


Expression of paraoxonase types 1, 2 and 3 in reproductive tissues and activity of paraoxonase type 1 in the serum and seminal plasma of bulls

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Summary

The paraoxonases types 1, 2 and 3 (PON1, PON2 and PON3, respectively) are enzymes that degrade lipid peroxides, preventing oxidative damages relevant for male reproductive function. This study determined the expression of those three paraoxonases in reproductive tissues of bulls and evaluated correlations among the activity of PON1 in the serum and seminal plasma with breeding soundness parameters in bulls. The expression of *PON1*, *PON2* and *PON3* was characterised by RT-PCR in samples of testicular parenchyma, vesicular glands and epididymis collected from three slaughtered bulls. All three paraoxonases were expressed in the testicular parenchyma, *PON2* and *PON3* were both expressed in the epididymis head and *PON3* was also expressed in the epididymis tail. The PON1 activity was determined in samples of serum and seminal plasma from 110 bulls submitted to breeding soundness evaluation. There was a strong correlation ($r = .90$) between the activity of the PON1 in both serum and seminal plasma ($p < .0001$). The PON1 activity in the seminal plasma was positively correlated with ejaculate's colour, sperm mass activity ($p = .04$), motility, vigour and viability (all $p < .01$). Thus, PON1 may be a potential marker for sperm motility and viability in bulls.

KEYWORDS

acute phase proteins, bulls, reproductive tissues, sperm quality

1 | INTRODUCTION

The paraoxonases types 1, 2 and 3 (PON1, PON2 and PON3, respectively) are acute phase proteins capable to degrade lipid peroxides (Barranco, Roca et al., 2015), presenting antioxidant activity (Précourt et al., 2011). In rats, expression of *PON1*, *PON2* and *PON3* was reported in many tissues such as intestines, stomach, pancreas, hepatocytes, adipocytes, muscles, follicular fluid, seminiferous tubules and spermatozoa (Marsillach et al., 2008). The *PON2* expression was detected in fewer tissues compared to *PON1* and *PON3*, but it was already detected in the liver, lungs, placenta, heart and testicles of humans (Ng et al., 2001).

The PON1 is involved in male reproductive functions, as it was identified in sperm lineage cells, Sertoli cells and Leydig cells of rats and humans (Marsillach et al., 2008, 2011). Low activity of PON1 was observed in the seminal plasma of infertile or subfertile men (Lazaros et al., 2011; Tavilani et al., 2014; Verit, Verit, Ciftci, Erel, & Çelik, 2009) and in ejaculates of azoospermic men (Gulum et al., 2016). In boars, activity of PON1 was determined in the seminal plasma (Barranco, Roca et al., 2015), and its antioxidant activity was related to subsequent fertility in vivo (Barranco, Tvarijonavičute et al., 2015). Such antioxidant activity would be beneficial for sperm quality and function as spermatozoa are sensitive to oxidative stress induced by increased production of reactive oxygen species (ROS)

(Chatterjee & Gagnon, 2001; Guthrie & Welch, 2006), due to the lack of efficient antioxidant systems (Bucak, Atessahin, & Yuca, 2008),

Potential antioxidant activity of PON1 was reported in bull semen (Dedeoglu, Arslan, & Erzenin, 2014), but the presence of paraoxonases in reproductive tissues of bulls and associations among PON1 activity and reproductive traits were not determined yet. Nevertheless, at field level, potential bull fertility is most commonly estimated through breeding soundness evaluations, which includes conventional evaluations of semen quality (Kastelic & Thundathil, 2008; Waldner, Kennedy, & Palmer, 2010). However, after censoring bulls with obvious physical and reproductive flaws, ejaculates classified within generally recommended levels do not necessarily differentiate bulls with distinct fertility, which requires the evaluation of several parameters of sperm quality altogether (Gillan, Kroetsch, Maxwell, & Evans, 2008; Puglisi et al., 2012). Thus, the search of potential marker capable to detect highly fertile individuals still needs to be developed. The objectives of this study were as follows: to determine the expression of *PON1*, *PON2* and *PON3* mRNA in the testicular parenchyma, epididymis and vesicular glands of bulls; and to evaluate PON1 activity in the serum and seminal plasma and potential correlations with breeding soundness parameters in bulls.

2 | MATERIALS AND METHODS

The procedures involving bulls were approved by the Ethics in Animal Experimentation Committee (CEEA-UFPEL; process # 1943).

2.1 | Experiment 1

Samples of testicular parenchyma, vesicular glands and of the head, body and tail of the epididymis were collected from three bulls in a local slaughterhouse. Such bulls were the only ones sent to slaughter during a 6-month period and had no defined racial and genetic pattern. The samples were immediately stored in 2-ml cryotubes into liquid nitrogen (LN₂).

To identify the gene expression of the paraoxonases, the total RNA of the collected tissues was extracted using TRIzol[®] (Invitrogen), following the manufacturer's recommendations. The extracted RNA was quantified by spectrophotometry (NanoDrop, Thermo Scientific; Abs 260/280 nm ratio). The purity of the RNA was evaluated through the absorption rate

of the OD260/OD280 ratio, so values inferior to 1.8 were not used. The total RNA was treated with DNase (Promega) at 37°C for 5 min to digest any contaminant genomic DNA. After inactivation of the DNase at 65°C for 10 min, the reverse transcriptase reaction was performed using the iScript cDNA Synthesis kit (Bio-Rad), as recommended by the manufacturer. Gene expression was conducted through real-time PCR (CFX384 real-time PCR; Bio-Rad) using SsoFast™ EvaGreen[®] Supermix (Bio-Rad) and bovine-specific primers. The integrity of the cDNA samples was certified by amplification of the constitutive gene *GAPDH*. The primers used for the *PON1*, *PON2* and *PON3* genes (Table 1) were designed from sequences obtained in GenBank (NCBI) using the Primer-BLAST tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>). Only samples that amplified the *GAPDH* gene were used to confirm the results. The specificity of the obtained amplicons was evaluated through analyses of dissociation curves (melting) and the size of the amplicons. For such procedure, 10 µl of the PCR product of each gene was analysed by electrophoresis on a 2.0% agarose gel containing ethidium bromide, and the amplicon size was compared with a DNA ladder.

2.2 | Experiment 2

This experiment included 110 Braford bulls with approximately 2 years of age, from seven farms located in the Rio Grande do Sul State, Brazil (29°45'17"S latitude and 57°05'18"W longitude), all from the same breeding programme. Breeding soundness evaluations were conducted as described elsewhere (Kastelic & Thundathil, 2008; Waldner et al., 2010). Body weight and scrotal circumference were determined for all bulls. Ejaculates were collected using a manually controlled electro-ejaculator (Palmer et al., 2005). The ejaculate volume (ml) was recorded, and the ejaculate colour was classified through a score from 1 to 5, in which 1 means clear and 5 means creamy (McGowan et al., 2002). Motility, vigour and mass activity were evaluated for fresh spermatozoa, at the time of breeding soundness evaluation, through optical microscopy. Sperm motility was evaluated under a 200 × magnification, considering a scale of 0%–100%. Sperm vigour was classified through a score from 1 to 5, in which 1 means no progressive movement and 5 means maximum progressive movement. Sperm mass activity was classified through a score from 0 to 4, in which 0 means no activity and 4 means rapid swirling activity (Fitzpatrick et al., 2002). Normal sperm morphology was determined

Gene		Sequence	Amplicon bp	Access number
<i>PON1</i>	F	ACTCCATTGAAGTCTCTGGACTG	191	NM_001046269.2
	R	ACCACCGTAACTTTGGGTTCT		
<i>PON2</i>	F	TCTCTTTGTTGTAACCACCCA	148	NM_001013588.1
	R	ATGTTCTGGTCCAACAGCTATGA		
<i>PON3</i>	F	TGTTGATCCTGACACGGGAG	122	NM_001075479.1
	R	CAGACAAAGCGTTCCGGATG		
<i>GAPDH</i>	F	GATTGTCAGCAATGCCTCCT	94	NM_001034034.2
	R	GGTCATAAGTCCCTCCACGA		

TABLE 1 Initiators used for gene expression analyses—Experiment 1^a

^aF, forward primer; R, reverse primer.

TABLE 2 Descriptive statistics for parameters of breeding soundness evaluation for bulls—Experiment 2

Parameter	n	Mean	SD
Ejaculate colour (1–5)	95	2.8	1.0
Ejaculate volume (ml)	95	2.8	2.5
Sperm mass activity (0–4)	58	2.5	1.1
Sperm motility (%)	109	62.5	19.4
Sperm vigour (1–5)	109	3.1	0.9
Sperm viability (%)	110	75.1	24.5
Sperm normal morphology (%)	110	84.8	13.8
Scrotal circumference (cm)	110	37.6	3.1
Live weight (kg)	95	601.0	64.8

in samples extended in buffered formol saline (Fitzpatrick et al., 2002), and sperm viability was assessed in smears stained with eosin–nigrosin (Brito, Barth, Bilodeau-Goeseels, Panich, & Kastelic, 2003), both through optical microscopy. Subsequently, semen samples were stored in 15-ml conic tubes and centrifuged at $200 \times g$ for 5 min. The seminal plasma was fractioned in 1.5-ml tubes and frozen in LN₂.

At the time of the breeding soundness evaluations, blood samples were collected through puncture of the coccygeal vein using a BD Vacutainer® system with a 25 × 8 mm needle. The blood was placed in sterile 10-ml tubes with no anticoagulant. Blood samples were centrifuged at 1500 g for 10 min. Serum samples were conditioned in 1.5-ml cryotubes and frozen in LN₂.

The PON1 arylesterase activity in serum and in seminal plasma was determined as described by Browne et al. (2007). Briefly, samples were diluted 1:3 in a 20 mM Tris/HCl buffer solution (1 mM CaCl₂). Absorbance was measured at 270 nm through spectrophotometry (Cirrus 80ST), using 3.3 μl of the diluted samples in 500 μl of the buffer solution (containing 4 mM phenylacetate), for 60 s. The PON1 activity, expressed in U/ml, was determined by the formula: Δ absorbance*115*3.

2.3 | Statistical analyses

Data were tested for normality using the Shapiro–Wilk test. Scrotal circumference was normally distributed, whereas the activity of

TABLE 3 Correlations among the activity of the paraoxanase 1 (PON1) enzyme and parameters of breeding soundness evaluation for bulls—Experiment 2^a

Parameter	n	Serum PON1		Seminal plasma PON1	
		r	p	r	p
Ejaculate colour (1–5)	95	−0.03	.76	0.27	<.01
Ejaculate volume (ml)	95	0.20	<.05	0.17	.10
Sperm mass activity (0–4)	58	0.10	.46	0.27	.04
Sperm vigour (1–5)	109	0.03	.78	0.28	<.01
Sperm viability (%)	110	0.04	.70	0.24	<.01
Normal sperm morphology (%)	110	−0.14	.15	−0.12	.12
Scrotal circumference (cm)	110	−0.10 ^a	.30	−0.03 ^a	.76
Body live weight (kg) ^a	95	−0.14	.18	−0.02	.84

^aPearson's coefficient. All other correlations were determined using the Spearman's coefficient for nonparametric data.

PON1 in seminal plasma and serum was normalised using logarithmic transformation. Correlations among such variables were determined by the Pearson's coefficient. As the remaining breeding soundness parameters were either categorical or not normally distributed, correlations involving such variables were determined by the Spearman's coefficient for nonparametric data. All analyses were conducted using Statistix® (2013).

3 | RESULTS

3.1 | Experiment 1

The three paraoxonases were expressed in the testicular parenchyma. Expression of PON2 and PON3 was also observed in the head of epididymis. The PON3 was also expressed in the tail of the epididymis. None of those three enzymes was expressed in the vesicular glands. The GAPDH gene was expressed in all samples of all bulls.

3.2 | Experiment 2

The PON1 activity in the serum was 24.4 ± 16.7 U/ml, ranging from 2.2 to 80.7 U/ml. In the seminal plasma, mean PON1 activity was 7.4 ± 5.1 U/ml, with values ranging from 0.9 to 27.1 U/ml. The PON1 activity in serum was highly correlated ($r = .90$) with the PON1 activity in the seminal plasma ($p < .0001$).

The results of the breeding soundness evaluations are shown in Table 2. As shown in Table 3, the ejaculate volume was the only breeding soundness parameter positively correlated with the serum PON1 activity ($p < .05$). However, PON1 activity in the seminal plasma was positively correlated with the ejaculate colour ($p < .01$) and with sperm mass activity ($p = .04$), vigour and viability (all $p < .01$). Sperm motility was positively correlated ($p < .01$) with PON1 activity in the seminal plasma (Figure 1), but uncorrelated with PON1 activity in the serum ($r = .13$, $p > .05$).

4 | DISCUSSION

The expression of PON1, PON2 and PON3 in the testicular parenchyma and the PON1 activity in the seminal plasma and serum of bulls were characterised for the first time in this study. Expression of

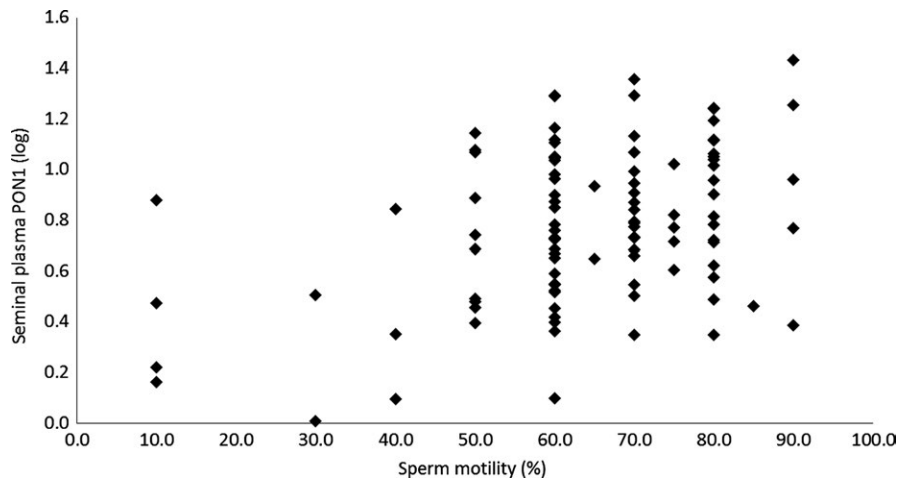


FIGURE 1 Correlation between the activity of the paraoxanase 1 (PON1) enzyme in the seminal plasma and sperm motility of bulls—Experiment 2*. * r (Pearson's coefficient) = 0.38; $p < .01$; $n = 106$

those three enzymes has been previously reported in mice, not only in the testicular parenchyma, but also in Leydig cells and in germ cells (Marsillach et al., 2008). Furthermore, those three paraoxanases were also identified by immunohistochemistry in the testicular parenchyma of subfertile men (Marsillach et al., 2011), although their concentration in semen was reduced, likely due to cell catabolism. Additionally, *PON2* and *PON3* were both expressed in the head of the epididymis and *PON3* was expressed in the tail of the epididymis. The *PON2* was previously identified in the head and in the tail of boar spermatozoa with intact membranes (Barranco, Roca et al., 2015). Those findings indicate that three paraoxanases are present in bull testicles and may play a role in protecting spermatozoa against oxidative stress, at both intra- and extracellular levels.

Although *PON1* activity was nearly three times greater in the serum than in the seminal plasma, the *PON1* activity in the seminal plasma was correlated with a greater number of parameters of ejaculate and sperm quality compared to the serum *PON1*. The seminal plasma includes a variety of proteins originated from the testicles, epididymis and accessory sex glands, which are involved in processes such as sperm metabolism, maturation, capacitation, acrosome reaction and interaction with oocytes (Rodríguez-Martínez, Kvist, Ernerudh, Sanz, & Calvete, 2011; Boe-Hansen et al., 2015; Rego et al., 2014). The fact that *PON1* activity was observed in the seminal plasma of bulls (in the present study) and in the sperm-rich portion of boar's ejaculates (Barranco, Tvarijonaviciute et al., 2015) suggests that *PON1* may be originated from either the testicles or the epididymis. Even though ejaculates of bulls do not have distinct portions, as occurs in boars, the positive correlation of the activity of serum *PON1* with the ejaculate colour (a subjective indicator of sperm concentration) apparently corroborates the hypothesis of the testicular/epididymal origin of the seminal plasma *PON1*, which is to be confirmed by future research. An unbalanced activity of *PON1* may result in oxidative stress at testicular level (Rego et al., 2014; Barranco, Roca et al., 2015). Therefore, excessive formation of ROS may occur, leading to damages in spermatozoa's membrane and DNA (Bucak et al., 2008). Such detrimental effects on spermatogenesis likely result in negative effects on seminal traits that are detectable in conventional breeding soundness evaluations

(Fitzpatrick et al., 2002). As suggested by our results, bulls with greater *PON1* activity in the seminal plasma may present ejaculates with increased volume and improved sperm mass activity, motility, vigour and viability. Such traits are correlated among themselves and have moderate repeatability (Fitzpatrick et al., 2002), but, individually, their correlations with field fertility are commonly poor (Johnson, 1997), indicating that precise evaluations of fertility should be based on several combined assays (Kastelic & Thundathil, 2008). Thus, although *PON1* may qualify as a candidate marker for bull sperm quality, potential associations with subsequent fertility still require further investigation.

The expression of the three paraoxanases was confirmed in all samples by the amplification of the gene *GAPDH*, and the specificity of the amplified fragments was confirmed by both the size of the amplicons estimated through the comparison with the DNA marker and by the inexistence of unspecific bands. Distinct levels of enzymatic activity may be genetically driven by polymorphisms in the promoting and encoding regions of the *PON1* gene, as observed in humans (Camps, Marsillach, & Joven, 2009; Padungtod et al., 1999) and in cows (Silveira et al., 2015). The *PON1* 55M, *PON1* 192R and *PON2* 311C alleles are associated with the activity of the *PON1* and with increased levels of oxidative stress in men's seminal plasma (Lazaros et al., 2011). However, the presence and the activity of the *PON2* and *PON3* in the seminal plasma and in spermatozoa of bulls still need to be determined, as only the expression of the mRNA of such enzymes was investigated in the present study.

5 | CONCLUSIONS

The *PON1*, *PON2* and *PON3* were expressed in the testicular parenchyma of bulls. As the *PON1* activity in the seminal plasma was positively correlated with some breeding soundness traits, *PON1* may be a potential marker for bull sperm motility and viability.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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