A Review on the Effect of Immunocastration Against Gonadal Physiology and Boar Taint

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Abstract: Castration is the disruption of testicular function, practiced for most economically important and pet animals; pigs, bovine, ovine, equine, caprine, canine and feline during puberty since ancient times. Gonadectomy practiced for centuries worldwide. Application of surgical castration with anesthesia/analgesia and temporary suppression of testicular function by vaccination are currently the most practical choices. Both surgical & chemical castration methods have drawback from the animal welfare point of view. Whereas the European Commission and the representatives of European farmers, scientist and veterinarians signed declaration to end surgical castration. For these reasons, immunocastration tested using peptides similar to GnRH, combined with proteins, to trigger antibodies that neutralize the function of GnRH. Australia & New Zealand use this method since 1998, Switzerland since January 2007 and Belgium since October 2010. Currently approved in over 60 countries. It is reported to be an advantageous alternative to improve aggressive behavior, smell and taste of meat and feed conversion efficiency. GnRH is composed of 10 amino acids which plays critical role in reproductive system by stimulating pituitary gland to release FSH/LH. These regulate gonadal functions, testes growth with spermatogenesis/steroidogenesis in Leydig cell. Androstenone and testosterone are sex hormones regulating reproductive physiology and sexual behaviors. Androstenone and skatole are the two compounds resulting taint odor of meat under the control of GnRH during puberty. These two compounds give urine-like and faecal-like odour. Skatole is a breakdown product of the amino acid tryptophan in large intestine by bacteria. Anti-GnRH antibodies produced by vaccination to neutralize GnRH. Deficiency of GnRH leads to castration. This novel technology needs further research on the mechanism and links with metabolism. Anti-GnRH has reproductive impacts on human being for self-injection. In general, in this review it has been attempted to give a highlight in understanding the mechanisms and benefits of immunocastration, boar taint prevention and the physiology in relation to its effect on the behavior, environment, meat production which have paramount importance in designing intervention methods in the control of undesired breeding, aggressiveness and taint in Boar. Finally, relevant recommendations are forwarded.
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Introduction

Castration is the disruption of testicular function, usually by removal of the testes of male animals (Albrecht, 2013) and has been a routine management procedure for most economically important animals and pets; pigs, bovine, ovine, equine, caprine, canine and feline to be prevented from breeding since ancient times. Surgical castration (gonadectomy) of young male pigs has been practiced for centuries in pig farming worldwide. It is estimated that about 100 million piglets are castrated annually in the 25 countries of the European Union (EU) (Thun *et al.,* 2006). Application of surgical castration with anesthesia and analgesia and temporary suppression of testicular function by vaccination are currently the most practical choices to avoid tainted meat (Zols *et al.*, 2008). Both surgical and chemical castration methods have drawbacks (Moreira *et al.,* 2015). Surgical castration must be performed by specialist personnel, is irreversible, causes infections can lead to inguinal hernias and immunosuppression, in some cases resulting in death. The administration of steroids causes side effects detrimental to animal health (Janett *et al.*, 2012).

For these reasons, animal immunocastration has been tested using peptides similar to gonadotropin releasing hormone (GnRH), combined with proteins, to trigger antibodies that neutralize the function of GnRH (Fuchs *et al.*, 2009). According to EU legislation, castration of male piglets can be performed without anaesthesia/analgesia within the first 7 days of life and without tearing of the tissue (Klont *et al.*, 2008). Nevertheless, this practice has been criticized from the animal welfare point of view. It has already been forbidden in Norway and Switzerland, whereas the European Commission (EC) and the representatives of European farmers, meat industry, retailers, scientist, veterinarians and animal welfare NGO's signed a declaration to end surgical castration of pigs (Moreira *et al.,* 2015).

Hormonal castration (immunocastration) typically involves the injection of

immuncontraceptives to induce antibody production against gonadotropin releasing hormone (GnRH), resulting in decreased production of endogenous reproductive hormones (AVMAAWD, 2009). The indications for castration include reduction of aggressive behavior, docile and prevention of the occurrence of boar taint, a distinctive unpleasant odor/flavor which can be perceived during cooking/eating of meat from entire male pigs (Park *et al.,* 2015). The most promising alternative method is use of an immunocastration vaccine composed of gonadotropin-releasing hormone (GnRH) that induces anti-GnRH antibodies (AVMAAWD, 2009).

Having it, Australia and New Zealand use this method since 1998. Switzerland has started the use of this method since January 2007 and Belgium since October 2010. Vaccine, improvac is approved in other countries: Brazil, Mexico, Korea, Thailand, Philippines, Guatemala, South Africa, Chile, Venezuela, Panama, Russia, El Salvador. Improvac is registered in Serbia, Slovenia and Croatia (Brunius *et al.,* 2011). The vaccine against GnRH, developed in Australia and produced by Zoetis (formerly Pfizer Ltd., formerly CSL Limited, Parkville, Victoria, Australia), For the time being it is approved in over 60 countries worldwide and has been in commercial use in the EU since 2009 (Zamaratskaiaa and Rasmussenb, 2015).

Although the synthetic vaccine is administered via injection it act by stimulating the natural active immunity of the animal for the formation and production of specific antibodies against the synthesis of gonadotropin releasing hormone (GnRH), which inhibit the liberation of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by the pituitary gland and thus suppress testicular function in males, with a subsequent decrease in the concentration of blood testosterone (Thun *et al*., 2006). The antibody neutralizes GnRH, and as a result hypothalamic-pituitary-gonadal axis is blocked and testes growth and sexual steroids synthesis are effectively inhibited. Physiologically immunocastration becomes effective in a week following second vaccination (V2). Within 4-6 weeks' interval between V2 and slaughter androstenone and skatole levels in fat tissue are already below the limit of sensory detection (Jelena *et al*., 2012).

Immunocastration is quite persistent although it should not be permanent (Škrlep *et al.*, 2010). Some animals do not react to it (called "non-responders") due to poor immunological response or improper vaccination; their number is low (1 -3%) and similar to the number of cryptorchids (Marjeta *et al.,* 2015). Skatole is a breakdown product of the amino acid tryptophan produced in the large intestine of the pig by intestinal bacterial it is absorbed and metabolized in the liver excreted with urine. These two compounds give a "boar taint" with odour as urine-like and faecal-like. Anti-GnRH antibodies produced by vaccination with GnRH neutralize GnRH in boars. According to Škrlep *et al.* (2010) justification the deficiency of GnRH leads to insufficient production of FSH and LH, which eventually induces castration effects. In general, in this review it has been attempted to give a highlight in understanding the mechanisms and benefits of immunocastration, boar taint prevention and the physiology in relation to its effect on the behavior, environment, meat production which have paramount importance in designing intervention methods in the control of undesired breeding, aggressiveness and taint in Boar.

Therefore, this review is prepared with the following objectives: to provide compiled scientific information about immunocastration, to contributes for the development of future immunocontraceptives or vaccines against hormone-dependent cancers, to provide information on mechanisms and pathophysiology of Anti-GnRH, to develop an integrated data on the physiology and biotechnological application, to have inductive information for responsibles on the application and to provide information towards the feature research areas.

1.1. Materials and Method

1.1.1. Literature Search

The required information was accessed, extracted and abstracted independently by using online search of the HINARI, Pubmed, EMBASE, Ovid, Cochrane databases and open access internet search from August 2017 to December 2017. The following key term; castration, immunocastration, immunovaccination, improvac, Gonadotropin releasing hormone (GnRH), GnRF, LHRH, GnRH antibody, boar taint, together with the terms "effect of immunocastration" and "physiology of boar taint" were used to access relevant publications without any study design and country restriction. 44 Journal articles, PhD theses, public policies, government and learned society reports are used. I also screened the references of retrieved articles to identify additional studies.

1.1.2. Inclusion and Exclusion Criteria

The eligible studies met the following criteria: (a) case control or cohort studies reporting pathophysiology of immunocastration and boar taint, (b) studies involving gonadotropin releasing hormone neutralization with vaccine, (c) studies providing sufficient information on the physiological effect of immunocastration was accessed. But the following as exclusion criteria a) abstracts and economic studies (b) studies in which the requested information not obtained (c) when multiple studies reported the same or overlapping data. I selected the latest one or the one providing more amount of information.

2. Immunological Castration

2.1. General Aim and Principles of Immunocastration

The aim of immunocastration (Chang, 2015) is to deactivate testicular functions by neutralization of the hormones of the hypothalamic-pituitary-gonadal axis with a basic principle; involves vaccination of animals against the hypothalamic gonadotropin releasing hormone (GnRH) also known as Luteinising hormone-releasing hormone (LHRH), key hormone regulating reproductive function (Sharma and Hinds, 2012). Anti-GnRH vaccination involves the injection of GnRH analog conjugated to a foreign protein and combined with an adjuvant, to initiate transient formation of anti-GnRH antibodies that can bind and inhibit the action of endogenous GnRH. The vaccination induced inhibition of LH and FSH with a sequel of absence of steroidogenesis and spermatogenesis (Zamaratskaiaa and Rasmussenb, 2015, Karaconji *et al.*, 2015). That is temporary castration to induce infertility and prevent body odor (boar taint) without the need of stressful and painful surgical castration (Chen *et al.,* 2007).

2.2. Luteinizing Hormone Releasing Hormone and its Applications

Luteinizing hormone-releasing hormone (LHRH), also known as gonadotropi-releasing hormone, is secreted from the base of hypothalamus and stimulates gonadotropic cells in the anterior pituitary gland to release LH and FSH (Chang, 2015). LH and FSH in turn regulate the gametogenic and steroidogenic functions of the gonads (Figure 1). Vaccination against LHRH leads to anti-LHRH antibodies that neutralize LHRH and prevent the secretion of LH and FSH, resulting in immunocastration. This response has led to studies that used peptide-based LHRH vaccines for the treatment of hormone-sensitive cancers or as immunocontraceptives (Sharma and Hinds, 2012).

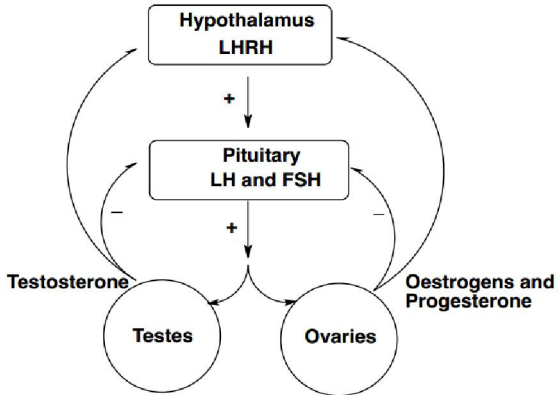

Figure 1: Control route of sex hormones in Male and Female (Chang, 2015).

2.3. Development of Immunocastration Vaccine

Immunocastration is based on the vaccination against gonadotropin releasing hormone (GnRH) by using the natural immune system of the animal to achieve the effects of surgical castration (Škrlep *et al.,* 2014). The first studies on immunocastration were performed more than twenty years ago (Falvo *et al.*, 1986, Škrlep *et al.*, 2010), but were not suitable for practical use due to the utilization of strong adjuvants. Commercial vaccine (Improvac) was first introduced in Australia and New Zealand in the nineties. Currently, the vaccine is registered in more than 60 countries around the world, including Brazil (where it is used the most extensively), Switzerland and EU countries since 2009. The new immunological product will be called Improvest® in Canada and the US, Vivax® in Brazil, Innosure® in Central America, Colombia and Venezuela, and Improvac® in most other countries (Škrlep *et al.*, 2014).

2.4. Formation and Nature of Vaccine

2.4.1. Generality of Formation

The vaccine, immunogenic material is synthetic (GnRH) conjugated to a carrier protein in a water adjuvant (Evans, 2006). Immunogenicity of GnRH is too low to produce enough antibodies, because it is a peptide composed of 10 amino acids. Therefore, most immunocastration vaccines contain multiple copies of GnRH conjugated with various adjuvants to overcome the low immunogenicity (Oonk *et al.*, 1998). Recognition of the pathogen associated molecular patterns (PAMPs) by members of the toll-like receptor (TLR) family is important for the induction of innate and adaptive immune responses (Park *et al.*, 2015). It has been suggested that administration of antigens with the PAMPs would provoke potent antigen-specific immune responses in mammals. Bacterial flagellin, a ligand of TLR5, functions as an adjuvant and enhances immunogenicity of antigens (Turley *et al.*, 2011). A new immunocastration vaccine composed of multiple copies of GnRH conjugated with *Salmonella* Typhimurium flagellin fljb (STF^2), the SFGnRH vaccine, was developed and showed very efficient immunocastration effects in males. If it is administered with two injections at least 4 weeks apart in to the base of the ear (Park *et al*., 2015).

Each 1 ml dose of vaccine contained 400 µg of modified GnRH peptide covalently conjugated to carrier protein, together with Advasure, an aqueous adjuvant (Zamaratskaia and Squires, 2009).

2.4.2. Synthetic Anti-LHRH /GnRH Vaccines

Chemical synthesis of LHRH is advantageous because it allows chemical modifications of the peptide (Partidos *et al.,* 2000). For instance, substitution of glycine at position 6 with D-lysine,

which renders further conjugation in the middle of the sequence. Conjugation of LHRH to carrier proteins such as tetanus toxoid (TT) or DT as vaccine has been trailed in several animal and human studies (Chang, 2015). In addition, different structural modifications of anti-LHRH were made to improve immunogenicity of the construct, for example, insertion of lipopeptide dipalmitoyal-s-glyceryl cysteine between LHRH and a T helper epitope of influenza virus haemagglutinin has shown its efficacy to confer an antifertility (Partidos *et al.,* 2000).

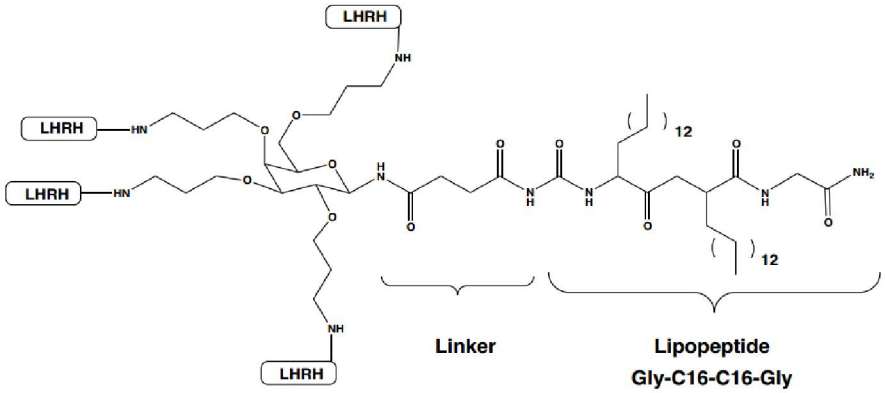

Figure 2: Structure of the anti-LHRH vaccine candidate containing lipopeptide (Chang, 2015).

2.4.3. Recombinant Aanti-LHRH Vaccines

Except for chemical ligation of LHRH to a carrier protein, recombinant protein expression is an effective approach to coexpress LHRH and carrier protein as one moiety. This method is considered to be more economic in large-scale industrial production (Chang, 2015). Partidos *et al.* (2000) expressed a recombinant protein containing 12 copies of LHRH with a carrier from the receptor-binding domain of Pseudomonas exotoxin A. Coexpression of four or five selected T cell specific epitopes with four or five copies of LHRH generated anti-LHRH antibodies in all animals, which caused the decline of testosterone to castration levels. Using four to five different T helper epitopes was proposed to enable communication with major histocompatibility complex (MHC) of subjects with genetic diversity (Sharma and Hinds, 2012).

2.4.4. T helper Epitope

T helper epitope is a peptide allows APC to display on an MHC class II and bind to immature CD4+ T cell, which leads to the maturation of the T helper cells. T helper cells are important for maturation of T cells to eliminate intracellular pathogens, and maturation of B cells to produce specific antibodies. T helper epitope enables conjugation with a target antigen via chemical ligation or recombinant protein expression technique (Chang, 2015). The combination of two specific peptides allows the whole peptide structure to be receptor specific. LHRH itself is an endogenous hormone and is recognized as a hapten. Development of an anti-LHRH requires it to be conjugated to a carrier protein or a promiscuous T helper epitope to enhance immunogenicity (Sharma and Hinds, 2012). Large inactivated toxins such as TT, DT and its nontoxic mutant, CRM197, are used as carriers in vaccines to develop strong immune responses. Several studies have shown variable T helper epitopes from influenza haemoglutinin and canine distemper virus fusion protein for conjugation with LHRH and induced significant specific antibody production and castration effect. Anti-LHRH antibodies induced from a vaccine containing the N-terminal of a modified LHRH (CHWSYGLRPG-NH2) conjugated to a TT gave high LHRH binding avidity antibodies and also exerted their effect on male reproductive organs and concentration of testosterone, LH and FSH (Partidos *et al*., 2000).

2.4.5. Branched Vaccine Structures

Lysine is a versatile amino acid when used as a building block in synthetic peptides (Chang, 2015). It provides two amine groups and a carboxylic group allowing for branch and/or dendrimer construction. B cell epitope and T cell epitope have different sites on one vaccine structure, which provides effective immunogenicity (Partidos *et al*., 2000). It was revealed that a chimeric peptide structure that contains a T cell epitope and a B cell epitope is an effective strategy against. The T cell epitope from surface glycoproteins of measles virus showed assistance to the B-cell epitope, an epitope from haemagglutination in B cell specific antibody production.

The orientation of both the T-cell epitope and the B-cell epitope affects immunogenicity and the affinity

of antibodies (Chang, 2015). In terms of antigen presentation, branched structures appeared better immune responses than linear structures (Partidos *et al.*, 2000). The advantage of one construct containing multiple identical epitopes is the ability to increase the chances of interaction with APCs. A branching core with multiple conjugation sites allows the attachment of multiple uniform epitopes to increase the portion of antigen in the vaccine construct. Multiple antigenic peptide (MAP) system containing a polylysine branching core possessing free terminal amines to which multiple identical epitopes were attached (Chang, 2015).

Four lipopeptide-based vaccine candidates were designed using four different components: a lipopeptide comprised of two copies of C16LAA and two copies of serine; a T helper epitope from the L chain of the influenza virus haemagglutinin; the B cell epitope, LHRH and a central lysine to permit the synthesis of branched structures. Compounds 2-3-4, were designed using a LHRH epitope with a free amino group at its N-terminus while the LHRH epitope in compound 1 was attached to the lysine through its N-terminus with a free amino group at the C-terminus. All compounds were synthesized via solid phase peptide synthesis using the Fmoc in situ neutralisation protocol and purified to a single peak (> 95% purity) (Chang, 2015).

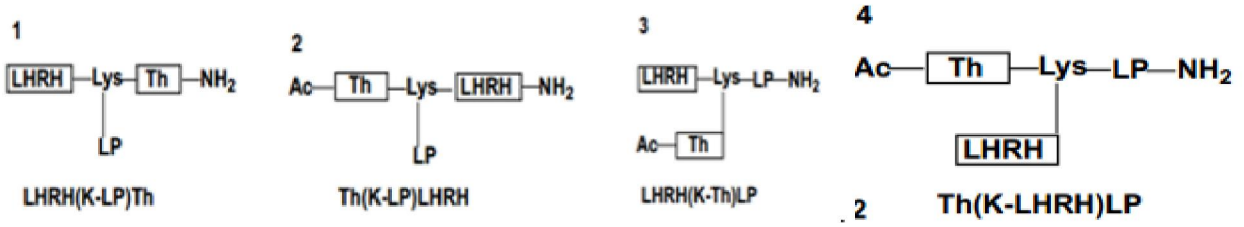

Figure 3: Schematic structures of anti-LHRH vaccine candidates. (Chang, 2015).

(Hint: Each vaccine candidate incorporates an influenza helper T cell epitope (GALNNRFQIKGVELKS), a LHRH epitope (PEHWSYGLRPG) and two copies of C16 LAA. Ac = acetyl group, LP = Lipopeptide, Th = T helper epitope)

These constructs stimulated significant anti-LHRH antibody titers after the second booster. The structure-activity relationship with LHRH on either the N-terminus (constructs 2 & 3) or on the branch between either termini (4), induced higher immunogenicity against LHRH than the construct with LHRH at the C-terminus (Sharma and Hinds, 2012). The lack of bulky residues in the N-terminus of constructs 2, 3 and 4 may have contributed to this observation. These constructs were non-toxic and no significant haemolytic effect (Chang, 2015).

2.4.6. Vaccine Adjuvants

An adjuvant is an immune response stimulator used in vaccine, which boosts the host's immune response and recognition of antigens (Chang, 2015). The adjuvant also increases the efficacy of vaccine by reducing the amount of antigen required. In subunit vaccine where minimum components of pathogen are used as antigen, a strong immunostimulatory is often required to increase its immunogenicity. LHRH is a decapeptide and endogenous hormone, which has been incorporated as an antigen in anti-LHRH vaccines (Albrecht, 2013). Nonetheless, in the absence of an adjuvant the LHRH peptide does not produce a potent immune response as it is a self-hapten and is highly conserved. Also, the LHRH sequence does not contain any T helper epitope and hence does not induce a T cell response. Modification of the vaccine structure is necessary in order to improve the efficacy of anti-LHRH vaccines. Selection of a suitable adjuvant and a carrier system, the molar ratio of the carrier and hapten, and conjugation methods are critical for developing an optimal vaccine against small peptides (Partidos *et al.,* 2000).

2.4.6.1. Mechanism of Adjuvants

The vaccine antigen is taken up by immature dendritic cells (DC) via two approaches: receptor-mediated endocytosis or fluid-phase pinocytosis. Through these methods, antigens are internalized by DCs which promotes their maturation. This maturation may occur with different kinds of antigens such as the components of microorganisms, or vaccine adjuvant (Chang, 2015). Microbial components and inflammatory chemokines mediate the recruitment of immature DCs, which migrate from peripheral vessels to draining lymph nodes (Partidos *et al.*, 2000).

For effective antibody production, persistent release of antigen from the injection site provides enough time for antigen present in the draining lymph node to be taken up by follicular DCs. Water-oil emulsion injections allow antigens and adjuvant to form a depot locally and gradually release the antigen in a sustained way. Microorganism sourced adjuvants provide their adjuvanticity via recognition by

pathogen recognition receptors (PRRs) existing on the surface of antigen-presenting cells (APCs). Without extra stimuli from conserved microbial structures, that are also known as pathogen-associated microbial patterns (PAMPs), to the PRRs on immune cells, the presentation of a specific antigen is not able to induce an effective immune response. PRRs are expressed by conserved genes (Chang, 2015). Adjuvant is recognized as a source of danger signal to cells. Like the danger signals sourced from damaged and infected cells. The immune cells detect these signals and trigger immune responses.(Sharma and Hinds, 2012). Adjuvant as a danger signal can induce inflammation at the injection site to trigger cells releasing cytokine in order to respond to the inflammation immediately (Partidos *et al.*, 2000).

An ideal adjuvant for peptide-based vaccines is one that induces both cellular (T helper, Th1), which is a CD4 immune response mainly to promote maturation of B cells for specific antibody production, and humoral (Th2) immune responses for maturation of macrophages that diminish pathogens by phagocytosis. Foreign invasive immunogens in vivo trigger cells to release cytokines, thus leading to Th1 and Th2 immune responses (Chang, 2015).

2.4.6.2. Classification of Adjuvants

Mineral-based adjuvants: Mineral-based adjuvants, for example, aluminium hydroxide and aluminium phosphate, are the most commonly used components of prophylactic vaccines. The alum based adjuvants are known to induce potent Th2 immune responses but less Th1 immune responses (Sharma and Hinds, 2012). In adult, long-term exposure to aluminium causes cognitive dysfunction and autoimmune responses. Alternatives to alum salt-based adjuvants are calcium, iron and zirconium salts, which are less potent in adsorption of antigens for efficient production of antibodies than alum-based adjuvants (Chang, 2015).

Oil-based adjuvants: Oil-based emulsions such as saponins can induce stronger cellular immune response than alum-based adjuvants, which is particularly required for the effect of some vaccines. These water-in-oil or oil-in-water type adjuvants act like a depot at the injection site to gradually release antigen. They also promote plasma cells for antibody production (Chang, 2015).

Microbial component-based adjuvants: Bacterial products are used as adjuvants. Peptidoglycan and lipopolysaccharide, sourced from Gram-negative bacteria, are able to enhance immunogenicity (Chang, 2015). Virulence and ability to revive to the pathogenic form of these adjuvants causes concerns in human use vaccines (Sharma and Hinds, 2012).

2.5. Protocol and Delivery of Immunization

The newly developed vaccine could be administered to pre-pubertal age to maintain immunocastration effects until the fattening period. Immuncastration, Improvac vaccine needs successful prescription and application to be injected with two doses for best pathophysiological effect on GnRH (Park *et al.*, 2015). The vaccine is administered at the age of eight weeks with the body weight between 30 - 60 kg while they are not stressed by weaning, moving or mixing (Škrlep *et al*., 2014).

The first dose of vaccination is injected subcutaneously and then the "Booster", second dose application at least 4 weeks later (2x2 ml for boar) or (1x1ml for cattle) about the dorsal part of the neck, behind the ear base (Jelena *et al.*, 2012) region of the ear at eight weeks of age for boar and on the lateral aspect of the left side of the neck (cattle) through a 12.5 mm 16 gauge needle inserted at a 45° angle to the surface of the neck with the needle directed cranially (Shalev *et al.*, 2003). The periodic time line of immunization, that is: 1) the first period is the time before the application of the first dose of GnRH protein conjugate that is 56 to 80 days of age, 2) second period is the time interval between the application of the first and the second dose of GnRH protein conjugate in the 81 to 110 days of age, and 3) the third period is after the application of the second dose of GnRH protein conjugate from 111 to 140 days of age of boar (Turley *et al.*, 2011). In addition to various immunological factors the type of antigen, formulation, dose, delivery aspects, route of administration, the need of vector or specific delivery system, and the nature of the target animal population and their habitat are very important (Chang, 2015).

2.6. Mechanism of Action of Anti-GnRH

The mechanism of immunocastration is through utilizing immunity against endogenous sex hormones in the body (Chang, 2015). Several immunocontraceptive vaccines have been classified based on their targets such as anti-sperm vaccines, anti-zona pellucida (ZP) vaccines, anti-human chorionic gonadotropin (hCG) vaccines and anti-LHRH vaccines (Chang, 2015).

The immunocastration vaccine antigen has no hormonal activity (Marjeta *et al.*, 2015), although efficiently produces specific antibodies against GnRH (Fuchs *et al.,* 2009). It stimulates the natural active immunity of the pig for the formation and production of specific antibodies against the synthesis of gonadotropins, anti-GnRH antibodies (Janett *et al.,* 2012). The antibody neutralizes GnRH, and as a result hypothalamic-pituitary-gonadal axis is blocked, testes growth (Marjeta *et al*., 2015) (induces a one-third reduction in the size of the testes and three-quarters reduction in the weight of the internal sexual glands compared with boars) (Moreira *et al*., 2015) and

sexual steroids synthesis are effectively inhibited. Physiologically the immunocastration becomes effective in a week following second vaccination (V_2). Within 4-6 weeks' interval between V_2 and slaughter androstenone and skatole levels in fat tissue are already below the limit of sensory detection. Immunocastration is quite persistent although it should not be permanent. Some animals do not react to it (called "non-responders") due to poor immunological response or improper vaccination; their number is low (1 -3%) and similar to the number of cryptorchids (Marjeta *et al.*, 2015).

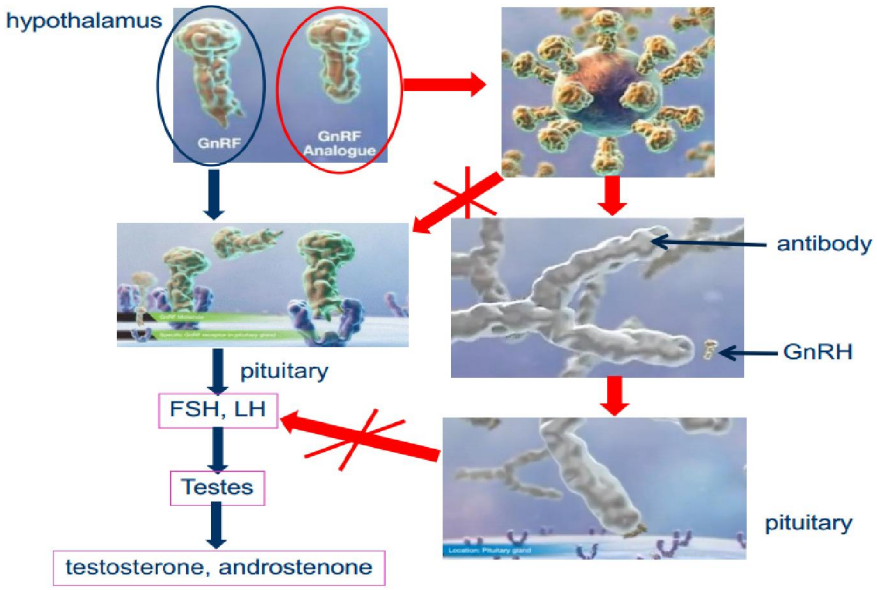

Figure 4: Schematics of mechanism action of Improvac against GnRH (Bonneau *et al.,* 1994).

2.7. Physiological Response for Immunocastration

Immunocastration is highly effective in castration and elimination of meat taint. In most animal species the effect is observed soon after the booster dose. Within a week after the treatment, a rapid increase of antibodies against GnRH (Pglu-His-Trp-Ser-Tyr-Gly-LEU-Arg-Pro-Gly-NH_2) occurs which causes, together with breakdown of HHG axis, a fast decrease of gonadal steroids including plasma androstenone (Claus *et al.*, 1994). A complete clearance of boar taint requires a longer period. According to vaccine producer, a minimum of 4 weeks between 2nd treatment and slaughter is recommended. Decline of androstenone and skatole in fat tissue below sensory detection observed two weeks after the successful immunization. The decrease of steroid hormones is reflected in size reduction of testes and accessory glands. The immunization has the strongest effect on vesicular gland followed by testes and bulbourethral gland. Changes on histological level concern the atrophy of Leydig cells and germinative epithelium, cessation of spermatogenesis (testes), reduced size of glandular acini and secretion of accessory glands (Einarsson *et al.,* 2009). The effect of immunocastration persists up to 22 weeks; however, it seems that it does not have a permanent effect. There is progressive restoration of testicular activity and increase of androstenone level after 12 weeks (Claus *et al.*, 1994). According to Moreira *et al.* (2015) irreversible loss of reproductive ability is most likely associated with earlier vaccination. It should be noted that some animals do not react to immunization (Škrlep *et al.*, 2014).

2.7.1. Immunocastration Against Reproduction

2.7.1.1. Physiology of GnRH and Reproduction

According to Moreira *et al*. (2015) reproductive physiology is controlled by mutual function of three main endocrine glands: the hypothalamus, the pituitary gland and testicles (hipotalamic-pituitary-gonadal axis) (Škrlep *et al*., 2014). As male animals approach puberty, the lower part of the brain (hypothalamus) releases gonadotrophin releasing hormone (GnRH) also known as luteinizing hormone releasing hormone (LHRH) which is composed of 10 amino acids playing a critical role in reproductive system development by stimulating the pituitary gland at the base of the brain to release 2 other hormones; FSH & LH (Park *et al*., 2015). These regulate gonadal functions and stimulate the growth of the testes and, in turn, the production of testicular steroids. FSH is critical for spermatogenesis in the seminiferous tubules and LH stimulates the

secretion of testosterone from the testis. LH stimulates secretion of steroid hormones (androgens, estrogens and androstens), which are essential for normal reproductive function, but also affect metabolism, behavior and development of the sexual odour *(Škrlep et al.*, 2014). One of steroid, androstenone, is a pheromone that concentrates in saliva. Anti-GnRH antibodies produced by vaccination with GnRH neutralize GnRH in boars. Deficiency of GnRH leads to insufficient production of FSH and LH, which eventually induces castration effects (Park *et al.,* 2015).

2.7.1.2. Immunocontraceptive Vaccines

With the increasing human population, food, water and resource shortages and diseases incidences are always of public concern. Additionally, excessive wild animal proliferation causes environmental issues such as damaging the ecosystem, hindering the growth of forests, and spreading animal-to-human transmitted diseases (Chang, 2015). Therefore, animal birth control is an adequate and effective way to address these problems. Immunocontraception has been proposed as an acceptable, effective and achievable way to control the wild animal population. It is also an alternative to traditional castration methods such as surgery and chemical castration (Partidos *et al.,* 2000). Several immunocontraceptive vaccines have been classified based on their targets such as anti-sperm vaccines, anti-zona pellucida (ZP) vaccines, anti-human chorionic gonadotropin (hcg) vaccines and anti-LHRH vaccines and these will be discussed in the following sections (Sharma and Hinds, 2012).

2.7.1.2.1. Contraceptive Vaccines Targeting Gamete Function

Vaccines targeting gamete function against sperm antigens and zona pellucida (ZP) are among the different possible approaches towards immunocontraception. Sperm has been found to possess auto- and isoantigen properties, which are able to induce antibody production in males and females. The antibody induced by sperm is known to inhibit fertilization in vivo. Fertilisation and fertility have been shown to be blocked by anti-sperm antibodies (ASA) both in vitro and in vivo (Chang, 2015), immunization with sperm stimulated the development of anti-sperm antibodies which led to infertility. However, the entire spermatozoon is not antigen-specific due to sharing the same antigens with various somatic cells. Targeting sperm requires sperm-specific antigens, which means that only a certain part of sperm can be used to produce ASA (Sharma and Hinds, 2012).

The ZP matrix that surrounds the mammalian oocyte plays a critical role in the recognition and binding of sperm to the oocyte during fertilization. Therefore, it can be targeted for the development of contraceptive vaccines. The ZP is composed of three glycoproteins, the 4 vital function of which is to provide a binding site for sperm as well as prevent polyspermy. A variable degree of conservation has been shown in certain regions of the cDNA of the ZP glycoproteins from different species (Chang, 2015). Therefore, there is cross-reactivity between the anti-ZP antibodies produced by a particular species to a different one (Sharma and Hinds, 2012). However, some disadvantages of utilizing ZP glycoproteins including limited amounts of ZP glycoprotein obtained from native sources, batch-to-batch variations in the quality of the purified product, and risk of contamination with other ovarian-associated proteins. But recombinant ZP proteins have proven their potential in antifertility (Chang, 2015).

2.7.1.2.2. Contraceptive Vaccines Targeting Gamete Production

Vaccination against LHRH, leads to anti-LHRH antibodies that neutralize LHRH and prevent the secretion of LH and FSH, resulting in an immunocastration condition (Partidos *et al.,* 2000). Due to having weak immunogenicity, a carrier protein or a T helper epitope is required for LHRH to elicit a potent immune response (Chang, 2015). Similar to many peptide-based vaccines, anti-LHRH vaccines require adjuvants to give effective immunity. Several carrier proteins or adjuvants have been examined with LHRH in a large number of domestic and wild animals (Sharma and Hinds, 2012). However, a range of local and systemic side effects hinder the clinical use of adjuvants both in animals and humans. Improvac is proven to be safe for use. It contains synthetic LHRH which is chemically conjugated to a large protein and is formulated with an aqueous nonoil based adjuvant. Improvac coadministered with porcine somatotropin was shown to increase food intake and reduce fat deposition in tissue in pigs. Two injections elicited a one-year long immune response (Fuchs *et al*., 2009).

Besides its application as a contraceptive, the anti-LHRH vaccine may also be used in the treatment of prostate cancer. Following clinical studies in India, Austria and the UK, the safety of LHRH linked to diphtheria toxoid (DT) vaccine was confirmed in prostate carcinoma patients. These studies further showed that testosterone declined to castration levels with concomitant decline of Prostate Specific Antigen (PSA) in patients generating adequate antibodies. This was a significant clinical benefit to patients (Chang, 2015).

2.7.2. Immunocastration Against Boar Taint

2.7.2.1. Characteristics of Taint Odors

Boar taint has distinctive sensory characteristics which can be described as urine-like, animal-like, sweat-like and faecal-like (Disjksterhuis *et al.,* 2000). It is widely recognized and accepted that the

contribution of androstenone to the unpleasant experience of boar taint is associated with the urine-like perception. However, androstenone is described in other many different ways ranging from unpleasant odour like ammonia, sweaty, dirty, acrid, silage smell, parsnip to a more pleasant sweet floral scent (Disjksterhuis *et al*., 2000). The vast majority of people, as high as 99%, are able to detect skatole and find it unpleasant mainly associated with a faecal-like, musty and naphthalene perception. Skatole at low concentrations is normally used in the making of fragrances and perfumes as it is described as pleasant and with flowery odour, but at high concentrations is associated with unpleasant odours. The use of trained sensory panels is very common to distinguish between androstenone and skatole in boar meat and to detect between different levels of these substances (Disjksterhuis *et al.*, 2000).

2.7.2.2. Responsible Compounds for Taint Odour

Odor, flavor and taste on the quality characteristics of boar meat together are known as "boar taint" (Goa, 2010). Tainted meat and its undesirable odour is perceived by nasal mucosa (Zamaratskaia and Squires, 2009) receptors especially when fat, meat and meat products are exposed to heating. For the appearance of boar taint numerous substances are responsible and of primary importance are androstenone (5-alpha-androst-16-en-3-one) and skatole (3-methylindole) (Substances such as indole (Doran *et al.,* 2002) and 4-phenyl-3-butene-2-one (Weiler *et al*., 2013) also contribute to the appearance of this meat disadvantage. Of particular importance are indole compounds such as indole-methanol (indole-3-methanol), indole-propionic acid (indole-3-propionic acid), indole-acetonitrile (indole-3-acetonitrile) and indoleethanol (indole-3-ethanol) (Zamaratskaiaa and Rasmussenb, 2015)

2.7.2.3. Responsible Metabolisms for Taint Oour

The accumulation of two compounds in pig fat with interrelated metabolism; skatole breakdown in the liver is hindered by androstenone. Androstenone, a pheromone produced in the testis and exhibiting a urine-like and perspiration odour/ smell and is produced by Leydig cells in testes of sexually mature male pigs (Shalev *et al.,* 2003). Skatole, a breakdown product of the amino acid tryptophan in the large intestine, exhibiting a faecal-like and naphthalene odour, and is a by-product of microbial breakdown of tryptophan in large intestine, originating mainly from gut mucosa cell debris (Goa, 2010). Indole (2, 3-benzopyrrole), like skatole, is formed in the large intestine of the pig by intestinal flora. It is generally accepted to be involved in boar taint even if its smell not strong and its contribution was minor compared to skatole. (Jelena *et al.,* 2012). Androstenone is principally related to the sexual development which is largely under the genetic influence, whereas skatole levels in addition to genetic background and hormonal status of the pigs are also controlled by nutritional and environmental factors (Oonk *et al.,* 1998).

2.7.2.3.1. The Biosynthesis and Metabolic Impact of Androstenone

Androstenone is a male sex steroid hormone derived from progesterone that reinforces masculine characteristics. It is produced in the Leydig cells of the testes in parallel with biosynthesis of other steroids during steroidogenesis. It acts as a pheromone, and influences also some metabolic pathways. Androstenone (5α-androst-16-ene-3one) is a pheromone, exhibiting a urine-like odour. The biosynthesis is controlled by the neuro-endocrine GnRH-LH axis (Oonk *et al.,* 1998). The release of GnRH induces the FSH and LH secretion during the pubertal development. The rise in LH levels induces spermatogenesis, with a resulting synthesis of androgens, oestrogens and androstenes. Androstenone is transported via the blood stream to the target tissue, the submaxillary salivary glands (Gower, 2016), where it binds to a specific binding protein, pheromaxein (Booth, 1984). The primary function of pheromaxein is to trap the pheromonal steroids from the blood and to transport them in the aqueous medium of saliva, since the androstenes are lipophilic. But in the salivary glands 21 portions of androstenone is converted to α-androstenol and to a lesser extend to β-androstenol (Gower, 2016).

When a mature boar is aroused by the presence of female pigs, it produces a copious amount of saliva that it is also deposited in the environment by the rubbing action of the boar's snout. The odour of androstenone and the other androstenes facilitate the adoption of the mating stand in oestrous pigs, also the smell of androstenone elicits oxytocin release in sows in oestrous (Gower, 2016).

The androstenone that is not accumulated in the salivary glands is catabolized by the liver. The enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD), also responsible of the production of androstenone in the testis, is responsible for the oxidation of androstenone, the first step in its hepatic catabolism (Doran *et al*., 2002). The resulting products of the first phase, mainly β-androstenol, are then conjugated by sulfotransferases (Sults) along with other specific enzymes, increasing the water solubility of the steroids allowing their excretion through the kidney (Sinclair *et al*., 2006). The variation in androstenone levels in adipose tissue between pigs could be due to differences in the biosynthesis or in the catabolism of the steroid in the liver, linked to a low activity and/or expression of the enzymes controlling them. It is still

not clear what is more important. It is generally accepted that a high production rate of androstenone will lead to an accumulation in the adipose tissue because of the incapacity of the liver to metabolise all the androstenone (Carter *et al.,* 2005).

The androstenone which has not been metabolized in the liver is easily transferred from plasma to adipose tissue, inside the adipocytes due to its lipophilic structure. The most commonly used cut-off levels to sort out tainted meat are 0.5 µg and 1.0 µg of fat androstenone levels are mostly affected by genetic factors controlling its (Sinclair *et al.*, 2006).

2.7.2.3.2. Synthesis and Metabolic Link of Skatole

The indolic compound, skatole is a breakdown product of the amino acid tryptophan produced in the large intestine of the pig by intestinal bacterial flora. Bacterial metabolism of tryptophan in the pig large intestine mainly lead to the production of two volatile lipophilic compounds, indole and skatole; with a third product of the tryptophan metabolism, indolic acetic acid (IAA) that is main precursor of skatole in the hind guts of pigs (Sinclair *et al*., 2006).

While many types of intestinal bacteria are responsible of the conversion of tryptophan to indole and IAA, the strains of only two of the genera containing common intestinal bacteria, the genera *Clostridium* and *Lactobacillus,* are capable of further degradation of IAA to skatole. During the years' different bacteria have been found to produce skatole: a strain of *Lactobacillus helveticus*, *Clostridium scatologenes*, and *Clostridium nauseum*. In contrast with these bacteria (Jensen *et al*., 1995) found that *C. Scatologenes* DSM 757 was able to generate 3-methylindole from tryptophan, but concluded that the organism producing skatole and causing boar taint in pigs is *Lactobacillus sp*. Strain 11201. These Skatole-producing bacteria are normally present in the colon of the pigs but they represent less than 0.01% of the total intestinal micro-flora These bacteria to produce skatole mainly utilize the tryptophan originates from the diet, but also the one that became available with the degradation of the intestinal mucosa (Thornton-Manning *et al*., 1993). This mucosa is characterized by a very high turnover and the resulting cell debris is a source of tryptophan for the IAA-producing bacteria (Jensen *et al.,* 1995).

The skatole produced in the gut can remain in the intestine and excreted through faeces or it can be metabolized in the liver and the degeneration products, as androstenone, are excreted with the urine. The skatole that is not excreted with the faeces is rapidly absorbed by the intestinal mucosa by the venous blood vessels and then it circulates through the peripheral blood stream with a half- life of approximately 60 minutes (Jensen *et al*., 1995). The liver is potentially capable to extract skatole from blood in quantities that greatly exceed what is found under physiological conditions, in some boars a proportion of skatole is accumulated in the adipose tissue because the liver is not able to metabolize all of it. The reason must be found in the influence of the sex steroids, androstenone in particular on the skatole metabolism in the liver (Thornton-Manning *et al.,* 1993).

Skatole metabolism in the liver takes place in two phases of; oxidation and conjugation. The enzymes cytochrome P4502E1 (CYP2E1) and the cytochrome P4502A6 (CYP2A6) are the main enzymes in the first step of the skatole metabolism; the second stage is regulated by the sulfotransferease A (SULT1 A), a hydroxisteroid sulfotransferases (HST) As the activities of these key enzymes are positively correlated with the skatole metabolism, a pig producing high levels of these liver enzymes will present low level of skatole in the fat (Zamaratskaia *et al*., 2005).

High levels of skatole in the fat can be related to high levels of androstenone, due to its effect on skatole metabolism. Demonstrated the competitive inhibition of androstenone on the formation of some skatole metabolites in liver microsomes. This antagonizing effect of androstenone was then confirmed by studying primary cultured hepatocytes. These authors found that while skatole induced the expression of the protein CYP2E1, androstenone antagonized this effect with a consequent accumulation of skatole in the fat due to a reduced metabolism. This was later explained by (Moreira *et al.,* 2015) whom found that CYP2E1 promoter is activated by the transcription factors COUP-TF1 and HNF-1 and that the promoter activity is decreased by androstenone, which inhibit the binding of COUP-TF1 to the promoter. The decreased levels of sex steroids, in particular androstenone, following castration was associated with an increased expression of P4502E1 with a consequent reduction in skatole levels in the fat. The increase in testicular steroid concentration initiates an increase in skatole levels at young age and there is a positive correlation between the levels of free androestrone and skatole level (Fuchs *et al.,* 2009).

Skatole easily transferred from plasma to adipose tissue and there is a correlation between plasma and fat levels of skatole, but it is yet not completely understood which is the physiological function of skatole and if it has any target tissues (Zamaratskaia *et al*., 2005).

Skatole has a toxic effect on many microorganisms; it has a bacteriostatic effect on gram-negative enterobacteria and it is toxic for rumen protozoa (Goa, 2010) demonstrated that skatole is the etiologic agent causing acute bovine pulmonary edema

and emphysema in cattle, acting as pneumotoxin that causes the degeneration of certain lung tissues. Also has the ability to affect the production of serotonin and in high concentration to cause the haemolysis of bovine erythrocytes. However, not toxic effect on pigs as it has in ruminants or microorganisms (Jelena *et al.*, 2012). It is not easy to define a precise limit to the level of skatole. The most common used threshold values are 0.2 and 0.25 μg/g of fat (Zamaratskaia and Berger, 2014)

2.7.2.4. Effect of Gender on Taint Odor

The reason why intact male pigs usually have higher concentrations of skatole in fat compared with female and castrated male pigs is probably related to gender differences in metabolic clearance of skatole (Jelena *et al.,* 2012). These differences are believed to be due to involvement of testicular steroids in skatole metabolism. The effect of testicular steroids on variations in skatole levels has been intensively studied in recent years both in vitro and in vivo. Although evidence has accumulated that at least some testicular steroids can inhibit hepatic skatole metabolism, the identity of the steroid or group of steroids that is the major inhibitor of skatole metabolism is unclear. In contrast to many other species, intact male pigs produce higher oestrogen levels compared with female pigs. In vivo skatole levels in fat are positively correlated with the levels of oestrogens (Jelena *et al.,* 2012). In vitro microsomal studies demonstrated that the activity of skatole-metabolising enzyme cytochrome P4502E1 (CYP2E1) is reduced in the presence of 17b-oestradiol This might be the cause for the increased skatole levels in intact male pigs (Chen *et al.,* 2007).

2.7.2.5. Perception of Taint Odour

The differences in meat acceptability by consumers, i.e. Large variations in the detection of tainted meat odour in different countries, are influenced by different genetic structures of pig populations, farm management, differences in culinary habits and methods of evaluation, a native origin of the consumers, age, gender and the fact that only a part of the population is sensitive to the smell of androstenone (Weiler *et al*., 2013). Most of people show higher sensitivity to the smell of skatole, compared to the smell of androstenone. Skatole could be perceived by 99% of consumers (Weiler *et al.*, 2013). Perception ability varies, decreases in men and increases in women with age. Sensitive people in Germany (32 %), Spain (46–48%), Norway (39%) and Belgium (45%) were lower than it was observed in France which ranged between 63% and 74% (Bonneau *et al.,* 1994).

2.8. Application of Immunocastration

The novel technology, Immunocastration (AVMAAWD, 2009) is particularly interesting when fattening animals to higher age and weight and represents good solution for special production systems (heavy pig, extensive outdoor, ecological) (Zamaratskaia and Berger, 2014). Immunocastration vaccines composed of GnRH induce meat-quality improvement and better daily growth rate (Park *et al.*, 2015) and the average yield of lean meat was significantly improved when compared to the effect of surgical castration (Carter *et al.,* 2005).

Immunocontraception has been proposed as an acceptable, effective and achievable way to control the wild animal population (Chang, 2015). Generally the scientific explanation on the significance of castration immunologically is to reduce the management problems related to aggressiveness, manage sexual behavior, prevent unwanted pregnancies, avoid the occurrence of meat taint and avoid an unpleasant odor present in meat (Doran *et al*., 2002), renders the animals more docile, mitigate sexual conduct such as sodomy and provide a better carcass finish with a higher percentage of marbling and a greater subcutaneous fat thickness, which is desired by the slaughter house industry because the carcass is protected against the effects of refrigeration, thus avoiding dark meats and meats with depreciative visual aspects. Anti-GnRH immunization also addresses boar taint from cryptorchid and intersex animals (Zamaratskaia and Squires, 2009).

Vaccination against LHRH leads to anti-LHRH antibodies that neutralize LHRH and prevent the secretion of LH and FSH. This results in a reduced level of sex steroid and thus anti-LHRH vaccines can be used as in the treatment of hormone-dependent cancers or as immunocontraceptives (Chang, 2015) in both male and female models in animals. A large number of studies have targeted LHRH for anti-cancer therapy due to the high level of expression of LHRH receptors in a number of different malignant human tumours, including breast, ovary, endometrium, and prostate cancers (Sharma and Hinds, 2012).

2.9. Problem on the Application of Immunocastration

The interesting effects of immunocastration is with a disadvantage of increasing fat deposition when compared with EM (Albrecht, 2013). Basically the main problem for wider use of immunocastration seems to be a fear related to consumer acceptance. Contrary to some parts of the world (Brazil, Australia, and New Zealand) where it is widely used, European consumers seem to be more prudent and conservative. However, consumer surveys conducted in Switzerland, Norway and Belgium indicate that the fear may be overrated and that consumers would accept it, if properly informed (Janett *et al.*, 2012). Accidental self-injection could negatively affect reproduction physiology of both men and women. Therefore

extreme caution should be exercised when administering the product (Jago *et al*., 1997).

2.10. Gaps in Researches Investigation

Although evidence has accumulated that, at least some testicular steroids can inhibit hepatic skatole metabolism, the identity of the steroid or group of steroids that is the major inhibitor of skatole metabolism is unclear (Jelena *et al.,* 2012, Turley *et al*., 2011). Since the metabolism and physiology relating to skatole is yet to be fully understood, it is difficult to draw conclusions from the research (Jelena *et al.*, 2012). The variation in androstenone levels in adipose tissue between pigs could be due to differences in the biosynthesis or in the catabolism of the steroid in the liver, linked to a low activity and/or expression of the enzymes controlling them. It is still not clear what is more important (Batorek *et al.*, 2015).

2.11. Future Perspectives

A great deal of progress has been made since the disappointment of the original vaccine clinical trials almost 20 years ago. Advancements in antigen design, improved formulations, inclusion of molecular adjuvants and physical methods of delivery have greatly enhanced the immunocastration. Castration which detoriates animal welfare as it causes pain and inflammation in the animal. It is therefore criticized as a production practice and a group of stakeholders have agreed to cease traditional castration in Europe by year 2018 (Niemi *et al*., 2015). A future direction for developing anti-LHRH persistent antibodies could involve inclusion of another T helper epitope to provide a longer lasting immune response for castration and treatment and prevention of hormone dependent cancers (Chang, 2015).

3. Conclusion and Recommendation

Immunocastration is the injection of GnRH analog conjugated to a foreign protein and combined with an adjuvant, to initiate transient formation of anti-GnRH antibodies that can bind and inhibit the action of endogenous GnRH. The vaccination induce inhibition of LH and FSH with a sequel of absence of steroidogenesis and spermatogenesis. That is temporary castration to induce infertility and prevent boar taint without the need of stressful and painful surgical castration. It is to deactivate testicular functions by neutralization of the hormones of the hypothalamic-pituitary-gonadal axis of agricultural and pet male animals. The antibody neutralizes GnRH, and as a result hypothalamic-pituitary-gonadal axis is blocked, testes growth and sexual steroids synthesis are effectively inhibited. Physiologically the immunocastration becomes effective in a week following second vaccination. Within 4-6 weeks' interval between V_2 and slaughter androstenone and skatole levels in fat tissue are already below the limit of sensory detection. Immunocastration is quite persistent although it should not be permanent. Some animals do not react to it due to poor immunological response or improper vaccination their number is low. Even if the first studies on immunocastration were performed more than twenty years ago, but its distribution and application were limited. Immunocastration have been proven to be effective in the castration and boar taint prevention effect. For effective application with worldwide distribution of immunocastration and control of the negative of impact of boar taint the following fundamental worthwhile measures are forwarded as recommendation**:** Conduct study/review on the treatment of hormone dependent cancer with the effect of immunocastration, carryout further research on the mechanisms of action of immunocastration on female, fill and contribute to the research gaps of the area and prefer immunocastration than surgical castration to apply.

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