Evaluation of DNA Fragments on Boar Sperm by Ligation-mediated Quantitative Real Time PCR

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ABSTRACT

Sperm chromatin integrity is essential for successful fertilization and development of an embryo. Reported here is a quantification of DNA fragments which is intimately associated with reproductive potential to provide one of criteria for sperm chromatin integrity. Three sperm populations were considered: CONTROL (no treatment), UV irradiation (48 mW/cm^2, 1 h) and H_2O_2 (oxidative stress induced by hydrogen peroxide, 10 mM, 50 mM and 100 mM). DNA fragments in boar sperm were evaluated by using ligation-mediated quantitative real-time polymerase chain reaction (LM-qPCR) assay, which relies on real-time qPCR to provide a measure of blunt 5' phosphorylated double strand breaks in genomic DNA. The results in agarose gel electrophoresis showed no significant DNA fragmentation and no dose-dependent response to H_2O_2. However, the remarkable difference in shape and position was observed in melting curve of LM-qPCR. This result supported that the melting curve analysis of LM-qPCR presented here, could be more sensitive and accurate than previous DNA fragmentation assay method.

(Key words : DNA fragmentation, ligation-mediated quantitative real-time polymerase chain reaction (LM-qPCR), boar sperm, sperm quality)

INTRODUCTION

Many reports show that animal reproductive disorders or fertility decreasing have been related to copulatory behavior, structural defects and defective sperm function (Meliska and Bartke, 1997; Maleszewski *et al.*, 1998). A DNA damage of sperm cell is dependent on two factors. Unlikely most of somatic cells, sperm chromatin is composed in a tight structure, resulting in sperm DNA being six times (Boe-Hansen *et al.*, 2005; Saravia *et al.*, 2007). Sperm chromatin condensation takes place during spermatid development when histones are removed from nucleosomes by transition nuclear proteins. The stripped DNA is coated with protamines and repacked in late-step spermatids in two transition phases (Gadella and Harrison, 2002; De Ambrogi *et al.*, 2006). Proper condensation probably stabilizes DNA, thus reducing its susceptibility to oxidative damage (Silva and Gadella, 2006). The other mechanism of defense is the presence of antioxidants in the seminal plasma that protect sperm against oxidative damages (to both sperm membrane and DNA) by reactive oxygen species (ROS). The damage that ultimately occurs to sperm DNA, despite the defense mechanisms, can only partially be repaired by the zygote after successful fertilization (Boe-Hansen *et al.*, 2005), as repair of DNA damage is not possible in the mature sperm (Silva and Gadella, 2006).

The DNA integrity is important in fertilization and development of an embryo (Karja *et al.*, 2004). Therefore, if the DNA integrity is damaged, it causes the increase of the number of DNA fragment and affects the living of organism. The Sperm DNA integrity is reduced by extended boar semen storage time (Pérez-Llano *et al.*, 2006; Boe-Hansen *et al.*, 2008; Byun *et al.*, 2008). While sperm DNA fragmentation is partially prevented by some sperm extenders. Also quantity of DNA fragment is increased in the freezing-thawing but this is somewhat prevented by input of heparin (Fraser and Strzezek, 2007; Hwang *et al.*, 2009). Recently, reproductive molecular biology is com-

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monly acceptable tools of sperm quality control (Enciso *et al.*, 2006).

Moreover, assessment of sperm DNA fragmentation may be new parameter of sperm quality (Fraser and Strzezek, 2007; Flores *et al.*, 2008; Hu *et al.*, 2008; Bryla *et al.*, 2010) and effective fertility indicator among in situ nick translation (ISNT), terminal deoxynucleotidyl transferase-mediated nick end labelling (TUNEL), comet assay, and mainly the Sperm Chromatin Structure Assay (SCSA) (Evenson *et al.*, 2002; Agarwal and Said, 2003; Hernández *et al.*, 2006; de la Torre *et al.*, 2007; Didion *et al.*, 2009).

DNA integrity is evaluated by Ligation-Mediated Real Time PCR (LM-qPCR). It is based on quantity of products by LM-qPCR. Quantity of products is dependent on DNA fragment because it inhibits PCR (Hwang *et al.*, 2009). The LM-qPCR is unified Method of Ligation-Mediated PCR and Real Time PCR. The LMqPCR has a merit that products are more accurate identified than previous method by melting curve analysis The purpose of this study was to evaluate the efficiency of LM-qPCR assay when used in boar sperm samples with different conditions.

MATERIALS & METHODS

1. Semen Collection and Preparation

The sperm-rich fractions were collected by gloved-hand technique from two mature boars of proven fertility and sperm samples were pooled. The sperm samples were diluted with modified-Modena to 10^8 spermatozoa/mL.

2. DNA Isolation from Boar Semen

Genomic DNA was extracted from boar spermatozoa. All reagents were from Sigma unless otherwise noted. A total of 1×10^9 sperm were diluted in 250 μl phosphate buffered saline (PBS, Invitrogen, Carlsbad, CA) and combined with an equal volume of lysis buffer #1 (20 mM Tris-HCl pH 8.0, 20 mM ethylenediaminetetraacetic acid, 200 mM NaCl, 4% sodium dodecyl sulfate, 80 mM dithiothreitol) and 20 μg proteinase K (Invitrogen). Then 500 μl of the tissue homogenates was added to an equal volume of lysis buffer #2 (10 mM Tris-HCl pH 8.0, 0.5% sodium dodecyl sulfate, 0.1M ethylenediaminetetraacetic acid pH 8.0 and 20 μg proteinase K. Samples were incubated overnight at 55℃ and then extracted with 25:24:1 phenol:chloroform:isoamyl alcohol. An equal volume of isoproganol was added to the aqueous phase. The DNA pellet was recovered, washed in 70% ethanol, and air-dried. The pellet was resuspended in 10 mM Tris plus 1 mM ethylenediaminetetraacetic acid pH 8.0 and incubated overnight at 4℃.

3. Ligation-mediated quantitative Real-time PCR and Melting Curve Analysis (MCA)

Real-time PCR was performed using an iQ^{TM}5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories) which monitored the PCR reactions, for two sets of primers simultaneously, and produced separate fluorescence amplification plots for each primer set product. The quantification was performed by experimental determination of the threshold cycle (Ct), defined as the PCR cycle number. The quantities relative to control sample were automatically estimated using the iQ^{TM}5 Optical System Software. To determine the normalized expression of the target gene, the relative quantity of target sequence was divided by relative quantity of the reference sequence. After completion of the PCR reaction, a melting curve was recorded by holding at 95℃ for 1 min, cooling to 55℃ for 1 min, and then heating slowly at 0.5℃/s up to 95℃ with a maximal ramp rate by default for the iQ^{TM}5 instrument (80 repeats of counts). The melting peaks were plotted as the -dF/dT versus T (F is fluorescence; T is temperature).

The procedure was adapted from a previously described method (Staley et al., 1997; Salas et al., 2007) and modified for this study. Genomic DNA of each sample was mixed with 0.5 nmol each of 24 bp (5'-AGCACTCTCGAGCCTCTCACC GCA-3') and 12 bp (5'-TGCGGTGAGAGG-3') oligonucleotides in 30 μl of T4 DNA ligase buffer (New England Biolabs). Oligonucleotides were annealed by heating to 55℃ for 10 min, the mixture was gradually cooled to 10℃ for 10 min. Then, 1.5 units of T4 DNA ligase (New England Biolabs) were added and the sample was incubated at 16℃ for 16h. The ligated product was diluted to a concentration of 5 ng/μl with TE buffer prior to use in the PCR assay. Each PCR reaction (20 μl) contained 10ng ligated DNA, 124 pmols of the 24 bp linker primer, 10 μl 2× iQ^{TM} SYBR® Green supermix (Bio-rad). The reaction mixture was incubated for a 5 min at 72℃ to allow filling of the protruding 5' ends of the ligated adapters. Samples were then amplified for 30 cycles of 1 min at 94℃ and 3 min at 72℃. A final 5 min extension step at 72℃ was also performed. To confirm the presence of amplicons and compare with melting curve analysis data, PCR products were separated by electrophoresis on a 1% agarose gel, stained with ethidium bromide, and photographed under UV light.

RESULT

In general method of identifying DNA fragmentation with Gel electrophoresis followed by ethidium bromide staining (Fig. 1). It's too difficult to identify DNA fragmentation. To evaluate DNA fragments on boar sperm samples containing damaged by UV irradiation, different concentration of H_2O_2 (10, 50, 100 mM), we performed previous LM-qPCR and carried out agarose gel electrophoresis.

Nucleosomal fragmented DNA in size 200~400 bp appeared on agarose gel. B lane is significant, but rest of C, D, E and F lanes are not couldn't observe any nucleosomal fragmented DNA in size 200~400 bp (Fig. 2).

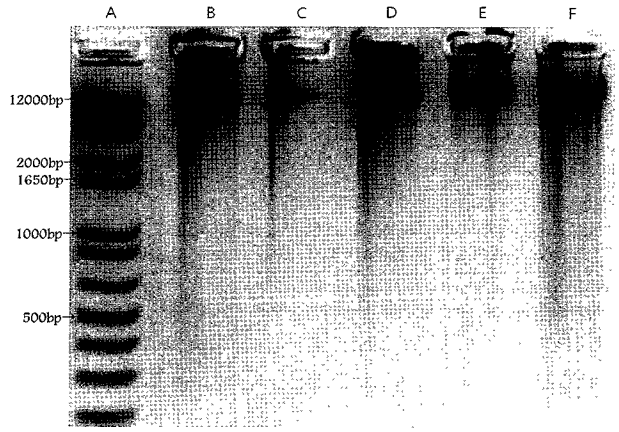


Fig. 1. To identify DNA fragmentation with Gel electrophoresis followed by ethidium bromide staining in 1 kb ladder (A), CONTROL (B), UV irradiation (C), 10 mM H_2O_2 (D), 50 mM H_2O_2 (E), 100 mM H_2O_2 (F).

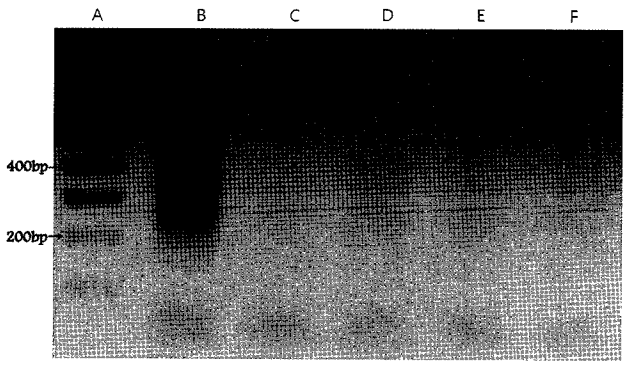
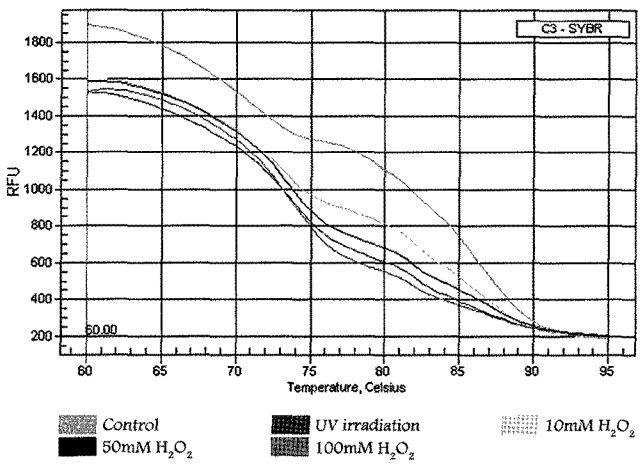


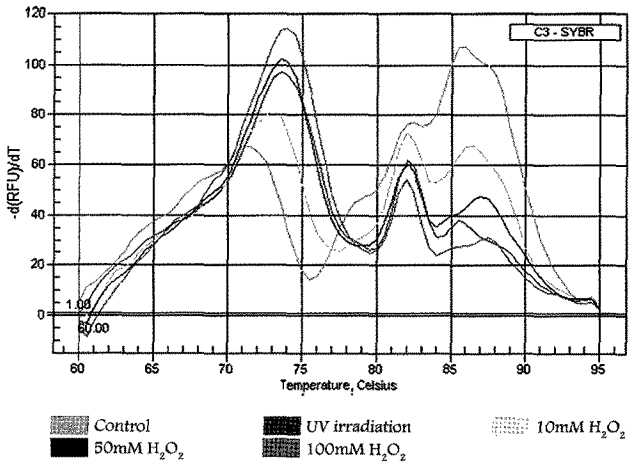
Fig. 2. Agarose gel electrophoresis of LM-qPCR for DNA fragmentation assay in 1 kb ladder (A), CONTROL (B), UV irradiation (C), 10 mM H_2O_2 (D), 50 mM H_2O_2 (E), 100 mM H_2O_2 (F). Only in B, nucleosomal ladder of 200~400 bp size is remarkably appeared.

In graph of LM-qPCR for DNA fragments derived melting curve, CONTROL group's LM-qPCR product seemed to be different from rest groups's LM-qPCR product (Fig. 3). CONTORL group's melting curve has more dynamic change in high temperature, but rest groups' melting curve has more sharp change in low temperature. In gragh of LM-qPCR for DNA fragments derived melting peak, CONTROLgroup showed low fluorescence decline at low temperature (melting temperature 71℃). Other damaged groups showed high fluorescence decline at low temperature (melting temperature UV irradiation 73.5℃, 10 mM H_2O_2 73℃, 50 mM H_2O_2 73.5℃, 100 mM H_2O_2 74℃). However the reverse aspect appeared at high temperature. As shown in Fig. 3, melting temperature CONTROL 85.5℃, 10 mM H_2O_2 86.5℃, 50 mM H_2O_2 87℃ (peak 3), respectively. Graph shows the decline of fluorescence is likely to H_2O_2-dose-dependent. Higher concentration of H_2O_2, DNA damage is more severe. It can be told higher concentration of H_2O_2, more amount of fragmented DNA. This means length of DNA fragments is most effective factor to melting temperature of LM-qPCR products to evaluate nucleosomal fragmented DNA.

DISSCUSION

Sperm DNA integrity is being considered as a new parameter of semen quality. To evaluate this potential fertility predictor, several techniques focus on sperm DNA fragmentation (Nakai *et al.*, 2007; López-Fernández *et al.*, 2008; Pérez-Llano *et al.*, 2010). In this study, we derived DNA damage to boar sperm by treating UV irradiation and H_2O_2. Using standard method, gel electrophoresis followed by ethidium bromide staining, the initial products of DNA fragmentation are not shown remarkably in figure 1. However the nucleosomal ladders in size 200~400 bp appeared in agarose gel electrophoresis for LM-qPCR because LM-qPCR can specifically amplified 5' phosphorylated blunt DNA ends. We could confirm that condition of DNA fragmentation among each sperm samples is different, but the LM-qPCR result was contrast to those we had expected. We expected that nucleosomal fragements were observed in dose dependent manner, but the result of agarose gel electrophoresis was completely opposed. We could solve this problem by analyzing the melting curve of LM-qPCR products which exactly presented what we had expected. Because the melting curve of a PCR product is dependent on GC content, length, and sequence, PCR products can be distinguished by their melting curves. Apoptotic DNA fragments induced by





CONTROL

Name	Melt temp.	Begin temp.	End temp.	Begin edit	End edit
Peak 1	71.0	60.0	73.5	60.0	73.5
Peak 2	82.5	73.5	83.0	73.5	83.0
Peak 3	85.5	83.0	94.0	83.0	94.0

UV irradiation

Name	Melt temp.	Begin temp.	End temp.	Begin edit	End edit
Peak 1	73.5	60.0	79.5	60.0	79.5
Peak 2	82.0	79.5	84.0	79.5	84.0

10mM H_2O_2

Name	Melt temp.	Begin temp.	End temp.	Begin edit	End edit
Peak 1	73.0	60.5	77.5	60.5	77.5
Peak 2	82.0	77.5	84.0	77.5	84.0
Peak 3	86.5	84.0	94.0	84.0	94.0

50mM H_2O_2

Name	Melt temp.	Begin temp.	End temp.	Begin edit	End edit
Peak 1	73.5	60.5	78.5	60.5	78.5
Peak 2	82.0	79.5	84.0	79.5	84.0
Peak 3	87.0	84.0	94.0	84.0	94.0

100mM H_2O_2

Name	Melt temp.	Begin temp.	End temp.	Begin edit	End edit
Peak 1	74.0	61.0	79.5	61.0	79.5
Peak 2	82.0	79.5	84.0	79.5	84.0

Fig. 3. LM-qPCR for DNA fragments derived melting curve (left column) and melting peaks (right column). Melting curve show LM-qPCR of CONTROL group and damaged by UV irradiation, 10, 50, 100 mM H_2O_2 group. Each axis in the melting curve represents Temperature, Celsius (x-axis) and RFU (y-axis). Melting peaks show LM-qPCR of CONTROL group and damaged by UV irradiation, 10, 50, 100 mM H_2O_2 group. Each axis in the melting peaks represents Temperature, Celsius (x-axis) and -d(RFU)/dT (y-axis).

UV irradiation and the oxidative reagent (H_2O_2) have 5' phosphorylated blunt DNA ends and 180~200 bp lengths commonly. If apoptotic cleavage events randomly, their GC contents are also arbitrary. Under this hypothesis, we could suppose that the length of DNA fragments is most effective factor to melting temperature of LM-qPCR products. In melting peaks graph which was plotted as the negative derivative of fluorescence with respect to temperature (-dF/dT vs T), the difference between CONTROL group and other DNA DAMAGE group (UV irradiation, 10, 50, 100 mM H_2O_2) was well-established. The CONTROL group showed low fluorescence decline at low temperature (peak 1), but other groups showed high fluorescence decline at low temperature (peak 1). On the other hand, the reverse aspect appeared at high temperature (peak 3). It seemed that the difference between agarose gel electrophoresis for LM-qPCR and melting curve analysis for LM-qPCR products was resulted from a limit of gel electrophoresis. Bands with 200~400 bp in CONTROL and DNA DAMAGE group consisted with decline of fluorescence at peak 2 or peak 3 in melting curve analysis, so we drew that the gel electrophoresis presented PCR products partly, which had over 200 bp length. These two explanations supported that

DNA DAMAGE group had more DNA fragments which were below 200 bp than the CONTORL group. In addition, the decline of fluorescence was likely to H_2O_2-dose-dependent. From these results, we concluded the melting curve analysis of LM-qPCR products is more accurate and sensitive method than previous DNA fragmentation assay.

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