E-screen assay validation: evaluation of estrogenic activity by MCF7 cell culture bioassay, in drinking water from different watersheds in state of São Paulo, Brazil

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Abstract— *Natural and synthetic estrogens have been detected in rivers, lakes and estuaries in many parts of the world. Primary sources of these compounds are domestic and industrial effluents, which are not deleted after the water treatment. Estrogen has been the endocrine disruptor most researched to be very active biologically and be the etiologic agent of diverse types of cancer and other conditions such as endometriosis, precocious puberty, feminization, masculinization, sterility. In this context, we use water of 36 natural reservoirs or dams, in a bioassay to characterize their estrogenicity in culture of MCF7 cells and obtained high concentration of estrogen in samples taken in Ibiúna and Equestrian Santo Amaro / SP. However, certain concentration in our samples for most water samples from different regions was very close to the limit of quantification by bioassay and estrogen was in fmol. It has been shown that e - screen assay with MCF7 cells is a sensitive and stable tool for quantitative analysis of estrogenicity of water and can easily be developed and implemented for routine for estrogen quantification also in animal food and man, aqueous and plastics etc.*

Keywords— endocrine disrupters, estrogen, breast cancer cells, (MCF7) bioassay: E-screen assay

I. INTRODUCTION

The speed of production and use of synthetic chemical products, since 1940, resulting in contamination ubiquitous of aquatic animals, land and the human population. Since 1960 observers noted an increase of changes in reproduction of animals across the globe as well as changes in reproduction and health of men by exposure to these synthetic products (VAN LABEKE et al, 2008)

In 1991 scientists from various fields met at Wingspread Conference Center for defining and structuring the Disruption Endocrine phenomenon. These researchers, in consensus, established that many synthetic chemicals were potentially able to disrupt the endocrine system of animals and man. At that time concluded that the characterization of exposure to Endocrine Disruptors was a crucial aspect for the prognosis of health effects as given in the below link.
(http://www.ourstolenfuture.org/Consensus/wingspread1.htm).

In the mid-90s there was a clear insight the possible negative impact of endocrine disruptors and the need for new legislation to really ensure protection to human and animal's health and the environment. But only two decades later the Society of Endocrinology renewed the request of the endocrine disruptors list and the effects of its exposure, considering as a priority research (DIAMANTI-KANDARAKIS et al, 2009).

The welfare of society is linked to many chemicals, essential components to everyday life, which are found everywhere, even in remote locations both in the environment and animals and men.

For millennia, human and animals' bodies adapted to EDs vegetables, cereals and fruit (apple, cherry, plum, potato. Carrots, peas, beans, soybeans, wheat, oats, barley, rye, parsley, garlic) and naturally excreted not accumulate in the body. The chemicals, however, accumulate mainly in adipose tissue mimicking glandular hormones (Colborn et al, 2002). Some of these substances are transplacental and may affect the fetus as lead (Bowler and Cone, 2010) or fixate the milk being

ingested by the newborn (Matuo, 1999) even toxic agents already accumulated in the maternal organism over the years. (Colborn et al, 2002).

Recent studies in several countries have shown that the aquatic environment can possess estrogenic activity capable of influencing the fauna. (Xeno) estrogens are believed to reach the aquatic environment mainly by means of municipal and industrial sewage outfalls. However, agricultural drainage may also be a route for (xeno) estrogens to enter the aquatic system.

Numerous natural and anthropogenic substances are known to exhibit estrogenic activity. In the aquatic environment, estrogenic activity has primarily been ascribed to the natural steroids, 17b-estradiol (E2), estrone (E1) and estriol (E3), and the synthetic estrogen, ethinylestradiol (EE2), used in contraceptives and hormone replacement. To a lesser extent xenoestrogenic chemicals, such as alkylphenols and bisphenol A, may also contribute to the estrogenic activity in the aquatic environment. (GRAY et al, 2000, SHAW and MCCULLY, 2002; AERNI et al ,2004)

Estrogen has been the endocrine disruptor most researched to be very active biologically and be the etiologic agent of diverse types of cancer and other conditions such as endometriosis, precocious puberty, feminization, masculinization, sterility (WELSHONS et al, 1999). Endocrine disruptors have recently been shown to promote an epigenetic transgenerational phenotype involving several disease states (e.g. male infertility) (GORE et al, 2013). The MCF-7 cell proliferation assay is potentially a simple and highly reproducible tool for the identification of estrogenic compounds. In the E-screen assay developed by SOTO et al. (1995), proliferation of MCF-7 cells as a response to estrogen is measured. The E-screen is based on the following three premises: (i) factors in human serum inhibit the proliferation of MCF-7 cells, (ii) estrogens induce cell proliferation by negating this inhibitory effect, and (iii) non-estrogenic steroids and growth factors do not neutralize the inhibitory signal present in human serum.

Numerous studies on animal's exposure of wildlife and laboratories such products have shown that endocrine disrupters affect various physiological processes such as brain activity, reproduction, immune response, development and metabolic rates. (Tyler, 1998; McLachlan, 2001; Guillette and Gunderson, 2001; Hayes et al., 2002; Markey et al., 2003).

1.1 Endocrine disruptors

Endocrine disruptors are chemicals or agents that promote changes in the human or animal endocrine system. Several of these substances remain in the environment accumulating in soil, river sediment being transported at great distance. They can accumulate in the food chain representing a health risk especially those who are at the top of the food chain. (Meyer, 1999). For example, in the region of the Great Lakes between the United States and Canada, on Lake Ontario, it was observed biomagnetization of polychlorinated biphenyls (PCBs) from phytoplankton and zooplankton to trout and sea-gulls. The concentration of PCBs in the sediment was established as the initial value and from that concentration analyzed the concentration in the other beings of this ecosystem. There was a considerable increase in the concentration of PCB: phytoplankton 250X; zooplankton 500X; Trout 2.800.000X and 25.000.000X gulls (Colborn et al, 2002).

The disruptor may be organic or inorganic substance and appear as a byproduct or waste of industrial use. They are found in landfills and thus contaminating soil, groundwater, water sources used for public supply. Incinerators products (medical waste and industries) also contribute to this contamination (XELEGATI and Robazzi, 2003)

TEVES in 2001 noted the presence of mercury and lead in waste of São Paulo and SISINO and OLIVEIRA in 2000 confirmed the presence of cadmium, lead, manganese and mercury in chorumem landfills and dumps.

Several species of animals have been affected by endocrine disruptors. For example, we have thyroid dysfunction in birds and fish, decreased fertility in birds, fish, crustaceans, mammals; Successful reduction in hatching in birds, fish, turtles; metabolic abnormalities in birds, fish and mammals, behavioral abnormalities in birds, desmasculinization and desfeminilization of fish and female birds and dangerous changes in the immune system.

It is believed, therefore, that the effects of endocrine disruptors on the endocrine and reproductive systems act by mimicking the endogenous hormone antagonizing the normal effects of endogenous hormones; stimulation or inhibition of the synthesis and metabolism of hormones natural or modifying the levels of hormone receptors. These compounds are widely used by modern society, being found in pharmaceuticals, personal products (like eg. The fragrances pesticides, antioxidants, plastics,

industrial products, surfactants and others). Some of endocrine disruptors can enter the human body by the dermal route. They are: Benzo (a) anthracene, benzo (a) pyrene, Benzene, Lead, Chlordane; dieldrin; DDT; Carbon disulfide, Heptachlor; HCH, Mercury, pentachlorophenol (Azevedo & CHASIN, 2003).

Heavy metals act by inhibiting certain enzymes, for example, glycolysis, lipolysis and protein synthesis. Cadmium binds to the sulfhydryl group (-SH) of the enzyme inhibiting its action. Lead inhibits the action of ζaminolivólico acid dehydrase enzyme required for heme synthesis leading anemia. Arsenic form complex with enzyme inhibitors of adenosine triphosphate (ATP). Mercury has affinity with the sulfhydryl group of proteins, enzymes, serum albumin, hemoglobin (Patnaik 2002; Ferreira, 2003).

The particular hormone action starts by binding to a specific receptor of a cell. The resulting complex binds specific regions of DNA in the nucleus activating or deactivating particular gene. KOIFMAN et al in 2002 showed that in some Brazilian states is correlation between the use of pesticides and endocrine changes in the exposed population as infertility, testicular, breast, prostate and ovary cancer.

The breast cancer is the most frequent cancer of women. The risk to develop it can be genetic, but according FENTON, 2006, 70% of women diagnosed had no hereditary or sporadic cancer. The increased risk of breast cancer and early puberty owes much to the lifestyle and environment, exposure to certain chemicals that mimic hormones such as endocrine disruptors. These products can increase the incidence of cancer by altering the flow or level-dependent tissue hormones; altering the expression of glandular receptors, transporters of hormones or growth factors. These hormones have the best conformation recognized by receptors and therefore result in maximum responses and are considered as responsible for most disruptors effects caused by the disposal of effluents (Gray et al, 2000; SHAW and McCully, 2002; AERNI et al, 2004).

Understanding the complexity of exposure to synthetic chemicals is difficult by the large number of compounds and limitations of analytical techniques. Researchers thus have focused on a few products. Elucidating the whole universe including chemical unknown compounds is yet to happen.

Bioanalytical techniques (bioassays) may help to clarify this fact by characterizing the actual biological effects in a complex sample and thus incorporate the effects of unidentified components and mixtures.

The ecotoxicology has done in vitro bioassays to assess the endocrine activity in environmental samples.

In this context, we can use water of our natural or built reservoirs in a bioassay to try to characterize their estrogenicity in MCF7 cell culture (human cancer cell line). Cells of Mammary gland cancer have been used as a model of the effects of estrogen on the growth of breast cancer and the synthesis of specific proteins.

The E-SCREEN assay was developed to evaluate the estrogenicity of chemical agents present in the environment using the proliferative effect of estrogens on their target cells as an endpoint. This quantitative assay compares the number of cells obtained by similar inoculum MCF-7 cells in the absence of estrogen (negative control) and presence of 17 beta-Estradiol (positive control) in a range of suspected chemical concentrations have estrogenic function

the introduction of the paper should explain the nature of the problem, previous work, purpose, and the contribution of the paper. The contents of each section may be provided to understand easily about the paper.

II. MATERIAL AND METHOD

Cells were seeded in plates with 96 wells, 1500 cell / well in culture medium (Dulbeccos Modified Eagle Medium / Sigma) and incubated for 24 hours.

Following incubation, the wells were washed with 150 uL of phosphate buffer (PBS Gibco) and the medium changed to 150 ul of DMEM without phenol red (Sigma) and 5% FCS (charcoal dextran stripped / Sigma), 100 U / ml penicillin (Sigma), 0.1 mg / ml streptomycin (Sigma) and 2,5µg / ml amphotericin (Sigma).

After another 48 hrs, the medium was discarded and replaced with fresh water, experimental medium containing different concentrations of standard (17-β estradiol) or experimental means to extract different samples.

After 24 hrs of incubation (to cells adhere to the well) was exchanged to culture media with different concentrations of beta estradiol (controls: white, negative, positive (10nm to 1FM) and solvents) and samples of the extracts (repeat where, sample 3 X).

After 6 days, the cells were fixed with cold trichloroacetic acid (Sigma), 10% (w / v) for 30 min at -4oC.

Washed 5 times in tap water and allowed to dry. Stained with 0.4% sulforhodamine B (SRB / Sigma) in 1% acetic acid (Gibco).

Unbound SRB was removed by washing with 1% acetic acid and allowed to dry in air.

The bound SRB was solubilized with 10 nM Tris (pH 10.4) in a shaker.

After dilutions with estrogen on the MCF-7 cell proliferation, was made lecture in Elisa reader It measured the color intensity plate reader (absorbance) at 550nm.

In possession of the results, the readings and concentration, were plotted in graph of standard curve in logarithm.

The equation of the line was used to calculate the concentrations of estrogen in the water samples from distinct locations.

III. RESULTS AND DISCUSSION

3.1 Analysis with standard in different estrogen concentrations on the MCF-7 cell proliferation

Cells when grow in culture medium without estrogen showed proliferation with lecture in Elisa reader with 107cells However, when the medium was supplemented with water, the lecture presented 1013cells. These results showed that almost no cells were proliferated. Wells called white presented only water or Tris, and that showed results at minus of 1013cells, that can demonstrate negative proliferation.

Standart solution initiated with 10mmol and diluted it to micromole, picomol and fentomol. In each well were put 1500cells and 0.15mL of culture medium and after the period of reaction was made lecture in Elisa reader.

The standart curve was plotted in logarithm graph and demonstrated a high value in lecture with 0.01mmol corresponding at 105 in concentration and decreased lecture at dilutions with estrogen until 0.01fmol or 1013 of concentration. When have picomol the lecture decreased rapidly indicating less intensity on cell growth. The curve equation was y=-5,148ln(x) + 12,979 (Fig 1).

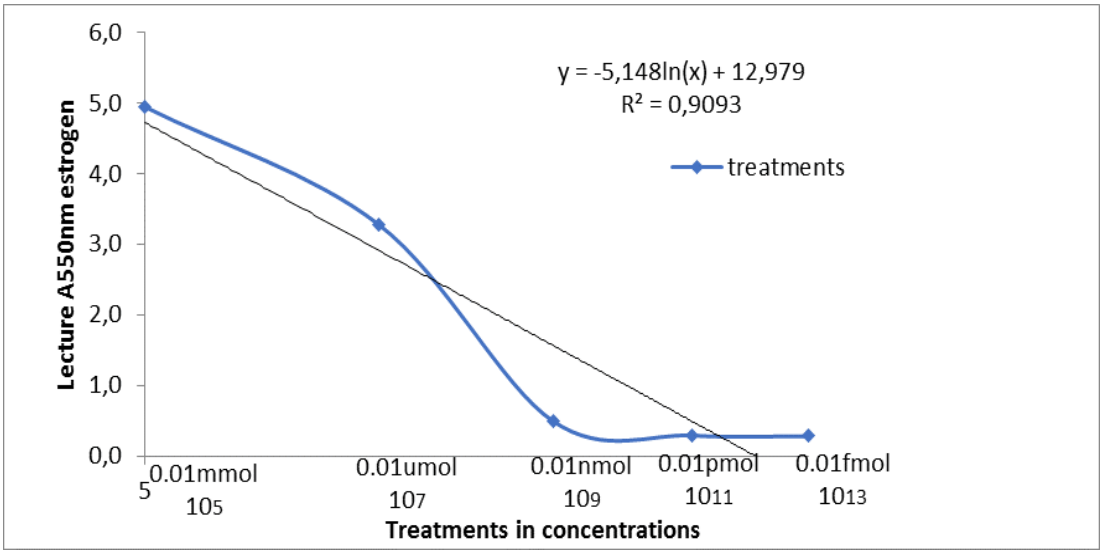

FIG 1: STANDART CURVE OF ESTROGEN PRESENT IN MEDIUM. CONCENTRATION OF ESTROGEN AT mmol, PICOMOL AND FENTOMOL.

3.2 Analysis of waters collected from lakes, rivers and lakes for breeding of animals and human sources.

All lecture from samples after check in Elisa reader, was used the equation curve for determinate the concentration of estrogen. Were studied 36samples of water and in all most water samples the result demonstrated estrogen present in fentomol (Table 1).

TABLE 2: CONCENTRATION OF ESTROGEN PRESENT IN SAMPLES OF WATER COLLECTED IN LAKES, RIVERS AND LAKES FOR BREEDING ANIMALS AND HUMAN SOURCES (EQUIVALENT A ESTROGEN IN A STANDARD CURVE WITH LOGARITHM).

samples number	concentration of estrogen			samples number	Concentration of estrogen		
1	0,895	$.10^{13}$	Fentomol*	21	0,833	$.10^{13}$	Fentomol
2	0,880	$.10^{13}$	Fentomol	22	0,806	$.10^{13}$	Fentomol
3	0,900	$.10^{13}$	Fentomol	23	0,811	$.10^{13}$	Fentomol
4	0,834	$.10^{13}$	Fentomol	24	0,838	$.10^{13}$	Fentomol
5	0,870	$.10^{13}$	Fentomol	25	0,902	$.10^{13}$	Fentomol
6	**0,970**	**$.10^{7}$**	**Micromol**	26	0,749	$.10^{13}$	Fentomol
7	0,906	$.10^{13}$	Fentomol	27	0,803	$.10^{13}$	Fentomol
8	0,920	$.10^{13}$	Fentomol	28	0,877	$.10^{13}$	Fentomol
9	0,933	$.10^{13}$	Fentomol	29	0,854	$.10^{13}$	Fentomol
10	0,829	$.10^{13}$	Fentomol	30	0,723	$.10^{13}$	Fentomol
11	0,894	$.10^{13}$	Fentomol	31	0,811	$.10^{13}$	Fentomol
12	0,824	$.10^{13}$	Fentomol	32	0,744	$.10^{13}$	Fentomol
13	0,877	$.10^{13}$	Fentomol	33	0,868	$.10^{13}$	Fentomol
14	0,891	$.10^{13}$	Fentomol	34	0,832	$.10^{13}$	Fentomol
15	0,898	$.10^{13}$	Fentomol	35	**0,215**	**$.10^{7}$**	**Micromol**
16	0,838	$.10^{13}$	Fentomol	36	**0,598**	**$.10^{7}$**	**Micromol**
17	0,825	$.10^{13}$	Fentomol				
18	0,859	$.10^{13}$	Fentomol				
19	0,857	$.10^{13}$	Fentomol				
20	0,853	$.10^{13}$	Fentomol				

Three samples of water the estrogen was present in micromol that corresponding in samples from Ibiúna and Equestrian Santo Amaro/SP.

The only samples that showed a great amount of estrogen were sent samples of Ibiuna and Hipica of Santo Amaro / SP. In Ibiúna, the water comes from a mine being raised fish in ponds receiving this water. The Equestrian comes from well and belongs to the Hípica. These samples should take another repeated time analysis to confirm the results, there may be changes in the analysis caused by the weather station, currently in São Paulo: heat and lack of water or it may be that actually have estrogenicity by the presence of waste on site, presence of algae and other factors.

According Ouyanga et al, 2006 and Eun-Joung et al, 2007, seasonal variations can alter water quality and concentration of DEs. During the drier station, the streams may include less water or no water is not silted to lakes high concentrations of estrogens and thus detects the estrogenicity is little water. In our study, the E2 was determined only in periods of drought, where the sewage inflow, constant throughout the year, is enhanced with the decrease in river flows slowly over the region rain events. E2 is the most potent estrogen, followed by E1 and E3. Estriol, however is the major secreted form, since the biosynthesis of 17β-estradiol in the body, other hormones, together with E2 are converted into estrone and estriol in later before excretion (Osterlund and Hurd, 2001).

Countries like Italy, Holland, Belgium and the United States reported concentrations of these estrogens in their watersheds between 0.2 and 21.7 ng L-1 (Verliefde, et al, 2007; Benotti et al, 2009). On the other hand, the recorded history for some

Brazilian sources, including Atibaia and Capivari, it is more worrying. Concentrations in ug L-1 levels are reported to oestrogens in rivers of Campinas, Jaboticabal and Belo Horizonte (Montagner and Jardim, 2011; Lopes et al, 2010; Moreira et al., 2009).

The concentrations determined in our samples, in most cases, were very close to the limit of quantification by bioassay, which only shows a tendency to detect synthetic estrogen in the bodies of water studied. The Endocrine Society has recently expressed its concern about the presence and chronic exposure to traces of endocrine disruptors concentrations with high potential estrogenic, as EE2 and other substances used as contraceptives and hormone replacement therapy as well as the chemical industry by-products, because of causing adverse effects human and animal health (Diamanti-Kandarakis et al., 2009).

MCF7 cells, tumor cells of human breast are easily cultivated and have estrogen receptors and stable estrogen dependence. We were able to demonstrate the proliferative response of these MCF7 in the control and the presence of 17B-estradiol (E2). A simple answer, reproducible. So, this is a sensitive system for the detection and quantification of estrogenic activity present in water or other substrate.

Accordingly, the results of e-screen assay were also found by different groups such as, Sonnenschein et al, 1998, Soto et al, 1995, etc. Different classes of substances such as steroids, alkylphenols, bisphenols, polyphenols, phthalates, hydrocarbons, chlorine, Xenoestrogen) act as estrogens which allow a quantitative extraction and application, also this e-screen assay.

IV. CONCLUSION

With this work, we were able to demonstrate that the E-screen assay with MCF7 cells is a sensitive and stable tool for quantitative analysis of estrogenicity of water in watersheds in the State of São Paulo and can easily be developed and applied in routine for animals and man food, aqueous extracts, plastics, etc.

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