Histol Histopathol (2007) 22: 869-872

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Lipid peroxidation was associated to the impairment of the fertilizing capability of gilthead sperm exposed to surfactants

M. Rosety[1], I. Rosety[1], L. Frias[2], J.M. Rosety[1], F.J. Ordoñez[1] and M. Rosety-Rodríguez[1]

[1]School of Medicine, University of Cadiz, Spain and [2]School of Veterinary, University of Cordoba, Spain

Summary. The present study was designed to determine whether lipid peroxidation was associated with the impairment of the fertilizing capability of gilthead sperm after acute exposure to anionic surfactant Sodium Dodecyl Sulphate (SDS).

Spawned eggs and sperm were collected from adult giltheads. Sperm suspensions (10^8 spermatozoa/mL) were dosed separately with different concentrations of SDS (0.6, 1.5, 3 and 6 mg/L) for 60 minutes. After this period, sperm samples were randomly distributed for both outcome measurements: fertilization percentage or lipid peroxidation assessment. On one hand, exposed sperm and unexposed eggs were combined for 20 minutes during which fertilization took place. Fertilization, defined as the presence of a fertilization envelope, was assessed by microscopic observation. On the other hand lipid peroxidation on exposed gilthead sperm was determined by estimating the production of malondialdehyde (MDA).

Acute exposure to SDS caused a significant inhibitory effect on fertilization success in gilthead. It also increased significantly lipid peroxidation in exposed sperm. Furthermore, a strong but negative statistical association was found between fertilizing capability and lipid peroxidation gilthead sperm exposed to SDS.

Although extrapolation from the laboratory to the field requires caution, the results of this work demonstrated that the impairment of fertilization was significantly associated with lipid peroxidation induced by acute exposure to SDS. Consequently lipid peroxidation may be recommended as an early-warning bioindicator of exposure to surfactants. Further studies are required.

Key words: Surfactant, Fish, Sperm, Fertilization, Lipid Peroxidation

Offprint requests to: M. Rosety-Rodríguez, Medicine Department, School of Medicine. University of Cádiz, Plaza Fragela s/n 11003 Cádiz, Spain. e-mail: manuel.rosetyrodriguez@uca.es

Introduction

The wide variety of processes in which surfactants are incorporated has resulted in a spectacular increase in their consumption, which has grown from about $13x10^6$ tons in 1977 to $18x10^6$ tons in 1995 (Granados, 1996). Due to their widespread use, there has been increasing concern about the environmental behaviour of these compounds.

Accordingly, acute toxic effects of anionic surfactants on aquatic species have been studied at different life stages in fish (Ribelles et al., 1995; Rosety et al., 1997, 2001a). On the other hand, the effects of this phenomenon on free gametes have received little attention (Nipper et al., 1993; Rosety et al., 2001b,c, 2003).

In this line, the selection of sperm seemed of particular interest, since it is widely accepted that they are highly sensitive to xenobiotics (Kime, 1995; Rosety et al., 2001b). Furthermore, the sperm cell toxicity test requires only 60 minutes of toxicant exposure, whereas other marine embryo and larval tests require 2 to 4 days of exposure, both having similar sensitivities (Dinnel, 1995).

In any case, the question arises as to how surfactant exerts its toxic action. Recent studies have reported oxidative stress may play an important role in toxicological effects induced by acute exposure to anionic surfactants such as SDS (Nunes et al., 2006).

For the reasons already mentioned, the present study was designed to determine whether oxidative damage was associated with the impairment of the fertilizing capability of gilthead sperm induced by acute exposure to anionic surfactant SDS.

Materials and methods

Gilthead seabream, *Sparus aurata,* L, was chosen as a test species since it represents a widespread fish on the Atlantic and Mediterranean coast of Spain and also for its traditional importance in the aquaculture industry (Sitja-Bobadilla et al., 2005). In this case, adult giltheads

were collected from a culture unit at San Fernando (Cádiz).

Three males were chosen for collection by stripping the sperm stock for our bioassay. It was kept at 4°C in a dry undiluted state until the beginning of the experiments. Sperm viability was assessed by observing (not quantifying) their mobility in filtered seawater on a microscope slide under x400 magnification. Sperm stock was diluted to 10^8 sperm/mL using a Neubauer chamber.

To prevent adhesion of sperm, the tubes were precoated by dipping in 1% polyvinyl alcohol (average molecular weight 30000-70000; Sigma) solution and dried at 60°C (Perchec et al., 1995).

In the experiments on the determination of surfactant toxicity for sperm, 0.1 mL of the sperm suspensions were exposed for 1 hour in individual test tubes containing SDS in concentrations of 0 (controls), 0.6, 1.5, 3 and 6 mg/L as was reported previously (Rosety et al., 2003). This stock was randomly distributed for both outcome measurements: fertilizing or lipid peroxidation assessment.

Spawning was artificially induced by an injection of 1.5 ml of KCl 0.5 M into the coelomic cavity. We selected three females, whose eggs had showed previously a 90% fertilization success or more, to contribute to the pool of eggs for each bioassay. Released eggs were filtered through a relatively coarse screen to remove debris and washed 3 times in 300 mL of filtered seawater before being used in the bioassay. The egg stock was diluted to 2000 eggs/mL, using a Sedgwick-Rafter chamber to count them.

On one hand, 1 mL of the unexposed egg suspension was added to each test tube containing exposed sperm after being washed in FSW to prevent SDS from affecting the eggs. After 20 minutes exposure during which fertilization took place, the tests were stopped by adding 2 ml of a 10% solution of formaldehyde in filtered seawater. Fertilization rate in a 100-egg subsample per replicate was analyzed under a microscope. It may be noted that we prepared 3 replicates per treatment.

In our study, sperm/egg ratios of 3500 were selected to evaluate the effects of SDS on the sperm fertilizing capability. Using this ratio, fertilization rate in controls was higher than 90%. Furthermore, the potential problem of high sperm concentrations masking the toxicity of surfactant was avoided.

In this respect, the criterion used to judge exposure effectiveness was fertilization success after being combined with unexposed eggs. Fertilization, defined as the presence of a fertilization envelope, was assessed by microscopic observation. While Dinnel et al. (1982) considered eggs unfertilized if they had partially formed membranes, we considered them fertilized since the test endpoint is the evaluation of the fertilizing capability of the sperm. Partial membranes indicate the existence of enough viable sperm to fertilize the eggs, in spite of the eggs inability to raise the whole fertilization membrane (Nipper et al., 1993).

On the other hand, lipid peroxidation on gilthead sperm exposed to SDS (0 [controls], 0.6, 1.5, 3 and 6 mg/L) was determined by estimating the production of malondialdehyde (MDA) as was reported previously by Placer et al. (1996).

Stock solutions of Sodium Dodecyl Sulphate (SDS) were diluted with sperm-free seawater in order to obtain the desired concentrations. The seawater used for dilution and control tubes was filtered through 0.5 um cellulose filters prior to use. Its physiological characteristics were as follows: pH 7.4, temperature 20°C, surface tension 72.7 mN/m, dissolved oxygen 8-8.6 mg/L, hardness 100 mg $CaCO_3$ mg/L and salinity 30‰. It should be mentioned that to carry out *in vivo* or *in vitro* fertilization of gilthead eggs, water salinity should be more than 9 ppt (Ratto et al., 1997).

Results were expressed as mean ± sd and 95% confidence interval. For statistical analysis of our data, one-way analysis of variance (ANOVA) followed by Tukey´s test post hoc were performed. Pearson´s correlation coefficient (r) was used to measure the strength of the association between the variables fertilizing capability and lipid peroxidation. In any case, significance was ascertained at $p<0.05$. The SPSS statistical software package was used.

Results

All SDS tested concentrations caused significant

Table 1. Effects of acute exposure to different concentrations of sodium dodecyl sulphate (SDS) on fertilizing capability and lipid peroxidation of gilthead sperm.

	FERTILIZING CAPABILITY	LIPID PEROXIDATION
Controls	93.8±2.9 [90.2-97.4]	0.96±0.14 [0.94-0.98]
0.6 mg/L SDS	72.3± 3.1 [68.2-76.4] [a]	2.10±0.28 [2.09-2.11] [a]
1.5 mg/L SDS	60.7±3.2 [57.5-64.1] [a,b]	4.15±0.40 [3.99-4.31] [a,b]
3 mg/L SDS	42.6±2.8 [40.1-45.1] [a,b,c]	6.90±0.48 [6.65-7.15] [a,b,c]
6 mg/L SDS	7.7±2.0 [6.9-8.5][a,b,c,d]	8.30±0.56 [7.98-8.62] [a,b,c,d]

Results are expressed as mean ± sd and 95% Confidence Interval. Controls: 0 mg/mL SDS. Fertilizing capability (%). Lipid peroxidation expressed as malondialdehyde content (nmol MDA/108 spermatozoa). Significant differences at $p<0.05$ when compared to controls a; 0.6 mg/l SDS b; 1.5 mg/l SDS c ; 3 mg/l SDS d.

reduction in the fertilizing capability of gilthead sperm. We also found a significant increase of lipid peroxidation in terms of malondiahdehyde content after acute exposure to SDS. These results are listed in Table 1 and Figure 1. Moreover, we found a significant but negative correlation between the assessed variables fertilizing capability and lipid peroxidation ($r=-0.68$; $p<0.05$)

Discussion

As was hypothesized, our results clearly indicated that acute exposure to anionic surfactant SDS impaired the fertilizing capability of gilthead sperm. A stronger negative effect on this parameter was reported by Rosety et al. (2001b) after the exposure of gilthead sperm to another anionic surfactant such as linear alkylbenzene sulphonate (LAS). In any case, a clear dose-response relationship for the reduction on percentage fertilization of gilthead *Sparus aurata* was observed for both SDS and LAS (Rosety et al., 2001b). Later studies also demonstrated that exposure to LAS significantly decreased sperm motility which may explain, at least in part, the impairment of fertilization success (Rosety et al., 2003).

Recent studies have also reported that oxidative stress may be directly associated with reproductive impairment in fish. In this respect, Zhou et al. (2006) have already reported that lipid peroxidation was associated with reproductive impairment of the common carp (*Cyprinus carpio*) exposed to duroquinone. Similar results were reported by Oakes et al. (2003a) in the gonads of white sucker (*Catostomus commersoni)* exposed to pulp and paper mill effluent.

Although differences in experimental procedures and conditions between the above mentioned studies suggest proceeding with caution, these data demonstrated the high sensitivity of fish sperm to oxidative stress induced by xenobiotics.

Accordingly, sperm seemed to be particularly susceptible to oxidative damage. This may be explained by the fact that the majority of the cytoplasm in the spermatozoa was lost during the final stage of spermatogenesis, leading to a low activity of cytoplasmic antioxidant enzymes (Donnelly et al., 2000).

In previous studies to explain how surfactants exerted their toxic action, Thorhaug (1992) and Singer and Tjeerdema (1993), reported that they may disrupt biological membranes and subcellular organelles. These findings may be explained, at least in part, by the fact that these membranes contain high levels of polyunsaturated fatty acids (PUFA) which are highly susceptible to reactive oxygen species (Agarwal and Prabakaran, 2005).

The lipid metabolism in sperm cells is important both for cell structure and for energy production. In this respect it should be pointed out that sperm membranes also contain a high level of polyunsaturated fatty acids (PUFA) both in mammal (Lenzi et al., 2002) and fish sperm (Pustowka et al., 2000). Consequently, lipid peroxidation assessment may be recommended as a bioindicator of oxidative damage (Mansour et al., 2006).

It should be emphasized that we have determined lipid peroxidation by assessing malondialdehyde content instead of thiobarbituric acid reactive substances (TBARS), as reported by previous studies (Oakes et al., 2003a,b). This fact seemed of great interest since it is generally accepted that there are methodological problems with the TBARS assay, including lack of specificity and generation of artifactual TBARS under various assay conditions (Janero, 1990; Mylonas and Kouretas, 1999).

Although extrapolation from the laboratory to the field requires caution, the results of this work suggested that gilthead reproduction may be impaired by lipid peroxidation induced by acute exposure to anionic surfactants such as sodium dodecyl sulphate (SDS). Consequently, lipid peroxidation assessment may be recommended as an early-warning bioindicator of acute

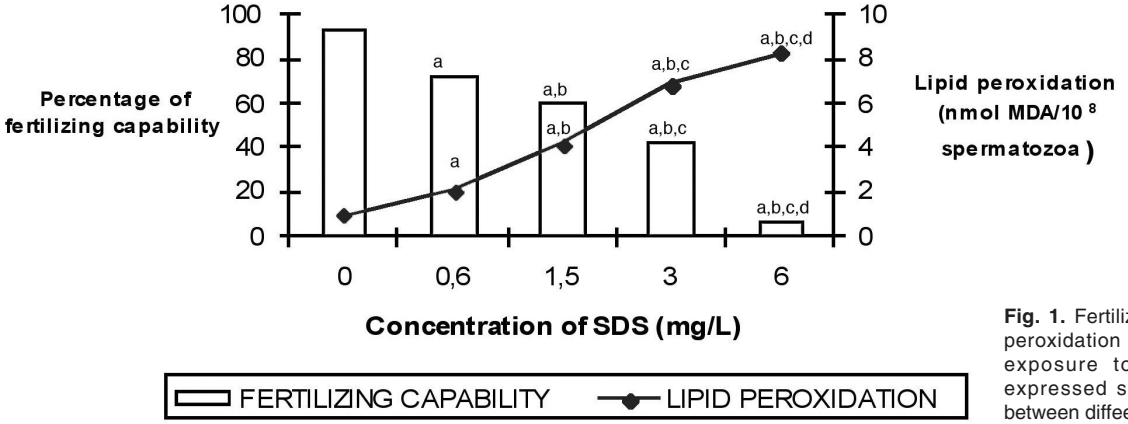

Fig. 1. Fertilizing capability and lipid peroxidation of gilhead sperm after exposure to SDS. Superscripts expressed signnificant diffeences between diffeent SDS concentrations.

exposure to surfactant. Further studies on this topic are required.

References

Agarwal A. and Prabakaran S.A. (2005). Mechanism, measurement, and prevention of oxidative stress in male reproductive physiology. Indian. J. Exp. Biol. 43, 963-974.

Dinnel P.A. (1995). Evolution and present status of the sea urchin sperm test. Russ. J. Mar. Biol. 21, 344-350.

Dinnel P.A., Stober Q.J., Crumley S.C. and Nakatami R.E. (1982). Development of a sperm cell toxicity test for marine waters. In: Aquatic toxicology and Hazard assessment. Pearson J.G., Foster RB, Bishop W.E. (eds.). Fith Conference, ASTM STP 766, American Society for Testing and Materials. Philadelphia. pp 82-98.

Donnelly E.T., Mcclure N. and Lewis E.M. (2000). Glutathione and hypotaurine: effects on human sperm motility, DNA integrity and production of reactive oxygen substances. Mutagenesis 15, 61-68.

Granados J. (1996). Surfactants raw materials. Constant evolution and a solid future. 4th World Surfactant Congress, Barcelona. 1, 100-123.

Janero D.R. (1990). Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. Free. Radic. Biol. Med. 9, 515-540.

Kime D.E. (1995). The effects of pollution on reproduction in fish. Rev. Fish. Biol. Fish. 5, 52-96.

Lenzi A., Gandini L., Lombardo F., Picardo M., Maresca V., Panfili E., Tramer F., Boitani C. and Dondero F. (2002). Polyunsaturated fatty acids of germ cell membranes, glutathione and glutathione-dependent enzyme-PHGPx: from basic to clinic. Contraception 65, 301-304.

Mansour N., McNiven M.A. and Richardson G.F. (2006). The effect of dietary supplementation with blueberry, alpha-tocopherol or astaxanthin on oxidative stability of Arctic char (*Salvelinus alpinus*) semen. Theriogenology. 66, 373-382.

Mylonas C. and Kouretas D. (1999). Lipid peroxidation and tissue damage. In Vivo 13, 295-309.

Nipper M.G., Prosperi V.A. and Zamboni A.J. (1993). Toxicity testing with coastal species of southeastern Brazil. Echinoderm sperm and embryos. Bull. Environ. Contam. Toxicol. 50, 646-652.

Nunes B., Carvalho F. and Guilhermino L. (2006). Effects of widely used pharmaceuticals and a detergent on oxidative stress biomarkers of the crustacean Artemia parthenogenetica. Chemosphere 62, 581-94.

Oakes K.D. and Van der Kraak G.J. (2003a). Utility of the TBARS assay in detecting oxidative stress in white sucker (Catostomus catostomus) populations exposed to pulp mill effluent. Aquat. Toxicol. 63, 447-463.

Oakes K.D., McMaster M.E., Pryce A.C., Munkittrick K.R., Portt C.B., Hewitt L.M., MacLean D.D. and Van Der Kraak G.J. (2003b). Oxidative stress and bioindicators of reproductive function in pulp and paper mill effluent exposed white sucker. Toxicol. Sci. 74, 51-65.

Perchec G., Jeulin C., Cosson J., Andre F. and Billard R. (1995). Relationship between sperm ATP content and motility of sperm spermatozoa. J. Cell. Sci. 108, 747-753.

Placer Z.A., Cushman L.L. and Johnson B.C. (1966). Estimation of product of lipid peroxidation (malonyldialdehyde) in biochemical systems. Anal. Biochem. 16, 359-364.

Pustowka C., McNiven M.A. Richardson G.F. and Lall S.P. (2000). Source of dietary lipid affects sperm plasma membrane integrity and fertility in rainbow trout Oncorhynchus mykiss (Walbaum) after cryopreservation. Aquacult. Res. 31, 297-305

Ratto A., Dádamo R., Pelosi S., Martello A., Barbieri V. and Perruci G. (1997). Seabream (*Sparus aurata*) spermatozoa activation at low salinity. Biol. Mar. Mediterr. 4, 343-345.

Ribelles A., Carrasco M.C., Rosety M. and Aldana M. (1995). Morphological and histochemical changes in the liver and pancreas of gilthead, *Sparus auratus* L., induced by acute action of the anionic detergent, sodium dodecyl sulphate. Histol. Histopathol. 10, 781-787.

Rosety M., Ribelles A. and Carrasco C. (1997). A morphological study in the kidney and spleen of gilthead, *Sparus aurata*, L. caused by sodium dodecyl sulphate. Histol. Histopathol. 12, 925-929.

Rosety M., Ordonez F.J., Ribelles A., Rosety-Rodriguez M., Dominguez A., Carrasco C. and Rosety J.M. (2001a). Morpho-histochemical changes in the liver and intestine of young giltheads (fish-nursery), *Sparus aurata*, L., induced by acute action of the anionic tensioactive alkylbenzene sulphonate. Eur. J. Histochem. 45, 259-265.

Rosety M., Ordoñez F.J., Rosety-Rodriguez M., Rosety J.M., Rosety I., Carrasco C. and Ribelles A. (2001b). Acuate toxicity of anionic surfactants Sodium Dodecyl Sulphate and Linear Alkylbenzene Sulphonate on the fertilizing capability of Gilthead (*Sparus aurata, L.*) sperm. Histol. Histophatol. 16, 839-843.

Rosety M., Ordoñez F.J., Rosety-Rodriguez M., Rosety J.M., Rosety I., Carrasco C. and Ribelles A. (2001c). Comparative study of the acute toxicity of anionic surfactants AlkylBenzene Sulphonate (ABS) and Sodium Dodecyl Sulphate (SDS) on Gilthead, *Sparus aurata*, L., eggs. Histol. Histopathol. 16, 1091-1095.

Rosety M., Ordóñez F.J., Rosety-Rodriguez M., Rosety J.M., Rosety I. (2003). In vitro acute toxicity of anionic surfactant linearalkylbenzene sulphonate (LAS) on the motility of gilthead (*Sparus aurata*, L) sperm. Histol. Histopatol. 18, 475-478.

Singer M.M. and Tjeerdema R.S. (1993). Fate and effects of the surfactant sodium dodecyl sulfate. Rev. Environ. Contam. Toxicol. 133, 95-149.

Sitja-Bobadilla A., Padros F., Aguilera C. and Alvarez-Pellitero P. (2005). Epidemiology of *Cryptosporidium molnari* in Spanish gilthead sea bream (*Sparus aurata* L.) and European sea bass (*Dicentrarchus labrax* L.) cultures: from hatchery to market size. Appl. Environ. Microbiol. 71, 131-139.

Thorhaug A. (1992). Oil spills in the tropics and subtropics. In: Pollution in tropical aquatic systems. Connel D.W. and Hawker D. (eds). CRC Press. London. pp 99-127.

Zhou B., Liu W., Siu W.H.S., Otoole D., Lam P.K.S. and Wu R.S.S. (2006). Exposure of spermatozoa to duroquinone may impair reproduction of the common carp (*Cyprinus carpio*) through oxidative stress. Aquat. Toxicol. 77, 136-142.

Accepted February 16, 2007