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Effects of cortisol on testicular apoptosis in goldfish (*Carassius auratus*)

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Abstract: Apoptosis is a programmed cell death that has been attributed to the action of stress hormones, such as cortisol. We investigated the *in vitro* effect of cortisol in goldfish (*Carassius auratus*) mature (GSI: 2.75-4.65) or immature (GSI: 1.94-2.4) testes. Testes were taken out and transferred into culture medium (M 199), cut into 20µm thick slices and supplemented with fetal bovine serum (FBS), added different doses of cortisol (0, 50, 100, 200, 400, 750 and 800 ng/ml) and incubated for 24h, 48h or 72h at 18°C. Samples were rinsed and homogenized with phosphate buffered saline (PBS) after incubation period. Duplicates or triplicates processed for caspase-3 activity. The results showed a significant increase in caspase-3 activity in immature testes after 72h treatment with 750ng/ml of cortisol ($P<0.05$), and also at 400ng/ml ($P<0.05$). In contrast, no significant differences were observed between non-treated and cortisol-treated samples from mature goldfish testes. In this study we have demonstrated, for the first time that the stress hormone cortisol induces apoptosis in the goldfish testis in a stage-specific manner (immature testis).

Keywords: Apoptosis, Caspase 3, Cortisol, Goldfish, Stress, Testis

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Introduction

The type of cell death today known as apoptosis was first observed by Carl Vogt in 1842 (Hsu & Hsueh, 2000). Apoptosis or programmed cell death, displays morphological and biochemical characteristics that differ from those of pathological cell death or necrosis (Kerr *et al.*, 1972; Wyllie *et al.*, 1980).

Caspases are apoptogenic proteases (Kroemer *et al.*, 1998) that play critical roles in the initiation and execution of apoptosis (Earnshaw *et al.*, 1999). Caspases are present in all living cells as inactive pro-enzymes (Rotonda *et al.*, 1996; Hirata *et al.*, 1998), which, once activated, will drive and execute the apoptotic program by cleaving critical cellular proteins in cytosol and nucleus (Wolf *et al.*, 1999). Although apoptosis can be initiated in different ways, in most cases its program converges into the activation of the so-called effector caspases, such as caspase-3 (Earnshaw *et al.*, 1999; Strasser *et al.*, 2000; Kaufmann & Hengartner, 2001; Shi, 2002).Caspases have been shown to be well-conserved proteins (Johnson & Bridgham, 2000; Nakajima *et al.*, 2000; Yabu *et al.*, 2001; Laing *et al.*, 2001; Lamkanfi *et al.*, 2002) and to be present in mammalian testes (Kim *et al.*, 2000; Giannattasio *et al.*, 2002; Omezzine *et al.*, 2003). The presence of caspase-like proteins has also been described in teleosts, although their tissue distribution in adult tissues has not been investigated to date (Yabu *et al.*, 2001; Laing *et al.*, 2001; Guo *et al.*, 2003; Masumoto *et al.*, 2003).

In the testis, apoptosis has been shown to occur during normal spermatogenesis in mammals as well as in cartilaginous fish (Prisco *et al.*, 2003). The occurrence of spontaneous or induced testicular apoptosis has been also described in both cartilaginous and teleost fish (Kwak *et al.*, 2001; Kuwahara *et al.*, 2002; Prisco *et al.*, 2003). Beside gonadotropine releasing hormone has been shown to induce apoptosis in a stage dependent manner in the goldfish testis (Andreu-Vieyra & Habibi, 2000, 2001a,b).

High levels of cortisol were shown to decrease testosterone production by direct action on guinea pig testis (Fenske, 1997). Similarly, high cortisol levels were shown to delay puberty and decrease the levels of active androgens via a direct action on carp testes (Consten *et al.*, 2002 a,b). Indeed, expression of glucocorticoid receptors has been described in the trout testis (Takeo *et al.*, 1996).

The aim of the present studies was to evaluate the response of goldfish testicular tissues to cortisol (Bahmani, 2002), to test the hypothesis that high levels of stress hormones may detrimentally affect gametogenesis by inducing apoptosis.

Materials and Methods

Animals:

Male goldfish (*Carassius auratus*) of the comet variety (length 8-10cm) were purchased from Aquatic Imports (Calgary, AB, Canada). Fish were maintained in a 1500-L semi-recirculating aquarium (60% replacement per day) at 18°C on a 16h light: 8h dark photoperiod and fed a commercial fish diet (Nutrafin floating pellets; Rolf C. Hagen Inc., Montreal, QC, Canada).

Hormone and chemicals:

Cortisol (11α,17β, 21-trihydroxypregn-4-ene-3, 20-dione or hydroxycortisone) was purchased from SIGMA (St. Lois, MO), solubilized in 1:1 absolute ethanol and propylene glycol (propane-1,2-diol) mixture and stored at -20°C until use. Appropriate concentrations of the hormone were prepared by diluting the stock solution in sterile culture medium immediately prior to use. Caspase-3 colorimetric substrate (Ac-DEVD-pNA; pNA: p-nitroanilide) and p-nitroanilide standard were purchased from SIGMA (St. Lois, MO) and solubilized in dimethyl sulfoxide (DMSO) (SIGMA, St. Lois, MO) to prepare a 20mM and 1mg/ml stock solutions respectively that were stored protected from light at –20°C until use.

Isolation of testes:

Animals were killed by a blow in the head, in accordance with the principles and guidelines of the Canadian Council of Animal Care, followed immediately by spinal transection. Mature testes (gonadosomatic index: 2.75-4.65) or immature (GSI: 1.94-2.4) were dissected out and transferred into M199 culture medium (SIGMA, St. Lois, MO) containing 7.5% sodium bicarbonate (SIGMA), pH=7 on ice. Testes were washed with medium and cut into 20μm thick slices by using a tissue chopper (McIlwain, The Mickle Laboratory of Engineering, Co., Ltd.).

Incubation of testes:

Slices from mature or immature testes grouped by size (24 pieces/well) were transferred to sterile 24-deep-well culture dishes (Falcon; Becton Dickinson,

Franklin Lakes, NJ) containing 1ml M199 medium (pH=7) supplemented with 2% FBS (fetal bovine serum). Cultures were carried out per duplicated in the case of testis. Different doses of cortisol (0,50,100,200,400,750 and 800ng/ml) were added immediately and the tissue slices were incubated for 24h, 48h or 72h at 18°C. In order to minimize tissue degradation, tissues were kept on ice during the dissection procedure. The experiments were carried out 4 (males) times and in each experiment, gonads from one fish were used for all treatments, to take into account individual variability. At the end of each incubations period, duplicates or triplicates were pooled and processed for caspase-3 activity.

Caspase-3 activity assay:

After the incubation period, samples were rinsed with PBS (phosphate buffered saline). Testis slices were pooled and homogenized through different gauze needles in 300µl (testis) extraction/substrate caspase Buffer [50mM Pipes-KOH pH=7.4, 220mM mannitol, 68mM sucrose, 50mM KCl, 5mM EGTA, 1mM MgCl, 1mM DTT, 10µg/ml proteinase inhibitor phenylmethylsulfonyl fluoride (PMSF)]. Samples were incubated for 30 min on ice and frozen at -80°C for 3-4h, after which they were thaw and re-frozen overnight. Before use, samples were spun down at 14,000g at 4°C for 15 min and the supernatant was assessed for caspase-3 activity. Total protein content in testicular samples was determined by Bradford reagent (Bio-Rad, CA, USA) at 595nm, using BSA as standard. Samples (20µg total protein for testis) were run per duplicate on 96 well-plates (Falcon). Caspase-3 colorimetric substrate (10µl 2mM) and extraction/substrate caspase buffer were added to a final volume of 100µl/well. Samples were incubated at 37°C for 2h and read immediately in a plate reader (Molecular Devices) at 405nm. A standard curve was prepared by performing serial dilutions of the colorimetric product p-nitroanilide in extraction/substrate caspase buffer and read concomitantly with the samples. Results were expressed as caspase-3 activity (mg of product/mg total protein/h) after correction for protein content and incubation time and referred to the enzyme activity of the control samples (no cortisol), taken as 100.

Data analysis:

Statistical analysis was performed by one-way analysis of variance (ANOVA) and significant differences between treatment groups were determined by the Fisher test. Statistical significance was inferred at $P<0.05$.

Results

We initially tested the time-related effect of two different concentrations of cortisol (100 and 750ng/ml) on immature goldfish testes. Samples were harvested after 24h, 48h and 72h treatment and assessed for caspase-3 activity, a marker for apoptosis. The results presented here show a significant increase in caspase-3 activity in immature testes after 72h treatment with 750ng/ml of cortisol (Fig. 1A, $P<0.05$). We subsequently expanded the concentration response curve for cortisol and tested its effects on both mature (GSI: 2.75-4.65%) and immature (GSI: 1.94-2.4%) testes after 72h treatment. Treatment of immature testes with cortisol significantly increased caspase-3 activity at 400ng/ml (Fig. 2A, $P<0.05$). This activity appeared to be maximal, since no further increases were observed at higher concentrations of cortisol (800ng/ml). In contrast, no significant differences were observed between non-treated and cortisol-treated samples from mature goldfish testes (Fig. 2B.).

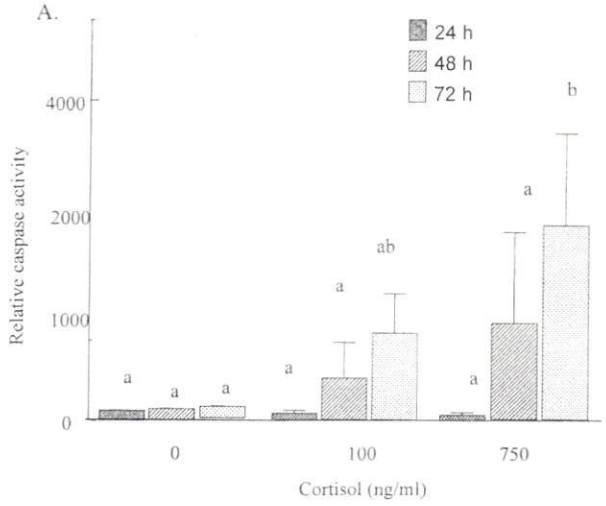

Figure 1: Time and concentration related effects of cortisol on immature goldfish testes.

Slices (20-μm thick) from immature testes (GSI<2.4) were cultured at 18°C for 24h, 48h or 72h in M199 media supplemented with 2%FBS in the presence of different cortisol concentrations , which was used as positive control. Caspase 3 activity was determined as indicated in materials and methods. Results were referred to the control and expressed as the mean +/- S.E. from 4 separate experiments. The results were analyzed by one-way ANOVA and Fisher test. Values displaying different letters are significantly different ($P<0.05$).

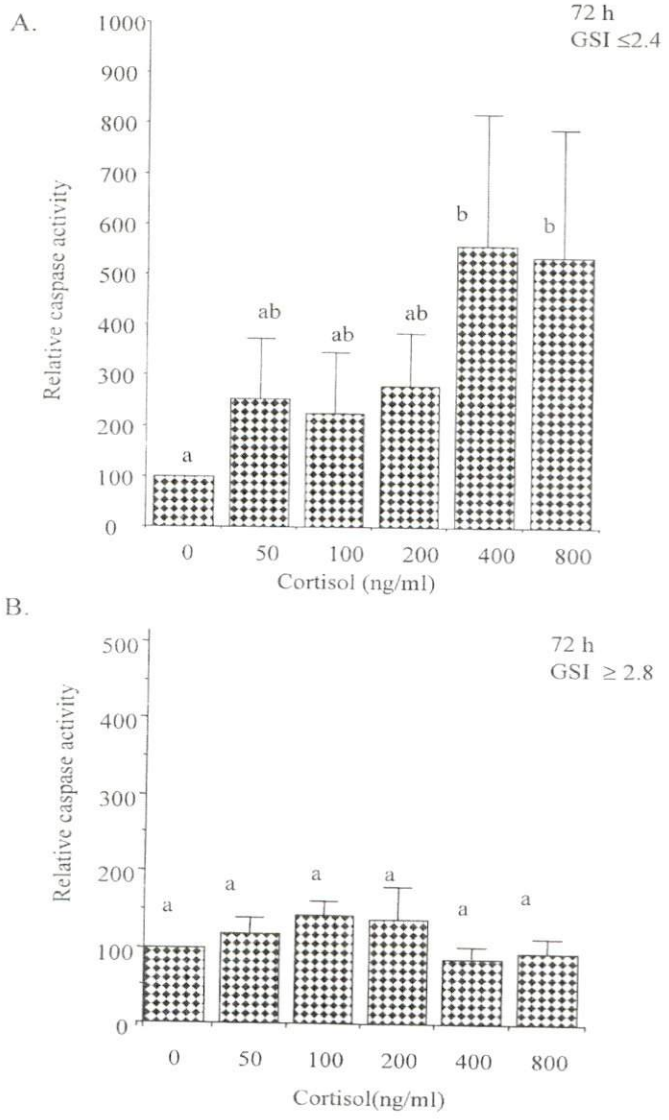

Figure 2: Concentration-related effects of cortisol on immature (A); vs. mature (B) goldfish testes after 72h treatment.

Slices (20-µm thick) from immature (GSI < 2.4) or mature (GSI > 2.4) testes were cultured at 18°C for 72h in M199 media supplemented with 2%FBS in the presence of different cortisol concentrations. Caspase 3 activity was determined as indicated in materials and methods. Results were referred to the control and expressed as the mean +/- S.E. from 4 separate experiments. The results were analyzed by one-way ANOVA and Fisher test. Values displaying different letters are significantly different ($P<0.05$).

Discussion

Prolonged and elevated cortisol levels, which are involved in stress adaptation, have been shown to inhibit the onset of puberty in male fish. In this context, cortisol has been shown to decrease testicular androgen production in male fish via a direct gonadal effect (Consten *et al.*, 2002a,b).

In the testis, an early apoptotic wave appears to be essential for normal mature spermatogenesis, probably because it maintains the appropriate ratio between germinal to Sertoli cells (Rodriguez *et al.*, 1997; Lee *et al.*, 1997). The relevance of apoptosis in the mammalian male gonad is highlighted by the fact that dysregulation of apoptosis leads to infertility (Pentikainen *et al.*, 2002). The control of apoptosis in the mammalian testis appears to be exerted by both gonadotropin hormones and locally produced factors such as gonadal steroids (Tapanainen *et al.*, 1993; Billig *et al.*, 1995, 1996; Erkkila *et al.*, 1997; Pentikainen *et al.*, 2000).

The occurrence of spontaneous or induced testicular apoptosis has been also described in both cartilaginous and teleost fish (Kwak *et al.*, 2001; Kuwahara *et al.*, 2002; Prisco *et al.*, 2003). However, to our knowledge, there is no information about the role of stress hormones, such as cortisol, in the induction of apoptosis in fish gonadal tissues, although apoptosis has been shown to occur in response to cortisol in fish lymphocytes (Weyts *et al.*, 1997, 1998). In the present study, we demonstrated that *in vitro* treatment with cortisol induced an increase in the levels of apoptotic marker, namely caspase-3 activity, in the immature goldfish testis. The results presented here are in agreement with previous studies performed in rats, in which *in vivo* treatment with stress hormones was shown to induce spermatogonia apoptosis (Yazawa *et al.*, 2000; Sasagawa *et al.*, 2001). Indeed, spermatogonias are the main germ cell type present in immature testis (Billard, 1986). At present, it is not known whether the stage-specific effect of cortisol is mediated by a decrease in steroid hormone production, or the inhibition of growth factors required for the proliferation of spermatogonias. In this context, estradiol has been shown to induce spermatogonia cell proliferation in *Rana sculenta* and to increase the levels of anti-apoptotic Bcl-2 like proteins in goldfish mature testis (d'Istria *et al.*, 2003). Thus, a decrease in Bcl-2 proteins may offset the balance of anti- to pro-apoptotic proteins

(Antonsson & Martinou, 2000; Borner, 2003) in the testis and induce apoptosis. Alternatively, it is possible that cortisol may directly decrease the levels of anti-apoptotic Bcl-2 related proteins, an effect that has been shown to be cell type specific in mammals (Amsterdam *et al.*, 2002; Sasagawa *et al.*, 2001). The findings presented here provide evidence that the induction of apoptosis maybe controlled by different factors during gametogenesis.

These results are in agreement with *in vivo* studies, which have shown that treatment with glucocorticoids decreased testosterone production only in peri-ovulatory trout females (Pankhurst & Van Der Kraak, 2000). The sensitivity of peri-ovulatory follicles to cortisol increased with the time in culture and it was shown to be higher for the ovary than the testis (Bahmani, 2002). The results presented here argue against the hypothesis that during folliculogenesis, increased levels of 11-beta-hydroxysteroid dehydrogenase, which is the enzyme responsible for the conversion of cortisol to the inert metabolite cortisone, may protect the teleost ovary against the detrimental effects of glucocorticoids (Kusakabe *et al.*, 2003).

In summary, the present studies provide support for the hypothesis that high levels of stress hormones may detrimentally affect gametogenesis by inducing apoptosis. The results show that the induction of apoptosis by cortisol in goldfish testicular tissues is stage-dependent. Furthermore, the stages affected by this hormone differ in females and males, suggesting a possible sexual dimorphism in the control of the hypothalamic-pituitary-gonadal axis by stress hormones in goldfish. Overall, the results presented here provide support for the hypothesis that stress may cause impaired fertility in fish due to the production of stress hormones such as cortisol, which in turn may lead to the onset of gonadal cell apoptosis at critical stages of gametogenesis.

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References

Amsterdam, A. ; Tajima, K. and Sasson, R. , 2002. Cell-specific regulation of apoptosis by glucocorticoids: implication to their anti-inflammatory action. Biochem. Pharmacol. **64:**843-850.

Andreu-Vieyra, C.V. and Habibi, H.R. , 2000. Factors controlling ovarian apoptosis. Can. J. Physiol. Pharmacol. **78:**1003-1012.

Andreu-Vieyra, C.V. and Habibi, H.R. , 2001a. Effects of salmon GnRH and chicken GnRH-II on testicular apoptosis in goldfish *(Carassius auratus)*. Comp. Biochem. Physiol. Part B 129, pp.483-487.

Andreu-Vieyra, C.V. and Habibi, H.R., 2001b. Effect of testosterone on GnRH-induced apoptosis in the Goldfish testis. *In*: Perspective in Comparative Endocrinology: Unity and Diversity. International Proceedings Division, Bologna, Italy, pp.943-950.

Antonsson, B. and Martinou, J.C. , 2000. The Bcl-2 protein family. Exp. Cell Res. **256:**50-57.

Bahmani, M. , 2002. The effects of stress hormones on testis and ovarian follicular apoptosis in fishes. Final report of Post-Doctoral Research Fellow project in Comparative Reproductive Endocrinology. Biological Sciences Department. University of Calgary. Canada. 475P.

Billard, R. , 1986. Spermatogenesis and spermatology of some teleost fish species. Repro. Nutr. Develop. **26:**877-890.

Billig, H. ; Furuta, I. ; Rivier, C. ; Tapanainen, J. and Parvinen, M. , 1995. Apoptosis in testis germ cells: developmental to selective tubule stages. Endocrin-ology. **136:**5-12.

Billig, H. ; Chun, S.Y. ; Eisenhauer, K. and Hsueh, A.J. , 1996. Gonadal cell apoptosis: hormone-regulated cell demise. Hum Reprod. Update, **2:**103-117.

Borner, C. , 2003. The Bcl-2 protein family: sensors and checkpoints for life-or-death decisions. Mol. Immunol. **39:**615-647.

Consten, D. ; Keuning, E.D. ; Bogerd, J. ; Zandbergen, M.A. ; Lambert, J.G. ; Komen, J. and Goos, H.J., 2002a. Sex steroids and their involvement in the cortisol-induced inhibition of pubertal development in male common carp *(Cyprinus carpio* L.). Biology of Reproduction. **67:**465-472.

Consten, D. ; Lambert, J.G. ; Komen, H. and Goos, H.J. , 2002b. Corticosteroids affect the testicular androgen production in male common carp (*Cyprinus carpio* L.). Biology of Reproduction. **66:**106-111.

d'Istria, M. ; Palmiero, C. ; Serino, I. ; Izzo, G. and Minucci, S. , 2003. Inhibition of the basal and oestradiol-stimulated mitotic activity of primary spermatogonia by melatonin in the testis of the frog, *Rana esculenta, in vivo* and *in vitro*. Reproduction. **126:83**-90.

Earnshaw, W.C. ; Martins, L.M. and Kaufmann, S.H. , 1999. Mammalian Caspases: structure, activation, substrates, and function during apoptosis. Annu. Rev. Biochem. **68:**383-424.

Erkkila, K. ; Henriksen, K. ; Hirvonen, V. ; Rannikko, S. and Salo, J. , 1997. Testosterone regulates apoptosis in adult human seminiferous tubules *in vitro*. J. Clin. Endocrinol. Metab. **82:**2314-2321.

Fenske, M. , 1997. Role of cortisol in the ACTH-induced suppression of testicular steroidogenesis in guinea pigs. J. Endocrinol. **154:**407-414.

Giannattasio, A. ; Angeletti, G. ; De Rosa, M. ; Zarrilli, S. and Ambrosino, M., 2002. RNA expression bcl-w, a new related protein Bcl-2 family, and caspase-3 in isolated sertoli cells from pre-pubertal rat testes. J. Endocrinol. Invest. 25, RC23-25.

Guo, Y.X. ; Wei, T. ; Dallmann, K. and Kwang, J. , 2003. Induction of caspase-dependent apoptosis by betanodaviruses GGNNV and demonstration of protein alpha as an apoptosis inducer. Virology. **308:**74-82.

Hirata, H. ; Takahashi, A. ; Kobayashi, S. ; Yonehara, S. ; Sawai, H. ; Okazaki, T. ; Yamamoto, K. and Sasada, M., 1998. Caspases are activated in a branched protease cascade and control distinct downstream processes in Fas-induced apoptosis. J. Exp. Med. **187:**587-600.

Hsu, S.Y. and Hsueh, J.W. , 2000. Tissue-specific Bcl-2 protein partners in apoptosis: An ovarian paradigm. Physiol. Rev. **80:**593-614.

Johnson, A.L. and Bridgham, J.T. , 2000. Caspase-3 and -6 expression and enzyme activity in hen granulosa cells. Biology Reproduction. **62:**589-598.

Kaufmann, S.H. and Hengartner, M.O. , 2001. Programmed cell death: Alive and well in the new millennium. Trends Cell Biol. **11:**526-534.

Kerr, J.F.R. ; Wyllie, A.H. and Currie, A.R. , 1972. Apoptosis: A basic biological phenomenon with wide-ranging implication in tissue kinetics. Br. J. Cancer. **26:**239-257.

Kim, J.M. ; Luo, L. and Zirkin, B.R. , 2000. Caspase-3 activation is required for Leydig cell apoptosis induced by ethane dimethanesulfonate. Endocrinology. **141:**1846-1853.

Kroemer, G. ; Dallaporta, B. and Resche-Rigon, M. , 1998. The mitochondrial death/life regulation in apoptosis and necrosis. Annu. Rev. Physiol. **60:**619-642.

Kusakabe, M. ; Nakamura, I. and Young, G. , 2003. 11beta-hydroxysteroid dehydrogenase complementary deoxyribonucleic acid in rainbow trout: cloning, sites of expression, and seasonal changes in gonads. Endocrinology. **144:**2534-2345.

Kuwahara, Y. ; Shimada, A. ; Mitani, H. and Shima, A. , 2002. A critical stage in spermatogenesis for radiation-induced cell death in the medaka fish, *Oryzias latipes*. Radiat. Res. **157:**386-392.

Kwak, H.I. ; Bae, M.O. ; Lee, M.H. ; Lee, Y.S. and Lee, B.J. , 2001. Effects of nonylphenol, bisphenol A, and their mixture on the viviparous swordtail fish (*Xiphophorus helleri*). Environ. Toxicol. Chem. **20:**787-795.

Laing, K.J. ; Holland, J. ; Bonilla, S. ; Cunningham, C. and Secombes, C.J. , 2001. Cloning and sequencing of caspase 6 in rainbow trout, *Oncorhynchus mykiss*, and analysis of its expression under conditions known to induce apoptosis. Dev. Comp. Immunol. **25:**303-312.

Lamkanfi, M. ; Declercq, W. ; Kalai, M. ; Saelens, X. and Vandenabeele, P. , 2002. Alice in caspase land. A phylogenetic analysis of caspases from worm to man. Cell Death Differ. **9:**358-361.

Lee, J. ; Richburg, J.H. ; Younkin, S. and Boekelheide, K. , 1997. The fas system is a key regulator of germ cell apoptosis in the testis. Endocrinology. **138:**2081-2088.

Masumoto, J. ; Zhou, W. ; Chen, F.F. ; Su, F. and Kuwada, J.Y. , 2003. Caspy, a zebrafish caspase, activated by ASC oligomerization is required for pharyngeal arch development. J. Biol. Chem. **278:**4268-4276.

Nakajima, K. ; Takahashi, A. and Yaoita, Y. , 2000. Structure, expression, and function of the *Xenopus laevis* caspase family. J. Biol. Chem. **275:**10484-10491.

Omezzine, A. ; Chater, S. ; Mauduit, C. ; Florin, A. and Tabone, E. , 2003. Long-term apoptotic cell death process with increased expression and activation of caspase-3 and -6 in adult rat germ cells exposed in utero to flutamide. Endocrinology. **144:**648-661.

Pankhurst, N.W. ; Van Der Kraak, G. , 2000. Evidence that acute stress inhibits ovarian steroidogenesis in rainbow trout *in vivo*, through the action of cortisol. Gen.Comp. Endocrinol. **117:**225-237.

Pentikainen, V. ; Erkkila, K. ; Suomalainen, L. ; Parvinen, M. and Dunkel, L., 2000. Estradiol acts as a germ cell survival factor in the human testis *in vitro*. J. Clin. Endocrinol. Metab. **85:**2057-2067.

Pentikainen, V. ; Sumoalainen, L. ; Erkkila, K. ; Martelin, E. ; Parvinen, M.; Pentikainen, M.O. and Dunkel, L. , 2002. Nuclear factor-kβ activation in human testicular apoptosis. Am. J. Pathol. **160:**205-218.

Prisco, M. ; Liguoro, A. ; Comitato, R. ; Cardone, A. and D'Onghia, B. , 2003. Apoptosis during spermatogenesis in the spotted ray *Torpedo marmorata*. Mol. Reprod. Dev. **64:**341-348.

Rodrigues, I. ; Ody, C. ; Araki, K. ; Garcia, I. and Vassalli, P. , 1997. An early and massive wave of germinal cell apoptosis is required for the development of functional spermatogenesis. The EMBO J. **6:**2262-2270.

Rotonda, J. ; Nicholson, D.W. ; Fazil, K.M. ; Gallant, M. ; Gareau, Y. ; Labelle, M. ; Peterson, E.P. and Rasper, D.M. , 1996. The three-dimensional structure of apopain/CPP32, a key mediator of apoptosis. Nat. Struct. Biol. **3:**619-625.

Sasagawa, I. ; Yazawa, H. ; Suzuki, Y. and Nakada, T. , 2001. Stress and testicular germ cell apoptosis. Arch Androl. **47:**211-216.

Shi, Y. , 2002. Mechanisms of caspase activation and inhibition during apoptosis. Mol. Cell. **9:**459-470.

Strasser, A. ; O'Connor, L. and Dixit, V.M. , 2000. Apoptosis signaling. Annu. Rev. Biochem. **69:**217-245.

Takeo, J. ; Hata, J. ; Segawa, C. ; Toyohara, H. and Yamashita, S. , 1996. Fish glucocorticoid receptor with splicing variants in the DNA binding domain. FEBS Lett. **389:**244-248.

Tapanainen, J.S. ; Tilly, J.L. ; Vihko, K.K. and Hsueh, A.J. , 1993. Hormonal control of apoptotic cell death in the testis: gonadotropins and androgens as testicular cell survival factors. Mol. Endocrinol. **7:**643-650.

Weyts, F.A. ; Verburg-van Kemenade, B.M. ; Flik, G. ; Lambert, J.G. and Wendelaar Bonga, S.E. , 1997. Conservation of apoptosis as an immune regulatory mechanism: effects of cortisol and cortisone on carp lymphocytes. Brain. Behav. Immun. **11:**95-105.

Weyts, F.A. ; Flik, G. ; Rombout, J.H. and Verburg-van Kemenade, B.M. , 1998. Cortisol induces apoptosis in activated B cells, not in other lymphoid cells of the common carp, *Cyprinus carpio* L., Dev. Comp. Immunol. **22:**551-562.

Wolf, B.B. ; Schuler, M. ; Echeverri, F. and Green D.R. , 1999. Caspase-3 is the primary activator of apoptosis DNA fragmentation via DNA fragmentation factor-45/inhibitor of caspase-activated Dnase. J. Biol. Chem. **274:**30651-30656.

Wyllie, A.H. , 1980. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature. **284:**555-556,

Wyllie, A.H. ; Kerr, J.F.R. and Currie, A.R. , 1980. Cell death: The significance of apoptosis. Int. Rev. Cytology. **68:**251-306.

Yabu, T. ; Kishi, S. ; Okazaki, T. and Yamashita, M. , 2001. Characterization of zebrafish caspase-3 and induction of apoptosis through ceramide generation in fish fathead minnow tailbud cells and zebrafish embryo. Biochem. J. **360:**39-47.

Yazawa, H. ; Sasagawa, I. and Nakada, T. , 2000. Apoptosis of testicular germ cells induced by exogenous glucocorticoid in rats. Hum. Reprod. **15:**1917-1920.