TRT. Our results showed no significant difference between low and high fertility bulls in lipid peroxidation. However, there was a negative correlation between lipid peroxidation and high fertility following thawing (PT group) and SS + TRT. Sperm have high levels of polyunsaturated fatty acids in their membranes, making them susceptible to oxygen-induced damage and, consequently, lipid peroxidation (Aitken et al., 2007). A rapid loss of ATP, due to lipid peroxidation, leads to axonal damage, decreased viability, and mid-piece morphological defects, which contribute to decreased sperm motility (Bansal & Bilaspuri, 2011; Gharagozloo1 & Aitken, 2011). In this sense, high fertility bulls appeared to be more susceptible to lipid peroxidation than low fertility bulls since high fertility bulls showed lower motility and a negative correlation between fertility and lipid peroxidation. Therefore, it is probable that lipid peroxidation was responsible for the lower motility of the sperm of high fertility bulls. In this study, the reduction in lipid peroxidation was only observed in high fertility bulls, suggesting that high fertility bull sperm can prevent the damage induced by oxidative stress.

SOD is one of the main enzymes involved in detoxification of ROS in mammalian sperm, and its effects differ among species (Hitit et al., 2020). SOD spontaneously dismutase superoxide anion to form oxygen and hydrogen peroxide, protecting mature sperm against excessive superoxide anion accumulation. Our results showed that SOD activity in sperm was significantly lower in high fertility bulls after thawing (PT group) compared to that in low fertility bulls. A comparative study monitoring the activities of some antioxidant enzymes in cattle and buffalo sperm reported that the SOD activity successively declined

in sperm and increased in the seminal plasma over a period of 72 h at refrigeration temperatures (Nair et al., 2006). According to Nair et al. (2006), decreased SOD activity in sperm could be related to the leakage of intracellular enzymes into the seminal plasma following sperm membrane damage from lipid peroxidation, thereby increasing SOD activity in the seminal plasma. Similar results have been reported in boar (Zakošek Pipan et al., 2014) and fowl (Partyka et al., 2012), where SOD activity during storage decreased in sperm and increased in seminal plasma. In our study, it is probable that the high fertility bulls could have had higher SOD activity in the seminal plasma than in sperm. In addition, mature sperm have little capacity for repairing oxidative damage because their cytoplasm contains low concentrations of antioxidants (Alvarez & Storey, 1989). Therefore, there was no significant difference between low and high fertility bulls in total antioxidant capacity measured in sperm. Seminal plasma is endowed with many enzymatic and non-enzymatic antioxidants that protect the sperm against oxidative stress (Kim & Parthasarathy, 1998). Therefore, further studies that include measurement of SOD activity and FRAP levels in seminal plasma are needed to confirm these predictions.

Sperm selection in IVF is extremely important to ensure a healthy embryo. However, sperm selection procedures result in high levels of ROS production (Arias et al., 2017), predisposing the cells to irreversible damage that could reduce sperm motility. Seminal plasma contains many antioxidant systems, which act as free radical scavengers that protect sperm against oxidative stress. In contrast, sperm possess few cellular ROS defense systems (Bilodeau et al., 2000). Therefore, after Percoll™ centrifugation, the sperm have little capacity for repairing oxidative damage (Bilodeau et al., 2002). In this study, the sperm concentrations were the same in all evaluations after thawing (PT); therefore, the sperm selection process was responsible for the reduction in ROS originating from cellular debris and dead sperm.

The correlations between individual *in vitro* diagnostic tests and the adjusted fertility (IFert™ index) of six Aberdeen Angus bulls are given in Table 1. In samples only thawed (PT group) of high fertility bulls, we observed a positive correlation between the fertility index and the linearity parameter (r = 0.9989; P = 0.029), and a negative correlation between the fertility index and the parameters TBARS (r = -0.996; P = 0.051) and FRAP (r = -0.9956; P = 0.059). In samples from the SS + TRT group of high fertility bulls, we observed a negative correlation between the fertility index and the VSL parameters (r = -0.9947; P = 0.065), linearity (r = -0.9887; P = 0.095), BCF (r = -0.9901; P = 0.0893), and TBARS (r = -0.9951; P = 0.0629). In samples from the SS + TRT group from low fertility bulls, we observed a positive correlation between the fertility index and the VSL parameter (r = 0.9960; P = 0.0564).

Table 1
Pearson pair-wise correlations (r) between individual in vitro diagnostic tests and the adjusted fertility (IFert™ index) of six Aberdeen Angus bulls

Variable	Post-thaw group (PT)		SS + TRT group	
	High fertility bulls	Low fertility bulls	High fertility bulls	Low fertility bulls
Total motility (%)	0.2097	-0.6242	-0.5205	-0.7997
Progressive motility (%)	03185	0.5668	-0.6918	0.6312
VCL (μm.s^{-1})	0.6901	0.7837	-0.2390	0.8534
VSL (μm.s^{-1})	0.9336	0.7842	**-0.9947***	**0.9960***
VAP (μm.s^{-1})	0.9693	0.7935	-0.9930	0.9524
Linearity (%)	**0.9989****	0.8402	**-0.9887***	0.9069
STR (%)	0.3620	0.7560	-0.9782	0.9569
WOB (%)	0.9672	0.8987	-0.9780	0.9029
ALH (μm)	-0.8744	0.7451	-0.8558	0.6624
BCF (hz)	-0.2917	0.5632	**-0.9901***	0.9330
Hyperactivity (%)	-0.7795	0.7359	-0.2931	0.5993
SOD (UI)	-0.2539	-0.4866	-0.2419	-0.9051
ROS (UF)	0.2986	-0.9844	0.7638	-0.9072
FRAP (nmol Fe^{+2}PTZ/g)	**-0.9956***	-0.9840	0.9828	-0.2101
TBARS (nmol MDA/mg protein)	**-0.9967***	-0.4654	**-0.9951***	-0.6363

Legends: VCL (curvilinear velocity); VSL (straight-line velocity); VAP (average path velocity); STR (straightness); WOB (wobble); ALH (amplitude of lateral head displacement); BCF (beat cross-frequency); SOD (superoxide dismutase); ROS (Reactive oxygen species); FRAP (Ferric reducing antioxidant potential); TBARS (thiobarbituric-acid-reactive substances). High fertility bulls = 59.08 ± 7.10 % pregnancy rate; Low fertility bulls = 35.61 ± 6.36 % pregnancy rate. Pearson's correlations: *P < 0.10, **P < 0.05. Three replicates (straws) were performed from each of three ejaculates for each bull. All parameters were correlated back to individual pregnancy rates. PT: Post-thaw; SS: Sperm selection; TRT: Thermo resistance test.

We assessed the correlation between *in vitro* sperm kinetic parameters, oxidative stress assessments, and field fertility to identify suitable parameters to provide a reliable prediction of fertility. Eleven kinetic parameters were measured (MTOT, MP, VCL, VSL, VAP, LIN, STR, WOB, ALH, BCF, and HYP). *In vitro* assessment of sperm kinetics using CASA has shown a correlation between motility parameters and *in vivo* fertility (Broekhuijse et al., 2012). However, our results showed higher MTOT in low fertility bulls compared to high fertility bulls after SS + TTR. Although motility is the most used parameter in routine assessments performed in AI centers, its correlation with fertility is not universally accepted. Studies report conflicting associations between fertility and motility. According to Bailey et al. (2000), excessive motility may be an unfavorable fertility trait, because sperm would advance rapidly through the oviduct

before ovulation and would not reach and fertilize a viable oocyte. Similarly, LIN have shown a positive correlation with pregnancy rates in some studies (Verstegen et al., 2002), and a negative correlation in others (Mortimer, 2020; Pereira et al., 2021). In our study, VCL was greater in high fertility bulls after SS + TRT. High values of VSL, VCL, and VAP have been correlated with higher fertility (Misra & Fridovich, 1972; Loetchutinat et al., 2005). However, these velocity parameters should not be individually related to fertility. Vianna et al. (2009), evaluated the efficiency of rapid (46 °C for 30 min) and slow (38 °C for 5 h) TRT tests in predicting the fertility of frozen semen, and reported low correlations in both tests with fertility. Thus, TRT tests, when used alone, are not reliable for estimating *in vivo* fertility. However, according to our results, the SS + TRT test was efficient at predicting bull fertility.

Conclusion

Our results showed that a combination of *in vitro* sperm kinetic parameters predicted *in vivo* fertility more accurately than individual kinetic parameters. Our data also indicate that lipid peroxidation of sperm is an important indicator of fertility in bulls. Also, sperm selection using discontinuous Percoll™ density gradient improves the parameters of oxidative stress for high and low fertility bulls. The *in vitro* assay parameters VSL, VAP, lipid peroxidation, and ROS levels are valuable tools for assessing the potential fertility after sperm selection using the Percoll™ gradient. These results further develop assessment tools to identify useful indicators of *in vivo* fertility. Finally, the cryopreservation of sperm may induce cellular damage and cause cells to lose their antioxidant defense systems. Thus, further studies using fresh, instead of frozen, sperm are required to assess the relationship between field fertility and oxidative stress.

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