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Short communication

Humic acid reduces gonadotropin activity and hormonal sensitivity of frog oocytes

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Abstract

The specific stimulatory effect of sturgeon *Acipenser güldenstädti* Br. gonadotropic hormone (GTH) on frog *Rana temporaria* L. oocyte maturation in vitro was investigated in relation to humic acid (HA) concentrations from 12.5 to 50 mg/l. HA was observed to bind to both the follicular membrane of the oocytes and the GTH molecule, reducing the oocytes' hormone sensitivity and maturation ability. It was also shown that HA inactivated GTH, lowering its specific ability to stimulate oocyte maturation.

Keywords: Humic acid; Frog oocytes; Gonadotropic hormone; Hormonal effects

Humic substances (HS) are an intermediate, but persistent decay product of living organic matter and as natural compounds they are abundant in soils, peat, sediments, some fossil fuels and waters all around the world. HS can be characterized by variables such as mean molecular mass, shape and conformation of molecules, degree of polidispersity and distribution of molecular masses varying in a broad range from 400 to 1 000 000 Da (Hoque et al., 2003; Seitzinger et al., 2005). Structural organisation can occur both at molecular and molecular aggregate levels (Schulten and Schnitzer, 1993; Klavins, 1997). The chemical network structure of HS contains hydrocarbons, aromatic and heterocyclic structures, carboxyl groups, phenolic and alcoholic hydroxyls and possesses many active hydrogen bonding sites making them very chemically reactive.

HS can control variety of processes in freshwater ecosystems (Klavins, 1997). They can impact bioavailability or release of trace elements and may have influence on enzyme activities in fishes, invertebrates and macrophytes (Pflugmacher et al., 2001; Steinberg et al., 2004). Also, some hormone-like effects of HS have been shown in nematode *Caenorhabditis elegans* (Steinberg et al., 2002).

The aim of the study was to determine whether humic acid can modify the freshwater amphibian reproductive process. This was investigated employing the interaction of the frog hormone-sensitive oocytes with

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the sturgeon *Acipenser güldenstädti* Br gonadotropic hormone in a very specific and a highly sensitive in vitro biotest system "oocyte-hormone". Commercially available humic acid (HA) was purchased from Aldrich and additionally purified according to the recommendations by the International Humic Substances Society (Hayes and Clapp, 2001).

Chromatographically pure standard sturgeon gonadotropic hormone (GTH, $M_r = 25$ kDa) was isolated from acetone-dried pituitary glands according to a standard procedure (Zenkevics, 1992). The grass frog *Rana temporaria* L. oocyte in vitro maturation test (Thornton, 1971) was employed to assess the gonadotropic impact of GTH on the oocytes. The frogs were purchased in September and were kept in the dark cold room at 5 °C. In total, 10 frogs were used in the study. The oocyte sensitivity to the hormone was expressed as the minimal dose of the hormone that produced 50% test-oocyte maturation (D_{50}).

Two sets of experiments were carried out. In the first three separate frog oocyte portions (30 oocytes) were preincubated for 48 h with HA concentrations of 12.5, 25 and 50 mg l^{-1} at 5 °C in Petri dishes in Ringer solution (pH 7.5). After the exposure to HA, the oocytes were washed five times with fresh Ringer solution to wash off the residual HA and then tested in three parallel replicates with GTH concentrations of 0.1, 0.3, 0.5 and 1.0 μg l^{-1} to estimate the hormone sensitivity of the HA-pretreated oocytes. In the second set of experiments, three replicates of intact fresh oocytes were tested for 48 h using the same standard GTH concentrations in the same test system which contained the above HA concentrations in the test medium throughout the experiment. The effect of HA on oocyte GTH sensitivity in both cases was evaluated by the hormone concentration producing 50% oocyte maturation (D_{50}). The mean values of responses are reported.

The preincubation of the test oocytes with the HA concentrations of 12.5, 25 and 50 mg l^{-1} lowered the oocyte response to GTH depending on used HA concentrations at all hormone concentrations used (Fig. 1). The inhibitory effect produced by HA in the test oocytes was almost proportional to the used HA concentrations. The oocytes exposed to12.5 and 25 mg l^{-1} HA concentrations exhibited almost similar decrease in the hormone sensitivity—D_{50} value increased by about 1.7 times in comparison with that of the standard. The sensitivity level of the oocytes exposed to

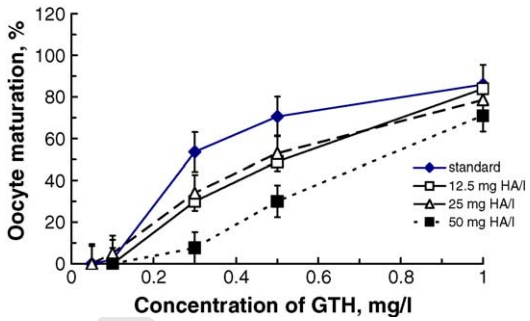

Fig. 1. Response of frog oocytes (preincubated for 48 h with humic acid in concentrations of 12.5, 25 and 50 mg l^{-1}), to standard GTH stimulatory concentrations up to 1.0 mg l^{-1}.

50 mg HA l^{-1} decreased more in comparison with that of the intact oocytes. The relative gonadotropic activity of GTH decreased almost three-fold, as indicated by the increase of D_{50} values (Fig. 1).

Using the intact oocytes in the Ringer test solution containing the same HA concentrations (without preincubation but continuous presence of HA) also considerably lowered the oocyte sensitivity to the hormone (Fig. 2). The minimal effective concentration of GTH producing the oocyte 50% maturation turned out to be slightly higher than that in the first set of exposures in which the preincubated oocytes were tested in HA-free medium. In the presence of 12.5 mg HA l^{-1} the relative GTH activity dropped approximately two-fold, at HA concentration of 25 mg l^{-1} it decreased almost three-fold, and at HA concentration of 50 mg/l about four-fold in comparison with that of the standard (Fig. 2).

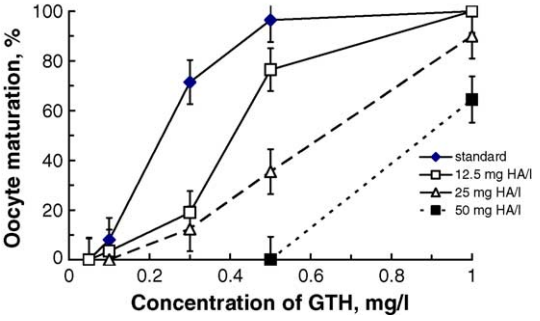

Fig. 2. Effect of humic acid (concentration 12.5 and 50 mg l^{-1}) on fresh intact frog oocyte maturation at stimulatory concentrations of GTH up to 1.0 mg l^{-1} in Ringer solution.

Thus, D_{50} of GTH increased by 20–30% in the tests using intact oocytes in Ringer solution in the continuous presence of HA in comparison with HA-preincubated oocytes, which consequently were hormone-treated in HA-free medium.

Humic substances are traditionally considered to be inert or at least refractory and too large to be taken up by aquatic organisms. In the same time many recent studies have demonstrated that dissolved humic substances are taken up and directly and/or indirectly interfere with freshwater organisms (Steinberg et al., 2004; Timofeyev et al., 2004). Recent investigations of humic substances have shown quite clearly that these natural compounds may impact number of physiological effects in plants and animals, including humans (Hseu et al., 2002; Timofeyev et al., 2004; Meems et al., 2004). Among them are induction of stress proteins, modulation of biotransformation enzymes, production of internal oxidative stress, chemosensititation of the multixenobiotic resistance mechanism, feminisation of fish and amphibians, interference within the thyroid system and action as chemical attractant.

The present results show that humic acid significantly decreased the sensitivity of frog oocytes to sturgeon GTH, which is a highly specific and effective stimulator of the oocyte in vitro maturation. Our experiments showed that the relative decline of GTH activity (D_{50}) occurred almost directly proportional to the concentrations of HA to which the test oocytes had been exposed for 48 h. The washing off HA from the test oocytes with Ringer solution after preincubation did not regenerate their standard ability to respond to the stimulatory impact of the hormone. The greatly lowered stimulatory impact of GTH on the test oocytes indicates quite clearly that most likely the hormone receptor sites in the follicular membrane are partly blocked by HA, preventing the transduction of the specific hormonal signal through the membrane to induce the oocyte maturation process. Similar interaction pattern between humic substances and biological membranes has recently been described (Steinberg et al., 2003) and it can significantly change the permeability of follicular membrane to GTH.

In order to determine, whether HA may have any direct effect on GTH glycoprotein molecule function, the experiments were performed both with intact oocytes and standard GTH in HA-free and HA-containing test medium. The results indicate that in the presence of HA in the test medium, the sensitivity to GTH decreased further by 20–30% in comparison to experiments with oocytes preincubated with the same HA concentrations, but hormone-treated in HQA-free medium. Based on this finding, it is apparent that HA may affect not only the oocyte follicular membrane as demonstrated previously (Campbell et al., 1997), but also the hormone molecule thus producing deeper negative influence on the oocyte-hormone interaction. As a conclusion, HA may have an impact on the endocrine regulation of reproduction in frogs, suggesting that aquatic frog communities, and their reproduction, may be influenced by the concentration of HSs in the water.

References

Hayes, M.H.B., Clapp, C.E., 2001. Humic substances: considerations of compositions, aspects of structure and environmental influences. Soil Sci. 166, 723–737.

Hoque, E., Wolf, M., Teichmann, G., Peller, E., Schimmack, W., Buckau, G., 2003. Influence of ionic strength and organic modifier concentrations on characterization of aquatic fulvic and humic acids by high-performance size-exclusion chromatography. J. Chromatogr. A 1017, 97–105.

Hseu, Y.C., Huang, H.W., Wang, S.Y., Chen, H.Y., Lu, F.J., Gau, R.J., Yang, H.L., 2002. Humic acid induces apoptosis in human endothelial cells. Toxicol. Appl. Pharmacol. 182, 34–43.

Klavins, M., 1997. Aquatic Humic Substances: Characterisation, Structure and Genesis. University of Latvia, Riga.

Meems, N., Steinberg, C.E.W., Wiegand, C., 2004. Direct and interacting toxicological effects on the waterflea (*Dapnia magna*) by natural organic matter, synthetic humic substances and cypermethrin. Sci. Total Environ. 391, 123–136.

Pflugmacher, S., Tidwell, L.F., Steinberg, C.E.W., 2001. Dissolved humic substances can directly affect freshwater organisms. Acta Hydrochim. Hydrobiol. 29, 34–40.

Schulten, H.R., Schnitzer, M., 1993. A state of the art structural concept for humic substances. Naturwissenschaften 80, 29–30.

Seitzinger, S.P., Hartnett, H., Lauck, R., Mazurek, M., Minegishi, T., Spyres, G., Styles, R., 2005. Molecular-level chemical characterization and bioavailability of dissolved organic matter in stream water using electrospray-ionization mass spectrometry. Limnol. Oceanogr. 50, 1–12.

Steinberg, C.E.W., Hoss, S., Brűggemann, R., 2002. Further evidence that humic substances have the potential to modulate the reproduction of the nematode, *Caenorhabditis elegans*. Int. Rev. Hydrobiol. 87, 121–133.

Steinberg, C.E.W., Paul, A., Pflugmacher, S., Meinelt, T., Klöcking, R., Wiegand, C., 2003. Pure humic substances have the potential to act as xenobiotic chemicals—a review. Fresenius Environ. Bull. 12, 391–401.

Steinberg, C.E.W., Höss, S., Kloas, W., Lutz, I., Meinelt, T., Pflugmacher, S., Wiegand, C., 2004. Hormone-like effects of humic substances on fish, amphibs, and invertebrates. Environ. Toxicol. 19, 409–411.

Thornton, V.F., 1971. A bioassay for progesterone and gonadotropins based on meiotic division of *Xenopus* oocytes in vitro. Gen. Comp. Endocrinol. 16, 599–605.

Timofeyev, M.A., Wiegand, C., Burnison, B.K., Shatilina, Z.M., Pflugmacher, S., Steinberg, C.E.W., 2004. Impact of natural organic matter (NOM) on freshwater amphipods. Sci. Total Environ. 319, 115–121.

Zenkevics, H., 1992. Fish Gonadotrophic Hormone: Isolation and Properties. Zinatne, Riga (in Russian).