Theses of a doctoral (PhD) dissertation

INTERACTIONS BETWEEN NUTRITIONAL STATUS, METABOLIC BIOMARKERS AND REPRODUCTIVE FUNCTION IN DOGS

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Table of contents

1. Background and objectives

The common incidence of obesity, consequential metabolic disorders and related reproductive problems has given a huge momentum to the research about the reproductive role of hormones and mediators which are involved in metabolic processes, like leptin, insulin, or insulin-like growth factor (IGF1). The reproductive effect of the most important metabolic parameters has been widely investigated in human studies, the most common laboratory species (mice and rats) and in cattle and sheep, where the primary goal is to improve the reproductive parameters.

Nowadays there is an increased emphasis on the diagnosis and prevention of obesity related metabolic problems in companion animals as well. Moreover, due to the genetic and physiological similarities, dogs are commonly used as model species in human studies as well. Despite the fact that obesity is a growing issue in cats and dogs- mirroring the tendency in human population-, we know less about the correlation between body condition and nutritional factors, like leptin and their role in reproduction, than in other species. One of the main goals of our study was to start investigating the most important biomarkers in connection with nutritional status, and metabolism, which proved to have effect on reproduction in other species.

Based on the review of human studies, the range of parameters to be examined as part of the condition determination, turned out to be much wider than the indicators used in everyday veterinary practice. Due to this, first we aimed to examine those condition parameters, that haven't been studied in dogs yet. In bitches, we **investigated the applicability of morphometric and bioimpedance measurements and the relationship between body fat percentage and serum leptin concentration determined from the measurements (1.).**

Applying human studies to dogs, a more detailed research about the types of obesity, its regionality, and the development of methods for the detection of subcutaneous and visceral adipose tissue distribution may be of great importance. In this context, in dogs, we examined the presence of different obesity types, based on body fat distribution. We aimed to **develop a method for determining the distribution of adipose tissue in dogs and to investigate the effect of animal-specific adipose tissue regionality on serum leptin levels and changes in insulin sensitivity (2.).**

Besides the distribution of adipose tissue, the number and morphology of adipose-forming cells can also fundamentally affect the metabolic activity of fat tissue, therefore we examined **the size of adipocytes in fat depots, as an unknown parameter in the canine species (3).**

In the second part of our work we investigated the role of leptin and IGF1 (hormones which reflect nutritional status and play an important role in the local (autocrine / paracrine) regulatory processes of the reproductive organs) in reproductive functions of male and female dogs. Leptin

is one of the major regulatory factors of food intake. Its blood level is primarily determined by the degree of fat store saturation, and is therefore a biomarker suitable for determining body condition. The nutritional status of the body affects reproductive biological functions at several points in both genders. Leptin is one of the most important messenger molecules of nutritional status' effect on reproductive biological processes. However, the precise understanding of its role is complicated by the fact that in addition to adipose tissue, many other organs and tissues, including genital cells, produce this protein and express its receptor.

Thanks to this, it is likely, that the peripheral leptin also has auto-or paracrin effect in addition to the effect of circulating leptin levels. To **clarify the reproductive role of leptin in the canine species, we described the cellular localization of leptin and the leptin receptor in canine-derived ovaries based on our own study (4).**

In addition, in bitches we examined the combined **effect of body condition and hormonal changes on serum leptin concentration (5).**

The level of IGF1 in the circulation, which is the most important effector molecule of growth hormone (GH) produced by the liver, is a potential biomarker of short-term changes in nutritional status, but its blood levels can vary significantly depending on the size of dogs. In addition to the GH/IGF1 axis, IGF1, locally produced in the periphery in various tissues, such as mammary tissue, and then entering the blood stream, can cause systemic hormonal changes and effects. Produced in the ovaries, it can also affect follicular maturation or corpus luteum function through auto-/paracrine pathways. These processes are still poorly explored areas in dogs, therefore we first investigated gene and **protein-level expression of IGF1 and IGF1R on ovaries, corpus luteum from pregnant and non-pregnant animals, and intracellular localization of these proteins in preovulatory follicles (6).**

Some metabolism regulating molecules, including leptin and IGF1, are expected to be involved in the regulation of sexual function at several points in males as well, however, their influencing role is still considered less clear. During our work, we **investigated the presence and possible role of leptin and insulin-like growth factor-1 in testicular tissues from healthy juvenile and sexually mature dogs. The expression of leptin, leptin receptor, IGF1, and IGF1 receptor was examined in the testes and epididymis of intact males and deslorelin-implanted animals modeling infertility (7).**

2. Summary

The effect of nutritional status and certain metabolic factors on reproductive function has long been studied in both sexes. First, We aimed to investigate the parameters for body condition estimation known in human practice and not yet used in dogs. In the case of female dogs, we investigated the applicability of morphometric and bioimpedance measurements, as well as the relationship between body fat percentage determined on the basis of these and serum leptin concentration. Based on the analysis of CT scans, we examined the distribution of body fat and its effect on serum leptin levels and insulin sensitivity. After that, we studied the size of fat cells in larger fat depots using our own method developed for the analysis of native fat samples. A strong positive correlation was described between serum leptin concentration and body fat percentage calculated from morphometric measurements, however, the result of the bioimpedance measurement showed no correlation with serum leptin concentration. In some individuals, there was a fundamental difference in adipose tissue regionality, and blood levels of leptin were correlated with subcutaneous and visceral fat at lumbar region, whereas visceral adipose tissue had a stronger effect on leptin levels. We showed that the size of adipocytes in subcutaneous and visceral stores increases in parallel with condition and leptin concentration. Nevertheless in some individuals the average adipocyte size was higher or lower than the average for the condition group, which makes it probable that hyperplastic or hypertrophic-type obesity also exists in dogs. In the second part of our work, we investigated the role of leptin and insulin-like growth factor-1 (IGF1) as factors reflecting nutritional status in reproductive function in bitches and males.

Although leptin expression was detected in some ovarian cell types, the effect of sexual cycle stage and serum progesterone levels on serum leptin levels could not be demonstrated. We were the first to investigate gene expression and protein expression of IGF1 and its receptor in the ovaries, corpus luteum of pregnant and non-pregnant animals, and the cellular localization of these proteins in preovulatory follicles. Based on our results, we conclude that IGF1 may have a para- /autocrine effect on granulosa and theca cells of preovulatory follicles in dogs, stimulating their proliferation and steroid production. We have shown that IGF1 and its receptor are most strongly expressed in the early luteal phase, i.e. in the developing corpus luteum, which is not yet strongly gonadotropin-dependent, thus confirming the theory that corpus luteum development, initial growth, and progesterone production are most affected by locally produced para-/autorkrine factors such as IGF1. A decrease in IGF1 expression later in the luteal phase suggests a decrease in the role of IGF1. The appearance and possible role of leptin and IGF1 in the testes and epididymis of animals from healthy juvenile and mature dogs and infertility modeling animals

treated with a deslorelin-containing implant were also studied. Based on our results, we hypothesize that the leptin and IGF1 systems play a variable role in testicular function in dogs as well, depending on age and developmental stage. Significant expression of leptin and IGF1 genes and/or increased protein expression in certains cell types of immature testis indicate the role of these hormones in the maturation and proliferation of gonocytes and Sertoli or Leydig cells. In adult male dogs, leptin may play a role in the maturation and differentiation of spermatocytes and spermatitis, while IGF1 is likely to play a role in controlling spermatogonial proliferation and steroidogenesis of Leydig cells. In mature animals, the strongest leptin expression was shown in the corpus epididymis, suggesting that ductal epithelium plays a different role from region to region. In the case of prepubertal and deslorelin-treated animals, leptin expression could not be detected in any of the regions of the epididymis, suggesting a testosterone-dependent or spermatozoa-regulated role of leptin in functions of ductal epididymal epithelium.

3. New scientific results

1. Applicability of morphometric and bioimpedance measurements and their relation to serum leptin concentration in dogs: A strong positive correlation was described between serum leptin concentration and body fat percentage calculated from morphometric measurements (PC = pelvic circumference, in cm; and HS = hock to stifle, length from right rear limb from the calcaneum tuber tothe mid-patellar ligament, in cm). The morphometric measurements show a stronger correlation with serum leptin concentration than the BCS score. The results obtained by bioimpedance measurement show a weaker correlation with body weight, BCS category and show no correlation with serum leptin concentration.

2. Fat distribution and its metabolic consequences in dogs: The method we have developed for the analysis of CT scans may help in the analysis of the effects of obesity, in particular in the examination of the relationships between fat distribution and metabolic diseases associated with obesity. In some individuals, there is a difference in the regionality of adipose tissue. In most of the studied animals, the fat stores occupied almost the same area on the images taken at the L2, L3 level, however, in some individuals the dominance of the visceral depot and in others the dominance of the subcutaneous depot was characteristic. Serum leptin levels correlate with the characteristic distribution of adipose tissue at the lumbar level (L2, L3), especially with the amount of visceral adipose tissue. The relationship between visceral fat and leptin levels is not significantly affected by fasting insulin levels or the degree of insulin sensitivity. There is a positive correlation between the ratio of visceral to subcutaneous fat and the blood insulin level, as well as the parameters of fat distribution and the degree of insulin resistance (HOMA-IR index value).

3. Significance of adipocyte size in canine species: Through the preparation of a novel native preparation, we have obtained a well-functioning and fast method of adipocyte size determination. The size of the fat cells that make up the two main fat stores correlates with body condition and leptin levels. There was no difference in the average size of the fat cells that make up the depots within each individual. In addition to fat cell sizes that are variable in synchronism with the condition, in some individuals, the average adipocyte size is greater than or less than the mean size characteristic of the condition group, which may indicate the appearance of hyperplastic or hypertrophic obesity.

4. Analysis of leptin and the leptin receptor (protein appearance) in canine ovaries by immunohistochemistry: Strong immunoreactivity for leptin was detected in granulosa cells. Theca interna cells show less strong staining, while the theca externa layer and the ova itself appeared to have weak sporadic leptin immunostaining. Strong immunoreactivity for IGF1R was detected in granulosa cells and theca interna cells of preovulatory follicles and in the ova.

5. Changes in serum leptin concentration during the estrous cycle of the female dog: Neither estrous cycle stage nor serum progesterone levels have an effect on serum leptin levels of dogs in either normal or higher BCS condition group.

6. Protein expression and localization of IGF1 and IGF1R in canine ovary: In ovaries removed in the follicular phase IGF1 and IGF1R protein was strongly expressed in granulosa cells and theca interna layers of the anthral follicles showing intense proliferation and luteinisation, however only sporadic, weak staining characterizes the wall layers of atreticizing follicles. IGF1 and its receptor are most strongly expressed in the early luteal phase, in developing corpus luteum that is not yet strongly gonadotropin-dependent, and then decreases later in the luteal phase. While in non-pregnant animals IGF1 mRNA expression gradually decreases from the middle of luteal phase, in pregnant bitches this decrease is shifted to the period of prepartum luteolysis. In non-pregnant bitches, IGF1R mRNA expression does not change during the luteal phase, whereas a significant increase in pregnant individuals is characteristic at the same time as prepartum luteolysis. In pregnant bitches, IGF1 expression in the corpus luteum decreases with prepartum luteolysis, whereas in case of the aglepristone-induced luteolysis at mid-gestation the luteal IGF1 and IGF1R mRNA expression remain unchanged.

7. The role of leptin and IGF1 in the reproductive function of males: Leptin gene expression in prepubertal (two months) animals does not change significantly with puberty. In the developing testis, the expression of protein bound to Sertoli and Leydig cells is shifted to sperm precursors after the transformation of gonocytes into spermatocytes and spermatids. Immature gonocytes appearing in prepubertal testes as well as elongated spermatoids present in mature animals did not show leptin protein expression, while in the testis of sexually mature dogs, IGF1 and IGF1R protein signals were found in spermatogonia within the seminiferous tubules. Testicles of mature animals treated with deslorelin show a marked decrease in leptin expression. In the epididymis of mature animals, leptin expression is characteristic in all regions, most strongly in the body region, which is not characteristic in any region of the epididymis in prepubertal or deslorelin-treated animals. Leptin receptor protein expression in prepubertal animals appears in Leydig cells, and then, after puberty, it most characterizes sperm precursors and appears on elongated spermatids. Expression of leptin and leptin receptor protein in Leydig cells is characteristic only in samples from prepubertal individuals. Protein expression of IGF1 as well as IGF1R in prepubertal individuals can be detected in Leydig cells as well as in gonocytes and then in Leydig cells and spermatognias after puberty. Sertoli cells are characterized by weaker expression of both proteins in the prepubertal age. In parallel with puberty, testicular IGF1 gene expression decreases. In contrast, germic epithelium induced by deslorelin treatment and decreased testosterone and

gonadotropin levels had no effect on expression. IGF1R gene expression is more intense not only in the prepubertal but also in the deslorelin-treated group.

4. Publications

4.1. Published/accepted publications in lectorated scientific journal with impact factor

Müller, L., Kollár, E., Balogh, L., Pöstényi, Z., Márián, T., Garai, I., Balkay, L., Trencsényi, G., Thuróczy, J.: **Body fat distribution and metabolic consequences - Examination opportunities in dogs.** Acta. Vet. Hung. 62. 169-79, 2014.

Müller, L., Kowalewski, M. P., Reichler, I. M., Kollár, E., & Balogh, O.: **Different expression of leptin and IGF1 in the adult and prepubertal testis in dogs**, Reprod. Domest. Anim., *52(2)*. 187–192, 2017.

Müller, L., Mester, L., Nagy, A., Hanácsek, R., Janett, F., Cseh, S., Reichler, I. M., Balogh, O.: **Deslorelin tartalmú implantátummal (Suprelorin 4,7 mg) végzett kémiai kasztráció hatása a spermaminőségre, a testtömegre, a vér egyes biokémiai paramétereire és a vérképre ivarérett Beagle kan kutyákban Irodalmi összefoglaló és saját tapasztalatok**, Magy. Állatorv. Lapja 140. 727-736, 2018.

Balogh, O., Müller, L., Boos, A., Kowalewski, M. P., Reichler, I. M.: **Expression of insulin-like growth factor 1 and its receptor in preovulatory follicles and in the corpus luteum in the bitch**, Gen. Comp. Endocrinol., 269. 68–74, 2018.

Müller, L., Kók, E., Kollár, E., Balogh, O.*, Thuróczy, J.*: **A vérszérum leptinkoncentrációjának változása az ivari ciklus és a testzsírmennyiség függvényében szuka kutyában: Irodalmi áttekintés és saját tapasztalatok**, Magy. Állatorv. Lapja 141. 411-424, 2019.

4.2. Conference presentation

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Müller L., Kollár E., Balogh L., Thuróczy, J.: **A hasi zsíreloszlás szerepe a metabolikus státusz kialakításában – vizsgálati lehetőségek és azok jelentősége kutya fajban**, MTA Akadémiai Beszámolók, Budapest, 2012.

Müller, L.*, Balogh, O.*, Kollár, E., Gürler, H., Kowalewski, M.P., Reichler, I.M.: **A pilot study on immunohistochemical detection of leptin and its receptor in the canine testis and epididymis**, Pilotstudie für immunhistochemische Erkennung von Leptin und dessen Rezeptor im kaninen Hoden und Nebenhoden. *Reprod Dom Anim* 50(Suppl.1). 55-56, 2015. *equal contribution

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Müller, L., Balogh, O.: **A leptin és az inzulinszerű növekedési faktor-1 szerepének vizsgálata egészséges kutyákból származó here szöveteken**, 23. Szaporodásbiológiai Találkozó, Cegléd, 2017.

Müller, L., Kowalewski, M.P., Reichler, I.M., Balogh, O.: **Leptin, leptin receptor, and androgen receptor expression in the testis and epididymis of adult male Beagles treated with a 4.7mg deslorelin implant**, Proceedings of the **21st** EVSSAR Congress, Venice, Italy, 2018.

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