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In vitro assessment of thyroidal and estrogenic activities in poultry and broiler manure

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HIGHLIGHTS

- Broiler and poultry manure show thyroidal activity.
- Poultry manure, but not broiler manure, exhibits estrogenic activity.
- Thyroidal and estrogenic activities did not pass through soil columns.
- Thyroidal and estrogenic activities were not retained in soil columns.
- Thyrogenic and estrogenic substances underwent degradation in soil.

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ABSTRACT

Among the many chemicals found in avian manure, endocrine disruptors (EDs), of natural or anthropogenic origin, are of special environmental concern. Nowadays, an increasing amount of estrogens is being released into the environment via the use of manure to fertilize agricultural land. While most research in this field has focused on estrogenic phenomena, little is known about alterations related to other endocrine systems, such as the thyroidal one. Here we simultaneously assessed the potential estrogenic and thyroidal activity of poultry and broiler litter manure using in vitro approaches based on estrogen receptor (Er) and thyroid receptor (Tr) transactivation assays. In addition, leaching experiments were performed to assess whether the EDs present in the manure pass through a soil column and potentially reach the groundwater. Manure from four broiler and four poultry farms was collected in two sampling campaigns carried out in two seasons (fall and spring). Extracts from broiler and poultry manure exhibited strong thyroidal activity. Only poultry manure showed estrogenic activity, which is consistent with the low levels of estrogens expected in hatchlings. Leakage experiments were performed in columns with two kinds of arable soils: sandy and loamy. No estrogenicity or thyroidal activity was detectable in soils treated with the manure or in the corresponding leachates. These results indicate that substances with estrogenic or thyroidal activity were degraded in the soil under our experimental conditions. However, the long-term effects associated with the constant and intensive application of manure to agricultural land in some regions require further research.

1. Introduction

The production of the poultry sector has increased by over 4% per year in the last decade. The 2011 census reported that the Spanish laying flock comprised 49 million hens (Ministerio de Agricultura, Alimentación y Medio Ambiente, 2013). As a result, approximately 2.7 million tons of broiler manure is generated each year. Taking into account poultry production, another million tons of manure should be added yearly (Ministerio de Medio Ambiente, 2003). It is commonplace to use animal manure to fertilize agricultural land. However, this practice also results in serious environmental problems because it could be a potential source of contaminants, such as natural hormones, antibiotics (Kwon, 2011), heavy metals (Zhang et al., 2012), detergents, flame retardants, and pesticides (Delgado et al., 2012; Sloan et al., 2003).

Among the plethora of chemicals present in manure, endocrine disruptors (EDs) are of special environmental concern. EDs are defined

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as exogenous substances or mixtures that alter the function(s) of the endocrine system, consequently causing adverse health effects on an intact organism, its progeny, or (sub) populations, even when present at very low concentrations (ng l^{-1}) (Sumpter, 1998; WHO/IPCS, 2002). EDs include natural substances such as reproductive hormones (e.g. estrogens, androgens, progestogens), thyroid hormones, and corticosteroids (Scholz et al., 2013), as well as a wide range of chemicals including synthetic hormones, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), dioxins, furans, alkylphenols, pharmaceuticals, and pesticides (Andersen et al., 1999; Combalbert et al., 2012).

When poultry manure is applied to agricultural land, the hormones present in it can be transferred to soil. These substances can make their way into surface and groundwater by leaching or by run-off, thereby contaminating aquatic ecosystems. Several studies have identified animal breeding activities as a major source of hormone input into the environment (Chen et al., 2010; Hanselman et al., 2006). To date, most research addressing the presence of EDs in manure has focused almost exclusively on reproductive hormones, particularly on estrogenic compounds (Hotchkiss et al., 2008). These substances can emulate the action of endogenous estrogens by activating the estrogen receptor (Er). This receptor acts as a ligand-activated transcription factor that induces the expression of estrogen-dependent genes (Beato and Klug, 2000), which are related mainly to reproduction, differentiation and growth.

Estrogen excretion associated with farm chicken amounts to 2.8 tons per year in the European Union (EU) and 2.7 tons in the USA (Lange et al., 2002). Steroid hormones show high chemical stability, and they are excreted in the free form or as conjugates, which are almost immediately biotransformed to the free form (Panter et al., 1999; Shore and Shemesh, 2003). 17β-Estradiol (E2) and estrone are the estrogens of greatest concern because they show a higher potency than other estrogens and can be found in the environment in concentrations above their lowest observable effect level (LOEL) for fish and plants (10 ng/l) (Miles-Richardson et al., 1999; Shore et al., 1993).

More recently, the thyroid axis has also been recognized as a target of EDs (Jugan et al., 2010; Schmutzler et al., 2007; Sciarrillo et al., 2008). The thyroid hormones (THs) triiodothyronine (T3) and thyroxine (T4) play a crucial role in the maintenance of a normal physiological state in vertebrates, notably in the control of development, growth, energy provision, reproduction and behavior (Yen, 2001). In amphibians and teleost fish, they also have essential functions in the regulation of metamorphosis, in the transition from larval to juvenile stages, and in smoltification in salmonids (Brown et al., 2004; Kloas, 2002; Power et al., 2001). In recent years, many chemicals, including some PCBs, tetrabromobisphenol A (TBBPA), and polybrominated diphenyl ethers (PBDEs) (Boas et al., 2006), have been suspected of acting as thyroid disruptors. These substances may compete with endogenous hormones for binding to transport proteins (i.e. transthyretin) and/or to TH receptors (Tr), thus acting as either agonists or antagonists and disrupting TH homeostasis (Boas et al., 2006; Kashiwagi et al., 2009). Trs, together with steroid receptors, belong to the nuclear receptor family and act as ligand-dependent transcription factors.

In contrast to estrogenic disruptors, studies devoted to thyroidal disruptors in environmental samples are scarce and have been limited to the detection of these compounds in rivers (Jugan et al., 2009), effluents from wastewater treatment plants (WWTPs) (Li et al., 2011; Murata and Yamauchi, 2008) and drinking water (Shi et al., 2012). To our knowledge, the thyroid-disrupting effects of manure have not been addressed, in spite of the detection of THs or products of their degradation in livestock residues. TH degradation is initiated by deiodination in peripheral tissues. Thereafter THs are decarboxylated and conjugated to hydrophilic products by means of glucuronidation or sulfatation and excreted via bile through feces or urine. Part of the conjugated THs can be hydrolyzed in the colon and excreted as free compounds like T3 and T4 (Brouwer et al., 1998; Schuur et al., 1999).

In addition to natural hormones, various chemicals with endocrine activity have been detected in manure, including PAH, dioxins, phthalates, bisphenol A and nonylphenol (Combalbert et al., 2012). All these substances affect the total hormonal activity of a manure sample. Although chemical analyses allow the detection of some of these products, even the high resolution methods currently available lack the sensitivity to detect all the substances present in manure, particularly when present at trace concentrations. In addition, chemical analysis does not have the capacity to evidence antagonistic or synergistic interplay among substances. To circumvent these limitations, the use of in vitro systems based on stably transfected cells with appropriate receptor and reporter genes is a valuable tool that allows the detection of global endocrine activity caused by the chemicals present in an environmental sample and that also reflects their interactions (additive, synergistic or antagonistic).

Accordingly, the aim of this study was to simultaneously assess the potential estrogenic and thyroidal activity of poultry and broiler litter manure using in vitro approaches based on Er and Tr transactivation assays with sea bass Erα (sbErα) and avian (av) Trα1, respectively. Although a link between animal farming and surface water or groundwater contamination with estrogens was already reported (Arnon et al., 2008; Shore et al., 1993), less attention has been paid to the risks associated with the use of manure in agricultural soils. Thus, in addition, we performed leaching experiments to assess whether the EDs present in manure pass through a soil column and potentially reach the groundwater.

2. Material and methods

2.1. Reagents

E2 (≥98% purity), T3 (≥98% purity), ethylene diamine tetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), $CaCl_2$, methanol (≥99.9% purity), and ethanol (≥99.9% purity) were purchased from Sigma-Aldrich (Madrid, Spain). Fetal bovine and horse serum (FBS and FHS), penicillin–streptomycin (10,000 U/ml), hygromycin, trypsin–EDTA, geneticin, ultraglutamine, and cell culture Dulbecco's minimal essential medium (DMEM) were obtained from Lonza (Barcelona, Spain). Phenol red-free DMEM was from Pan-Biotech (Zaragoza, Spain). The stock solutions of E2, and T3 were prepared in dimethyl sulfoxide (DMSO).

2.2. Sample collection

All the samples were taken from poultry farms located in the Autonomous Community of Castile Leon, Spain. Manure from four broiler and four poultry farms was collected in two samplings carried out in September of 2011 and in April of 2012. Manure is defined as a mixture of urine, feces and litter that accumulates on the ground of the farms. In each sampling, 500 g of manure was collected in amber glass bottles. All samples were kept in a cooler with ice in the field, and once in the laboratory they were immediately frozen at −20 °C until the extraction process.

2.3. Steroid and thyroid hormone extraction from manure

Manure samples from eight farms (4 broiler and 4 poultry) were tested for estrogenicity and thyroidal activity after each sampling. Estrogenic and thyrogenic substances were extracted using two methods, one with methanol and another with water at pH 11, following Combalbert et al. (2010). First, the manure was homogenized and 2 g of each manure sample was sonicated in 10 ml of methanol or in 10 ml of water pH 11 using a Vibra Cell TM ultrasonic probe (Sonic & Materials Inc., Newtown, CT, USA) at 18 kHz and 70% amplitude in three pulses of 15 s. Homogenates were then centrifuged at 1700 ×*g* for 10 min. Supernatants corresponding to the methanol extraction