Proposal:

Innovative Strategies for the Analysis of endocrine disrupting in Meats: Identification of their Metabolites in real matrices and studying their effect in vitro

Abstract

Food safety is the absolute highest priority and among the most significant international concern. It ensures the protection of consumers' health against any kind of hazards that may be present in the food matrix. In particular, the presence of endocrine disruptors represents an emergent risk factor. In this context, the development of a sensitive, specific and multi residue analytical method is necessary. The concentration level of these bioactive molecules and their metabolites will be performed using advanced analytical techniques, such as LC-MS/MS, GC/MS, NMR and HRMS. Furthermore, extraction methods parameters should be studied to improve the detection of hormones and will allow an analytical methods support for the identification of unknown compounds such as their metabolites in real meat samples. The development and validation of advanced analytical methods and of biological models could be very important in studying this issue.

Keywords: *Natural or Synthetic Hormones, LC/MS/MS, Reproductive System, Meat, In Vitro*

Introduction

In today's global marketplace, as foods are produced and distributed throughout the world, food quality and food safety have become increasing concerns for consumers, governments and producers. Chemical contaminants in food have been defined as "any chemical not intentionally added to food but present from many potential sources", including residues from endocrine disruptors. Providing consumer information about the relationship between diet and health over the last decade has been raised and consequently elevated the awareness and demand for functional food ingredients.

Improved testing methods now allow researchers to detect and monitor a strange brew of unpleasant chemicals in our food and bodies. Although the amounts are small and there's controversy about whether or not they're harmful, their presence alone is disturbing to many --especially parents of small children. In this way, the development of innovative analytical methods more suitable in the identification and characterization of these contaminants will be necessary. Therefore, the toxicological study covers all aspect and forms of xenobiotics (Contaminants and their metabolites) that can play roles in the effects and diseases (public health). The toxicological effect of these contaminants is essential to limit and regulate their impact. Experimental studies are therefore an indispensable link in understanding the cellular and molecular mechanisms and for future epidemiological studies. *In-vitro* studies on cellular models are relevant and necessary to know the action mode underlying pathophysiology involved in the toxicity of contaminants.

Farmers in the world wide use synthetic and natural growth hormones to promote rapid weight gain in cattle, increasing total meat volume when the cows are slaughtered and increase in milk production. The presence of hormones in milk and edible animals was discussed decades ago but rather more concerns attended to that with respect to finding hormones as biomarkers in milk and meat for diseases and pregnancy diagnosis. During the last couple of years, increasing body of evidence is indicating another property of hormones in animals products as possible impact on human health. These hormones enter the meat and milk, exposing children to potentially harmful chemicals and provoking of breast, prostate and endometrial tumors. However, further clinical studies are needed to determine the link between synthetic growth hormones in foods and disease risk. A review published in 2009 in "Medical Hypotheses" reported that drinking milk from treated cows during pregnancy may affect the health of the infant in its adult years.

Statement of Problem

The FDA's stamp of approval isn't likely to reassure those who worry that excess hormones in the food supply are contributing to cancer, early puberty in girls, and other health problems in humans. For years, consumer advocates and public health experts have fought to limit the use of hormones in cows, and some support a ban on the practice similar to the one in place in Europe, where food regulations are generally more stringent than in the U.S.

Toxicology is the scientific study of the harmful effects of chemicals on living organisms: humans, animals, and plants. Toxicological testing evaluates whether short-term exposure to chemical contaminants will produce acute effects (e.g., eye and skin irritation, death) and whether long-term, continual exposure will cause chronic effects (e.g., impaired liver function, reproductive abnormalities, cancer). The effect of contaminants coming from food, through changes in behavior, the nature of morphological and biochemical lesions, as well as the tracking of the metabolic disposition of hormones in the target sites of an organism. In vitro studies provide information regarding cellular responses and biochemical lesions with hormones and their metabolites. Toxicological profiles of hormones are cited using different species of animals for in vivo and in vitro studies. This information should help scientists and decision-makers reach conclusions regarding the toxicological effects of hormones on humans and the environment.

To reach our goal, adequate sample preparation and analysis equipment was needed. Interfering matrix compounds, such as proteins, lipids, salts, other endogenous and background compounds, should be removed in sample pretreatment, not only to avoid column clogging and instrument soiling, but also to improve the sensitivity, selectivity and reliability of analyses. Commonly and widely applied sample preparation techniques include protein precipitation (PP), liquid-liquid extraction (LLE), solid-phase extraction, Matrix solid phase dispersion (MSPD) and Quechers. These techniques were available in our Laboratory. For analysis the application of MS in combination with chromatography [GC or liquid chromatography (LC)] has been well recognized as the "gold standard" for both quantification and screening of food contaminants, such as pesticides, antibiotics and hormones. Although GC-MS continues to be used in the analyses of volatile, moderate to non-polar small molecules (e.g. PCBs, dioxins, other halogenated aromatic compounds and many pesticides), recent developments in both LC and MS have resulted in very powerful instrumentation for sensitive and selective determination of other more polar or ionic contaminants at trace levels in food including veterinary medicines, pesticides. Electrospray ionization (ESI) remains the most common ionization technique employed for the determination of chemical contaminants in food by LC-MS also APCI was used but a little bit.

Objectives

The objectives of this proposal is based on the development of the analytical techniques for the characterization, identification and quantification of the hormones and their metabolites in real matrix (Meat - Milk ...) and studying their potential negative effects on reproduction effect to public health especially the reproductive system. The development and validation of a suitable extraction method, according to the International standard guidelines, are important to improve the total recovery of hormones and provide suitable analytical methods to monitor and detect the illegal contamination of meat or milk. In particular, these procedures will be developed to monitor and detect the contamination with real meat or milk samples of the market. The determination and identification of metabolites that originate from native compounds in biological samples (real matrices) are not an easy to investigate, particularly for products that show low concentration levels. It requires expertise and good analytical

strategy that is based on all gathered information about the sample and potential sources of contamination. The elucidation of metabolites and the transformation products (TPs) formed requires the use of more powerful techniques and innovative nanotechnologies, capable to selectively identify the unknown compounds and their parent products. The analytical techniques applied to detect and quantify hormones, their metabolites and TPs are ^{13}C-NMR spectroscopy and mass spectrometry. LC-MS, high-resolution/accurate-mass measurements, using time-of-flight (TOF) or orbitrap MS instruments should be used for added selectivity, because high mass-resolving power (High Resolution Mass Spectrometry) allows discrimination between isobaric interferences and ions of interests. The analysis of hormones and their metabolites will be performed using LC-MS/MS, NMR, and HRSM techniques in order to identify metabolites structures and assess their toxicity to human health. The relationship between the endocrine disruptors and exogenous hormones detected in samples and effect of their concentration should be studied on the reproductive system both *in vitro*.

Research program planned for this proposal and contribution of the various partners

1) Firstly, we begin with a bibliographic data on the use of hormones molecules especially the most used families (steroid, as peptides…) in Lebanon for the selection of target molecules of our work. On the one hand in the experimental section, development of an analytical method for the analysis of Hormones in meat or milk was crucial to implement an innovative method for extraction to attend a very low concentration (1 µg/kg).
2) Secondly, the extraction of meat present in the Lebanese market will be performed using developed method that is selected for the extraction of solid matrices, followed by analysis by liquid chromatography-mass spectrometry (LC-MS/MS) for the determination of target molecules contaminating meat or milk. The metabolites of hormones (way of metabolisation) shall be further detected and quantified. The detection and identification of unknown metabolites is not an easy task and it will be done by LC-MS-MS, and supplemented by nuclear magnetic resonance (NMR) and high resolution mass spectrometry (HRMS) in order to determine their chemical structures.

3) Thirdly, the study of the effect of identified molecules on reproduction.

To assess the possible effects of molecules (or mix of molecules) identified in step I and II we will adopt an in vitro experimental approach on animal models with high predictive an translational value for human health (bovine and swine?).

In particular, we will use an in vitro fertilization (IVF) set-up carried out in traditional IVF systems (I) and in an innovative in vitro model (II).

I) The IVF will be carried out on in vitro matured oocytes and in vitro capacitated spermatozoa (either exposed or incubated in control conditions) in Petri dishes.

II) An innovative system will be used, consisting of oviductal epithelial cells cultured on a 3D printed scaffold, used as substrate for the IVF.

The IVF results will be expressed as fertilization rate (% of penetrated oocytes), incidence of polyspermy (% of polyspermic oocytes), and number of penetrating spermatozoa/polyspermic oocyte.

In parallel with the IVF experiments, we will carry out biochemical and biophysical analysis on spermatozoa. We will use this biological substrate because for many reasons these cells are an ideal candidate for this kind of analysis:

- first of all they are virtually transcriptionally silent, as a consequence their protein composition is stable. Indeed the most important problem in cell modelization is the continuous modification in cellular protein content and in molecular interactions due to the dynamical regulation of genes expression and protein transcription.
- differently from the most of other cellular types, it is possible to empirically evaluate the functional status of the system. In fact it is possible, using for instance an animal model, to verify if the spermatozoa completed their maturation process, testing the ability of spermatozoa to complete the capacitation and, subsequently, to undergo AR by in *in vitro* fertilization assay or by *in vivo* fertilization trials: only the spermatozoa that successfully fertilize an oocyte can be considered fully competent.
- finally the spermatozoa are the only cellular type, produced in an organism, that exert their function in another one. As a consequence they are capable of independent life (unlike the other cells) and it is possible to manage them outside the organism without loss of the cell function.

In detail, we will assess the effects of sperm in vitro exposure to the identified molecules by the following analysis:

1) morphological analysis: acrosome integrity (PSA/PNA) and cytoskeleton integrity, actin (phalloidin/tubulin)

2) functional analysis: mitochondrial function (MitoTracker); membrane potential (Bis-oxonol); calcium (Fura 2; Fluo 3 AM); cholesterol localization (Filipin III) membrane fluidity (FRAP with DilC12 and MC540); membrane anisotropy (laurdan); differential scanning calorimetry (DSC).

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