Clinical Sciences and Pathology Group Seminar

Abstract book

Auditório

Ciências Florestais

21th November

Universidade de Trás-os-Montes e Alto Douro

Associação de Estudantes de Medicina Veterinária da Universidade de Trás-os-Montes e Alto Douro

Fundação para a Ciência e a Tecnologia

Project PEst-OE/AGR/UI0772/2011

About the Clinical Sciences and Pathology group in CECAV

Program for the Pathology and Clinical Sciences Seminar

The 21th November 2012

Auditório Ciências Florestais – Campus da UTAD

9:00 – Opening of the Secretariat

9:30 – Seminar Opening

9:45 - Session 1 – Reproduction and Reproductive Pathology

Main Lecture by Professor John F. Edwards (DVM, PhD, DACVP) From Texas University:

"Ovarian Pathology for Veterinarians - Pathology 101"

11:00 - Short communications (10 minutes+5 for discussion for each presentation):

1. Single layer centrifugation (Androcoll-E™) improves stallion sperm motility and viability. Ana L. Costa, A.L. Martins-Bessa, A. Rebello de Andrade, T. Guimarães, M.R. Rebordão, P.P. Bravo, M.J. Correia, J. Colaço, I. Gaivão, A. Rocha
2. Oxidative stress enzymes in the canine endometrium during the oestrous cycle. Celso Santos, M.A. Pires, D. Santos, R. Payan-Carreira
3. TNF immunoreaction in canine cystic endometrial hyperplasia. Carla Santos, H. Vala, M.A. Pires, R. Payan-Carreira
4. Early embryo collection in dogs. Sónia Miranda, R. Payan-Carreira, R.M.L.N. Pereira
5. Contribution for the Study of Endometrial Adenocarcinomas of the Queen. Ana Laura Saraiva, R. Payan-Carreira, F. Gärtner, M. Tavares Pereira, M. Cunha, M.A. Pires
6. Reproductive pathology in Laying Hens. Sónia Saraiva, A. Esteves, C. Saraiva, F. Seixas

12:30 - Lunch Break

14:30 - Session 2 – Clinical pathology

Main Lecture by Professor John F. Edwards (DVM, PhD, DACVP):

"Lessons about Schmallenberg Virus Learned with Cache Valley Virus"

15:30 - Short communications (10 minutes+5 for discussion for each presentation):

1. Lymphocyte population in the granulomatous lesions of wild-boars (*Sus scrofa*) and red-deer (*Cervus elaphus*) suspected of tuberculosis. A.M. Matos, S. Andrade, M.A. Pires, A.C. Coelho, M.L. Pinto
2. The role of synaptic brain mitochondria dysfunction in aging and Alzheimer's disease. Vera F. Monteiro-Cardoso, M.M. Oliveira, F. Peixoto, R.A. Videira.

16:00 - Coffee Break

16:15 - Session 3 – Oncology:

Main Lecture by Professor José Manuel Costa from the Instituto Nacional de Saúde Dr Ricardo Jorge, Director of the Center for the Study of Animal Science (CECA)

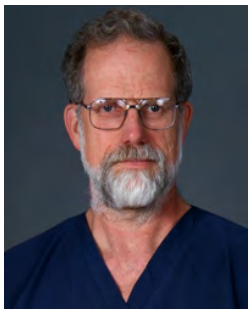
"Helminthes as Class I Carcinogens: lessons from the scientific work in CECA, a renewed FCT R&D Unit."

17:15 - Short communications (10 minutes+5 for discussion for each presentation):

1. Estudo da expressão de TGF β1 e TGF β2 alterações e lesões de mama da gata. F. Seixas, M.J. Novais, M.A. Pires
2. Application of everolimus in three bladder cancer cell lines: evaluation of its effectiveness. Rosário Pinto-Leite, R. Arantes-Rodrigues, C. Palmeira, A.A. Colaço, P.A. Oliveira, L. Santos
3. Development of a monoclonal antibody against the canine CCR2. Teresa Raposo, D.J. Argyle, I. Pires, F.L. Queiroga
4. *In vivo* and *in vitro* effects of meloxicam on bladder cancer. R. Arantes-Rodrigues, R. Pinto-Leite, C. Palmeira, L. Santos, A.A. Colaço, P.A. Oliveira
5. Tumour-associated macrophages in canine melanocytic tumours. Isabel Pires, L. Coelho, J. Oliveira, F.L. Queiroga
6. Caracterização de uma população de células com propriedades estaminais em cancro de mama de cadela. Daniela Pereira, A. F. Vieira, F. Schmitt, J. Paredes, A. Gama

18:45 – Closing remarks

Short Resume for the Main Speakers

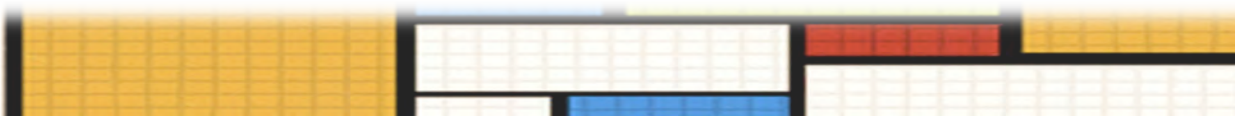


Professor John F. Edwards:

Professor at the Department of Veterinary Pathobiology (College of Veterinary Medicine and Biomedical Sciences) at Texas A&M University, he is diplomate by the American College of Veterinary Pathologists. With large experience in teaching and post-graduate formation, Professor John Edwards constant participation on Seminars and Courses on Food Safety and Pathology worldwide and its numerous publications together with his research expertise gives testimonial of his high achievements as teacher and researcher.
Professor John F. Edwards is also a member of the Scientific Advisory Committee for CECAV.

Professor José Manuel Costa:

Session 1 - Reproduction and Reproductive Pathology



Ovarian Pathology for Veterinarians, "Pathology 101"

Edwards JF (*DVM, PhD, DACVP*)

College of Veterinary Medicine and Biomedical Sciences. Texas A&M University. College Station, TX, USA

This presentation is a review of basic ovarian pathology aimed at the expected audience of Veterinary Students, Graduate Veterinarians and Veterinary Faculty. Whether you are a student, pathomorphologist or a field theriogenologist, we are all biologists that must deal with a variety of species. Fortunately, many principles of anatomy, physiology. endocrinology and pathology are shared among the species; therefore, a one-hour review of the ovarian pathology of domestic species is possible. However, anatomic variations do exist and physiologic specialties evolved as species diverged. Unfortunately, erroneous observations from the past have persisted and old, accurate observations have been rediscovered and given a new name sometimes with inaccurate interpretation. Husbandry changes produce new lesions, a form of job security for the future. Some conditions are unpublished and known only to experts. Perhaps most important, many new observations have yet to be made. The presenter will review the pathology exposing his common, uncommon, misinterpreted, unpublished observations on ovarian Veterinary Pathology.

Single layer centrifugation (ANDROCOLL-E™) improves stallion sperm motility and viability

Costa AL[1], Martins-Bessa AL[1], Rebello de Andrade A[2], Guimarães T[3], Rebordão MR[4], Bravo PP[4], Correia MJ[5], Colaço J[1], Gaivão I[1], Rocha A[3]

[1]CECAV, UTAD, Vila Real; [2]MIMV- UTAD; [3]CECA, UP, Porto; [4]ESAC-Coimbra; [5]Fundação Alter Real, Alter do Chão

A significant number of stallions produce low quality ejaculates with high sensibility to chilling. Single Layer Centrifugation (SLC) with Androcoll-E™ has been presented as an efficient method of selecting good quality spermatozoa.

The aims of this study were to apply the SLC protocol to semen samples and observe its effects on sperm motility, viability, acrosome and DNA integrity. Samples were preserved at 5°C for a maximum of 72 hours.

For that, one single ejaculate from 12 stallions was collected, extended 1:1 in Kenney® extender and split in two aliquots: SLC-selected and control at a final concentration of 50×10^6/mL. The SLC-selected aliquot was centrifuged at 500×g/20 minutes and, after that, the *pellet* was ressuspended in the same extender. Both aliquots were chilled and stored at 5°C and spermatozoa were evaluated for motility (0, 24, 48 and 72h), viability (with fluorochromes SYBR-14, propidium iodide and PE-PNA at 24, 48 and 72h) and DNA integrity (comet assay at 48h). Results were analyzed with ANOVA and Independent Samples T-test (SPSS 19.0; IBM) depending on the type of sample.

In the SLC-selected aliquots, there was a significant improvement in terms of progressive motility (0 h: $P = 0.005$; 24 h: $P < 0.001$; 48 h: $P < 0.001$; 72 h: $P < 0.001$) and percentage of live spermatozoa with intact acrosome (24 h: $P = 0.003$; 48 h: $P = 0.003$; 72 h: $P = 0.004$). The DNA damage (in Arbitrary Units) was not different between SLC- selected and control samples ($P > 0.05$).

In conclusion, SLC with Androcoll-E™ improved semen quality prolonging sperm longevity of chilled semen ($P = 0.012$). Therefore, this method reveals to be a useful technique for selecting spermatozoa and maintain sperm quality during storage.

Acknowledgemnts:

FCT, PTDC/ CVT/108456/2008 *"Development of methods to increase the fertilizing ability of chilled and frozen stallion semen: a multidisciplinary approach".*

Oxidative stress enzymes in the canine endometrium during the oestrous cycle

Santos C[1,2], Pires MA [2], Santos D[1], Payan Carreira R [2]

[1]*CITAB - ECVA – UTAD, Vila Real, Portugal;* [2]*CECAV, ECAV - UTAD, Vila Real, Portugal*

The oxidative stress is often defined as the imbalance between oxidants and antioxidants, either by overproduction of reactive oxygen species or by dysfunction of the antioxidant systems. The ovarian cycle correlates with changes in cell death, proliferation and differentiated products secretion both in the endometrial and stroma tissues. In order to support endometrial homeostasy antioxidant molecules have an important role minimizing cell damage caused by oxidant molecules such as reactive oxygen species (ROS). The enzymatic antioxidant defence compromises enzymes such as superoxide dismutases, catalases, glutathione, and thioredoxin peroxidasis. In this study, we pretend to study the variation of the antioxidant enzymes along the ovarian cycle.

Twenty five post-pubertal, healthy non-pregnant bitches with ages ranging from 10 months to 6-year old, submitted to elective ovariohysterectomy (OVH) were used in this study, and grouped according to their oestrus cycle stage (anoestrus, prooestrus, oestrus, early dioestrous and full dioestrus), on the basis of the progesterone concentration in blood samples, in vaginal cytology and in ovarian and uterine morphology. From each uterine horn, at its middle portion, one fragment with 1,5 cm in length was collected immediately after the surgery and snap frozen in liquid nitrogen during the transport until the laboratory, and thereafter maintained frozen at -80°C until analysis. From frozen unthawed sample tissues, dissected endometrium was obtained and homogenized in phosphate buffer saline, centrifuged and the resulting supernatant was used for enzymatic analysis (SOD, CAT, GST, GR, GPX).

A reduction in Superoxide dismutase (SOD) accompanies the stages were the blood progesterone are over the 2ng/ml. Yet a small decrease was found in early dioestrus stage in comparison to oestrus and full dioestrus. Catalase (CAT) activity shows an inverse behaviour, being marked increased during progesterone-associated stages, in particular during the full dioestrus. A small decrease in CAT values was found in early dioestrus in comparison to the observed for oestrus and dioestrus. The Glutathione S-tranferase (GST) activity was found to be higher at oestrus, thereafter decreasing, with the lowest levels recorded for anoestrus and prooestrus. Glutathione reductase (GR) has similar low levels of activity from anoestrus to oestrus and higher levels at both early dioestrus and dioestrus. The highest value for Glutathione peroxidase (GP) was found in prooestrus whilst in progesterone-associated stages it tend to show lower values, in particular for the early dioestrus, when GP activity reached its lowest value.

Accumulation of oxidant molecules in the cell or tissue may induce severe damages unless there is an imbalance with antioxidant defence molecules or pathways, such as the enzymatic. The expression of these various antioxidants physiologically varies along the endometrial cycle, with the early dioestrus (where implantation occurs) showing a clear distinctive pattern. The present study also provided reference data for potential use in pathologic conditions.

TNF immunoreaction in canine cystic endometrial hyperplasia

Santos CA[1], Vala H [1], Pires MA [2], Payan Carreira R [2]

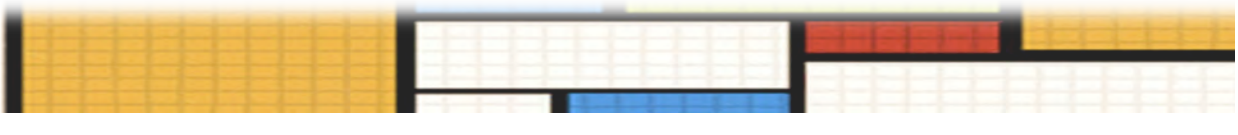
[1] *Center for Studies in Education, and Health Technologies. Agrarian School of Viseu, IPV, Portugal;* [2] *CECAV, University of Trás-os-Montes e Alto Douro, Vila Real, Portugal*

Canine pyometra, one of the most important pathologic conditions of the canine uterus, is frequently accompanied by endometrial glandular hyperplasia and cystic transformation of the endometrial gland (cystic endometrial hyperplasia - CEH). TNF (Tumor Necrosis Factor), a pleiotropic inflammatory cytokine, has been identified in the uterus of several species and its expression was shown to change in some pathological conditions. The aim of this study was to evaluate TNF immunoexpression in canine endometrium with CEH/pyometra, and to compare it with late postpartum and control samples from the different stages of the canine oestrous cycle.

In our study, canine uteri presenting CEH/pyometra were submitted to histological classification according to Dow´s (I, II, III, IV; 10 samples in each stage) and 10 samples corresponding to late involution uteri (PP) (≥ 250 postpartum days). For immunohistochemistry a specific monoclonal primary antibody raised against canine TNF molecule (sc-80386; Santa Cruz Biotechnology), was used at a 1:50 dilution. The distribution of TNF immunoexpression was analyzed in the superficial and glandular epithelia and compared o the one registered for the cystic epithelium, and also in the stroma. The immunostaining intensity was scored as weak (1), moderate (2) and strong (3). The results were compared with previous published data concerning the TNF immunoexpression on the canine endometrium, during the oestrous cycle.

Our results showed a remarkable loss of homogeneity in TNF expression in the HQE samples, particularly evident in areas with more irregular glandular contour, compared with the uteri in involution and in the different stages of the oestrous cycle. There was also found an increase in the TNF immunoreaction in initial stages of HQE. These results suggest that, without commitment for its possible involvement in the mechanisms of local immunity, TNF might be related with the pathology of this process, probably via modulation of the interactions between some matrix metalloproteinases and their inhibitors, favoring the growth of cystic areas.

Early embryo collection in dogs. Sónia Miranda, R. Payan-Carreira, R.M.L.N. Pereira



Contribution for the Study of Endometrial Adenocarcinomas of the Queen

Saraiva AL[1,2], Payan-Carreira Rita [2,3], Gärtner F[4,5], Tavares Pereira M[3], Cunha M[3], Pires MA[2,3]

[1]EUVG, Coimbra, Portugal; [2]CECAV – Centro de Ciência Animal e Veterinária, Vila Real, Portugal; [3]ECAV – UTAD, Vila Real, Portugal. [4]- ICBAS – Universidade do Porto, Porto, Portugal. [5]IPATIMUP Porto, Portugal

In domestic animals, endometrial adenocarcinomas are considered to be rare with the exception of cattle and rabbits. Although the uterus is pointed as the most common site of genital tract for the occurrence of tumours, the reported low incidence of endometrial adenocarcinomas in cats may be related to its underdiagnose, since post mortem evaluation of female genital tract is not always performed and the submission of surgical specimens for morphological diagnose is still not common in clinical practice. In our study, we pretend to achieve a more precise characterization of the molecular expression of these tumours, in comparison to the normal endometrium. Fifty feline endometrial adenocarcinomas selected from the archives of four laboratories, during a period of sixteen years, were identified by a minimum of three pathologists on conventional H&E-stained sections. For control, samples of histologically normal feline uterus in progestagenic and oestrogenic phases were used.

Immunolabelling was performed by the indirect avidin-biotine-peroxidase immunohistochemistry method, using antibodies against ER-α (clone ER12; Cell Marker®; 1:40), PGR (clone NCL-L-PGR; Novocastra®; 1:30), COX-2 (clone SP21; Neomarkers & LabVision Corporation®; 1:75), CK7 (clone R17-S; DB Biotech®; 1:100), CK20 (clone Ks20.8; Eurodiagnostica®; 1:100) macrophages (clone MAC 387; AbSerotec®; 1:100), T cells (clone CD3; Dako®; 1:50) and B cells (clone CD79α; Cell Marque®; 1:75).

Feline endometrial adenocarcinomas were morphologically classified as papillary serous adenocarcinomas, clear cell adenocarcinomas and *in situ* carcinomas. With the exception of clear cell adenocarcinomas, tumours under study for hormonal receptors labelling tended to loose ER-α, but maintained PGR expression, when compared with normal endometrium. There were no significant differences in COX-2 score of expression between normal and neoplastic endometrium, but tumour cells exhibited cytoplasmic, membranar (without polarity) and perinuclear labelling, contrary to normal endometrium where there was a tendency for apical membrane labelling. When compared with normal endometrium, the tumours showed a tendency for a decrease in CK7 expression and an increase in CK20 expression. Finally, the immune cells infiltrates differed among stages and cell type. Both macrophages and T lymphocytes´ infiltrate increased on tumours, when compared with controls; with Macrophages and B lymphocytes showing higher increases when co-existing with pyometra.

Feline endometrial adenocarcinomas might be more common than we presume and three different morphologies can occur. Molecular characterization of feline endometrial adenocarcinomas might provide important information on carcinogenesis and diagnosis. Further studies must be performed in order to achieve the clinical impact of our results, in terms of progression and prognosis of the disease.

Reproductive Pathology in Laying Hens

Saraiva S[1], Esteves A[2], Saraiva C[2], Seixas F[2]

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This study was performed to evaluate the incidence of lesions of the reproductive system in commercial laying hens (*Gallus domesticus*). Twelve flocks from different farms were slaughtered at ages ranging from 74 to 118 weeks of life. During the *post mortem* examination of 33839 laying hens at the slaughterhouse line, 1188 (3.5%) were rejected by different causes. From these, the reproductive pathology concerning the ovary and oviduct disorders (developmental, inflammatory, and neoplastic) corresponded to 727 (61.2%) of the *post mortem* rejections. Salpingitis was observed in 213 (17.9%) cases, peritonitis in 276 (23.2%) cases, leiomyoma in 84 (7.1%) cases and other neoplastic were found in 154 (13.0%) cases. Samples were collected for histopathological examination. In this study we present some macro and microscopic findings observed in slaughtered laying hens.

Session 2 - Clinical Pathology

Lessons about Schmallenberg Virus Learned with Cache Valley Virus

Edwards JF (*DVM, PhD, DACVP*)

College of Veterinary Medicine and Biomedical Sciences. Texas A&M University. College Station, TX, USA

Both Akabane virus (AKV) in the Japan and Cache Valley virus (CVV) in the USA were known for over 20 years before an arthrogryposis and hydranencephaly syndrome (AGH) was associated with these pathogens. AKV was the first, arthropod-borne virus causing AGH in cattle and sheep. In 1985, an identical AGH outbreak occurred in San Angelo Texas in sheep caused by CVV. It has been shown that several other Orthobunyaviruses can cause AGH in sheep and cattle. In 2011, Schmallenberg virus (SBV) was identified in cattle and isolated from the blood of a sick cow. Subsequently, this virus was associated with AGH especially in small ruminants. The rapidity of the characterization of SBV is a tribute to the European team of scientists using metagenomics and the collaboration of European Community field veterinarians. The discovery of Schmallenberg virus is not cause for alarm. A review of the CVV story will show, that SBV is not totally unique. Rather, it merely represents a new chapter in the Orthobunyavirus book. Over 160 Orthobunyaviruses are known, and it is expected that AGH will be found to be caused by a variety of endemic, orthobunyaviruses around the world whenever the right constellation of epidemiologic events come together.

Lymphocyte population in the granulomatous lesions of wild-boars (*Sus scrofa*) and red-deer (*Cervus elaphus*) suspected of tuberculosis.

Matos AC, Andrade S, Pires MA, Coelho AC, Pinto ML

CECAV – Centro de Ciência Animal e Veterinária; ECAV – Escola de Ciências Agrárias e Veterinárias, Vila Real, Portugal.

Man has been affected by tuberculosis for hundreds of years. This infectious disease threatens the lives of millions of people both in developing and developed countries. In humans, tuberculosis is caused mainly by the microorganism of the species *Mycobacterium tuberculosis*, and according to data from the World Health Organization, one third of world population is affected by *M. tuberculosis*. However, tuberculosis also affects other animal species and these are often responsible for the contamination of humans.

This study is part of a larger part set of studies conducted to evaluate the chronic inflammatory response in animals with a presumptive diagnosis of tuberculosis. In order to better understand the pathogenesis of the disease and further characterizise it's lesions, it was our aim to investigate the expression of antibodies anti-CD3 and anti-CD79α in mesenteric lymph nodes of wild boar and in the kidneys of deer, through the immunohistochemistry technique.

Histological analysis allowed the observation of granulomatous lesions in both species and in the particular case of deer, other lesions including chronic interstitial nephritis, chronic perivascular nephritis and pyelonephritis were also observed.

Through immunohistochemical analysis we observed the lymphocyte cells population in 11 mesenteric nodes (55%) for the anti-CD79α antibody, in 15 mesenteric nodes (75%) for the anti-CD3 antibody and 15 kidneys (71,43%) for the anti-CD3 antibody. The quantification of these same cells allowed grouping of lesions depending on the percentage of lymphocyte cells that they exhibit.

The lesions of this study showed relatively similar percentages of B and T lymphocytes, thus suggesting a cellular and humoral response and a similar immune response triggered by the organism against the entry of the pathogen.

The role of synaptic brain mitochondria dysfunction in aging and Alzheimer's disease

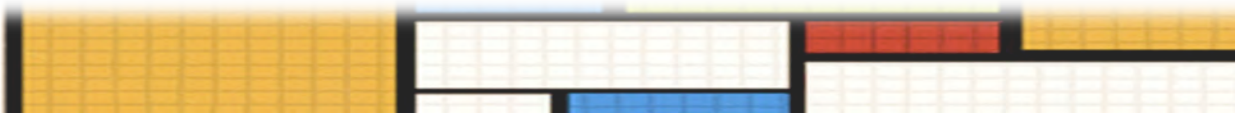
Monteiro-Cardoso VF[1], Oliveira MM[2], Peixoto F[3], VideiraRA[1]

[1]CECAV, Chemistry Department, University of Trás-os-Montes e Alto Douro, 5001-801 Vila Real, Portugal; [2]CQ-VR, Chemistry Department, University of Trás-os-Montes e Alto Douro, 5001-801 Vila Real, Portugal; [3]CITAB, Chemistry Department, University of Trás-os-Montes e Alto Douro, 5001-801 Vila Real, Portugal

Mitochondria play a critical role in cell health and their dysfunction has been implicated in the pathogenesis of various human disorders, including Alzheimer's disease. Neurons contain two mitochondria populations which fall into synaptic and non-synaptic, which differ not only in cellular localization but also in their bioenergetics activity indicating a regionalization of cellular metabolism. In this study we are investigate our hypothesis that Alzheimer's disease (AD) results from mitochondrial dysfunction, mainly on the synaptic mitochondria population. Thus, age-dependent bioenergetics activity of synaptic and non-synaptic brain mitochondria, using triple-transgenic (3xTg-AD) mice as Alzheimer model and wild-type (WT) mice as age-matched controls, was characterized to evaluate it putatively correlation with onset and AD development. 3xTg-AD mitochondrial dysfunction is early evidenced at 3 months old by a significant decrease in complex I activity of synaptic mitochondria (WT 1.16±0.40 *vs* AD 0.61±0.050) without alterations in the bioenergetics activity of non-synaptic brain mitochondria. The impairment of complex I activity is extended to non-synaptic mitochondria at 6 months old, while additional inhibition of complex II, complex IV is detected in synaptic mitochondria population. At 12 months old, synaptic mitochondria complex II and FoF_1-ATPase activity are significant lower in 3xTg-AD. However, the effects detected at 6 months old in the complex I and IV are abolished mainly due to an additional enzymes-activity reduction in the WT mice mitochondria. All these data together indicate that synaptic mitochondria dysfunction precedes the non-synaptic mitochondria impairment, suggesting an important role in the synaptic deterioration characteristic of AD brains.

Acknowledgments
This work was supported by the Foundation for Science and Technology (FCT), FEDER and COMPETE, research grants PTDC/SAU-NMC/115865/2009.
Vera F. Monteiro-Cardoso was supported by FCT grant BI/PTDC/SAU-NMC/115865/2009.

Session 3 - Oncology



Helminthes as Class I Carcinogens: lessons from the scientific work in CECA, a renewed FCT R&D Unit.

Correia da Costa JM

Investigador Coordenador, INSA & CECA/ICETA-U. Porto.

TGF β1 and TGF β2 expression in feline mammary lesions

Seixas F, Novais MJ, Pires MA

CECAV – Centro de Ciência Animal e Veterinária; ECAV – Escola de Ciências Agrárias e Veterinárias, Vila Real, Portugal

Transforming growth factor beta (TGFβ) is a pleiotropic cytokine involved in a variety of important cellular processes, including cell growth and differentiation, motility, apoptosis, immune function and angiogenesis. It has a dual role in tumorigenesis; in early tumour outgrowth it acts as a potent tumour suppressor whereas at late stages it becomes a tumour promotor, involved in malignant transformation, invasiveness and metastasis. TGFβ is an important regulator of normal mammary gland development and function, and it is frequently dysregulated in woman breast cancers. In this study we analysed the imunoexpression of TGFβ1 and TGFβ2 in 35 spontaneous feline mammary lesions.

Our results show that TGFβ is commonly expressed in hyperplasic and neoplastic mammary lesions; the expression is higher in malignant tumours suggesting that TGFβ may be an unfavourable prognostic feature in feline mammary tumours.

Application of everolimus in three bladder cancer cell lines: evaluation of its effectiveness

Pinto-Leite R[1], Arantes-Rodrigues R[2], Palmeira C[3], Colaço A[2], Oliveira PA[2], Santos L[3]

[1]*Genetic Service, Cytogenetic Laboratory, Hospital Center of Trás-os-Montes and Alto Douro, Vila Real, Portugal;* [2]*Department of Veterinary Sciences, CECAV, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal;* [3]*Experimental Pathology and Therapeutics Group, Portuguese Institute of Oncology, Porto, Portugal.*

Recently it has been reported that everolimus could have an important role to play in bladder-cancer treatment and that mTOR inhibitors may restore chemosensitivity in resistant tumours. The aim of this study was to assess everolimus (RAD001) *in vitro* ability to enhance cisplatin (CDDP) cytotoxicity in three human bladder-cancer cell lines.

Over the course of 72 hours, the cells were exposed to different concentrations of CDDP and RAD001, isolated or combined.

Results: Treatment with CDDP statistically ($p<0.05$) decreased cell proliferation in cell lines in a dose-dependent manner. The anti-proliferative activity of CDDP used in combination with RAD001 was statistically significant ($p<0.05$) in the cell lines at all concentrations tested.

RAD001 had a therapeutic effect when used in combination with CDDP and could therefore be a useful anti-cancer drug combination for patients with bladder cancer.

Development of a monoclonal antibody against the canine CCR2

Raposo T[1], Argyle DJ[2], Pires I[1], Queiroga FL[1]

1 Department of Veterinary Sciences - CECAV,UTAD, Portugal; 2 The Royal (Dick) School of Veterinary Sciences and The Roslin Institute, University of Edinburgh, Scotland - UK.

In this project, it is proposed that the clinical application of monoclonal antibody against CCR2, the receptor of CCL2, also known as the Monocyte Chemotactic Protein-1, would be helpful in hindering the progression to advanced stages of cancer disease, while inhibiting TAMs recruitment to the tumour site. For this purpose, canine mammary tumours will be used as a model of human breast cancer, in face of the vast similarities presented between both species, considering molecular, histologic and clinical aspects of the disease. The tumour microenvironment in canine mammary tumours will also be further characterized, analysing the up and down-regulated genes associated with TAMs infiltration, the distinction of M1 (classically activated) and M2 (alternatively activated), respectively tumour progression opposing and favourable TAMs and how these populations are balanced within the tumours.

RNA was isolated from canine bone marrow, CCR2 cDNA was synthesized and cloned into a plasmid vector, and the cloning confirmed through DNA sequencing. Transformed BL21 DE3 *E.coli* were used as a system of bacterial expression. The bacterial culture obtained was lysed and purified through an affinity chromatography column, to isolate the CCR2 protein which was subsequently dialyzed and verified through mass spectrometry.

The DNA sequencing of the canine CCR2 revealed a point mutation when matched against the predicted sequence published in the NCBI database. Throughout the purification process, the inclusion of urea in the lysis, wash and elution buffers was crucial to withdraw the most of the protein from the bacterial lysates. In order to prepare the protein for immunization, a buffer exchange through a dialysis membrane was carried out, so as to reduce the urea molarity, refold and concentrate the protein.

Up till now, the initial stage of the antigen production has been successfully concluded. The CCR2 protein was synthesized using a bacterial system of expression purified through an affinity chromatography column and is now ready for the immunization of mice. Hence, it is now possible to progress to the following phases in the development of a monoclonal antibody.

In vivo and *in vitro* effects of meloxicam on bladder cancer

Arantes-Rodrigues R[1], Pinto-Leite R[2], Palmeira C[3], Santos L[3], Colaço A[1], Oliveira PA[1]

[1]*Department of Veterinary Sciences, CECAV, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal;* [2]*Genetic Service, Cytogenetic Laboratory, Hospital Center of Trás-os-Montes and Alto Douro, Vila Real, Portugal;* [3]*Experimental Pathology and Therapeutics Group, Portuguese Institute of Oncology, Porto, Portugal.*

To assess the efficacy of meloxicam, a non-steroidal anti-inflammatory drug (NSAID), on mice bladder cancer chemically induced by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) and its influence on three human bladder-cancer cell lines (HT1376, T24 and 5637).

Over the course of 12 weeks, Hsd:ICR male mice were exposed to BBN in drinking water. Subsequently, animals were treated with meloxicam by intra-peritoneal route, for 6 consecutively weeks. Tumour development was evaluated by haematoxylin and eosin staining. The *in vitro* effects of meloxicam was assessed by optical microscopy, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and flow cytometry.

Meloxicam inhibited invasive bladder tumours development, without toxicity signs. *In vitro,* meloxicam induced a significant ($p<0.05$) decrease of cell proliferation and cell cycle arrest on G_0/G_1 phase in all the cell lines. A slight but significant increase of sub-G_0/G_1 fraction on T24 ($p=0.006$) and 5637 ($p<0.001$) cells was found.

Meloxicam is effective on *in vivo* and *in vitro* models of bladder cancer. These findings support that meloxicam deserves more attention on bladder cancer treatment and it strengthen the proposal that NSAIDs should be considered as new possible approach to cancer treatment.

Tumour-associated macrophages in canine melanocytic tumours

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Inflammation in tumour stroma greatly influences its development. In human cancer it has been described that tumour associated macrophages (TAMs) may promote tumour cell invasiveness and potentiate metastatic diffusion. Among the various inflammatory mediators generated by TAM, assume particular relevance the arachidonic acid metabolites, which are known to influence several biological responses involved in tumour progression, such as inflammatory and immune reactions, haemostasis and angiogenesis. Melanoma is a devastating disease frequently encountered within both veterinary and human medicine. Although the scientific evidence of an association between TAM and histological aggressiveness in human melanoma, in the dog there are few studies concerning this subject.

In order to evaluate the number and the distribution of TAMs in canine melanocytic lesions; and the associations between TAMs and several clinicopathological characteristics, we analyzed 40 canine melanocytic tumors. Macrophages were characterized by immunohistochemical techniques using MAC387.

TAMs were observed in all the samples analyzed. The number of tumoural-associated macrophages in malignant melanoma was significantly higher than the mean values in the benign counterparts ($p=0,017$). The number of macrophages also showed a statistically significant association with some clinicopathologic features, such tumor location ($p=0,003$), presence of ulceration ($p=0,002$), presence of necrosis ($p=0,001$) and presence of tumoural embolus ($p=0,048$). Most of these characteristics are classically linked to higher tumoural aggressiveness and poor clinical prognosis in these neoplasias.

Our study suggests that TAMs could constitute an important marker of canine melanocytic tumour aggressiveness.

Characterization of Cancer Stem-Like Properties in Canine Mammary Cancer

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Canine mammary gland tumours represent an important disease in female dogs, attracting considerable attention over the last decades. Currently, one of the most motivating concepts that is being explored in the human cancer research field is the cancer stem cell hypothesis, which states that a minority of transformed cells, with acquired stem or progenitor properties, are the source of tumour cell renewal and thereby determine tumour behaviour. However, in canine mammary cancer, only few studies have focused on the existence of cancer stem cells.

By using an *in vitro* approach (three mammary tumour cell lines), we have isolated cells with stem-like properties, by using a mammosphere formation assay. A phenotypical characterization by flow cytometry, immunofluorescence and western blot has been performed, as well as functional assays. In addition, we have evaluated the immunohistochemical expression of CSC markers (ALDH1, CD24, CD44) in canine mammary carcinomas (CMC).

Our results show that canine mammary cell lines seem to reflect mammary cancer heterogeneity, as they presented distinct phenotypical and functional properties. Cells were able to grow as spheroids in anchorage-independent conditions, presented ALDH1 activity (ALDEFLUOR assay) and expressed CSC markers.

With regard to CMC, the majority expressed CD44, in opposition to CD24, which was rarely expressed; in addition, ALDH1 expression was frequently observed. No association was observed between CSC phenotype and aggressive tumour behaviour. In fact, ALDH1 expression was significantly associated with higher survival rates.

Our results further consolidate the stem cell theory in this animal model; however, additional *in vitro* e *in vivo* studies are essential in order to unravel its significance in this complex disease.